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Article type : Original Article

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

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/MEC.15838](https://doi.org/10.1111/MEC.15838)

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42 **Keywords:** visual ecology, visual pigment, molecular evolution, relaxed selection, protein
43 evolution

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46
47 **Abstract**
48 Natural variation in the number, expression, and function of sensory genes in an organism's
49 genome is often tightly linked to different ecological and evolutionary forces. Opsin genes, which
50 code for the first step in visual transduction, are ideal models for testing how ecological factors
51 like light environment may influence visual system adaptation. Neotropical cichlid fishes are a

52 highly ecologically diverse group that evolved in a variety of aquatic habitats, including black
53 (stained), white (opaque), and clear waters. We used cross-species exon capture to sequence
54 Neotropical cichlid short wavelength sensitive (SWS) opsins, which mediate ultraviolet to blue
55 visual sensitivity. Neotropical cichlid SWS1 opsin (UV-sensitive) underwent a relaxation of
56 selective constraint during the early phases of cichlid diversification in South America, leading to
57 pseudogenization and loss. Conversely, SWS2a (blue-sensitive) experienced a burst of episodic
58 positive selection at the base of the South American cichlid radiation. This burst coincides with
59 SWS1 relaxation and loss, and is consistent with findings in ecomorphological studies
60 characterizing a period of extensive ecological divergence in Neotropical cichlids. We use
61 ancestral sequence reconstruction and protein modelling to investigate mutations along this
62 ancestral branch that likely modified SWS2a function. Together, our results suggest that variable
63 light environments played a prominent early role in shaping SWS opsin diversity during the
64 Neotropical cichlid radiation. Our results also illustrate that long-term evolution under light-
65 limited conditions in South America may have reduced visual system plasticity; specifically, that
66 early losses of UV sensitivity may have constrained the evolutionary trajectory of Neotropical
67 cichlid vision.

68

69 **Introduction**

70 Detecting, perceiving, and responding to environmental stimuli is essential for the survival of
71 most organisms, and the genes underlying complex traits such as sensory systems can offer
72 unique insight into the molecular basis of adaptation (Baldwin et al. 2014; Vandewege et al.
73 2016; Marques et al. 2017; Baldwin and Ko 2020). The visual system permits the near-
74 instantaneous relay of information between organisms and their environments, and the study of
75 opsin genes, which code for the first step in visual transduction and are maximally sensitive to
76 different wavelengths of light, has greatly enhanced our understanding of the genetic mechanisms
77 underlying adaptation (Davies 2012; Hauser and Chang 2017). Cone opsins, mediating bright
78 light (photopic) and colour vision, have undergone extensive duplication and reduction
79 throughout vertebrate evolution, and comparative studies of these genes offer important insight
80 into how genes may become lost, inactivated, or duplicated in response to different ecological

81 variables, since opsin gene repertoire, expression, and genetic variation are often tightly linked to
82 ambient light environment (Fuller and Claricoates 2011; Phillips et al. 2015; Torres-Dowdall et
83 al. 2017; Härer et al. 2018; Musilova et al. 2019; Carleton et al. 2020).

84 In fishes, short wavelength-sensitive vision (ranging from ultraviolet to blue; ~360-480
85 nm; Bowmaker 2008) is critical for a variety of important fitness-related tasks including foraging
86 (Novales Flamarique 2016), species recognition (Siebeck et al. 2010), and mating (Pauers et al.
87 2016). The expression of different opsins involved in short wavelength vision can differ
88 depending on ontogenetic stage (Sabbah et al. 2012; Cortesi et al. 2015). For example, in many
89 freshwater fishes UV-sensitive SWS1 is often expressed in the larval stage (Novales Flamarique
90 2012; Hunt and Peichl 2014) where it may aid in the detection of small suspended particles
91 (Jordan et al. 2004). Violet-sensitive SWS2b may be expressed in both fry and adult fishes, while
92 blue-sensitive SWS2a is frequently expressed only in adults (O'Quin et al. 2010). Expression and
93 repertoire of short wavelength-sensitive opsins and loss or retention of opsin genes is also known
94 to be highly dependent on habitat. In some cases, rather than SWS1 expression being restricted to
95 larval fish, it is also expressed in adult fishes living in habitats where UV light is abundant, such
96 as the upper portion of lakes (Hofmann et al. 2010), or shallow coral reefs (Cortesi et al. 2020).
97 Fishes that traverse different habitats highlight the usefulness of labile short-wavelength vision:
98 juvenile surface or freshwater-dwelling salmonids express a suite of opsins tuned to shorter
99 wavelengths (including a UV-sensitive SWS1 opsin) and upon smoltification (accompanied by
100 transition to deeper or marine waters) lose ultraviolet sensitivity (Allison et al. 2003).

101 Beyond opsin gene expression, evolutionary gains and losses of opsins from the genomes
102 of fishes also may affect (or even eliminate) short wavelength vision. Recent surveys of fish
103 opsins note that non-reef marine fishes tend to have lost SWS1 opsins encoding UV sensitivity,
104 likely due to the rapid depth-dependent attenuation of UV light in pelagic ocean habitats (Lin et
105 al. 2018). Nocturnal reef fishes were also found to lack SWS1 while retaining expression of other
106 cone opsins (Luehrmann et al. 2019). Conversely, freshwater fishes appear more likely to retain
107 SWS1 if they inhabit shallower, clearwater environments where UV light is able to penetrate (Lin
108 et al. 2018). Despite the utility of UV vision in many freshwater habitats, recent efforts to more
109 extensively sample freshwater fish opsin diversity have suggested that loss of UV sensitivity may
110 be more widespread (Weadick et al. 2012; Escobar-Camacho et al. 2019; Escobar-Camacho et al.

111 2020). This may be a result of variation in the pattern and degree of attenuation of light across the
112 visual spectrum in different freshwater habitats.

113 Neotropical cichlid fishes comprise one of the most species-rich freshwater fish clades in
114 South and Central America and inhabit a variety of photic and ecological niches (Astudillo et al.
115 2015; Arbour and Lopez-Fernandez 2014; Hauser et al. 2017; Arbour et al. 2020). While the
116 African rift lake radiation took place relatively recently, Neotropical cichlids have diversified
117 over much longer timescales in South and Central America into diverse riverine habitats
118 (reviewed in Matschiner 2018), allowing for more opportunity for structural variation to their
119 opsin genes to accumulate as a result (Weadick et al. 2012; Hauser et al. 2017). Previous work
120 examining the cichlid opsin repertoire suggests a reduction in the number of opsins available for
121 vision in Neotropical cichlids with evidence for both loss (Weadick et al. 2012); and
122 pseudogenization (Escobar-Camacho et al. 2017) of SWS1 in two different South American
123 species. Based on these studies, Neotropical cichlids likely possess up to 8 opsins: (SWS1,
124 SWS2a, SWS2b, RH2a α , Rh2a β , RH2b, RH1, LWS), but the extent of absence/pseudogenization
125 of these opsins across the Neotropical cichlid tree remains unclear. In a functional study of
126 Neotropical cichlid rhodopsin (mediating vision in dim light), we also found accelerated
127 evolutionary rates in Central American cichlids, as well as repeated functional transitions in the
128 protein consistent with adaptation to clearwater habitats in Central America (Hauser et al. 2017).
129 Given this pattern of molecular evolution in rhodopsin, it is possible that a combination of
130 phylogeny and biogeography (the transition of some lineages from South to Central America)
131 and habitat (transitions between black or white and clear water) have shaped the evolution of
132 opsin genes in Neotropical cichlids.

133 Several recent studies of Neotropical cichlid opsin gene expression note the consistent
134 expression of a long-wavelength sensitive opsin “palette” consisting of the blue (SWS2a), green
135 (Rh2a), and red (LWS) opsins (Escobar-Camacho et al. 2017; Härer et al. 2018; Schneider et al.
136 2020). Interestingly, this palette appears relatively inflexible in Neotropical cichlids, especially
137 when compared with the highly plastic opsin expression in African cichlids (e.g. Hofmann et al.
138 2010; reviewed in and Carleton and Yourick 2020). However, it remains an intriguing question
139 whether this restricted palette might be the result of ancestral adaptations to ancient riverine
140 environments, particularly given the conflicting geological evidence surrounding the

141 paleoenvironments in which the initial diversification of cichlids in South America may have
142 taken place (Lundberg et al. 1998; Godoy et al. 1999; Albert and Reis 2011).

143 Neotropical freshwater habitats are notable for their striking variation in light
144 environments, consisting of clear, blackwater, or whitewater rivers (Sioli 1984; Costa et al. 2013;
145 Figure 1). Clearwater habitats show high transparency of wavelengths across the visible
146 spectrum, and unlike other river habitats do not rapidly attenuate shorter wavelength light
147 (including UV wavelengths); whereas blackwater environments tend to have large amounts of
148 dissolved tannins, producing an environment dominated by orange/red wavelengths (Albert and
149 Reis 2011). Whitewater habitats are generally turbid and sediment-rich, with overall low
150 transparency. Both white and blackwater habitats are thus characterized by the disproportionate
151 attenuation and scattering of short wavelength light available for vision (Costa et al. 2013).
152 Neotropical fish species may traverse starkly different water types while others are restricted to
153 drainages of a particular water type (e.g. Winemiller et al. 2008), and recent work has highlighted
154 how these water types may shape Neotropical freshwater fish community composition and
155 biology (Bogotá-Gregory et al. 2020). Did evolution in these diverse riverine habitats result in
156 changes in selection (e.g., a relaxation of constraint) on Neotropical cichlid short wavelength
157 vision, and has selection imposed by different water types resulted in pervasive changes in opsin
158 evolutionary rates during this group's diversification? More broadly, has the evolution of
159 Neotropical cichlid vision been constrained by strong selection against short wavelength vision at
160 the early stages of their South American radiation? These open questions invite a closer
161 examination of the evolution of short wavelength vision in Neotropical cichlids from the
162 perspective of gene loss and inactivation, as well as functional evolution particularly in relation to
163 light environment.

164 Here, we investigate how the evolutionary trajectory of Neotropical cichlid short
165 wavelength vision was shaped by light environment and gene loss in this diverse clade. We use
166 cross-species exon capture to examine three cone opsins mediating short-wavelength vision in the
167 UV (SWS1), violet (SWS2b), and blue (SWS2a) range of the visible spectrum across a broad
168 sampling of South and Central American cichlids, and test whether cichlids dwelling in light-
169 limited habitats experienced a release of evolutionary constraint on these opsins. In river types
170 known to be lacking short-wavelength light we found elevated evolutionary rates consistent with

171 release of selective constraint for SWS1, but not SWS2 genes. We found evidence for widespread
172 loss and inactivation of SWS1 in a highly diverse clade of South American cichlids. However,
173 these losses in SWS1 were accompanied by a surprising and strong burst of positive selection in
174 the blue-sensitive opsin (SWS2a) at the base of the South American radiation. Ancestral
175 reconstruction and protein modeling of positively selected mutations along this SWS2a branch
176 are suggestive of structural adaptations that may have enabled later shifts in spectral tuning
177 during the course of this cichlid radiation.

178

179 **Materials and Methods**

180 *Opsin sequencing and assembly*

181 Genomic DNA was isolated from muscle tissue from 83 Neotropical cichlids using a Qiagen
182 DNEasy Blood and Tissue Kit. Samples were obtained from the wild and supplemented with
183 some specimens from the aquarium trade, and are listed in detail in Table S1. A representative
184 genus-level sampling of Neotropical cichlids was obtained, spanning South and Central America,
185 as well as a diversity of aquatic habitats and life histories. RNA probes (100bp long) were
186 designed from African riverine cichlid tilapia (*Oreochromis niloticus*) cone opsin exons (SWS1,
187 SWS2a, and SWS2b) and used to enrich extracted Neotropical cichlid gDNA for the regions of
188 interest with an annealing temperature 58°C. We used tilapia for opsin probe design because it
189 had the most comprehensive genomic resources among cichlids and possessed all known intact
190 opsin sequences. Additional details on this capture method are outlined in previously published
191 work (Ilves and López-Fernández 2014; Ilves et al. 2018). Neotropical cichlid opsin sequences
192 were assembled using a custom assembly pipeline with BWA (Li 2013) for guided assembly
193 against the *Oreochromis niloticus* SWS sequences and the mpileup-bcf-vcfutils (Samtools)
194 pipeline for consensus generation (Schott et al. 2017). For SWS1, the *Astronotus ocellatus*
195 pseudogene, which contains a four bp frame shifting insertion in exon two and a codon insertion
196 in exon three (Escobar-Camacho et al. 2017), was also used for guided assembly to test for any
197 effect on assembly performance in cases where no SWS1 sequence was assembled using the *O.*
198 *niloticus* reference. Opsin sequences were aligned using MUSCLE codon alignment implemented
199 in MEGA (Kumar et al. 2016). Where applicable, opsin sequences were compared against
200 sequences from the same species obtained by independent studies via transcriptome and genome

201 sequencing to ensure accuracy (e.g., Escobar-Camacho et al. 2017). In addition to the exon
202 capture approach, we also validated potential instances of SWS1 opsin gene loss and
203 pseudogenization using Sanger sequencing. We designed exon-specific primers for putative
204 pseudogenes recovered via sequence capture and sequenced the region containing the stop codon
205 in the forward and reverse direction. PCR conditions and primers are described in Table S2.

206

207 *Identifying shifts in selection pressure in cichlid SWS opsins*

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

217 We tested whether shifts in selection in SWS opsins were associated with
218 pseudogenization (in the case of SWS1), biogeography, phylogeny, and ecology using Clade
219 Model C in PAML (CMC; Bielawski and Yang 2004). CMC allows for a class of codon sites that
220 experiences a shift in selective pressure in pre-selected “foreground” or “test” branches (which
221 can consist of entire clades or individual branches). This model allowing for a shift in selection
222 was compared to the null model M2a_rel (Weadick et al. 2012). We tested the following
223 partitioning schemes in SWS1 for evidence of selective shifts: pseudogene vs. intact sequences,
224 clear vs. black/whitewater dwelling cichlids, South vs. Central American cichlids (the current
225 geographic distribution of extant cichlids takes into account subsequent re-invasion of some
226 lineages from Central into South America, *sensu* Hauser et al. 2017), and cichlids in the
227 Geophagini/Chaetobranchini/Astonotini tribes (the clade where the majority of SWS1
228 pseudogenization and loss events appear to have occurred) vs. remaining cichlids. We also tested
229 individual ancestral branches leading to major Neotropical cichlid groups to examine whether
230 punctuated shifts in selection occurred along these branches (Figure 2a, roman numerals). These

231 same tests, with the exception of the pseudogene vs. intact sequence test, were also performed in
232 the other short wavelength-sensitive opsins (SWS2a, SWS2b). In all cases, model pairs were
233 compared with a likelihood ratio test (LRT) with a χ^2 distribution and Akaike Information
234 Criterion (AIC). Information on cichlid habitat was obtained from Fishbase (Froese and Pauly
235 2019) and the literature (e.g., Albert and Reis 2011) and used to infer the water type(s) commonly
236 encountered by the cichlids examined in this study (Table S1). We compared CMC results with
237 RELAX tests implemented in HYPHY (Wertheim et al. 2015). RELAX estimates ω among three
238 rate classes for each branch using a BS-REL (branch site random effects likelihood) model, and
239 then fits a parameter estimating the strength of selection (k). ω values are transformed by k (ω^k),
240 which means $k > 1$ drives site classes with high or low ω values away from 1 (suggesting an
241 intensification of selection); conversely, $k < 1$ shifts ω rate classes with high or low ω values
242 towards 1 (suggesting relaxed selection; Wertheim et al. 2015). A model estimating a single k for
243 all branches is compared against a model estimating k for the two branch classes corresponding to
244 test (foreground) and reference (background) lineages in the RELAX analyses.

245 Foreground branches inferred to have undergone significant episodic selection using
246 PAML's CMC were also tested with the HYPHY model BUSTED, which tests for episodic
247 diversifying selection along user-specified branches, and estimates which sites are under
248 selection along the lineage(s) in question, in a similar manner to PAML's branch-site method
249 (Murrell et al. 2015).

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

253 254 *Ancestral sequence reconstruction of SWS2a and protein homology modeling*

255 Specific branches inferred to be experiencing a burst of selection in the opsin genes were
256 further examined to pinpoint the amino acid changes occurring along the branch that may be
257 driving elevated positive selection. As no branch-specific bursts of selection were identified in
258 SWS1 or SWS2b, we focused on ancestral sequence reconstruction of SWS2a. To examine
259 amino acid changes occurring along ancestral branches in the Neotropical cichlid blue-sensitive
260 opsin SWS2a, we used PAML's best fitting model for SWS2a (M8) to reconstruct all ancestral

261 nodes at the codon level. We used Chimera (Pettersen et al. 2004) and Modeller (Webb and Sali
262 2016) to homology-model two Neotropical ancestral SWS2a opsins using bovine rhodopsin
263 (PDB ID: 1u19) as a reference. In all cases, the model with the lowest DOPE score (indicating
264 the most accurate model) was selected for further protein visualization (Webb and Sali 2016).

265

266 **Results**

267 *SWS opsin sequence assembly*

268 SWS sequences were assembled with over 100X depth of coverage on average. After controlling
269 for absent sequences (i.e., completeness of sequence recovered is 0 in the genera *Crenicichla* and
270 *Teleocichla*, and the Chaetobranchini cichlid tribe), SWS1 sequences used in molecular
271 evolutionary analyses (58 total) were on average 91% complete. For SWS2b, 54 sequences with
272 an average completeness of 76% were used (as exon 1 in SWS2b was not successfully captured
273 in this experiment for some species in our dataset). SWS2a sequences were 91% complete, and
274 61 sequences were used for molecular evolutionary analyses. While SWS2a was captured most
275 reliably of the three opsins, the final exon was not captured, likely due to its short length.

276

277 *Pseudogenization and loss of the UV-sensitive opsin in South American cichlids*

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

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

305

306 *Evidence for positive selection in Neotropical cichlid SWS opsins*

307 We attempted to retain a balanced genus-level taxon sampling across the three opsins for
308 molecular evolutionary analyses, with the exception of *Crenicichla*, *Chaetobranchus*, and
309 *Chaetobranchopsis*, which are present in the SWS2b and SWS2a datasets, but not in the SWS1
310 dataset due to the absence of assembled reads (Table S1).

311 The SWS1 opsin exhibits the highest instances of inactivation and loss among the SWS
312 opsins. PAML random sites analyses did not detect any evidence for positive selection in the
313 dataset when South and Central American cichlids were analyzed together (Table S3; $\omega_{M0} = 0.20$,
314 PAML M8 vs. M8a $P=0.31$). Conversely, both SWS2a and SWS2b were under positive selection
315 when the entire Neotropical cichlid group was analyzed (Table S3; S13-S14). Subdividing SWS
316 opsins into South and Central American cichlid datasets revealed that SWS1 was highly
317 conserved among Central American cichlids (Table S4; $\omega_{M0} = 0.2$, but under positive selection in
318 South American cichlids (Table S4; M8 vs M8a $P=0.03$)). While generally highly conserved,
319 SWS1 spectral tuning sites were more variable in South American cichlids (Table S12). This
320 same pattern of conservation in Central American cichlids but positive selection in South

321 American cichlids was found in SWS2b (Table S5; Table S13). By contrast, the SWS2a opsin
322 was under positive selection in both South and Central American cichlids (Table S6).

323
324 *Shifts in selective pressure in SWS1 are associated with pseudogenization and light environment*

325
326 Consistent with our prediction that SWS1 pseudogenes would show evidence for a release of
327 constraint, we found that SWS1 pseudogenes selected as foreground branches in Clade Model C
328 (CMC) analyses had elevated ω (Figure 2b; Table 1; $\omega_{\text{background}} = 0.14/\omega_{\text{pseudogenes}} = 0.55$; $P < 0.01$).
329 These same lineages showed evidence for relaxed selection when selected as test branches in
330 HYPHY's RELAX model (Figure 2a,b; $k = 0.23$; $P < 0.01$; Table S7). To pinpoint where the initial
331 release of constraint in SWS1 may have occurred, we tested whether specific ancestral branches
332 leading to major Neotropical cichlid clades showed evidence for a release of selective constraint
333 (highlighted with roman numerals in Figure 2a). While none of these branch-based tests were
334 significant, both Clade Model C and RELAX identified the Geophagini/Astronotini clade as
335 experiencing significantly elevated ω (Figure 2c; Tables 1 and S7). This suggests that cichlid
336 SWS1 experienced a widespread release of constraint during the early diversification of South
337 American cichlids, prior to pseudogenization and gene loss in certain lineages.

338 We explicitly tested whether ambient light environment (in this case, clear vs. black/white
339 water) influenced SWS1 opsin evolution. Cichlids dwelling in black or white water were
340 highlighted as a foreground partition in CMC, and were found to have elevated ω (Figure 2d;
341 Table 1; $\omega_{\text{clear}} = 0.04/\omega_{\text{black/white}} = 0.7$) and also show evidence for relaxed selection on SWS1
342 (Table S7; $k = 0.65$; $P = 0.03$). While we found that molecular evolutionary rate was associated
343 with water type, a phylogenetic correlation test did not demonstrate that presence/absence of
344 SWS1 was significantly associated with water type (Pagel's λ ; $P = 0.4$). In contrast to SWS1,
345 neither SWS2b nor SWS2a showed evidence for shifts in selection inferred with CMC associated
346 with biogeography (Central vs. South America) or light environment (black/white vs. clearwater
347 habitats) (Table 1). Similarly, neither SWS2b nor SWS2a showed evidence for relaxed selection
348 (Table S7; species tree topology for SWS2b shown in Figure S2).

349
350 *Episodic selection in SWS2a occurs at the base of the South American cichlid radiation*

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

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

373
374 *Homology modeling of ancestral cichlid SWS2a suggests structural reorganization prior to the*
375 *accumulation of spectral tuning variation*

376
377 We used ancestral sequence reconstruction and protein modeling to examine amino acid changes
378 in the ancestral South American cichlid blue-sensitive opsin and the subsequent evolution of
379 spectral tuning sites in this group. We inferred five nonsynonymous and one synonymous codon
380 change occurring in the positively selected SWS2a branch leading from the ancestor of all

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

392 Based on our ancestral reconstruction, variation in SWS2a spectral tuning sites appear to
393 have accumulated after episodic selection on (largely non-tuning) residues along the ACG
394 ancestral branch (Figure 3). SWS2a spectral tuning sites are highly variable among South
395 American cichlids, with independent evolution of putative red-shifting mutations (that were
396 experimentally determined in other fish groups) in several South American lineages: M44T (Li et
397 al. 2009), F46L (Yokoyama et al. 2007), and S292A (Yokoyama et al. 1999; Yokoyama et al.
398 2007) (Figure 3; Figure 4; Table S14). Interestingly, we also found a possible blue-shifting
399 mutation in SWS2a: The A109G mutation has been shown to distinguish SWS2a from
400 blackwater dwelling sticklebacks (A109) vs. clearwater sticklebacks (G109; Marques et al.
401 2017). In Neotropical cichlids, the A109G substitution occurs three times – once in Central
402 American cichlids, and twice in South American cichlids – and is inferred to be under positive
403 selection. In Central America, A109G follows an analogous pattern to sticklebacks, occurring
404 once in a largely clearwater dwelling clade (Figure 3). Conversely, in South America, A109G
405 occurs in cichlids dwelling in black/whitewater habitats, rather than clear habitats (Figure 3).

406 407 **Discussion**

408 We investigated whether the diverse light-limited riverine habitats of the Neotropics mediated
409 changes in selection affecting the short-wavelength vision of Neotropical cichlids using exon-
410 based targeted sequencing of cone opsins, codon-based analyses of selective constraint, and

411 ancestral protein homology modeling. We found evidence for widespread loss and independent
412 inactivating mutations in SWS1 in South American cichlids, and demonstrated that both
413 pseudogenization and river types with limited short-wavelength light (white or blackwater
414 habitats) resulted in reduced selective constraint (elevated rates) in SWS1. We found surprising
415 evidence for a burst of positive selection at the base of the South American cichlid radiation in
416 SWS2a, but, interestingly, this was not accompanied by any shifts in selection associated with
417 ambient light environment in either SWS2a or SWS2b opsins. By modeling the amino acid
418 substitutions under episodic selection along this branch we identified several mutations that may
419 have enhanced opsin dimerization and therefore downstream signaling effectiveness. These
420 mutations were followed by several independent substitutions at key spectral tuning residues
421 within South American cichlids that likely contribute to variable SWS2a sensitivities across the
422 group. Here we discuss how early selective shifts (and losses) in Neotropical cichlid SWS opsins
423 may have altered the evolutionary trajectory of their visual system, resulting in many South
424 American cichlids having a constrained opsin palette relative to their African counterparts.

425
426 *SWS1 loss contributes to the evolution of a long wavelength-sensitive opsin palette in South*
427 *American cichlids*

428
429 There are several plausible explanations for why SWS1 may be inactivated (or, in the case of
430 *Crenicichla/Teleocichla*, seemingly absent) in the South American cichlids examined in this
431 study. Most intuitively, light environment has a substantial impact on which opsin genes are
432 retained, duplicated, or inactivated in fishes. White and blackwater habitats found in many South
433 American riverine environments may have exerted substantial influence on which opsins were
434 under selection early in the Neotropical cichlid radiation (Costa et al. 2013). Indeed, our findings
435 are consistent with the “sensitivity hypothesis”, which posits that visual sensitivities evolve to
436 best match their habitats (Crescitelli et al. 1985). The rapid attenuation of UV light in many
437 South American cichlid habitats may have resulted in reduced selection and the eventual loss of
438 UV sensitivity in the South American lineages where we found pseudogenization and gene loss,
439 while violet and blue sensitivity are retained

440 Although interspecific opsin expression data for Neotropical cichlids is limited, the few
441 species examined to date consistently expressed the same three cone opsins (SWS2a, RH2a,
442 LWS), suggesting their visual system is adapted to maximize performance in habitats dominated
443 by orange or red wavelengths (Carleton et al. 2008; Hofmann and Carleton 2009; Escobar-
444 Camacho et al. 2017; Escobar-Camacho et al. 2019; Schneider et al. 2020). Schneider and
445 colleagues (2020) also note that Neotropical cichlid vision may be constrained by phylogenetic
446 history, with Amazonian cichlids in particular expressing these same three cone opsins, and
447 Central American cichlids having a comparatively wider breadth of opsin expression. The fact
448 that we did not find a strong correlation between SWS1 presence/absence and water type
449 (*Crenicichla* is an excellent example, where several species that have contemporary distributions
450 in clear water appear to still be missing SWS1), lends additional support to this historical
451 constraint.

452 Recent evidence for substantial turbidity-driven variation in visual sensitivity of the
453 invasive cichlid *Cichla monoculus* in Panama suggests that Neotropical cichlid visual systems
454 remain flexible at an individual level despite a reduced opsin complement relative to African
455 cichlids (Escobar-Camacho et al. 2019). However, in the case of *Cichla* much of the visual
456 system variation appears to be driven by A1/A2 retinal chromophore (the light-sensitive ligand
457 bound to the opsin protein) differences, rather than differential opsin expression across habitats
458 (Escobar-Camacho et al 2019). Specifically, the A2 chromophore can produce a substantial red
459 shift (up to 20nm) in a visual pigment relative to the A1 chromophore, and this switch often
460 occurs in high turbidity and red-shifted aquatic habitats (Escobar-Camacho et al. 2019). Changes
461 in opsin gene expression in both African cichlids (Nandamuri et al. 2017) and Central American
462 Midas cichlids (Härer et al. 2017) can occur rapidly (over a period of days or months) in response
463 to different light environments. Härer and colleagues (2017) found a significant effect of ambient
464 light on opsin gene expression during early stages of cichlid development. Exposure to a short-
465 wavelength shifted lighting regime caused retention of the larval opsin gene expression
466 phenotype (i.e., expression of short-wavelength sensitive opsin genes, including SWS1, referred
467 to as a pedomorphic visual phenotype; Rice 1997). Conversely, exposure to long wavelength
468 light caused a more rapid progression of cone opsin expression towards a long wavelength-
469 shifted palette through ontogeny (Härer et al. 2017). This suggests that evolution in long

470 wavelength-shifted habitats over long timescales may have accelerated the ontogenetic shift from
471 a larval to adult opsin expression profile in Neotropical cichlids. That is, the developmental stage
472 at which SWS1 opsins are expressed and used by larval fish is abbreviated relative to fish living
473 in habitats with more available short wavelength light. This reduced period in which SWS1
474 opsins were expressed may have relaxed selection on the UV opsin, leading to its inactivation in
475 some lineages and outright loss in others, and overall a much stronger functional emphasis placed
476 on the expression of a long wavelength-sensitive opsin palette. Interestingly, this contrasts with
477 other dim light-adapted fish groups missing SWS1, where substantial variability in expression
478 among the remaining opsins is retained (e.g., nocturnal reef fish; Luehrmann et al. 2019).
479 Although the extent of SWS1 inactivation in South American cichlids remains an open question
480 given the presence of some intact sequences in this group, the identification of a SWS1
481 pseudogene in *Cichla vazzoleri* (an early diverging Neotropical cichlid) by Escobar-Camacho and
482 colleagues (2019), as well as the presence of an additional putative SWS1 pseudogene in the
483 South American genus *Uaru* suggests at least three independent SWS1 inactivation events have
484 occurred across South American cichlids. Investigations into noncoding sequences in Neotropical
485 cichlids linked to SWS1 expression (e.g., if a promoter has been lost or inactivated) would shed
486 further light onto the evolutionary history of these inactivations. It is also important to note that
487 because Neotropical cichlids are often found in the ornamental fish trade, we cannot rule out that
488 gene loss may be associated with inbreeding in aquarium specimens. In the present study, SWS1
489 loss was found in a combination of aquarium and wild-caught specimens, so future examinations
490 of additional wild-caught specimens would provide further support for gene loss in the wild.

491

492 *Timing and magnitude of selective pressure shifts in Neotropical cichlid SWS opsins*

493 We found changes in selective pressure in SWS1 are associated with pseudogenization
494 and a clade-wide release of constraint among black and whitewater-dwelling geophagine cichlids.
495 Relaxation of selective constraint (i.e., elevated d_N/d_S) in ecologically important genes often
496 occurs in organisms that transition to habitats or lifestyles where the gene is no longer essential.
497 For example, release of constraint has also been found in amblyopsid cavefish rhodopsin
498 (Niemiller et al. 2012), but SWS1 pseudogenes (Wertheim et al. 2015), but vomeronasal receptor
499 pseudogenes (in lineages where social communication may be mediated by the olfactory rather

500 than vomeronasal system; Yohe et al. 2017), and certain digestive enzymes of primates with low
501 insect consumption (Janiak et al. 2017). Given that geophagine cichlids as a whole showed
502 evidence for a release of selective constraint on SWS1, this group-wide release likely led to
503 subsequent losses in independent South American lineages, while SWS1 function was retained in
504 Central American lineages. Central American cichlid habitats range from fast-flowing streams, to
505 lake and lagoons, as well as occasionally brackish habitats. Based on literature review and
506 species records, we inferred most Central American cichlids to inhabit a combination of turbid or
507 clear waters (e.g., Torres-Dowdall et al. 2017, Härer et al. 2017; Escobar-Camacho et al. 2019),
508 which may favor retention of SWS1 in these species. However, more detailed measurements (via
509 spectrometer) of the spectral compositions of various Neotropical cichlid habitats would provide
510 valuable information for future large-scale studies of associations between water type and opsin
511 content in this group.

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

520 In contrast to the clade-wide shift in selection pressure among Geophagine SWS1 opsins,
521 clade model analyses did not reveal a similar pattern in SWS2a. Instead, our analyses identified a
522 burst of positive selection along a branch near the base of the South American cichlid radiation,
523 which was also supported by the BUSTED analysis. Reconstruction of the nonsynonymous
524 amino acid substitutions along this branch revealed two codons with multiple differences (or
525 multinucleotide mutations; Venkat et al. 2018). Our results were robust when we excluded all of
526 the possible MNMs along the branch under episodic selection (e.g., Dapper and Peyseur 2019).
527 This SWS2a result highlights the importance of closely scrutinizing branches in a phylogeny
528 experiencing episodic selection, and that while conservative removal of multinucleotide
529 mutations may improve the reliability of statistical inferences of selection, amino acid

530 substitutions resulting from MNMs occurring along positively selected branches may still be
531 biologically relevant, rather than statistical artifacts (Appendix I).

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

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

560 rivers. Since SWS2a is the most highly expressed SWS opsin in adult South American cichlid
561 retinas studied to date, it is plausible that amino acid variation in this gene may reflect adaptation
562 to photic environment in some lineages and to diminished sensitivity at UV or violet wavelengths
563 in others.

564
565 *Are South American cichlids locked into a long wavelength sensitive opsin palette?*

566 The evolution of SWS1 and SWS2a opsins in Neotropical cichlids suggests that many
567 South American cichlids may have adopted different strategies for tuning visual sensitivity
568 compared to their African relatives. To date, opsin expression studies of adult South American
569 cichlids have revealed little variation in the set of opsin genes they express (SWS2a, RH2b, and
570 LWS, with occasional minimal expression of SWS2b; Escobar-Camacho et al. 2017; 2019; Härer
571 et al. 2018; Schneider et al. 2020). This starkly contrasts with the flexibility of the African cichlid
572 visual system, where adult visual palettes may express entirely different suites of opsins (O'Quin
573 et al. 2010). This suggests that ancient riverine habitats of South America may have been light-
574 limited, resulting in the entrenchment of the South American cichlid visual system into
575 expression of a primarily long-wavelength sensitive opsin palette, perhaps via the acceleration of
576 ontogenetic opsin palette shifts (Härer et al. 2017). Tuning of opsins in South American cichlids
577 to match ambient light environment via opsin expression may therefore be an evolutionary
578 avenue that has been eliminated by a combination of phylogenetic history and long-term
579 evolution in short-wavelength filtering habitats. Instead, modulation of visual sensitivity in these
580 cichlids may be governed primarily by a combination of gene inactivation, structural differences
581 (i.e., the rapid evolution and spectral tuning variation found in SWS2a in this study), and A1/A2
582 chromophore ratios (Escobar Camacho et al. 2019).

583

584 **Conclusions**

585 We found evidence for extensive, and potentially independent, losses of the ultraviolet-sensitive
586 opsin across a highly diverse clade of South American cichlids. A clade-wide release of
587 constraint on SWS1 likely occurred during the early diversification of South American cichlids in
588 short wavelength light-limited riverine habitats, which subsequently led to gene loss and a
589 relatively constrained evolutionary trajectory for the visual system in these fishes. The loss of

590 SWS1 in many South American cichlids may indicate that long-term evolution in these habitats
591 accelerated the ontogenetic trajectory of opsin expression such that the developmental window of
592 SWS1 expression narrowed, and the gene became increasingly unnecessary. A relatively
593 inflexible opsin palette heavily biased towards long wavelengths then came to dominate South
594 American cichlid visual systems. Prior to the release of constraint in SWS1, we found that
595 episodic selection in SWS2a resulting in several amino acid substitutions occurred at the base of
596 the South American cichlid clade where the bulk of SWS1 loss has occurred. SWS2a, the SWS
597 opsin with the highest expression in adult cichlids, may largely mediate visual sensitivity at short
598 wavelengths in South American cichlids. Interspecific structural variability at known spectral
599 tuning sites in SWS2a may therefore be the primary means of tuning visual sensitivity at short
600 wavelengths in these cichlids, as opposed to differential expression among SWS1, SWS2b, and
601 SWS2a. Future work examining the ontogenetic trajectory of Neotropical cichlid SWS opsin
602 expression, as well as a broader survey of Neotropical cichlid opsin expression profiles, will
603 enable further insight into how ecological and evolutionary factors have influenced the evolution
604 of these opsins in cichlids.

605

606 **Acknowledgements**

607 This work was supported by Vision Science Research Fellowships to FEH and RKS, a Raney
608 Fund Award from the American Society of Ichthyologists and Herpetologists to FEH, a Rebanks
609 Postdoctoral Fellowship from the Royal Ontario Museum to KLI, Discovery grants from the
610 Natural Sciences and Engineering Research Council of Canada to HLF and BSWC, and grants
611 from the National Geographic Society and the University of Michigan to HLF. Emma Hauser
612 designed the map, and Alex Van Nynatten made a version of the water type spectra in Figure 1.
613 We thank Karen Carleton for feedback on an earlier version of this manuscript, and three
614 anonymous reviewers for helpful comments and suggestions.

615

616 **Author Contributions**

617 FEH, KLI, HLF, and BSWC conceptualized the project. FEH, KLI, and EA collected the data.
618 FEH, KLI, RKS, and EA analyzed the data. FEH, HLF, and BSWC wrote the manuscript.

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847 **Data Accessibility**

848

849 Opsin sequences are deposited in Genbank [Accessions MW401034-MW401206]. Scripts for

850 assembly of data are available on Github ([https://github.com/ryankschott/Targeted-Capture-](https://github.com/ryankschott/Targeted-Capture-Assembly-Pipeline)

851 [Assembly-Pipeline](https://github.com/ryankschott/Targeted-Capture-Assembly-Pipeline))

852

853 **Supplemental Info**

854 Appendix I – Caveats and considerations with interpretation of multinucleotide mutations

855 Table S1: List of opsin sequences, habitat information, and genbank accession IDs (Excel file)

856 Table S2: Primers/PCR

857 Table S3: Random Sites analyses on each SWS opsin

858 Table S4: SWS1 Central and South America Random Sites
859 Table S5: SWS2b Central and South America Random Sites
860 Table S6: SWS2a Central and South America Random Sites
861 Table S7: RELAX results on each SWS opsin
862 Table S8: BUSTED analyses on SWS2a
863 Table S9: SWS2a codon changes along the Astronotini/Chaetobranchini/Geophagini ancestral
864 branch
865 Table S10: SWS2a results with MNMs removed
866 Table S11: IDs for photographs in Figure 2
867 Table S12: SWS1 Amino acid alignment and spectral tuning sites (Excel file)
868 Table S13: SWS2b Amino acid alignment and spectral tuning sites (Excel file)
869 Table S14: SWS2a Amino acid alignment and spectral tuning sites (Excel file)
870
871 Figure S1: SWS1 stop codon chromatograms
872 Figure S2: SWS2b species tree topology
873
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875

876 **Table 1. Results of Clade Model C (CMC) analyses testing for shifts in selection on**
877 **Neotropical cichlid SWS opsins**
878

879 NOTE.—np, number of parameters; lnL, ln likelihood; *K*, transition/transversion ratio; df, degrees of freedom; n/a, not
 880 applicable.^{§§}
 881 †Partitions listed are explained in Figures 2, 3 and Table S1. In all cases, an additional partition exists that contains the remaining

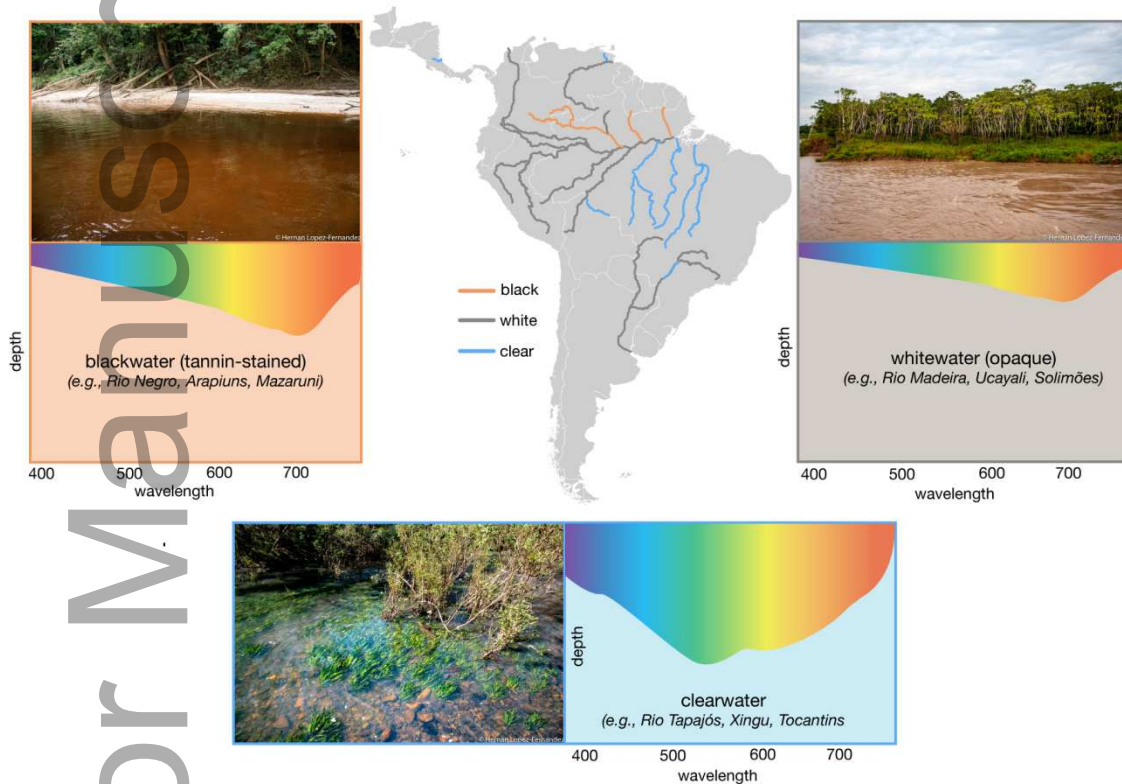
Opsin	Model & Foreground Partitions†	np	lnL	<i>K</i>	Parameters‡			ΔAIC§	Null	LRT	df	<i>P</i>
					ω_0	ω_1	ω_2/ω_4					
SWS1	M2a_rel	118	-5290.2	3.1	0.09(76%)	1(6%)	0.48(18%)	20.0				
	Pseudogenes	119	-5279.2	3.0	0.28(35%)	1(10%)	0.14(44%)	0	M2a_rel	22.0	1	<0.01
	Black/white water	119	-5287.3	3.0	0.1(80%)	1(7%)	0.04(12%)	16.2	M2a_rel	5.6	1	<0.01
	Black/white: 0.70											
	Geophagini/ Astronotini Clade	119	-5285.9	3.0	0.1(61%)	1(8%)	0.23(32%)	13.4	M2a_rel	8.6	1	<0.01
	Geo/Astro:0.53											
South American cichlids	119	-5290.2	2.5	0.48(18%)	1(6%)	0.09(76%)	22.0	M2a_rel	0.0	1	1.0	
SA: 0.09												
Branch leading to major Neotropical cichlid tribes (i)	119	-5290.2	2.5	0.48(18%)	1(6%)	0.09(76%)	22.0	M2a_rel	0.0	1	1.0	
Foreground branch: 0.09												
Branch leading to Geophagini/ Astronotini (ii)	119	-5290.2	2.5	0.48(18%)	1(6%)	0.09(76%)	22.0	M2a_rel	0.0	1	1.0	
Foreground branch: 0.09												
Branch leading to Geophagini (iii)	119	-5290.2	2.5	0.48(18%)	1(6%)	0.09(76%)	22.0	M2a_rel	0.0	1	1.0	
Foreground branch: 0.09												
SWS2b	M2a_rel	112	-4752.2	2.3	0.04(80%)	1(19%)	3.6(1%)	0				
	Black/white water	113	-7452.1	2.3	0.44(80%)	1(19%)	2.7(1%)	1.8	M2a_rel	0.2	1	0.65
Black/white: 3.5												
South America	113	-4752.15	2.3	0.44(80%)	1(19%)	3.4(1%)	1.98	M2a_rel	0.02	1	0.88	
SA: 2.9												
SWS2a	M2a_rel	124	-4987.5	2.9	0.03(85%)	1(9%)	3.2(6%)	9.4				
	Branch leading to Geophagini/ Astronotini (i)	125	-4981.8	3.1	0.03(85%)	1(8%)	3(7%)	0	M2a_rel	11.4	1	<0.01
	Geo_branch: 999											
Black/white water	125	-4987.4	2.9	0.03(85%)	1(8%)	2.8(7%)	11.2	M2a_rel	0.2	1	0.65	
Black/white: 3.2												
South America	125	-4987.5	3.0	0.03(85%)	1(8%)	2.97(7%)	11.4	M2a_rel	0	1	1	
SA: 3.1												

882 taxa (e.g., outgroups).

883 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
884 separate value for each partition.

885 §The difference in AIC values was calculated compared with the overall best-fitting model for each gene: SWS1 Pseudogenes vs.
886 Intact = 10796.4; SWS2b M2a_rel = 9728.3; SWS2a Geophagini/Astronotini Branch = 10213.6

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

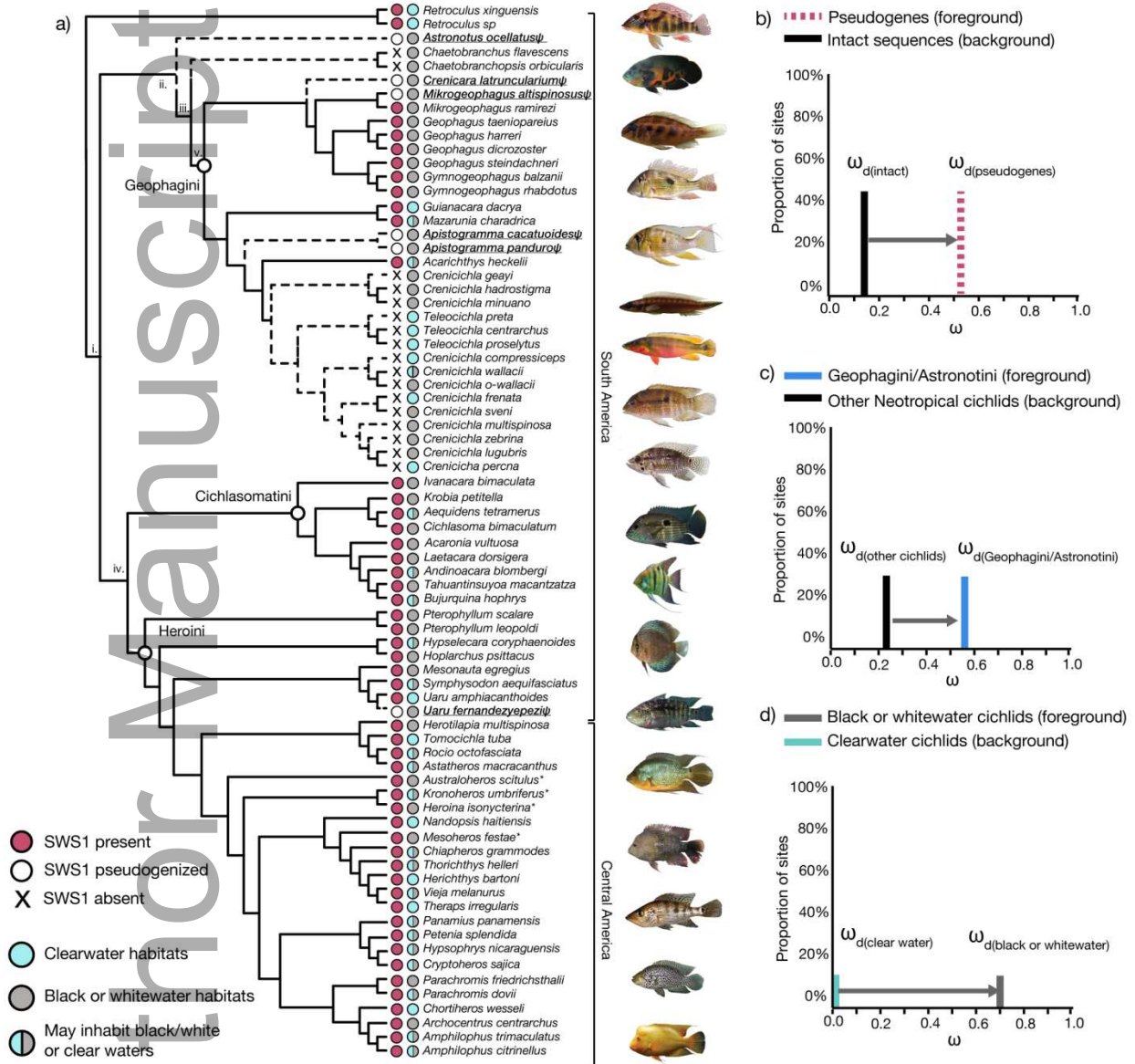


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890 Figure 1. Cichlids are abundant in Neotropical riverine environments. These environments are
891 often classified into three water types, each with distinct physicochemical (including spectral)
892 properties. Blackwater rivers are rich in dissolved organic matter, and often appear tea-colored.
893 Blackwater habitats are translucent but their spectral composition is dominated by red
894 wavelengths, and at increasing depths attenuates short wavelength light. Whitewater riverine
895 environments appear opaque due to suspended sediments, and scatter as well as absorb short
896 wavelength light. Clearwater rivers allow a broad spectrum of light (including UV and shorter
897 wavelengths) to penetrate at greater depths, and may appear blue or green-tinged in colour.

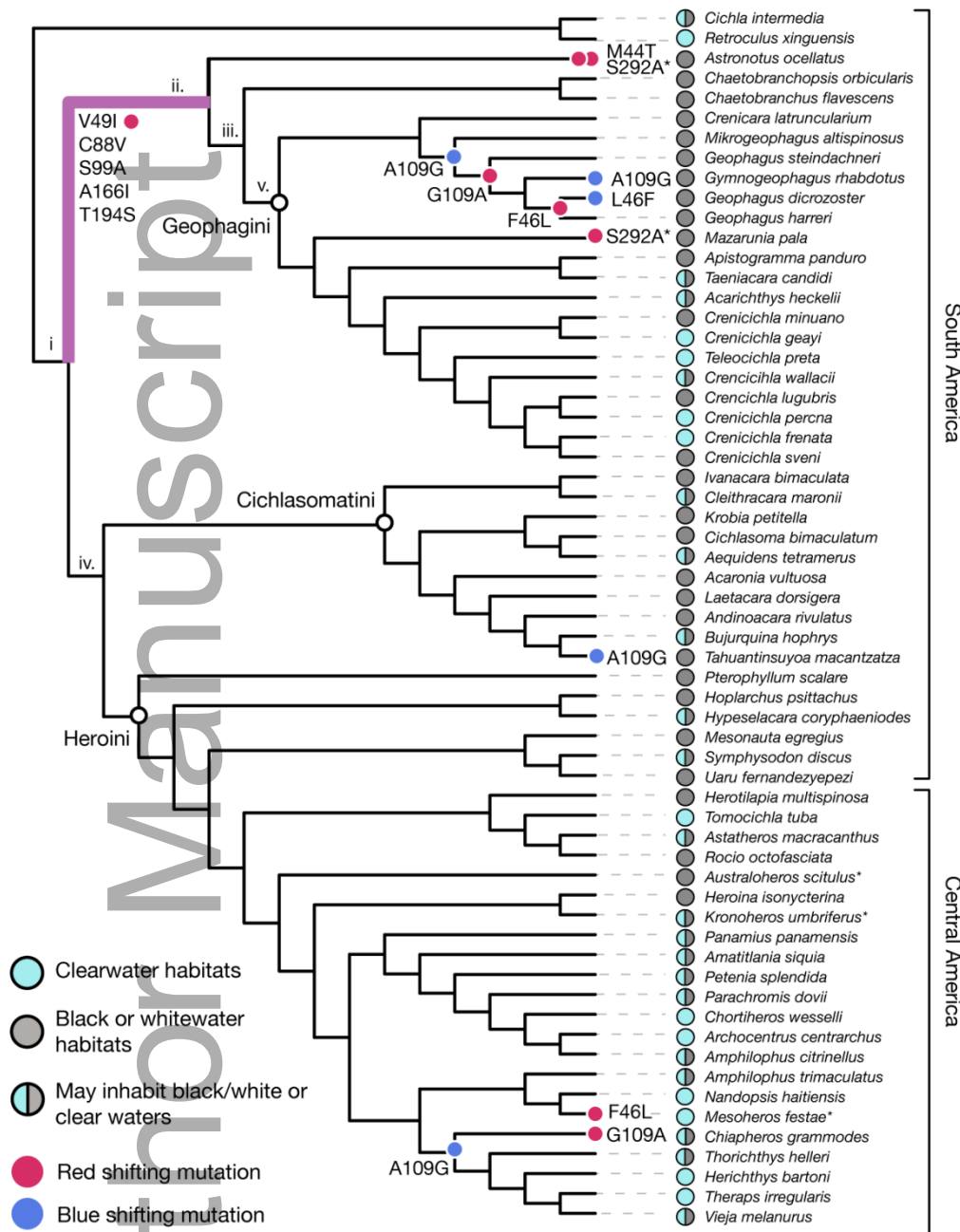
898 Photos taken by Hernán López-Fernández. Spectra schematics are adapted from Costa et al.
 899 (2013).



900
 901 **Figure 2. Pseudogenization, loss, and evolution of Neotropical cichlid UV-sensitive (SWS1)**
 902 **opsins.** a) Species tree illustrating losses and inactivations of SWS1 across Neotropical cichlids
 903 in relation to water type. Dotted lines represent either a nonsense mutation resulting in a
 904 premature stop codon or insertions/deletions rendering the protein non-functional via frameshift
 905 (open circle). An X indicates no SWS1 reads recovered. (b) Pseudogene sequences, (c) the

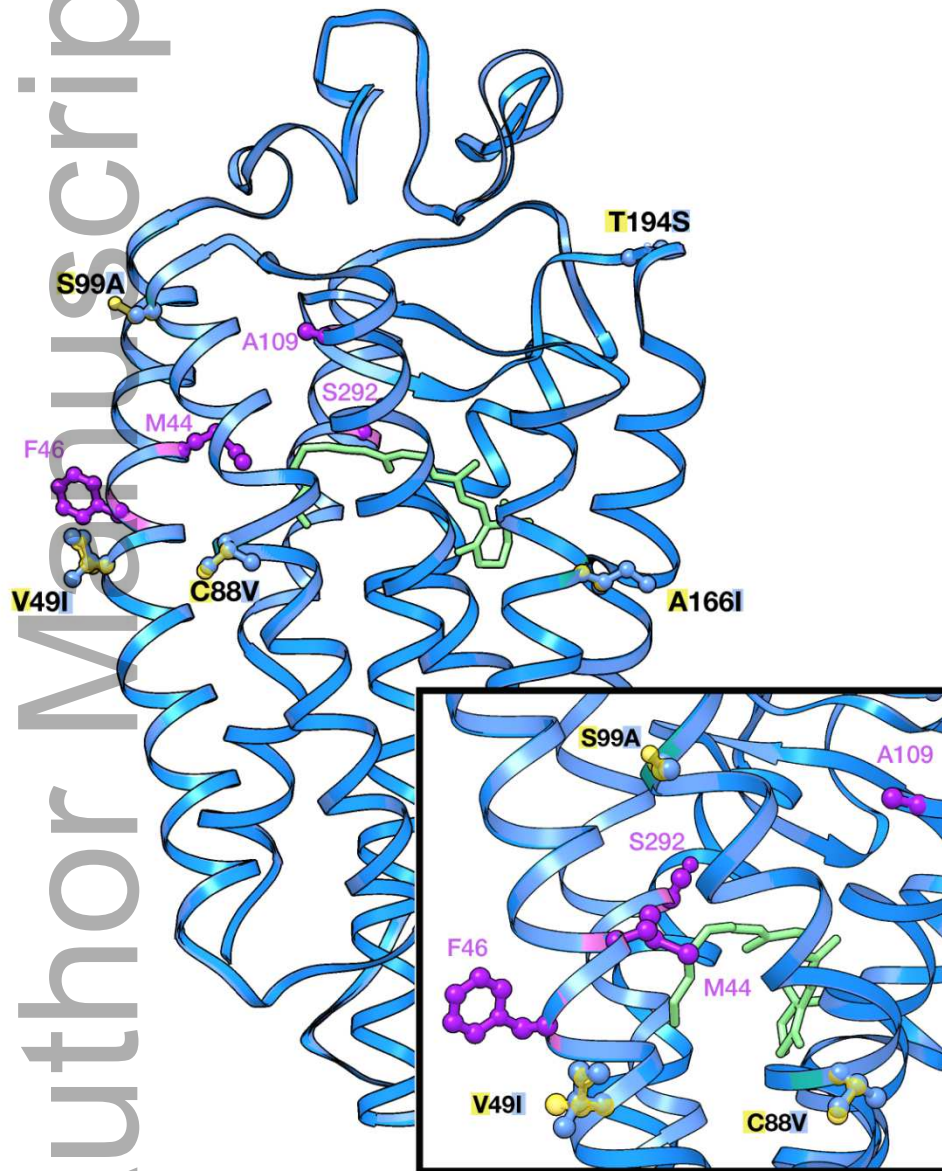
906 Geophagini/Astronotini tribe, (d) black/whitewater-dwelling cichlids experienced a significant
907 elevation in ω (d_N/d_S) inferred with CMC. The partition isolating pseudogene lineages was the
908 best fitting of the three significant models. Species with an SWS1 pseudogene are bolded and
909 underlined. Central American species marked with an asterisk secondarily re-invaded South
910 America. Roman numerals indicate ancestral branches that were tested for evidence of episodic
911 selection, the results of which are reported in Table 1 and S7. Photos were taken by Hernán
912 López-Fernández and Jessica Arbour, and species names associated with the photos are listed in
913 Table S11.
914

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915
 916 **Figure 3. Episodic selection and evolution of spectral tuning sites in the Neotropical cichlid**
 917 **SWS2a opsin.** A burst of positive selection in the blue-sensitive (SWS2a) opsin occurs at the
 918 base of the South American cichlid radiation, specifically the branch leading to the Geopagini,
 919 Chaetobranchini, and Astronotini tribes (ii). Variation in SWS2a spectral tuning sites inferred
 920 through ancestral reconstruction is illustrated by blue dots (indicating a blue-shifting mutation) or
 921 red dots (indicating a red-shifting mutation). Roman numerals indicate ancestral branches that

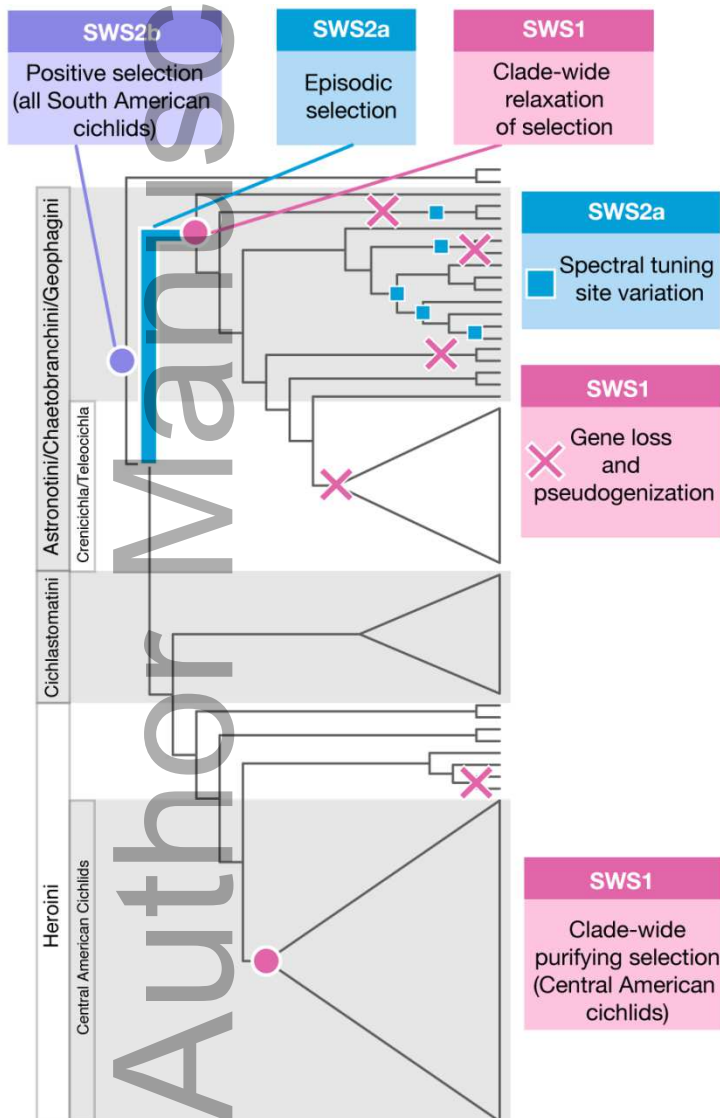
922 were tested for evidence of episodic selection, the results of which are reported in Table 1 and S7.
923 The 3D structures of ancestral nodes i and ii was subsequently modeled. Central American
924 species marked with an asterisk secondarily re-invaded South America.
925



926
927 **Figure 4. Homology model of ancestral Neotropical cichlid SWS2a opsin.** The
928 Astronotini/Chaetobranchini/Geophagini (node ii, Figure 3) ancestor is overlaid onto the
929 structure of node i. Variable SWS2a spectral tuning sites in Neotropical cichlids are shown in
930 purple. Amino acid substitutions along the SWS2a branch under episodic selection are

931 highlighted: the original amino acid identity is translucent yellow, and the amino acid identity in
 932 the Astronotini/Chaetobranchini/Geophagini ancestor is solid blue. Inset: the V49I transition in
 933 the ancestral Neotropical cichlid opsin occurs in close proximity to spectral tuning site F46 and
 934 introduces a larger amino acid residue at site 49. The retinal chromophore is depicted in light
 935 green. Numbering is standardized to bovine rhodopsin.

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 937



938
 939 Figure 5. Schematic of SWS opsin evolution superimposed onto Neotropical cichlid evolution.
 940 Episodic selection on SWS2a accompanied by nonconservative amino acid substitutions occurs

941 at the base of the Neotropical cichlid tree. A clade-wide relaxation of selection occurs in SWS1,
942 which likely led to independent gene loss events throughout
943 Astronotini/Chaetobranchini.Geophagini (ACG). Within the ACG clade, SWS2a (blue sensitive)
944 accumulates variation in spectral tuning sites. SWS1 in cichlids colonizing Central America
945 undergoes purifying selection and we detected no gene loss or inactivation events in this group.

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