




Evolution, inactivation and loss of short wavelength-sensitive opsin genes during the diversification of Neotropical cichlids

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Abstract

Natural variation in the number, expression and function of sensory genes in an organism's genome is often tightly linked to different ecological and evolutionary forces. Opsin genes, which code for the first step in visual transduction, are ideal models for testing how ecological factors such as light environment may influence visual system adaptation. Neotropical cichlid fishes are a highly ecologically diverse group that evolved in a variety of aquatic habitats, including black (stained), white (opaque) and clear waters. We used cross-species exon capture to sequence Neotropical cichlid short wavelength-sensitive (SWS) opsins, which mediate ultraviolet (UV) to blue visual sensitivity. Neotropical cichlid SWS1 opsin (UV-sensitive) underwent a relaxation of selective constraint during the early phases of cichlid diversification in South America, leading to pseudogenization and loss. Conversely, SWS2a (blue-sensitive) experienced a burst of episodic positive selection at the base of the South American cichlid radiation. This burst coincides with SWS1 relaxation and loss, and is consistent with findings in ecomorphological studies characterizing a period of extensive ecological divergence in Neotropical cichlids. We use ancestral sequence reconstruction and protein modelling to investigate mutations along this ancestral branch that probably modified SWS2a function. Together, our results suggest that variable light environments played a prominent early role in shaping SWS opsin diversity during the Neotropical cichlid radiation. Our results also illustrate that long-term evolution under light-limited conditions in South America may have reduced visual system plasticity; specifically, early losses of UV sensitivity may have constrained the evolutionary trajectory of Neotropical cichlid vision.

KEYWORDS

molecular evolution, protein evolution, relaxed selection, visual ecology, visual pigment

1 | INTRODUCTION

Detecting, perceiving and responding to environmental stimuli is essential for the survival of most organisms, and the genes underlying complex traits such as sensory systems can offer unique insight into the molecular basis of adaptation (Baldwin & Ko, 2020; Baldwin et al., 2014; Marques et al., 2017; Vandeweghe et al., 2016). The visual system permits the near-instantaneous relay of information between organisms and their environments, and the study of opsin genes, which code for the first step in visual transduction and are maximally sensitive to different wavelengths of light, has greatly enhanced our understanding of the genetic mechanisms underlying adaptation (Davies, 2012; Hauser & Chang, 2017). Cone opsins, mediating bright light (photopic) and colour vision, have undergone extensive duplication and reduction throughout vertebrate evolution, and comparative studies of these genes offer important insight into how genes may become lost, inactivated or duplicated in response to different ecological variables, since opsin gene repertoire, expression and genetic variation are often linked to ambient light environment (Carleton et al., 2020; Fuller & Claricoates, 2011; Härer et al., 2018; Musilova et al., 2019; Phillips et al., 2015; Torres-Dowdall et al., 2017).

In fishes, short wavelength-sensitive vision (ranging from ultraviolet [UV] to blue; ~360–480 nm; Bowmaker, 2008) is critical for a variety of important fitness-related tasks including foraging (Novales Flamarique, 2016), species recognition (Siebeck et al., 2010) and mating (Pauers et al., 2016). The expression of different opsins involved in short-wavelength vision can differ depending on ontogenetic stage (Cortesi et al., 2015; Sabbah et al., 2012). For example, in many freshwater fishes UV-sensitive short wavelength-sensitive 1 (SWS1) is often expressed in the larval stage (Hunt & Peichl, 2013; Novales Flamarique, 2012) where it may aid in the detection of small suspended particles (Jordan et al., 2004). Violet-sensitive SWS2b may be expressed in both fry and adult fishes, while blue-sensitive SWS2a is frequently expressed only in adults (O'Quin et al., 2010). The expression and repertoire of SWS opsins and loss or retention of opsin genes is also known to be highly dependent on habitat. In some cases, rather than SWS1 expression being restricted to larval fish, it is also expressed in adult fishes living in habitats where UV light is abundant, such as the upper portion of lakes (Hofmann et al., 2010), or shallow coral reefs (Cortesi et al., 2020). Fishes that traverse different habitats highlight the usefulness of labile short-wavelength vision: juvenile surface- or freshwater-dwelling salmonids express a suite of opsins tuned to shorter wavelengths (including a UV-sensitive SWS1 opsin) and upon smoltification (accompanied by transition to deeper or marine waters) lose UV sensitivity (Allison et al., 2003).

Beyond opsin gene expression, evolutionary gains and losses of opsins from the genomes of fishes also may affect (or even eliminate) short-wavelength vision. Recent surveys of fish opsins note that nonreef marine fishes tend to have lost SWS1 opsins encoding UV sensitivity, likely due to the rapid depth-dependent attenuation of UV light in pelagic ocean habitats (Lin et al., 2018). Nocturnal reef

fishes were also found to lack SWS1 while retaining expression of other cone opsins (Luehrmann et al., 2019). Conversely, freshwater fishes appear more likely to retain SWS1 if they inhabit shallower, clearwater environments where UV light is able to penetrate (Lin et al., 2018). Despite the utility of UV vision in many freshwater habitats, recent efforts to more extensively sample freshwater fish opsin diversity have suggested that loss of UV sensitivity may be more widespread (Escobar-Camacho et al., 2019, 2020; Weadick et al., 2012). This may be a result of variation in the pattern and degree of attenuation of light across the visual spectrum in different freshwater habitats.

Neotropical cichlid fishes comprise one of the most species-rich freshwater fish clades in South and Central America and inhabit a variety of photic and ecological niches (Arbour & Lopez-Fernandez, 2014; Arbour et al., 2020; Astudillo-Clavijo et al., 2015; Hauser et al., 2017). While the African rift lake radiation took place relatively recently, Neotropical cichlids have diversified over much longer timescales in South and Central America into diverse riverine habitats (reviewed in Matschiner, 2018), allowing for more opportunity for structural variation to their opsin genes to accumulate as a result (Hauser et al., 2017; Weadick et al., 2012). Previous work examining the cichlid opsin repertoire suggests a reduction in the number of opsins available for vision in Neotropical cichlids with evidence for both loss (Weadick et al., 2012) and pseudogenization (Escobar-Camacho et al., 2017) of SWS1 in two different South American species. Based on these studies, Neotropical cichlids probably possess up to eight opsins (SWS1, SWS2a, SWS2b, RH2a α , Rh2a β , RH2b, RH1, LWS), but the extent of absence/pseudogenization of these opsins across the Neotropical cichlid tree remains unclear. In a functional study of Neotropical cichlid rhodopsin (mediating vision in dim light), we also found accelerated evolutionary rates in Central American cichlids, as well as repeated functional transitions in the protein consistent with adaptation to clearwater habitats in Central America (Hauser et al., 2017). Given this pattern of molecular evolution in rhodopsin, it is possible that a combination of phylogeny and biogeography (the transition of some lineages from South to Central America) and habitat (transitions between black or white and clear water) have shaped the evolution of opsin genes in Neotropical cichlids.

Several recent studies of Neotropical cichlid opsin gene expression note the consistent expression of a long-wavelength-sensitive opsin "palette" consisting of the blue (SWS2a), green (Rh2a) and red (LWS) opsins (Escobar-Camacho et al., 2017; Härer et al., 2018; Schneider et al., 2020). Interestingly, this palette appears relatively inflexible in Neotropical cichlids, especially when compared with the highly plastic opsin expression in African cichlids (e.g., Hofmann et al., 2010; reviewed by Carleton and Yourick, 2020). However, it remains an intriguing question whether this restricted palette might be the result of ancestral adaptations to ancient riverine environments, particularly given the conflicting geological evidence surrounding the palaeoenvironments in which the initial diversification of cichlids in South America may have

taken place (Albert and Reis, 2011; Godoy et al., 1999; Lundberg et al., 1998).

Neotropical freshwater habitats are notable for their striking variation in light environments, consisting of clear, blackwater or whitewater rivers (Costa et al., 2013; Sioli, 1984; Figure 1). Clearwater habitats show high transparency of wavelengths across the visible spectrum, and unlike other river habitats do not rapidly attenuate shorter wavelength light (including UV wavelengths); by contrast, blackwater environments tend to have large amounts of dissolved tannins, producing an environment dominated by orange/red wavelengths (Albert and Reis, 2011). Whitewater habitats are generally turbid and sediment-rich, with overall low transparency. Both white and blackwater habitats are thus characterized by the disproportionate attenuation and scattering of short-wavelength light available for vision (Costa et al., 2013). Neotropical fish species may traverse starkly different water types while others are restricted to drainages of a particular water type (e.g., Winemiller et al., 2008), and recent work has highlighted how these water types

may shape Neotropical freshwater fish community composition and biology (Bogotá-Gregory et al., 2020). Did evolution in these diverse riverine habitats result in changes in selection (e.g., a relaxation of constraint) on Neotropical cichlid short-wavelength vision, and has selection imposed by different water types resulted in pervasive changes in opsin evolutionary rates during this group's diversification? More broadly, has the evolution of Neotropical cichlid vision been constrained by strong selection against short-wavelength vision at the early stages of their South American radiation? These open questions invite a closer examination of the evolution of short-wavelength vision in Neotropical cichlids from the perspective of gene loss and inactivation, as well as functional evolution, particularly in relation to light environment.

Here, we investigate how the evolutionary trajectory of Neotropical cichlid short-wavelength vision was shaped by light environment and gene loss in this diverse clade. We use cross-species exon capture to examine three cone opsins mediating short-wavelength vision in the UV (SWS1), violet (SWS2b) and blue

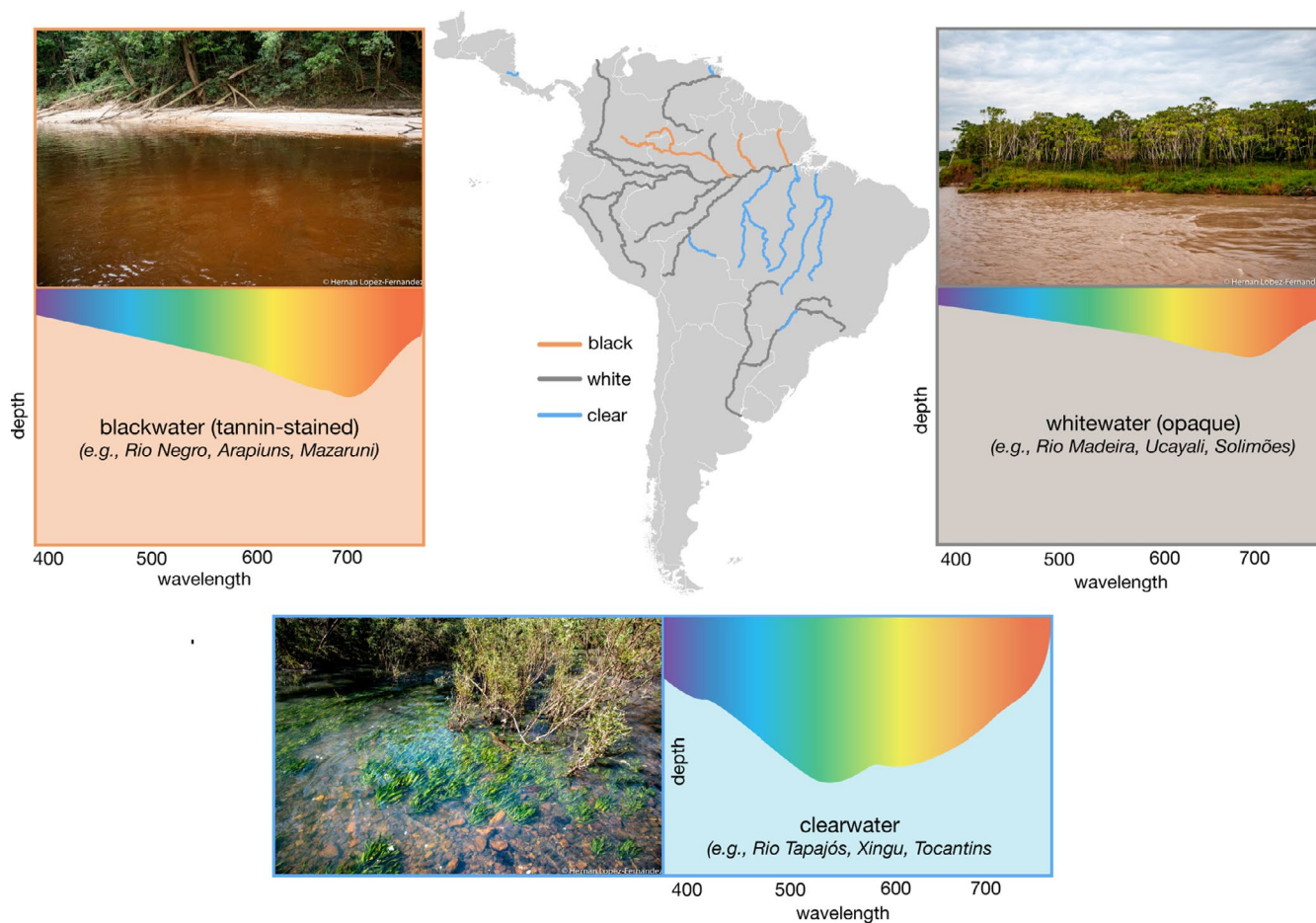


FIGURE 1 Cichlids are abundant in Neotropical riverine environments. These environments are often classified into three water types, each with distinct physicochemical (including spectral) properties. Blackwater rivers are rich in dissolved organic matter, and often appear tea-coloured. These habitats are translucent but their spectral composition is dominated by red wavelengths, and at increasing depths attenuates short-wavelength light. Whitewater riverine environments appear opaque due to suspended sediments, and scatter as well as absorb short wavelength light. Clearwater rivers allow a broad spectrum of light (including UV and shorter wavelengths) to penetrate at greater depths, and may appear blue or green-tinged in colour. Photos taken by Hernán López-Fernández. Spectra schematics are adapted from Costa et al. (2013) [Colour figure can be viewed at wileyonlinelibrary.com]

(SWS2a) range of the visible spectrum across a broad sampling of South and Central American cichlids, and test whether cichlids dwelling in light-limited habitats experienced a release of evolutionary constraint on these opsins. In river types known to be lacking short-wavelength light we found elevated evolutionary rates consistent with release of selective constraint for SWS1, but not SWS2 genes. We found evidence for widespread loss and inactivation of SWS1 in a highly diverse clade of South American cichlids. However, these losses in SWS1 were accompanied by a surprising and strong burst of positive selection in the blue-sensitive opsin (SWS2a) at the base of the South American radiation. Ancestral reconstruction and protein modelling of positively selected mutations along this SWS2a branch are suggestive of structural adaptations that may have enabled later shifts in spectral tuning during the course of this cichlid radiation.

2 | MATERIALS AND METHODS

2.1 | Opsin sequencing and assembly

Genomic DNA (gDNA) was isolated from muscle tissue from 83 Neotropical cichlids using a Qiagen DNEasy Blood and Tissue Kit. Samples were obtained from the wild and supplemented with some specimens from the aquarium trade, and are listed in detail in Table S1. A representative genus-level sampling of Neotropical cichlids was obtained, spanning South and Central America, as well as a diversity of aquatic habitats and life histories. RNA probes (100 bp long) were designed from African riverine cichlid tilapia (*Oreochromis niloticus*) cone opsin exons (SWS1, SWS2a and SWS2b) and used to enrich extracted Neotropical cichlid gDNA for the regions of interest with an annealing temperature of 58°C. We used tilapia for opsin probe design because it had the most comprehensive genomic resources among cichlids and possessed all known intact opsin sequences. Additional details on this capture method are outlined in previously published work (Ilves and López-Fernández, 2014; Ilves et al., 2018). Neotropical cichlid opsin sequences were assembled using a custom assembly pipeline with BWA-MEM (Li, 2013) for guided assembly against the *Oreochromis niloticus* SWS sequences and the mpileup-bcf-vcfutils (SAMTOOLS) pipeline for consensus generation (Schott et al., 2017). For SWS1, the *Astronotus ocellatus* pseudogene, which contains a 4-bp frame shifting insertion in exon 2 and a codon insertion in exon 3 (Escobar-Camacho et al., 2017), was also used for guided assembly to test for any effect on assembly performance in cases where no SWS1 sequence was assembled using the *O. niloticus* reference. Opsin sequences were aligned using MUSCLE codon alignment implemented in MEGA (Kumar et al., 2016). Where applicable, opsin sequences were compared against sequences from the same species obtained by independent studies via transcriptome and genome sequencing to ensure accuracy (e.g., Escobar-Camacho et al., 2017). In addition to the exon capture approach, we also validated potential instances of SWS1 opsin gene loss and pseudogenization using Sanger sequencing. We designed exon-specific primers

for putative pseudogenes recovered via sequence capture and sequenced the region containing the stop codon in the forward and reverse direction. Polymerase chain reaction (PCR) conditions and primers are described in Table S2.

2.2 | Identifying shifts in selection pressure in cichlid SWS opsins

To ensure the monophyly of the major Neotropical cichlid tribes, molecular evolutionary analyses were performed on a species tree topology based on established relationships and pruned to match the taxa in a given opsin alignment (Ilves et al., 2018). To estimate the strength and form of selection acting on each SWS opsin, the alignments, along with the species-level phylogeny, were analysed with the codeml package of PAML 4 (Yang, 2007) using the random sites models (M0, M1a, M2a, M2a_rel, M3, M7, M8a and M8), which estimate the rates of nonsynonymous to synonymous nucleotide substitutions (d_N/d_S , or ω). SWS1 pseudogene sequences >800 bp were retained, but insertions were removed and premature stop codons were converted to gaps to enable their inclusion in the analyses (e.g., Yohe et al., 2017; Janiak et al., 2018).

We tested whether shifts in selection in SWS opsins were associated with pseudogenization (in the case of SWS1), biogeography, phylogeny and ecology using Clade Model C in PAML (CMC; Bielawski and Yang, 2004). CMC allows for a class of codon sites that experiences a shift in selective pressure in preselected “foreground” or “test” branches (which can consist of entire clades or individual branches). This model allowing for a shift in selection was compared to the null model M2a_rel (Weadick & Chang, 2012). We tested the following partitioning schemes in SWS1 for evidence of selective shifts: pseudogene vs. intact sequences, clear vs. black/whitewater-dwelling cichlids, South vs. Central American cichlids (the current geographical distribution of extant cichlids takes into account subsequent re-invasion of some lineages from Central into South America, *sensu* Hauser et al., 2017), and cichlids in the Geophagini/Chaetobranchini/Astonotini tribes (the clade where the majority of SWS1 pseudogenization and loss events appear to have occurred) vs. remaining cichlids. We also tested individual ancestral branches leading to major Neotropical cichlid groups to examine whether punctuated shifts in selection occurred along these branches (Figure 2a, roman numerals). These same tests, with the exception of the pseudogene vs. intact sequence test, were also performed in the other SWS opsins (SWS2a, SWS2b). In all cases, model pairs were compared with a likelihood ratio test (LRT) with a χ^2 distribution and Akaike Information Criterion (AIC). Information on cichlid habitat was obtained from Fishbase (Froese and Pauly, 2019) and the literature (e.g., Albert and Reis, 2011) and used to infer the water type(s) commonly encountered by the cichlids examined in this study (Table S1). We compared CMC results with RELAX tests implemented in HYPHY (Wertheim et al., 2015; Pond et al., 2015). RELAX estimates ω among three rate classes for each branch using a BS-REL (branch site random effects likelihood) model, and then

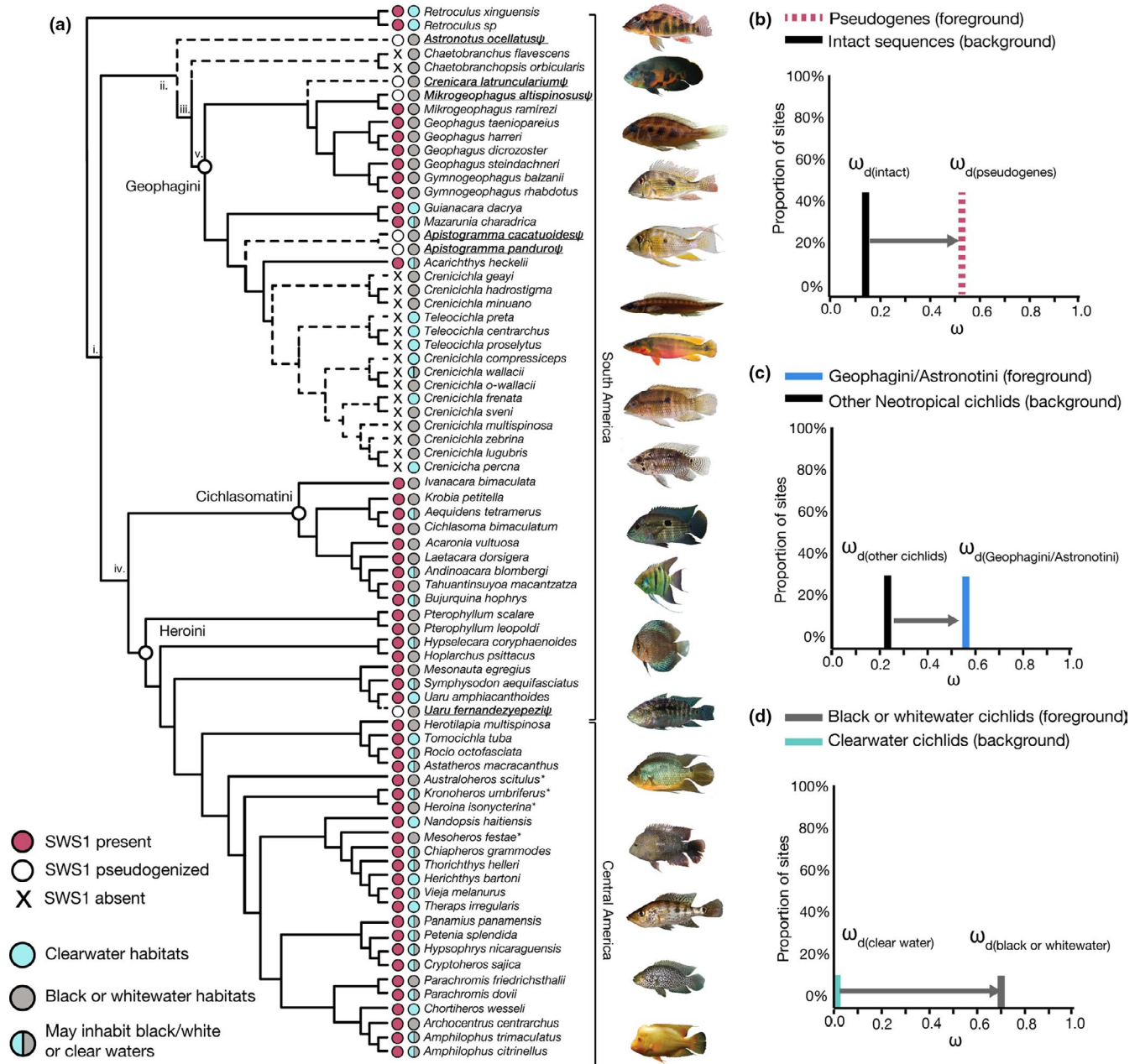


FIGURE 2 Pseudogenization, loss and evolution of Neotropical cichlid UV-sensitive (SWS1) opsins. (a) Species tree illustrating losses and inactivations of SWS1 across Neotropical cichlids in relation to water type. Dotted lines represent either a nonsense mutation resulting in a premature stop codon or insertions/deletions rendering the protein nonfunctional via a frameshift (open circle). An X indicates no SWS1 reads recovered. (b) Pseudogene sequences, (c) the Geophagini/Astronotini tribe, and (d) black/whitewater-dwelling cichlids experienced a significant elevation in ω (d_N/d_S) inferred with CMC. The partition isolating pseudogene lineages was the best fitting of the three significant models. Species with an SWS1 pseudogene are in bold type and underlined. Central American species marked with an asterisk secondarily re-invaded South America. Roman numerals indicate ancestral branches that were tested for evidence of episodic selection, the results of which are reported in Table 1 and Table S7. Photos were taken by Hernán López-Fernández and Jessica Arbour, and species names associated with the photos are listed in Table S11 [Colour figure can be viewed at wileyonlinelibrary.com]

fits a parameter estimating the strength of selection (k). ω values are transformed by k (ω^k), which means $k > 1$ drives site classes with high or low ω values away from 1 (suggesting an intensification of selection); conversely, $k < 1$ shifts ω rate classes with high or low ω values towards 1 (suggesting relaxed selection; Wertheim et al., 2015). A model estimating a single k for all branches is compared

against a model estimating k for the two branch classes corresponding to test (foreground) and reference (background) lineages in the RELAX analyses.

Foreground branches inferred to have undergone significant episodic selection using PAML's CMC were also tested with the HYPHY model BUSTED, which tests for episodic diversifying selection along

TABLE 1 Results of Clade Model C (CMC) analyses testing for shifts in selection on Neotropical cichlid SWS opsins

Opsin	Model and foreground partitions ^a	np	lnL	K	Parameters ^b			Null	LRT	df	p	
					ω_0	ω_1	ω_2/ω_d					
SWS1	M2a_rel	118	-5,290.2	3.1	0.09 (76%)	1 (6%)	0.48 (18%)					
	Pseudogenes	119	-5,279.2	3.0	0.28 (35%)	1 (10%)	0.14 (44%)	0	M2a_rel	22.0	1	<.01
	Black/white water	119	-5,287.3	3.0	0.1 (80%)	1 (7%)	0.04 (12%)	16.2	M2a_rel	5.6	1	<.01
	Geophagini/Astronotini Clade	119	-5,285.9	3.0	0.1 (61%)	1 (8%)	Black/white: 0.70	13.4	M2a_rel	8.6	1	<.01
	South American cichlids	119	-5,290.2	2.5	0.48 (18%)	1 (6%)	Geo/Astro: 0.53	22.0	M2a_rel	0.0	1	1.0
	Branch leading to major Neotropical cichlid tribes (i)	119	-5,290.2	2.5	0.48 (18%)	1 (6%)	SA: 0.09	22.0	M2a_rel	0.0	1	1.0
	Branch leading to Geophagini/Astronotini (ii)	119	-5,290.2	2.5	0.48 (18%)	1 (6%)	Foreground branch: 0.09	22.0	M2a_rel	0.0	1	1.0
	Branch leading to Geophagini (iii)	119	-5,290.2	2.5	0.48 (18%)	1 (6%)	Foreground branch: 0.09	22.0	M2a_rel	0.0	1	1.0
SWS2b	M2a_rel	112	-4,752.2	2.3	0.04 (80%)	1 (19%)	3.6 (1%)	0				
	Black/white water	113	-7,452.1	2.3	0.44 (80%)	1 (19%)	2.7 (1%)	1.8	M2a_rel	0.2	1	.65
	South America	113	-4,752.15	2.3	0.44 (80%)	1 (19%)	Black/white: 3.5	1.98	M2a_rel	0.02	1	.88
						SA: 2.9						
SWS2a	M2a_rel	124	-4,987.5	2.9	0.03 (85%)	1 (9%)	3.2 (6%)	9.4				
	Branch leading to Geophagini/Astronotini (i)	125	-4,981.8	3.1	0.03 (85%)	1 (8%)	3 (7%)	0	M2a_rel	11.4	1	<.01
	Black/white water	125	-4,987.4	2.9	0.03 (85%)	1 (8%)	2.8 (7%)	11.2	M2a_rel	0.2	1	.65
	South America	125	-4,987.5	3.0	0.03 (85%)	1 (8%)	Black/white: 3.2	11.4	M2a_rel	0	1	1
						SA: 3.1						

np, number of parameters; lnL, ln likelihood; K, transition/transversion ratio; df, degrees of freedom; n/a, not applicable.

Test branches leading to major cichlid clades are denoted with a letter, and correspond to the branches marked in Figure 2.

^a Partitions listed are explained in Figures 2 and 3 and Table S1. In all cases, an additional partition exists that contains the remaining taxa (e.g., outgroups).

^b ω values of each site class are shown with the proportion of each site class in parentheses. ω_d is divergent site class that has a separate value for each partition.

^c The difference in AIC values was calculated compared with the overall best-fitting model for each gene: SWS1 Pseudogenes vs. Intact = 10,796.4; SWS2b M2a_rel = 9,728.3; SWS2a Geophagini/Astronotini Branch = 10,213.6.

Best-fitting models are shown in bolded text.

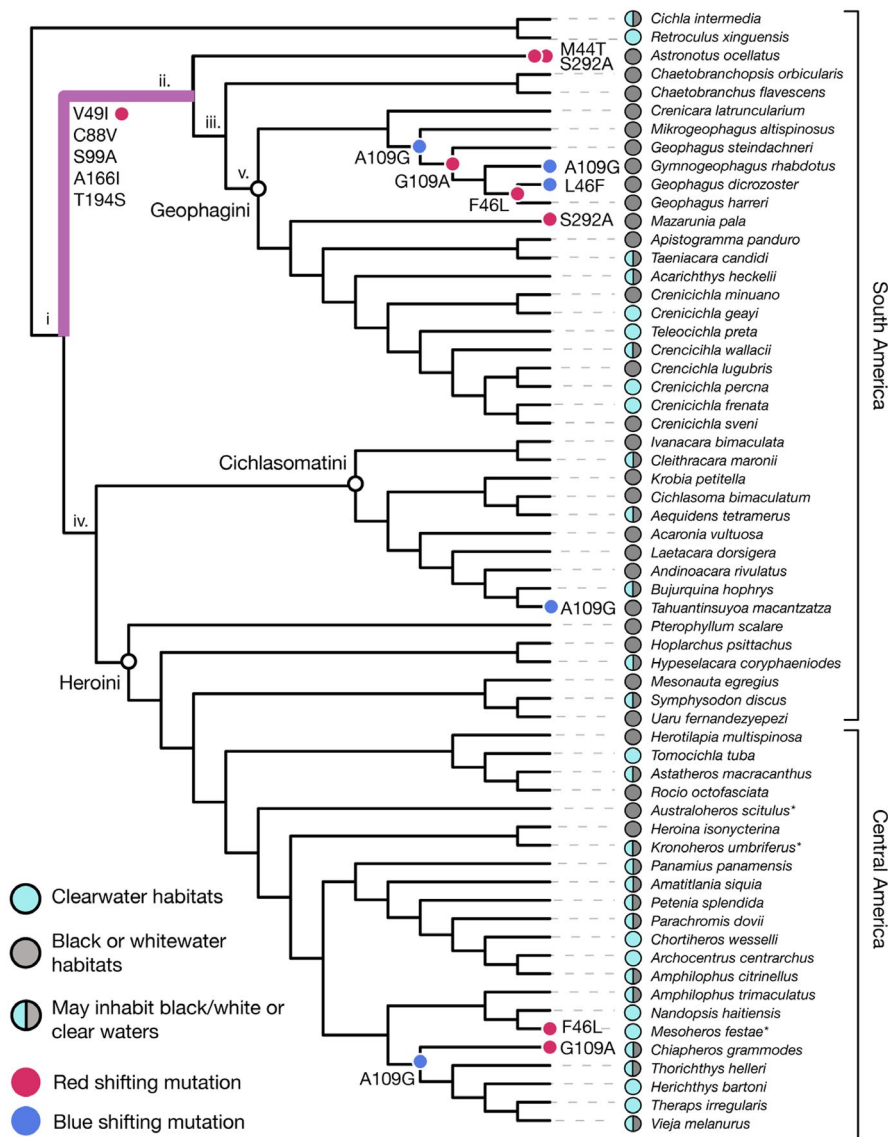


FIGURE 3 Episodic selection and evolution of spectral tuning sites in the Neotropical cichlid SWS2a opsin. A burst of positive selection in the blue-sensitive (SWS2a) opsin occurs at the base of the South American cichlid radiation, specifically the branch leading to the Geophagini, Chaetobranchini and Astronotini tribes (ii). Variation in SWS2a spectral tuning sites inferred through ancestral reconstruction is illustrated by blue dots (indicating a blue-shifting mutation) or red dots (indicating a red-shifting mutation). Roman numerals indicate ancestral branches that were tested for evidence of episodic selection, the results of which are reported in Table 1 and Table S7. The 3D structures of ancestral nodes i and ii were subsequently modelled. Central American species marked with an asterisk secondarily re-invaded South America [Colour figure can be viewed at wileyonlinelibrary.com]

user-specified branches, and estimates which sites are under selection along the lineage(s) in question, in a similar manner to PAML's branch-site method (Murrell et al., 2015).

In addition to molecular evolutionary models, for SWS1 we used Pagel's λ (1994) implemented in the R package phytools (Revell, 2013) to test for correlated evolution between presence/absence of SWS1 (0 = absence; 1 = presence) and water type (0 = clear; 1 = black/white).

2.3 | Ancestral sequence reconstruction of SWS2a and protein homology modeling

Specific branches inferred to be experiencing a burst of selection in the opsin genes were further examined to pinpoint the amino acid changes occurring along the branch that may be driving elevated positive selection. As no branch-specific bursts of selection were identified in SWS1 or SWS2b, we focused on ancestral sequence reconstruction of SWS2a. To examine amino acid changes along ancestral branches in the Neotropical cichlid blue-sensitive opsin SWS2a,

we used PAML's best fitting model for SWS2a (M8) to reconstruct all ancestral nodes at the codon level. We used CHIMERA (Peterson et al., 2004) and MODELLER (Webb and Sali, 2016) to homology-model two Neotropical ancestral SWS2a opsins using bovine rhodopsin (PDB ID: 1u19) as a reference. In all cases, the model with the lowest DOPE score (indicating the most accurate model) was selected for further protein visualization (Webb and Sali, 2016).

3 | RESULTS

3.1 | SWS opsin sequence assembly

SWS sequences were assembled with over 100× depth of coverage on average. After controlling for absent sequences (i.e., completeness of sequence recovered is 0 in the genera *Crenicichla* and *Teleocichla*, and the Chaetobranchini cichlid tribe), SWS1 sequences used in molecular evolutionary analyses (58 in total) were on average 91% complete. For SWS2b, 54 sequences with an average completeness of 76% were

used (as exon 1 in SWS2b was not successfully captured in this experiment for some species in our data set). SWS2a sequences were 91% complete, and 61 sequences were used for molecular evolutionary analyses. While SWS2a was captured most reliably of the three opsins, the final exon was not captured, probably due to its short length.

3.2 | Pseudogenization and loss of the UV-sensitive opsin in South American cichlids

Both Sanger and next-generation sequencing of Neotropical cichlid SWS1 support loss and pseudogenization of the UV-sensitive opsin across South American cichlids. Specifically, four species from three independent South American geophagine cichlid lineages (*Crenicara punctulatum*, *Apistogramma cacatuoides*, *Apistogramma panduro* and *Mikrogeophagus altispinosus*) were confirmed with Sanger sequencing to have nonsense mutations or frameshifting deletions resulting in premature stop codons in SWS1 (Figure 2; Table S1, Figure S1). Three additional South American cichlids outside Geophagini (*Astronotus ocellatus*, *Cichla intermedia* and *Uaru fernandezypezi*) were also found to have frameshifting mutations based on assembled SWS1 opsin reads. The reading frame of these SWS1 pseudogenes was otherwise intact, with the exception of *Cichla intermedia*, where only a partial (<50%) opsin sequence was recovered (due to this, the *Cichla intermedia* sequence was not included in subsequent molecular evolutionary analyses). The *Astronotus ocellatus* SWS1 pseudogene was previously identified by Escobar-Camacho and colleagues (2017).

In addition to independent pseudogenization events, we also found evidence for at least one instance of widespread gene loss in a large Neotropical cichlid group. We failed to recover any SWS1 reads for members in both genera of the Chaetobranchini tribe (*Chaetobranchopsis orbicularis* and *Chaetobranchus flavescens*), as well as all 14 sampled species of “pike cichlids” (genera *Crenicichla* and *Teleocichla*), despite guided assembly to both tilapia SWS1 and the *Astronotus ocellatus* pseudogene reference sequences. While failure to recover the sequence does not provide a definitive confirmation of gene loss in this group, capture of other SWS opsins in *Chaetobranchini* and *Crenicichla/Teleocichla*, the successful capture of SWS1 opsins in other Neotropical cichlids (as well as the failure to amplify SWS1 in *Crenicichla frenata* via Sanger sequencing in the present study and in Weadick et al., 2012), suggest that either SWS1 is absent from the genomes of these species, or its sequence is so divergent from the references that it cannot be captured with available homologous sequences from related cichlids. Genome sequencing or long-range PCR targeting the SWS1 region would provide additional evidence for SWS1 loss in these lineages.

3.3 | Evidence for positive selection in Neotropical cichlid SWS opsins

We attempted to retain a balanced genus-level taxon sampling across the three opsins for molecular evolutionary analyses, with

the exception of *Crenicichla*, *Chaetobranchus* and *Chaetobranchopsis*, which are present in the SWS2b and SWS2a data sets, but not in the SWS1 data set due to the absence of assembled reads (Table S1).

The SWS1 opsin exhibits the highest instances of inactivation and loss among the SWS opsins. PAML random sites analyses did not detect any evidence for positive selection in the data set when South and Central American cichlids were analysed together (Table S3; $\omega_{MO} = 0.20$, PAML M8 vs. M8a $p = .31$). Conversely, both SWS2a and SWS2b were under positive selection when the entire Neotropical cichlid group was analysed (Tables S3, S13 and S14). Subdividing SWS opsins into South and Central American cichlid data sets revealed that SWS1 was highly conserved among Central American cichlids (Table S4; $\omega_{MO} = 0.2$, but under positive selection in South American cichlids (Table S4; M8 vs. M8a $p = .03$). While generally highly conserved, SWS1 spectral tuning sites were more variable in South American cichlids (Table S12). This same pattern of conservation in Central American cichlids but positive selection in South American cichlids was found in SWS2b (Tables S5 and S13). By contrast, the SWS2a opsin was under positive selection in both South and Central American cichlids (Table S6).

3.4 | Shifts in selective pressure in SWS1 are associated with pseudogenization and light environment

Consistent with our prediction that SWS1 pseudogenes would show evidence for a release of constraint, we found that SWS1 pseudogenes selected as foreground branches in CMC analyses had elevated ω (Figure 2b; Table 1; $\omega_{background} = 0.14/\omega_{pseudogenes} = 0.55$; $p < .01$). These same lineages showed evidence for relaxed selection when selected as test branches in HYPHY'S RELAX model (Figure 2a,b; $k = 0.23$; $p < .01$; Table S7). To pinpoint where the initial release of constraint in SWS1 may have occurred, we tested whether specific ancestral branches leading to major Neotropical cichlid clades showed evidence for a release of selective constraint (highlighted with roman numerals in Figure 2a). While none of these branch-based tests were significant, both CMC and RELAX identified the Geophagini/Astronotini clade as experiencing significantly elevated ω (Figure 2c; Table 1; Table S7). This suggests that cichlid SWS1 experienced a widespread release of constraint during the early diversification of South American cichlids, prior to pseudogenization and gene loss in certain lineages.

We explicitly tested whether ambient light environment (in this case, clear vs. black/white water) influenced SWS1 opsin evolution. Cichlids dwelling in black or white water were highlighted as a foreground partition in CMC, and were found to have elevated ω (Figure 2d; Table 1; $\omega_{clear} = 0.04/\omega_{black/white} = 0.7$) and also show evidence for relaxed selection on SWS1 (Table S7; $k = 0.65$; $p = .03$). While we found that molecular evolutionary rate was associated with water type, a phylogenetic correlation test did not demonstrate that presence/absence of SWS1 was significantly associated with water type (Pagel's λ ; $p = .4$). In contrast to SWS1, neither SWS2b nor

SWS2a showed evidence for shifts in selection inferred with CMC associated with biogeography (Central vs. South America) or light environment (black/white vs. clearwater habitats) (Table 1). Similarly, neither SWS2b nor SWS2a showed evidence for relaxed selection (Table S7; species tree topology for SWS2b shown in Figure S2).

3.5 | Episodic selection in SWS2a occurs at the base of the South American cichlid radiation

Since the majority of SWS1 pseudogenization and loss occurred in the South American geophagine cichlids, we tested whether SWS2a would also show evidence for a shift in selection pressure during the diversification of the Geophagines. In contrast to the clade-wide shift in selection identified in SWS1, using CMC we found a burst of positive selection only along the branch leading to the major South American cichlid radiation (comprising Geophagine cichlids, the Chaetobranchini tribe and *Astronotus*, the only member of the Astronotini tribe; Figure 3; Table 1), rather than the Neotropical clade as whole. This result was also consistent when the branch was tested with BUSTED in HYPHY (Table S8).

Branch-based tests for positive selection, specifically PAML's branch-site test, have been shown to be sensitive to multinucleotide mutations (MNMs) (Venkat et al., 2018). MNMs occur when two mutations occur simultaneously within the same codon, resulting in a codon with multiple differences (e.g., GCT to ATT). We did not use PAML's branch site test for these analyses due to data set-wide positive selection in SWS2a, which has been shown in other studies to reduce the power of the branch-site test to detect positive selection (Schott et al., 2014). Nevertheless, we did find a high ω value along the SWS2a branch leading to the major South American cichlid radiation (Table 1). Using ancestral sequence reconstruction, we inferred all codon changes along the focal branch where episodic selection occurred (Figure 3, between nodes i and ii; Table S9), and found two codons with MNMs, which we removed from our SWS2a alignment (e.g., Dapper and Payseur, 2019). When the analyses were repeated without these codons, our results remained significant (Table S10), suggesting that the episodic selection along the South American cichlid ancestral branch is not a statistical artefact and is driven by several novel amino acid substitutions (Figure 3; Table S10).

3.6 | Homology modelling of ancestral cichlid SWS2a suggests structural reorganization prior to the accumulation of spectral tuning variation

We used ancestral sequence reconstruction and protein modelling to examine amino acid changes in the ancestral South American cichlid blue-sensitive opsin and the subsequent evolution of spectral tuning sites in this group. We inferred five nonsynonymous and one synonymous codon change occurring in the positively selected SWS2a branch leading from the ancestor of all Neotropical cichlids outside of *Retroculus* and *Cichla* (Figure 3, node i), to the ancestor

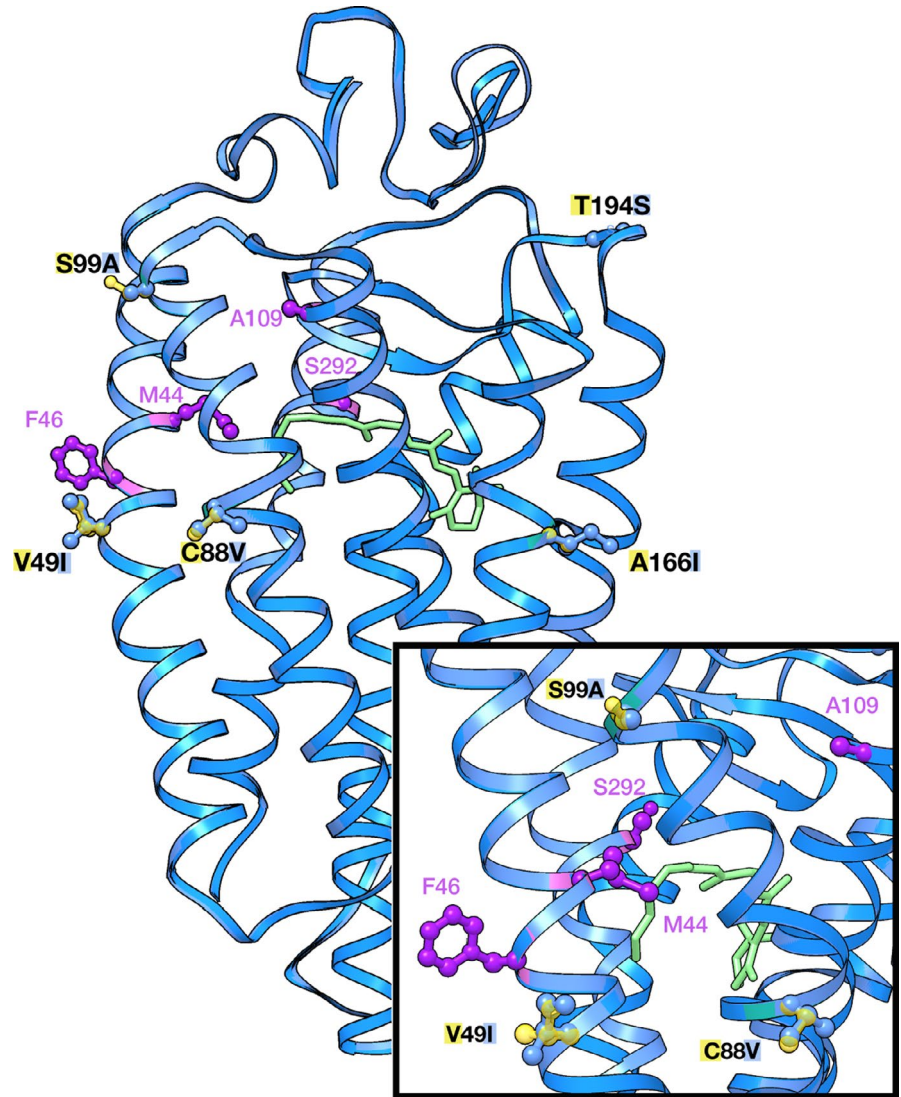
of Astronotini/Chaetobranchini/Geophagini (ACG; Figure 3, node ii). These substitutions are summarized in Table S9. Two of these amino acid sites occur in close proximity to known tuning sites that are variable in South American cichlids (Figure 4), and also result in increased hydrophobicity (Table S9). The V49I mutation, a replacement of a small hydrophobic residue with a bulkier one, is one turn of the opsin alpha-helix away from SWS2a spectral tuning site 46, and has been shown to red-shift other opsin pigments (Yokoyama and Tada, 2003; Figure 4). Beyond spectral tuning, three of these substitutions increase hydrophobicity and face outward on transmembrane domains I, II and IV, regions known to be involved in opsin dimerization/oligomerization and enhanced signalling (Table S9; Jasztrebska et al., 2017; Mallory et al., 2018; Ploier et al., 2019).

Based on our ancestral reconstruction, variation in SWS2a spectral tuning sites appear to have accumulated after episodic selection on (largely nontuning) residues along the ACG ancestral branch (Figure 3). SWS2a spectral tuning sites are highly variable among South American cichlids, with independent evolution of putative red-shifting mutations (that were experimentally determined in other fish groups) in several South American lineages: M44T (Li et al., 2009), F46L (Yokoyama et al., 2007) and S292A (Yokoyama et al., 1999, 2007) (Figures 3 and 4; Table S14). Interestingly, we also found a possible blue-shifting mutation in SWS2a: the A109G mutation has been shown to distinguish SWS2a from blackwater-dwelling sticklebacks (A109) vs. clearwater sticklebacks (G109; Marques et al., 2017). In Neotropical cichlids, the A109G substitution occurs three times—once in Central American cichlids, and twice in South American cichlids—and is inferred to be under positive selection. In Central America, A109G follows an analogous pattern to sticklebacks, occurring once in a largely clearwater dwelling clade (Figure 3). Conversely, in South America, A109G occurs in cichlids dwelling in black/whitewater habitats, rather than clear habitats (Figure 3).

4 | DISCUSSION

We investigated whether the diverse light-limited riverine habitats of the Neotropics mediated changes in selection affecting the short-wavelength vision of Neotropical cichlids using exon-based targeted sequencing of cone opsins, codon-based analyses of selective constraint, and ancestral protein homology modelling. We found evidence for widespread loss and independent inactivating mutations in SWS1 in South American cichlids, and demonstrated that both pseudogenization and river types with limited short-wavelength light (white or blackwater habitats) resulted in reduced selective constraint (elevated rates) in SWS1. We found surprising evidence for a burst of positive selection at the base of the South American cichlid radiation in SWS2a, but, interestingly, this was not accompanied by any shifts in selection associated with ambient light environment in either SWS2a or SWS2b opsins. By modelling the amino acid substitutions under episodic selection along this branch we identified several mutations that may have enhanced opsin dimerization and

FIGURE 4 Homology model of ancestral Neotropical cichlid SWS2a opsin. The Astronotini/Chaetobranchini/Geophagini (node ii, Figure 3) ancestor is overlaid onto the structure of node i. Variable SWS2a spectral tuning sites in Neotropical cichlids are shown in purple. Amino acid substitutions along the SWS2a branch under episodic selection are highlighted: the original amino acid identity is translucent yellow, and the amino acid identity in the Astronotini/Chaetobranchini/Geophagini ancestor is solid blue. Inset: the V49I transition in the ancestral Neotropical cichlid opsin occurs in close proximity to spectral tuning site F46 and introduces a larger amino acid residue at site 49. The retinal chromophore is depicted in light green. Numbering is standardized to bovine rhodopsin [Colour figure can be viewed at wileyonlinelibrary.com]



therefore downstream signalling effectiveness. These mutations were followed by several independent substitutions at key spectral tuning residues within South American cichlids that probably contribute to variable SWS2a sensitivities across the group. Here we discuss how early selective shifts (and losses) in Neotropical cichlid SWS opsins may have altered the evolutionary trajectory of their visual system, resulting in many South American cichlids having a constrained opsin palette relative to their African counterparts.

4.1 | SWS1 loss contributes to the evolution of a long-wavelength-sensitive opsin palette in South American cichlids

There are several plausible explanations for why SWS1 may be inactivated (or, in the case of *Crenicichla/Teleocichla*, seemingly absent) in the South American cichlids examined in this study. Most intuitively, light environment has a substantial impact on which opsin genes are retained, duplicated or inactivated in fishes. White and blackwater habitats found in many South American riverine environments may

have exerted substantial influence on which opsins were under selection early in the Neotropical cichlid radiation (Costa et al., 2013). Indeed, our findings are consistent with the “sensitivity hypothesis,” which posits that visual sensitivities evolve to best match their habitats (Crescitelli et al., 1985). The rapid attenuation of UV light in many South American cichlid habitats may have resulted in reduced selection and the eventual loss of UV sensitivity in the South American lineages where we found pseudogenization and gene loss, while violet and blue sensitivity are retained.

Although interspecific opsin expression data for Neotropical cichlids is limited, the few species examined to date consistently expressed the same three cone opsins (SWS2a, RH2a, LWS), suggesting their visual system is adapted to maximize performance in habitats dominated by orange or red wavelengths (Carleton et al., 2008; Escobar-Camacho et al., 2017, 2019; Hofmann and Carleton, 2009; Schneider et al., 2020). Schneider et al. (2020) also note that Neotropical cichlid vision may be constrained by phylogenetic history, with Amazonian cichlids in particular expressing these same three cone opsins, and Central American cichlids having a comparatively wider breadth of opsin expression. The fact that we did

not find a strong correlation between SWS1 presence/absence and water type (*Crenicichla* is an excellent example, where several species that have contemporary distributions in clear water appear to still be missing SWS1) lends additional support to this historical constraint.

Recent evidence for substantial turbidity-driven variation in visual sensitivity of the invasive cichlid *Cichla monoculus* in Panama suggests that Neotropical cichlid visual systems remain flexible at an individual level despite a reduced opsin complement relative to African cichlids (Escobar-Camacho et al., 2019). However, in the case of *Cichla* much of the visual system variation appears to be driven by A1/A2 retinal chromophore (the light-sensitive ligand bound to the opsin protein) differences, rather than differential opsin expression across habitats (Escobar-Camacho et al., 2019). Specifically, the A2 chromophore can produce a substantial red shift (up to 20 nm) in a visual pigment relative to the A1 chromophore, and this switch often occurs in high-turbidity and red-shifted aquatic habitats (Escobar-Camacho et al., 2019). Changes in opsin gene expression in both African cichlids (Nandamuri et al., 2017) and Central American Midas cichlids (Härer et al., 2017) can occur rapidly (over a period of days or months) in response to different light environments. Härer et al. (2017) found a significant effect of ambient light on opsin gene expression during early stages of cichlid development. Exposure to a short-wavelength-shifted lighting regime caused retention of the larval opsin gene expression phenotype (i.e., expression of SWS opsin genes, including SWS1, referred to as a pedomorphic visual phenotype; Rice, 1997). Conversely, exposure to long-wavelength light caused a more rapid progression of cone opsin expression towards a long-wavelength-shifted palette through ontogeny (Härer et al., 2017). This suggests that evolution in long-wavelength-shifted habitats over long timescales may have accelerated the ontogenetic shift from a larval to adult opsin expression profile in Neotropical cichlids. That is, the developmental stage at which SWS1 opsins are expressed and used by larval fish is abbreviated relative to fish living in habitats with more available short-wavelength light. This reduced period in which SWS1 opsins were expressed may have relaxed selection on the UV opsin, leading to its inactivation in some lineages and outright loss in others, and overall a much stronger functional emphasis placed on the expression of a long-wavelength-sensitive opsin palette. Interestingly, this contrasts with other dim-light-adapted fish groups missing SWS1, where substantial variability in expression among the remaining opsins is retained (e.g., nocturnal reef fish; Luehrmann et al., 2019). Although the extent of SWS1 inactivation in South American cichlids remains an open question given the presence of some intact sequences in this group, the identification of an SWS1 pseudogene in *Cichla vazzoleri* (an early diverging Neotropical cichlid) by Escobar-Camacho et al. (2019), as well as the presence of an additional putative SWS1 pseudogene in the South American genus *Uaru* suggests at least three independent SWS1 inactivation events have occurred across South American cichlids. Investigations into noncoding sequences in Neotropical cichlids linked to SWS1 expression (e.g., if a promoter has been lost or inactivated) would shed further light onto the evolutionary history

of these inactivations. It is also important to note that because Neotropical cichlids are often found in the ornamental fish trade, we cannot rule out that gene loss may be associated with inbreeding in aquarium specimens. In the present study, SWS1 loss was found in a combination of aquarium and wild-caught specimens, so future examinations of additional wild-caught specimens would provide further support for gene loss in the wild.

4.2 | Timing and magnitude of selective pressure shifts in Neotropical cichlid SWS opsins

We found changes in selective pressure in SWS1 are associated with pseudogenization and a clade-wide release of constraint among black- and whitewater-dwelling geophagine cichlids. Relaxation of selective constraint (i.e., elevated d_N/d_S) in ecologically important genes often occurs in organisms that transition to habitats or lifestyles where the gene is no longer essential. For example, release of constraint has also been found in amblyopsid cavefish rhodopsin (Niemiller et al., 2012), bat SWS1 pseudogenes (Wertheim et al., 2015), bat vomeronasal receptor pseudogenes (in lineages where social communication may be mediated by the olfactory rather than vomeronasal system; Yohe et al., 2017), and digestive enzymes of primates with low insect consumption (Janiak et al., 2018). Given that geophagine cichlids as a whole showed evidence for a release of selective constraint on SWS1, this group-wide release probably led to subsequent losses in independent South American lineages, while SWS1 function was retained in Central American lineages. Central American cichlid habitats range from fast-flowing streams, to lakes and lagoons, as well as occasionally brackish habitats. Based on literature review and species records, we inferred that most Central American cichlids inhabit a combination of turbid or clear waters (e.g., Escobar-Camacho et al., 2019; Härer et al., 2017; Torres-Dowdall et al., 2017), which may favour retention of SWS1 in these species. However, more detailed measurements (via spectrometry) of the spectral compositions of various Neotropical cichlid habitats would provide valuable information for future large-scale studies of associations between water type and opsin content in this group.

Contrary to our expectations, we did not find evidence for relaxed selection in SWS2b. This finding, coupled with the fact that SWS2b was under positive selection and had a small number of positively selected sites, suggests that despite its low expression levels in the Neotropical cichlid species studied to date, it may play a role in modulating short-wavelength vision in Neotropical cichlids during part of their lifespan (e.g., Torres-Dowdall et al. 2021). At least one blackwater-dwelling Neotropical cichlid, *Symphysodon discus*, has a pseudogenized copy of this opsin (Escobar-Camacho et al., 2017), which may indicate, much like SWS1, that SWS2b utility, retention and function is variable in this group.

In contrast to the clade-wide shift in selection pressure among Geophagine SWS1 opsins, clade model analyses did not reveal a similar pattern in SWS2a. Instead, our analyses identified a burst of positive selection along a branch near the base of the South

American cichlid radiation, which was also supported by the BUSTED analysis. Reconstruction of the nonsynonymous amino acid substitutions along this branch revealed two codons with multiple differences (or multinucleotide mutations; Venkat et al., 2018). Our results were robust when we excluded all of the possible MNMs along the branch under episodic selection (e.g., Dapper and Payseur, 2019). This SWS2a result highlights the importance of closely scrutinizing branches in a phylogeny experiencing episodic selection, and that while conservative removal of multinucleotide mutations may improve the reliability of statistical inferences of selection, amino acid substitutions resulting from MNMs occurring along positively selected branches may still be biologically relevant, rather than statistical artefacts (Appendix S1).

Although the major shifts in selective pressure in SWS2a and SWS1 are focused within the Geophagini clade, they do not occur simultaneously. Instead, a burst of episodic selection first occurs in the ancestral South American cichlid SWS2a opsin. This is followed by a widespread release of constraint in SWS1 and, concurrently, the accumulation of amino acid variation spectral tuning sites in SWS2a (Figure 5). These selective events suggest that a major reorganization of short-wavelength-sensitive vision occurred early during Geophagine diversification in South America, initiated by the five amino acid substitutions under episodic selection along the ACG ancestral branch. SWS2a also showed the highest number of positively selected sites as well as highest variability of spectral tuning sites among the three opsins. SWS2a spectral tuning residue variation suggests that South American cichlids have independently evolved mutations that either red- or blue-shifted pigment sensitivity in different lineages. Given that this opsin is the most highly expressed of the SWS opsins in adult Neotropical cichlids (Escobar Camacho et al., 2017, 2019), in addition to photic environment, amino acid variation in this opsin may reflect the rich diversity of life histories and trophic modes (e.g., substrate-sifting, invertebrate-picking, planktivory, piscivory) found in Neotropical cichlids. Moreover, this result is consistent with a pattern of increased jaw morphological diversification in South American cichlids (Arbour and López-Fernández, 2014) and with swimming-related (e.g., body shape) morphological diversification in geophagines (Astudillo-Clavijo et al., 2015).

Experimental examination of amino acid substitutions along the ancestral branch leading to the ACG clade, or within the Geophagini clade (e.g., via site-directed mutagenesis), would lend additional insight into the role of SWS2a variation in Neotropical cichlid evolution and visual adaptation. For instance, in lineages with inactivated or missing SWS1 opsins, mutations in SWS2a may promote a blue shift in sensitivity to compensate for absence of sensitivity at shorter wavelengths, as has been observed in opsin genes in other vertebrates (Schott et al., 2016). On the other hand, our reconstruction of an ancestral South American cichlid SWS2a revealed at least one mutation (V49I) that has been shown to red-shift other opsin pigments, which would probably allow SWS2a sensitivity to better match the red-shifted habitats of many South American rivers. Since SWS2a is the most highly expressed SWS opsin in adult South

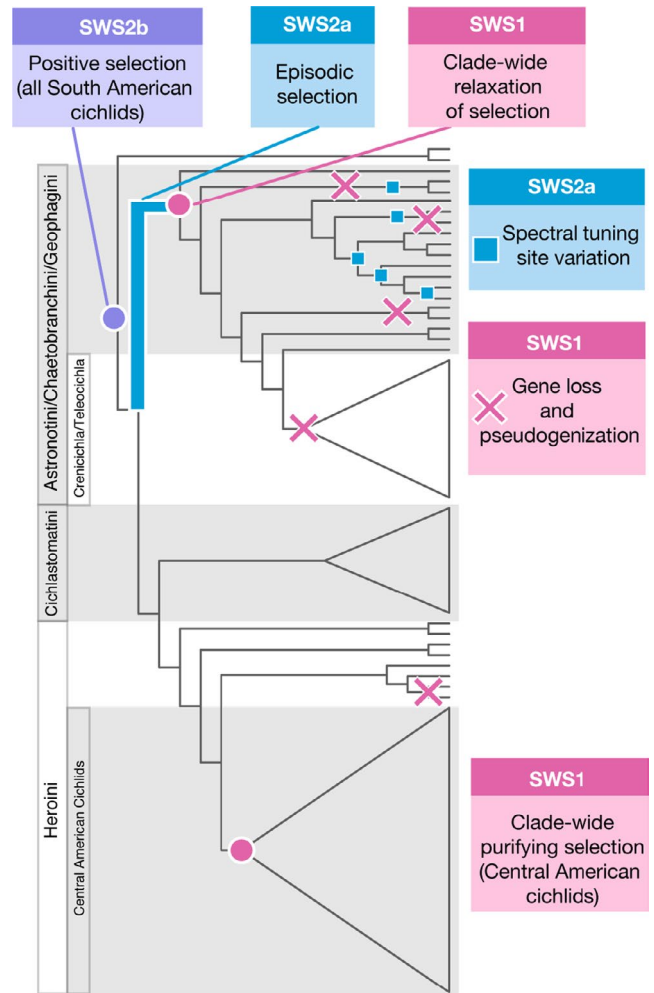


FIGURE 5 Schematic of SWS opsin evolution superimposed onto Neotropical cichlid evolution. Episodic selection on SWS2a accompanied by nonconservative amino acid substitutions occurs at the base of the Neotropical cichlid tree. A clade-wide relaxation of selection occurs in SWS1, which probably led to independent gene loss events throughout Astronotini/Chaetobranchini. Geophagini (ACG). Within the ACG clade, SWS2a (blue-sensitive) accumulates variation in spectral tuning sites. SWS1 in cichlids colonizing Central America undergoes purifying selection and we detected no gene loss or inactivation events in this group [Colour figure can be viewed at wileyonlinelibrary.com]

American cichlid retinas studied to date, it is plausible that amino acid variation in this gene may reflect adaptation to photic environment in some lineages and to diminished sensitivity at UV or violet wavelengths in others.

4.3 | Are South American cichlids locked into a long-wavelength-sensitive opsin palette?

The evolution of SWS1 and SWS2a opsins in Neotropical cichlids suggests that many South American cichlids may have adopted different strategies for tuning visual sensitivity compared to their

African relatives. To date, opsin expression studies of adult South American cichlids have revealed little variation in the set of opsin genes they express (SWS2a, RH2a and LWS, with occasional minimal expression of SWS2b; Escobar-Camacho et al., 2017, 2019; Härer et al., 2018; Schneider et al., 2020; but see Torres-Dowdall et al. 2021). This starkly contrasts with the flexibility of the African cichlid visual system, where adult visual palettes may express entirely different suites of opsins (O'Quin et al., 2010). This suggests that ancient riverine habitats of South America may have been light-limited, resulting in the entrenchment of the South American cichlid visual system into expression of a primarily long-wavelength-sensitive opsin palette, perhaps via the acceleration of ontogenetic opsin palette shifts (Härer et al., 2017). Tuning of opsins in South American cichlids to match ambient light environment via opsin expression may therefore be an evolutionary avenue that has been eliminated by a combination of phylogenetic history and long-term evolution in short-wavelength filtering habitats. Instead, modulation of visual sensitivity in these cichlids may be governed primarily by a combination of gene inactivation, structural differences (i.e., the rapid evolution and spectral tuning variation found in SWS2a in this study), and A1/A2 chromophore ratios (Escobar-Camacho et al., 2019).

5 | CONCLUSIONS

We found evidence for extensive, and potentially independent, losses of the UV-sensitive opsin across a highly diverse clade of South American cichlids. A clade-wide release of constraint on SWS1 probably occurred during the early diversification of South American cichlids in short-wavelength light-limited riverine habitats, which subsequently led to gene loss and a relatively constrained evolutionary trajectory for the visual system in these fishes. The loss of SWS1 in many South American cichlids may indicate that long-term evolution in these habitats accelerated the ontogenetic trajectory of opsin expression such that the developmental window of SWS1 expression narrowed, and the gene became increasingly unnecessary. A relatively inflexible opsin palette heavily biased towards long wavelengths then came to dominate South American cichlid visual systems. Prior to the release of constraint in SWS1, we found that episodic selection in SWS2a resulting in several amino acid substitutions occurred at the base of the South American cichlid clade where the bulk of SWS1 loss has occurred. SWS2a, the SWS opsin with the highest expression in adult cichlids, may largely mediate visual sensitivity at short wavelengths in South American cichlids. Interspecific structural variability at known spectral tuning sites in SWS2a may therefore be the primary means of tuning visual sensitivity at short wavelengths in these cichlids, as opposed to differential expression among SWS1, SWS2b and SWS2a. Future work examining the ontogenetic trajectory of Neotropical cichlid SWS opsin expression, as well as a broader survey of Neotropical cichlid opsin expression profiles, will enable further insight into how ecological and evolutionary factors have influenced the evolution of these opsins in cichlids.

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AUTHOR CONTRIBUTIONS

F.E.H., K.L.I., H.L.F. and B.S.W.C. conceptualized the project. F.E.H., K.L.I. and E.A. collected the data. F.E.H., K.L.I., R.K.S. and E.A. analysed the data. F.E.H., H.L.F. and B.S.W.C. wrote the manuscript.

DATA AVAILABILITY STATEMENT

Opsin sequences are deposited in GenBank (Accessions MW401034-MW401206). Scripts for assembly of data are available on Github (<https://github.com/ryanschott/Targeted-Capture-Assembly-Pipeline>).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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