Ecological and Lineage-Specific Factors Drive the Molecular Evolution of Rhodopsin in Cichlid Fishes

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Abstract

The visual system in the colorful cichlid fishes from the African great lakes is believed to be important for their adaptive radiations. However, few studies have attempted to compare the visual system of radiating cichlid lineages with that of cichlids that have not undergone recent radiations. One such study published in this journal (Schott RK, Refvik SP, Hauser FE, López-Fernández H, Chang BSW. 2014. Divergent positive selection in rhodopsin from lake and riverine cichlid fishes. *Mol Biol Evol.* 31:1149–1165) found divergent selection on rhodopsin between African lacustrine and riverine cichlid species and riverine Neotropical cichlids, concluding that ecology drives the molecular evolution of this opsin. Here, we expand this analysis by incorporating rhodopsin sequences from Neotropical lacustrine cichlids and show that both ecology and phylogeny are important drivers of the molecular evolution of rhodopsin in cichlids. We found little overlap of sites under selection between African and Neotropical lineages and a faster rate of molecular evolution in African compared with Neotropical cichlids. These results support the notion that genetic or population genetic features particular to African cichlids contributed to their radiations.

Key words: **codon substitution models, dim-light vision, Neotropical cichlids, vision, visual pigments.**

Letter

East African cichlid fishes have long attracted the interest of evolutionary biologists for their potential to help understand the mechanisms involved in adaptation and diversification (e.g., Kosswig 1947; Mayr 1984). Recently, special attention has been given to their visual system not only because of its impressive phenotypic and genotypic diversity (Carleton 2009) but also due to its potentially causal relationship to the mechanism of adaptive radiation of African cichlids (Carleton et al. 2005; Maan et al. 2006; Terai et al. 2006; Seehausen et al. 2008; Miyagi et al. 2012). Therefore, African cichlids have emerged as a model system for the study of vision evolution in vertebrates (Carleton 2009).

Much less is known about the ecology and evolution of the visual system of Neotropical cichlids, the sister lineage that diverged from African cichlids approximately 45–60 Ma (Friedman et al. 2013). Opsin genes, the protein component of visual pigments, have been sequenced in only a few species of Neotropical cichlids so far, all of which were riverine species (Weadick et al. 2012; Schott et al. 2014). Neotropical riverine species possess a reduced set of functional opsin genes (*SWS2A*, *SWS2B*, *RH1*, *RH2A*, and *LWS*) compared with the eight genes found in African cichlids (same as in Neotropical cichlids plus *SWS1*, *RH2B*, and two paralogs of *RH2A*). From these genes, just one cone opsin (*SWS2B*) and the dim-light sensitive rhodopsin (*RH1*) appear to be under selection in Neotropical cichlids (Weadick et al. 2012; Schott et al. 2014). One recent study compared *RH1* sequences among African and Neotropical cichlid species finding evidence for divergent selection, with African lacustrine cichlids showing stronger signature of positive selection than both African and Neotropical riverine cichlids (Schott et al. 2014). The proposed explanation for this pattern is that ecological differences between lakes and rivers strongly influence the molecular evolution of *RH1* (Schott et al. 2014). However, due to the lack of *RH1* sequences from Neotropical lacustrine cichlids, this explanation is confounded with an alternative, although not mutually exclusive hypothesis: That African lacustrine cichlids present a particularly rapid rate of molecular evolution of opsin genes (i.e., Brawand et al. 2014).

By incorporating sequences from lacustrine cichlid species inhabiting Nicaraguan lakes into the cichlid *RH1* alignment, we provide a second and independent contrast that allowed us to differentiate the contribution of ecological and lineage-specific factors in the rate of molecular evolution of the cichlid *RH1*. Our results show that a combination of ecological and lineage-specific factors drives the molecular evolution of this gene in cichlid fishes.

Results and Discussion

We recovered the full coding sequence of *RH1* (1,065 bp) from 14 species of Neotropical cichlids (1–18 individuals per species, supplementary table S1, Supplementary Material online) that inhabit the great and crater lakes of Nicaragua; 6 of which belong to the Midas cichlid species

complex that recently radiated in Nicaraguan crater lakes (Elmer et al. 2010). A comparison of the *RH1* sequences of these 14 species with sequences from 31 Neotropical riverine, 8 African riverine and 34 African lacustrine cichlid species (supplementary table S1, Supplementary Material online) revealed that across all lineages, 20.6% of the amino acids were variable and 15.0% were phylogenetically informative. Maximum-likelihood (ML) phylogenetic analyses of the *RH1* alignment resulted in a gene tree congruent with known phylogenetic relationships of Neotropical and African cichlids (Salzburger et al. 2005; López-Fernández et al. 2010; fig. 1). Just like in African cichlids, Nicaraguan lacustrine species are derived from ancestral riverine species; thus, riverine cichlids constitute paraphyletic groups including all but the lacustrine species (fig. 1). We used this topology for all subsequent molecular evolutionary analyses.

We tested for evidence of molecular evolution on cichlid *RH1* by using random site models as implemented in PAML (Yang 2007). We found evidence of positive selection in Neotropical lacustrine cichlid *RH1*, as it has been previously shown for both Neotropical riverine and African lacustrine and riverine cichlids (Spady et al. 2005; Weadick et al. 2012; Schott et al. 2014; table 1). The ratio of nonsynonymous to synonymous substitutions (ω) for Neotropical lacustrine cichlid *RH1* calculated under the model assuming only one site class ($\omega_{M0} = 0.32$; table 1) was similar to that of other cichlids ($\omega_{M0} = 0.25$; Schott et al. 2014), and relatively high compared with those seen in ray-finned fishes in general (0.074 in Rennison et al. 2012). This suggests that some amino acid sites are released from purifying selection. The M3/M0 random site model test on the subalignment of Neotropical lacustrine cichlid confirmed that there is variation among sites in ω; and the M2a/M1a, M8/M7, and M8/M8a site model tests found significant evidence of positive selection on some of those sites (table 1).

Three amino acid sites in the Neotropical lacustrine cichlids *RH1* alignment were assigned by M8 to the positive selected class with a high posterior probability (sites 172, 217, and 274; table 1). These sites were also assigned to the positive selected class by other models in PAML (M2) and in HYPHY (REL and FUBAR; Kosakovsky Pond et al. 2005; Kosakovsky Pond and Frost 2005; Murrell et al. 2013), with the exception of site 217 that was assigned to the positive selected site class only with intermediate posterior probability by FUBAR ($P = 0.713$; supplementary table S2, Supplementary Material online). These sites constitute a subset of those previously identified to be under selection in Neotropical riverine cichlid *RH1* (table 1), and their functional importance was already discussed in detail (Schott et al. 2014). Sites 172 and 217 are located, respectively, in transmembrane helixes IV and V, and are predicted to interact during dimerization of rhodopsin (Guo et al. 2005; Fotiadis et al. 2006; Schott et al. 2014). Site 274 is in close proximity to the retinal channel B and changes at this site from a nonpolar, hydrophobic amino acid (tryptophan) to a polar, hydrophilic one (tyrosine) might influence retinal uptake-release dynamic (Hildebrand et al. 2009; Schott et al. 2014).

Based on Clade model C (CmC; Bielawski and Yang 2004; Chang et al. 2012), we tested for evidence of divergent selection on *RH1* between African and Neotropical cichlids and/or between riverine and lacustrine cichlids. We defined a priori a set of eight models with different partitions testing for the role of lineage history and ecology on the molecular evolution of cichlid *RH1* (fig. 2). Compared with the null model that does not allow for divergence among cichlid lineage partitions (M2a_rel; Weadick and Chang 2012b), models that partitioned the *RH1* alignment fit the data significantly better (table 2).

Schott et al. (2014) importantly identified that the signature of positive selection is higher in lacustrine compared with riverine cichlids in African lineages. Here we augment that finding to show that selection is also higher in Neotropical lacustrine than riverine species, suggesting it is a general pattern among cichlids (tables 1 and 2; *Cichlids* vs. *R/L* and *Af/Neo* vs. *AfR/AfL/NeoR/NeoL*). The colonization of lakes by cichlid fish implied adaptation not only to a new photic environment but also potentially to a more variable one. This, in combination with the microhabitat partitioning observed in lake dwelling cichlids (Kocher 2004), might explain the differences in the estimates of positive selection between lacustrine and riverine species (Schott et al. 2014).

Interestingly, our data also suggest divergent selection on *RH1* between Neotropical and African cichlids, even after controlling for ecology within these lineages (table 2; *Cichlids* vs. *Af/Neo* and *R/L* vs. *AfR/AfL/NeoR/NeoL*). This pattern of divergence could result from peculiarities in the evolutionary history of the rhodopsin protein in Neotropical and African cichlids (fig. 1). This is supported by the little overlap of the specific sites under selection between both lineages. None of the positively selected sites identified by random site models was shared by the four different subalignments (although site 217 was shared by Neotropical cichlids and African lacustrine cichlids; table 1). However, within lineages, there was a high degree of overlap on the sites identified to be under positive selection on riverine and lacustrine cichlid *RH1* (table 1). This pattern will be expected if the evolutionary history of the protein constrains the specific sites that could vary without negatively affecting its functional dynamics (e.g., Tufts et al. 2014). This appears to be the case for rhodopsin, in which amino acid substitutions at particular sites are not restricted to local effects, but also have a global impact on protein folding and functional dynamics (Teller et al. 2003; Piechnick et al. 2012).

It has been repeatedly suggested that the visual system might have played a central role in the processes that led to the recent and rapid adaptive radiation in African cichlid fish (Terai et al. 2006; Seehausen et al. 2008; Brawand et al. 2014). Although it is not clear why other cichlid lineages did not radiate to the extent that African cichlids did, recent genomic and transcriptomic analyses have suggested the existence idiosyncrasies in the African rift lake cichlid genomes, showing a high rate of gene duplication, accelerated coding sequence evolution, and gene expression divergence (Brawand et al. 2014; reviewed in Henning and Meyer 2014). The visual system of African cichlids is an interesting

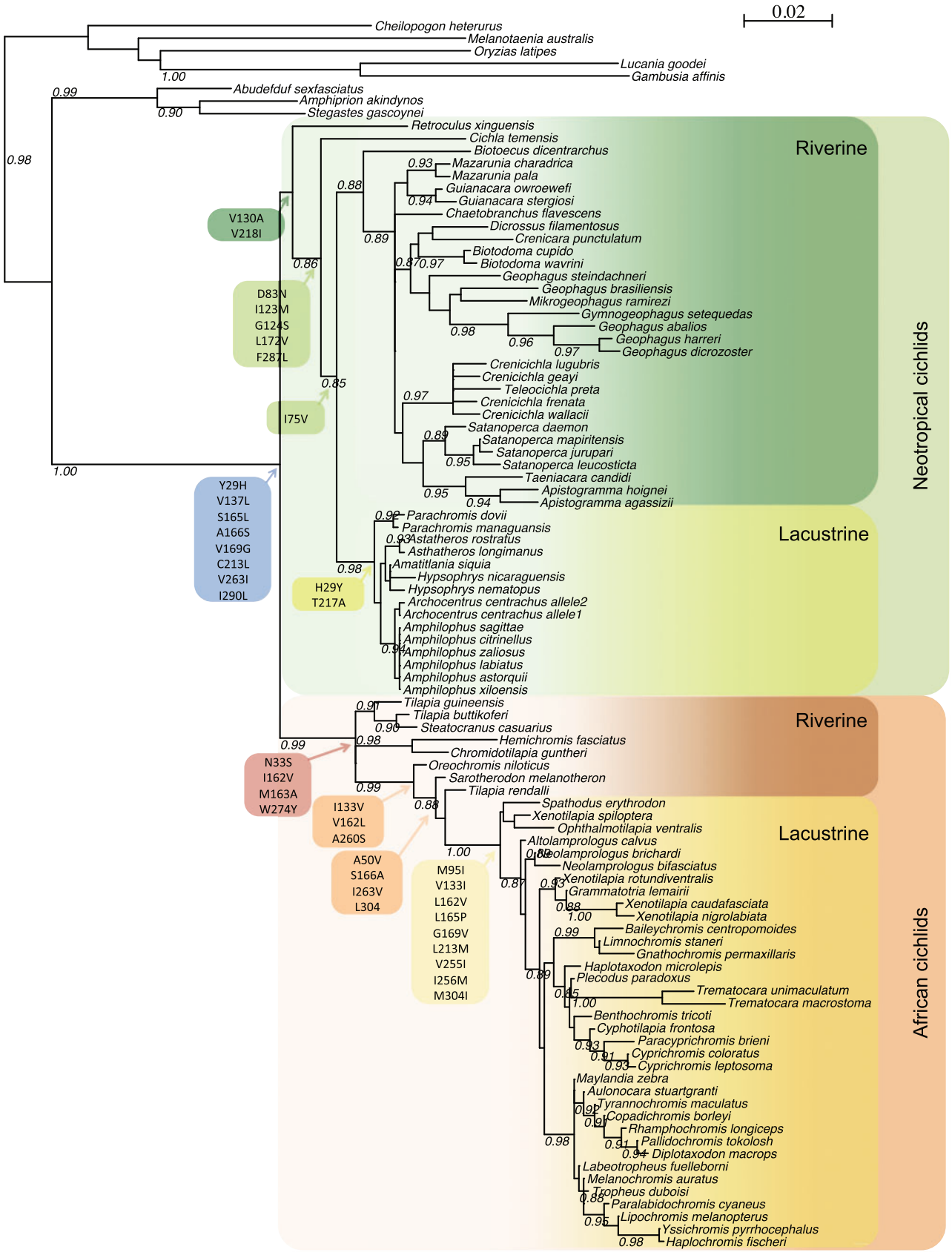

FIG. 1. ML rhodopsin gene tree. The nonparametric aLRT SH-like procedure is only showed when above 0.85. Insets show the amino acid substitution inferred to have occurred in the rhodopsin gene of the ancestor to all cichlids (blue inset), Neotropical cichlids (dark green inset), African cichlids (dark brown inset), Neotropical lacustrine cichlids (light green inset), and African lacustrine cichlids (light brown inset). Ancestral state reconstruction was performed as implemented in PAML.

Table 1. LRT of Positive Selection (Random Sites Model in PAML) in Different Subsets of Cichlid Rhodopsin Coding Sequence.

Biogeography	Ecology	ns	ls	M0 Tree Length	ω_{M0}	Log-LRTs				Parameters under M8	Positively Selected Sites (M8, BEB)[a]
						M3/M0	M2a/M1a	M8/M7	M8/M8a		
Neotropical	Lacustrine	15	354	0.085	0.319	19.85***	10.62**	9.23**	9.21**	*p*: 8.87; *q*: 99 ω_2: 11.29 (2.6%)	*172, 217, 274*
Neotropical	Riverine	31	354	1.188	0.258	245.99***	31.95***	37.29***	32.11***	*p*: 0.02; *q*: 0.10 ω_2: 3.66 (4.7%)	*156, 169,* <u>172</u>*,* <u>173</u>*, 217, 270,* <u>274</u>*, 281, 286*
Africa	Lacustrine	36	354	0.560	0.966	266.65***	142.13***	144.99***	142.09***	*p*: 0.006; *q*: 0.01 ω_2: 13.50 (5.6%)	<u>14</u>*, 22, 37, 41, 95, 133, 162, 165, 169, 213, 217, 218,* <u>259</u>*, 263,* <u>270</u>*, 297, 298, 299*
Africa	Riverine	8	354	0.245	0.175	32.08***	7.09*	8.35*	7.09**	*p*: 0.005; *q*: 3.91 ω_2: 3.15 (7.2%)	<u>49</u>*,* <u>133</u>*, 162, 297*

NOTE.—ns, number of sequences; ls, length of sequences.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[a]Only sites with a posterior probability higher than 80% are reported. If the posterior probability of a site belonging to the positively selected class (ω_2) is $0.8 > P > 0.9$ the site number is underlined and if it is $P < 0.9$ it is in italics. Sites are numbered following bovine *RH1*.

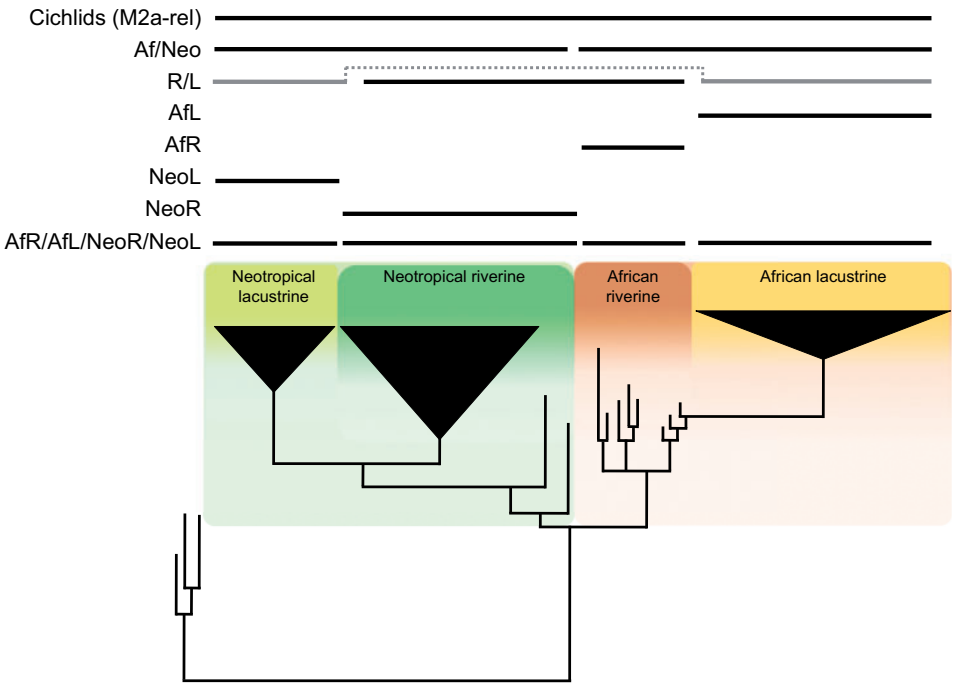

FIG. 2. Simplified representation of the rhodopsin gene tree indicating the a priori partitions among cichlids lineages tested using CmC. Abbreviations refer to riverine (R), lacustrine (L), African (Af), Neotropical (Neo), African riverine (AfR), African lacustrine (AfL), Neotropical riverine (NeoR), and Neotropical lacustrine (NeoL) cichlids. The AfL, AfR, NeoL, and NeoR models contain an implicit second partition that includes all cichlids but those in the depicted partition.

case for how these processes might potentially drive phenotypic diversity, as all these processes have been identified as important in the diversification of opsin genes as well (Carleton and Kocher 2001; Sugawara et al. 2002; Spady et al. 2005, 2006; Terai et al. 2006; Carleton 2009; Weadick and Chang 2012a). Thus, comparisons of African and Neotropical cichlid visual systems can further the understanding of the processes responsible for the differences in diversification rate between these lineages (Weadick et al. 2012).

By comparing the *RH1* alignments of African and Neotropical cichlids we found that within each lineage, the strength of selection among divergent sites was stronger in lacustrine cichlids than in riverine species. Yet, the signature of positive selection in divergent sites was overall stronger in the African lineage than in the Neotropical lineage. In particular, the signature of positive selection in African lacustrine cichlids was 3-fold higher than that seen in Neotropical lacustrine cichlids (ω_2 in table 2). The model testing for divergence between African lacustrine cichlids and the rest of cichlids (*AfL*) was the single-partition model that best fit the data. The *AfR/AfL/NeoR/NeoL* four-partition resulted only in a better fit than *AfL* (likelihood ratio test [LRT] = 5.62, df = 2, $P = 0.06$). This model shows that the signature of positive selection is extremely high in African lacustrine cichlids, even when

Table 2. Divergence Analysis (CmC in PAML) Using Different Partitions of Cichlid Rhodopsin Coding Sequence.

Model & Partition[a]				LRT			Parameters							
	n.p.	ln *L*	ΔAIC	Null	2Δ*ln L*	df	κ(ts/tv)	ω_0	(%)	ω_1	(%)		ω_2	(%)
M0	179	−5,510.72	717.98				2.96	0.37						
M1a	180	−5,236.40	171.35	M0	548.63***	(1)	2.51	0.02	(80.9)	1	(19.1)			
M2a	182	−5,170.51	43.58	M1a	131.77***	(2)	2.83	0.02	(80.3)	1	(13.8)		4.25	(5.9)
M3	182	−5,170.30	45.15	M0	680.84***	(4)	2.85	0.03	(81.2)	1.2	(13.8)		4.62	(5.1)
CmC: AfR/AfL/ NeoR/NeoL	*185*	*−5,145.72*	*0.00*	*M2a-rel*	*181.35****	*(3)*	*2.77*	*0.02*	*(80.2)*	*1*	*(14.3)*	*NeoR:*	*1.65*	*(5.5)*
				CmC: AfL	*5.62*[†]	*(2)*						*AfL:*	*11.50*	
				CmC: R/L	*12.81***	*(2)*						*NeoL:*	*3.25*	
				CmC: Af/Neo	*16.32****	*(2)*						*AfR:*	*3.58*	
CmC: AfL	183	−5,148.53	1.62	M2a-rel	175.73***	(1)	2.77	0.02	(80.2)	1	(14.5)		2.16	(5.3)
												AfL:	11.57	
CmC: R/L	183	−5,152.13	8.81	M2a-rel	168.54***	(1)	2.31	0.02	(80.1)	1	(14.6)	R:	2.26	(5.3)
												L:	9.42	
CmC: Af/Neo	183	−5,153.89	12.32	M2a-rel	165.03***	(1)	2.75	0.02	(80.2)	1	(14.8)	Neo:	2.04	(5.0)
												Af:	8.53	
CmC: NeoR	183	−5,154.36	13.27	M2a-rel	164.08***	(1)	2.75	0.02	(80.1)	1	(15.1)		7.91	(4.9)
												NeoR:	1.93	
CmC: AfR	183	−5,169.73	44.02	M2a-rel	133.33***	(1)	2.83	0.02	(80.3)	1	(13.8)		4.47	(5.9)
												AfR:	3.00	
CmC: NeoL	183	−5,170.51	45.57	M2a-rel	131.78***	(1)	2.83	0.02	(80.3)	1	(13.8)		4.26	(5.9)
												NeoL:	4.12	
M2a-rel: Cichlids	182	−5,236.40	175.35	M1a	131.66***	(2)	2.51	0.04	(81.8)	1	(15.6)	Cichlids:	4.41	(2.6)

NOTE.— R, riverine; L, lacustrine; Af, African; Neo, Neotropical; AfR, African riverine; AfL, African lacustrine; NeoR, Neotropical riverine; NeoL, Neotropical lacustrine cichlids.
[†]$P < 0.1$; **$P < 0.01$; ***$P < 0.001$.
[a]Most supported model is in italic letters.

compared with lake dwelling cichlids from the Neotropics. This finding is in line with the suggestions that there are molecular or population genetic features that contribute to the radiation of African cichlids (Brawand et al. 2014).

We conducted a series of a posteriori analyses using new partitions to determine how the extremely high rate of molecular evolution in African lacustrine cichlids *RH1* might have affected our results. First, we found that the *AfR/AfL/NeoR/NeoL* four-partition model fit the data significantly better than a model allowing for divergence between African and Neotropical lacustrine cichlids *RH1*, but not among riverine cichlids *RH1* (*R/NeoL/AfL*; LRT = 4.67, df = 1, $P = 0.03$). This provides further support to our conclusion that *RH1* has diverged between African and Neotropical cichlids, even when comparing just riverine taxa. Second, we found that a model allowing divergence among Neotropical cichlids, but not within the African lineage (*Af/NeoR/NeoL*), resulted in a better fit of the data than the more restricted model allowing for divergence only between African and Neotropical lineages (*Af/Neo*; LRT = 2.89, df = 1, $P = 0.09$). This supports the conclusion that ecological factors associated with the visual environment of lakes and rivers affected the evolution of *RH1* in Neotropical cichlids. This was not the case when comparing the *AfR/AfL/NeoR/NeoL* model to a more restricted model (*AfR/AfL/Neo*; LRT = 2.00, df = 1, $P = 0.15$), presumably because the improvement of the model fit due to partitioning Neotropical lacustrine and riverine cichlids is too small compared with the improvement that results from partitioning African lacustrine and riverine cichlids.

In summary, we found divergent selection on *RH1* between African and Neotropical cichlids, and that the proportion of sites under positive selection is higher (table 1) and the strength of positive selection in divergent sites is stronger (table 2) in the African than in the Neotropical lineage. In addition, we show that the signature of positive selection on *RH1* is higher in lacustrine than riverine Neotropical cichlids, as it has been found for African cichlids (Schott et al. 2014), suggesting that differences in the photic ecology of riverine and lacustrine environments are important drivers on the evolution of this visual pigment. Taken together, these data support the hypothesis that the rate of evolution on Neotropical cichlids visual system might be relatively lower than that seen on African cichlids, and this could be related to the overall difference in the rate of diversification between these lineages. Future analyses focusing on cone opsin evolution in Neotropical cichlids (e.g., Weadick et al. 2012) are needed to test this hypothesis.

Materials and Methods

Sequencing, Sequence Analysis, and Phylogenetic Reconstruction

Genomic DNA was isolated from one to 18 individuals of 14 species of cichlid fish inhabiting Nicaraguan lakes (supplementary table S1, Supplementary Material online), using

standard phenol–chloroform extractions. Genomic sequences of *RH1* were obtained by polymerase chain reaction. Primers were designed in PRIMER 3 (Rozen and Skaletsky 2000) using as a template the *Amphilophus citrinellus* draft genome (Elmer et al. 2014; primer list in supplementary table S2, Supplementary Material online). Samples were sequenced bidirectionally using a 3130xl Genetic Analyzer.

Sequence editing and assembly were performed and exported for analysis using SeqMan II (DNAstar). Rhodopsin gene trees were created using ML phylogenetic analyses in SeaView (Gouy et al. 2010), with a GTR+G model, a nonparametric Approximate Likelihood Ratio Test branch support based on Shimodaira–Hasegawa-like (aLRT SH-like) procedure, best of Nearest Neighbor Interchanges and Subtree Pruning and Regrafting tree searching operations, and five random starts.

Molecular Evolutionary Analyses

To detect positive selection in the *RH1* alignment, we used random site models in PAML (Yang 2007). We conducted the analyses on different subalignments including *RH1* sequences from 1) only Neotropical lacustrine (NeoL), 2) Neotropical riverine (NeoR), 3) African lacustrine (AfL), and 4) African riverine (AfR) cichlids. In each of these subalignments, using log-LRT, we compared different random site models to test for variation in ω across sites (M3/M0), and for the presence of positively selected sites (M2a/M1a, M8/M7, and M8/M8a; Yang 2007). We identified particular sites under positive selection using Bayes' Empirical Bayes in PAML (BEB in M2a and M8, Yang et al. 2005), and FUBAR and REL methods in HYPHY (Kosakovsky Pond et al. 2005; Kosakovsky Pond and Frost 2005; Murrell et al. 2013).

To test for divergent selection in *RH1* among cichlid clades, we used CmC in PAML (CmC; Bielawski and Yang 2004). CmC allows some sites to vary among a priori defined partitions in the alignment (background and foreground partitions). To test for divergence among partitions, this model was compared with a null model (M2a_rel; Weadick and Chang 2012b), which is similar to CmC but does not allow variation among partitions. Because we were not only interested in determining whether there was divergent selection among partitions, but also in determining what particular partition best fitted the data, we evaluated models using LRT for fully nested models and Akaike's Information Criteria (AIC) for non-nested models. Partitions were designed a priori based on the interaction of ecological (i.e., riverine vs. lacustrine species) and biogeographic factors (i.e., African vs. Neotropical species). This resulted in a set of eight models each with different partitions of the cichlid *RH1* alignment (table 2; fig. 2). The cichlid *RH1* alignment used in the clade analyses did not include noncichlid outgroups because we were interested in divergence among cichlids, and divergence between cichlids and other lineages has been already established (Spady et al. 2005; Weadick et al. 2012; Schott et al. 2014). Including noncichlid outgroups does not result in qualitative changes of our results (supplementary table S4, Supplementary Material online).

Supplementary Material

Supplementary tables S1–S4, and references are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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