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Long-wavelength sensitive opsin (*LWS*) gene variability in Neotropical cichlids (Teleostei: Cichlidae)

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ABSTRACT

Cichlid fishes are an important group in evolutionary biology due to their fast speciation. This group depends widely of vision for feeding and reproduction. During the evolutionary process it plays a significant role in interspecific and intraspecific recognition and in its ecology. The molecular basis of vision is formed by the interaction of the protein opsin and retinal chromophore. Long-wavelength sensitive opsin (*LWS*) gene is the most variable among the opsin genes and it has an ecological significance. Current assay identifies interspecific variation of Neotropical cichlids that would modify the spectral properties of the *LWS* opsin protein and codons selected. Neotropical species present more variable sites for *LWS* gene than those of the African lakes species. The *LWS* opsin gene in *Crenicichla britskii* has a higher amino acid similarity when compared to that in the African species, but the variable regions do not overlap. Neotropical cichlids accumulate larger amounts of variable sites for *LWS* opsin gene, probably because they are spread over a wider area and submitted to a wider range of selective pressures by inhabiting mainly lotic environments. Furthermore, the codons under selection are different when compared to those of the African cichlids.

Key words: convergence, ecology, evolution, visual system.

INTRODUCTION

The Cichlid family is an important freshwater group of fish that presents a varied color pattern and widely uses the visual system (Nelson 2006). They are also considered a model in evolutionary studies due to their fast radiation and speciation in several African lakes (Seehausen et al. 1999, Smith et al. 2011). In addition, Neotropical cichlids exhibit a diversity of ecological niches, behavioral and morphological adaptations (Maan and Sefc 2013), besides being a very diversified group (López-Fernández et al. 2010).

The genus *Cichla* has been used for phylogenetic and population structure studies (Oliveira et al. 2006, Willis et al. 2007, 2012) since it is one of the most basal (Poletto and Ferreira 2010) which forms a monophyletic group when compared to other Neotropical cichlids (López-Fernández et al. 2010). The body shape is a characteristic in *Cichla* and species are identified mainly on coloration

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(Kullander and Ferreira 2006). A breaking down of reproductive isolation was detected between these two species in the Paraná River basin through molecular evidence (Oliveira et al. 2006, Almeida-Ferreira et al. 2011), namely, *Cichla kelberi* and *Cichla piquiti*. However, there is no evidence of natural hybrids between the two species in the Tocantins-Araguaia basin where the two species coexist (Willis et al. 2007, 2012).

A modification of the isolation mechanisms that had existed between them where they were native may have occurred, but would not be functioning in the new environment. Thus, the color that differentiates the two species may also function as a pre-zygotic isolation and become a maintainer mechanism of sympatric species which are closely related, preventing gene flow and hybridization process (Miyagi et al. 2012).

The way coloring is perceived by aquatic organisms should be considered since the light wavelength reaching the environment and limnological characteristics act as a background for vision (Gray and McKinnon 2007). In this case, vision would play an important role among species that use coloring as a mechanism of recognition (Terai et al. 2002).

Visual sensitiveness is determined by the visual pigments formed from the interaction between opsin proteins and retinal chromophores (Schwanzara 1967), enabling the perception of light wavelengths according to opsin proteins which are expressed by opsin genes and, due to their plasticity, may also vary according to environment and life stage of fish (Spady et al. 2006, Yokoyama 2008, Hofmann et al. 2009, Hofmann and Carleton 2009, O'Quin et al. 2010, Smith et al. 2011).

In the case of studies on these genes in Neotropical cichlids, Weadick et al. (2012) investigated the molecular evolution of visual pigments in *Crenicichla frenata*, a species native to the Caribbean island of Trinidad, while Schott et al. (2014) studied the molecular evolution of the opsin gene *RH1* from 32 species of Neotropical cichlids and compared them with those of African cichlids.

African cichlids have seven cone opsin genes which express the opsin proteins sensitive to short-wavelength - *SWS1*, *SWS2B*, *SWS2A*; medium-wavelength - *RH2B*, *RH2Aβ*, *RH2Aα*; and long-wavelength - *LWS*. There is also a rod opsin gene - *RH1*, responsible for scotopic vision (Spady et al. 2005, Carleton et al. 2010). However, *LWS* presents the highest variability (Terai et al. 2002) and is related to ecological adaptation and sexual selection (Terai et al. 2006).

Variable sites of these sequences may cause small changes in the absorbed light wavelength due to the replacement of amino acids as long as they occur in specific regions of exons (Yokoyama 2008, Carleton 2009). Since water is an environment that requires adaptations in the visual system, the heterogeneity of light would represent a strong selection pressure on these genes. Any eventual variation may be associated with differences in sensitivity to light absorption spectra, similar to patterns shown by other studies (Yokoyama 2008, Seehausen et al. 2008).

Two hypotheses may be raised: (1) the occurrence of interspecific variation related to the visual sensitivity of the Neotropical species under analysis, and (2) different codons would be under selection when the studied groups of African and Neotropical cichlids are compared.

Amino acid and nucleotide sequences of coding regions of the *LWS* gene were analyzed and compared in ten species of Neotropical cichlids belonging to five genera. Subsequently, codon selection tests were performed to compare which codons were under selection between the African and Neotropical cichlids.

MATERIALS AND METHODS

STUDIED SPECIES

Current assay comprised ten Neotropical species and nineteen African species. The Neotropical species

were *Cichla kelberi* (n=9), *Cichla piquiti* (n=5), *Cichla monoculus* (n=5), *Crenicichla britskii* (n=4), *Crenicichla haroldoi* (n=4), *Crenicichla jupiaensis* (n=3), *Astronotus crassipinnis* (n=4) *Geophagus proximus* (n=4), *Satanoperca pappaterra* (n=3) (Figure 1). The *LWS* gene sequence of *Crenicichla frenata* was obtained from GenBank (JN990732).

The African species sequences were obtained from the GenBank: one species from the Nile River, *Oreochromis niloticus* (AF247128); four species from Lake Tanganyika, *Astatotilapia burtoni* (AY660540), *Opthalmotilapia ventralis* (AY780512), *Neolamprologus brichardi* (AY780513), *Tropheus duboisi* (AY780516); two species from Lake Victoria, *Pundamilia nyererei* (AY673688), *Pundamilia pundamilia* (AY673689); and eleven species from Lake Malawi, *Aulonocara heuseri* (AY780517), *Labeotropheus fuelleborni* (AF247127), *Metriaclima zebra* (AF247126), *Melanochromis auratus* (AY780518), *Lethrinops parvidens* (AY780519), *Tyrannochromis maculatus* (AY780520), *Cynotilapia afra* (AY780521), *Mylochromis lateristriga* (AY780522), *Labiochromis chisumulae* (AY780515), *Copadochromis borleyi* (AY780514), *Stigmatochromis modestus* (AY780523). The Nile River and Lake Victoria are characterized as turbid environments, whereas lakes Malawi and Tanganyika are clear water environments (Spady et al. 2005). Twenty-eight species were used in current analysis.

DNA EXTRACTION AND PCR

Muscle tissue samples, preserved in 96% ethanol and stored at -20 °C, were used for DNA extraction of species from the genus *Cichla*. Samples were provided by the tissue bank of the Genetics Laboratory of the Nucleus for Research in Limnology, Ichthyology and Aquaculture (Nupelia), Universidade Estadual de Maringá, Maringá, PR, Brazil. The other Neotropical species, collected within the Long Term Ecological

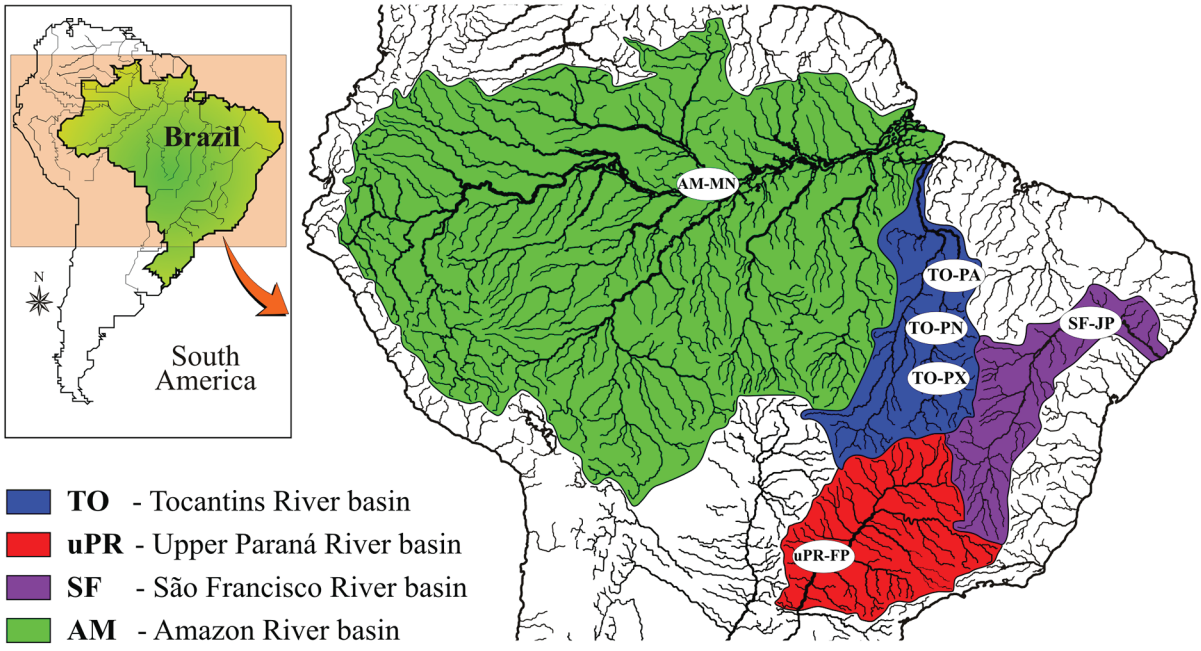

Figure 1 - Sampling locations of *Cichla* populations native from the Tocantins-Araguaia (TO) and Amazonas river (AM) basins and from Neotropical cichlids species from upper Paraná river (aPR). TO-PA: Tocantins river, near to Pedro Afonso city (08° 58′ S; 48° 10′ W); TO-PN: Lajeado's resevoir, in the Tocantins river, near to Porto Nacional city (09°45′ S; 48°22′ W); TO-PX: Tocantins river, near to Peixe city – TO (11° 52′ S; 48° 35′ W); AM-MN: Amazonas river (03° 07′ S; 59° 55′ W, near to Manaus city– AM; aPR-PL: Upper Paraná river floodplain, close to Porto Rico city - PR (22°47′ S; 53°19′ W).

Research Project (Projeto de Pesquisa Ecológica de Longa Duração, PELD), were also preserved in 96% ethanol and stored at -20 °C.

Genomic DNA was extracted with Promega kit (Wizard Genomic DNA Purification A1125) following manufacturer's instructions.

Primers described by Weadick et al. (2012) were used for PCR. The amplicons were purified (Rosenthal et al. 1993) and the sequencing reaction was performed with Big Dye Terminator kit with the ABI3730 automated DNA sequencer.

SEQUENCE ANALYSIS

The sequences were manually edited with the program BioEdit (Hall 1999). Sequences alignment data using the Clustal W algorithm (Thompson et al. 1994) and the construction of phylogenetic trees inferred from the Neighbor-Joining method were performed with MEGA 6 software (Tamura et al. 2013). The selected codons were estimated with the FUBAR (Murrell et al. 2013) and REL (Pond and Muse 2005) methods, available in the HyPhy software package (Pond et al. 2005) available in the Datamonkey web server (Delport et al. 2010), and CODEml, available in the PAML software (Yang 2007, Xu and Yang 2013).

So that variable sites and their relation to the spectral properties of the opsin protein could be identified, the sequence was aligned according to bovine rhodopsin (Bowmaker 1995, Hofmann et al. 2009, Smith and Carleton 2010, Schott et al. 2014).

RESULTS

VARIABLE SITES IN NEOTROPICAL CICHLIDS

Fragments comprised exons 2-4 (633 bp) of the *LWS* gene. Whereas the Neotropical species presented 64 nucleotide and 27 amino acid variable sites, the African cichlids exhibited 45 nucleotide and 24 amino acidic variable sites (Table I). Exon 2 was the most variable in the two groups.

TABLE I
Number of variable sites in *LWS* gene of Neotropical and African cichlids. E2 = exon 2; E3 = exon 3; E4 = exon 4; N = nucleotide variable sites; Aa = amino acidic variable sites; S = synonymous substitutions; nS = nonsynonymous substitutions.

	Neotropical cichlids			African cichlids		
	E2	E3	E4	E2	E3	E4
N	28	16	20	15	14	16
Aa	15	6	6	7	9	8
S	13	10	13	7	4	7
Ns	22	10	11	10	14	10

The amino acid and nucleotide genetic distances were calculated according to the Jones-Taylor-Thornton (Jones et al. 1992) and Kimura-2-parameters (Kimura 1980) models, respectively, among the Neotropical and African cichlids (Table SI - Supplementary Material). Figure 2 presents the phylogenetic trees based on the Neighbor-Joining method.

Two species of the genus *Crenicichla*, *C. britskii* and *C. frenata*, evidenced evolutionary convergence in one amino acidic site (site 119), and they rated a smaller genetic distance when compared to some African species rather than to species of the genus *Cichla*, which is a basal group for Neotropical cichlids (López-Fernández et al. 2010) (Table SII). Eight out of the 10 Neotropical species had a substitution at amino acid 164 which results in tuning in the maximum wave spectrum absorbed by *LWS* opsin protein (Yokoyama 2008).

CODON SELECTION

Selected codons were estimated by FUBAR, REL and M8 BEB model (available in CODEml) tests and were different between Neotropical and African cichlids, considering posterior probability above 80% to both models (Tables SIII to SVI). In the case of the REL evolution model, an evolution tree was built by the same model, taking into consideration REV substitution model and the neighbor-joining statistical method. There were nine different coding

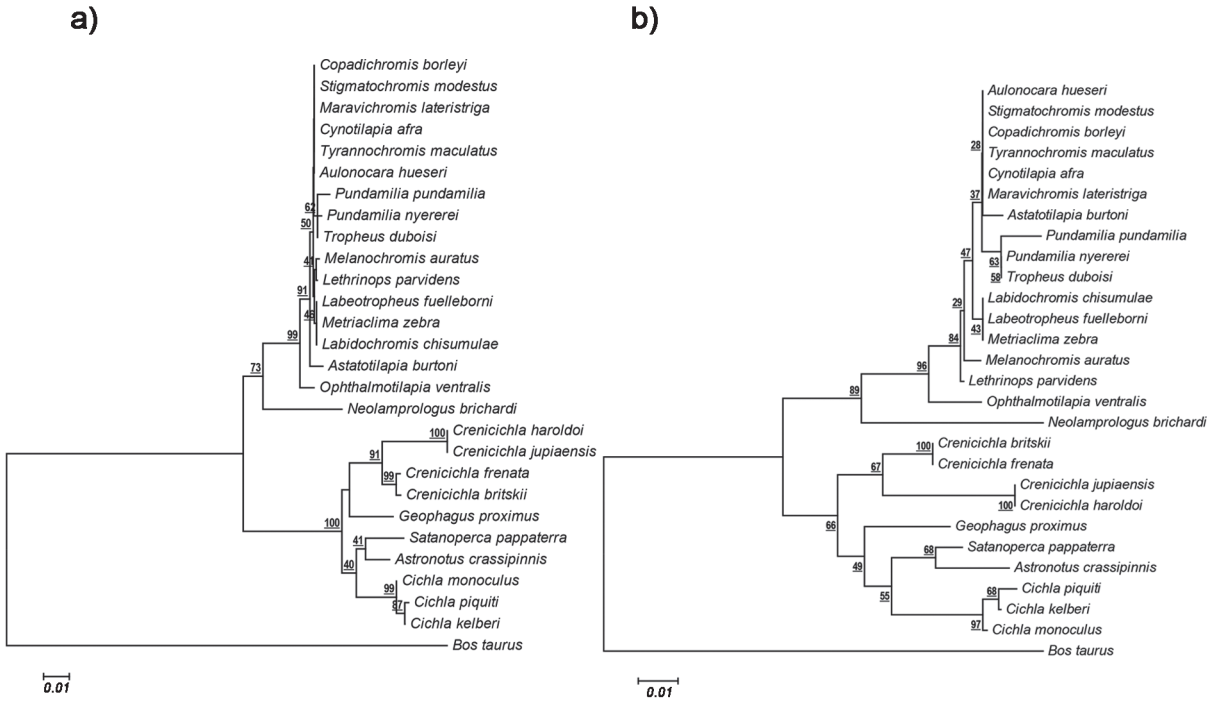

Figure 2 - Phylogenetic tree from fragment of the coding regions of the opsin gene *LWS* (633 bp) of Neotropical and African cichlids, according to **(a)** nucleotide substitution model Kimura-2-parameter with 1000 bootstrap resampling and **(b)** JTT + G substitution model with 1000 bootstrap resampling and statistical neighbor-joining method.

sequences among the ten Neotropical species selected, and twelve different coding sequences among the seventeen African species. The analyzed fragments comprised the codon 22-232 of the *LWS* gene.

REL model estimated eleven codons as positive selection, whereas the FUBAR and M8 models estimated nine and seven codons, respectively, for the Neotropical cichlids. All codons estimated by FUBAR and M8 models were also estimated by REL. In the case of the African lakes cichlids, REL test estimated eight codons under selection, FUBAR and M8 model estimated seven codons under selection. Only one codon under selection overlapped the two groups (site 119). Figure 3 shows posterior probabilities estimated by FUBAR model.

The position of the opsin protein codons *LWS* has been established according to the crystal structure of bovine rhodopsin (Stenkamp et al. 2002) (Table II).

DISCUSSION

DIVERGENT SELECTION OF *LWS* GENE IN NEOTROPICAL AND AFRICAN CICHLIDS

LWS presents the greatest variability among opsin genes (Terai et al. 2002, Spady et al. 2005, Miyagi et al. 2012). Fragments of exon 2-5 (872 bp) were used in other research work (Terai et al. 2006, Seehausen et al. 2008) since they encoded the transmembrane domains of the protein. The changes in specific sites of the opsin gene sequences which provide the longest (*LWS*) and shortest (*SWS1*) wavelength appear to be more critical (Carleton 2009, Carleton et al. 2010), especially those that occur between exon 1-3 (Terai et al. 2006). Further, the opsin genes seem to accumulate the highest amount of variation (Hofmann et al. 2012).

Papers on the gene in African cichlid species, comprising sequences of exons 2-5, reported ten variable sites in four species of three distinct genera inhabiting the same lake and thirteen variable

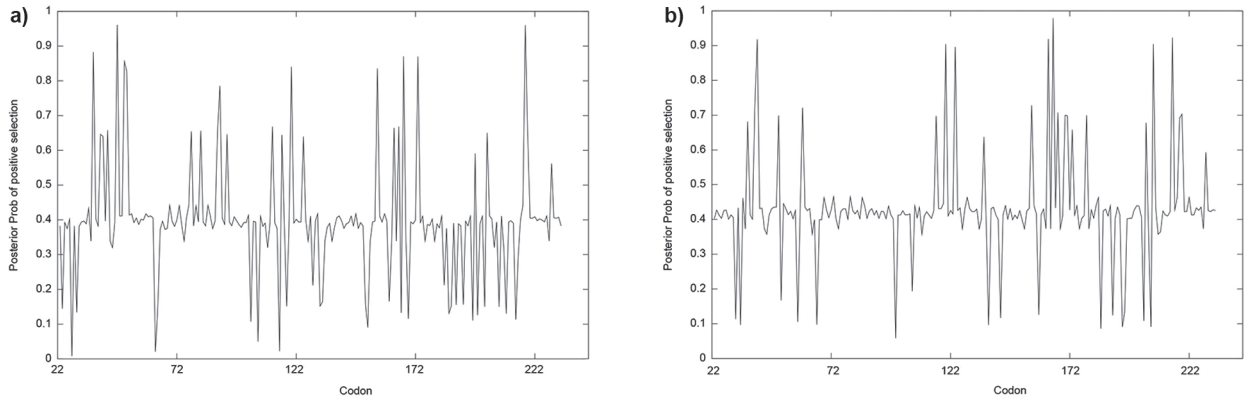

Figure 3 - Posterior probability of codons under selection estimated by FUBAR model (a = Neotropical cichlids; b = African cichlids).

TABLE II
Codons under positive selection and its position in the structure of the *LWS* opsin protein according to FUBAR (F), REL (R) and M8 BEB model (M).

	Codon	Model	Location	Possible effect on *LWS* opsin protein
Neotropical cichlids	36	FRM	TDe	
	46	FRM	TM1	
	49	FR	TM1	
	50	FR	TM1	
	89	R	TM2	
	119	FRM	TM3	Binding site to the retinal chromophore (Stenkamp et al. 2002)
	155	FRM	TM4	
	166	FRM	TM4	
	172	FR	TM4	
	217	FRM	TDe	
	218	RM	TM5	
African cichlids	40	FRM	TM1	
	119	FRM	TM3	Binding region to the retinal cromophore (Stenkamp et al. 2002)
	123	FRM	TM3	Binding region to the retinal cromophore (Stenkamp et al. 2002)
	135	R	TDc	
	162	FRM	TM4	
	164	FRM	TM4	-7nm λ_{max} shift (Yokoyama 2008)
	206	FRM	TM5	
	214	FRM	TM5	

sites in two sympatric species of the same genus (Terai et al. 2006), with most leads towards non-synonymous changes. Nine out of the ten studied species of Neotropical cichlids comprised new species that had fragments of their *LWS* opsin gene sequenced (exons 2-4) and five genera with a total of 64 nucleotide variable sites.

Neotropical cichlids presented a higher amount of variable sites for this gene when compared to African cichlids. Moreover, variation sites do not overlap, even though some relationship involving environment, nutrition and species behavior seems to exist. The species under analysis are very distinct from each other. Furthermore, it seems

that the period since speciation among Neotropical cichlids is superior (López-Fernández et al. 2010) than the time elapsed since speciation of cichlids from African lakes, due to their adaptive radiation (Seehausen et al. 1999). Thus, the Neotropical species would accumulate more substitutions over time.

However, a recent study has shown a positive selection on the rhodopsin gene *RH1* in Neotropical cichlids where selective pressure would be divergent between lotic and lentic environment and the encoding sites of the protein under selection (Schott et al. 2014). Current analysis corroborated the above. Among the codons under selection, estimated by FUBAR and REL models, only one codon overlapped and the others codons were divergent. Since Neotropical cichlids presented a greater number of codons under selection, this fact showed that the gene in this group would be under different evolutionary pressures.

Only one variable site was found when compared to sequences of *LWS* opsin gene between species from the African cichlids of Lake Malawi. However, when compared to *Oreochromis niloticus*, an African cichlid from lotic environment, 22 amino acids differed (Carleton and Kocher 2001). These genes seem to be under an intense selection pressure due to the meaning of their ecological and behavior (Bowmaker 1995, Hofmann et al. 2012). Moreover, they differ from the analyzed nucleotide sequence for the coding regions, in which African and Neotropical cichlids form two monophyletic groups.

Although the great African lakes comprise a wide and extensive region, the area in which the Neotropical cichlids are distributed is probably wider and more dynamic, since it is made of various types of environments, for example, floodplain environments (Thomaz et al. 1997). Therefore, these species would be under more types of selective pressures which would, consequently, lead to a greater divergence, especially among such diverse genera as the ones studied here (Graça and Pavanelli 2007).

AN EVOLUTIONARY CONVERGENCE EVIDENCE?

Due to the *Cichla* basal characteristics (López-Fernández et al. 2010) and the *Crenicichla* derived character, the *LWS* opsin gene shows a region (site 119) that would converge evolutionarily as the *Crenicichla* genus. Although they show a greater divergence than that in African species, the coding sequence of the *LWS* opsin protein is closer to that of some species of African lakes than *Cichla* are. For example, amino acidic divergence between *Cichla piquiti* and *Cynotilapia afra* is 11.91%, while between *Crenicichla britskii* and *C. afra* is 9.23%.

Taking into consideration the convergent evolution hypothesis due to the distance between the groups (Arendt and Reznick 2008), a strong evidence of pressure by natural selection mechanism is presumed (Nagai et al. 2011). It is actually a viable hypothesis (1) from the point of view of the two groups: African and Neotropical cichlids form monophyletic branches in which a group is mostly limited to lentic environments and the other is subjected to a greater diversity of environments, respectively. Both groups would be under different kinds of pressure; (2) the two genera *Cichla* and *Crenicichla* are very different: a great divergence of *LWS* opsin gene is expected since it is a gene of ecological and behavioral relevance. However, despite higher amino acid similarity between some African species and *Crenicichla britskii* and C. *frenata*, many variable sites do not overlap.

CONCLUSIONS

Neotropical cichlids seem to accumulate more *LWS* opsin gene variability than African cichlids from lentic environments, probably due to the differences between the African lakes and Neotropical regions. The latter factor would exert

different selective pressures (Schott et al. 2014), considering the divergence of the estimated codons to be under selection and the speciation time of both groups. Therefore, studies involving other opsin genes and more species of the Neotropical cichlids group should be performed. Moreover, given the variability of this gene in Neotropical cichlids, studies analyzing the possibility of its use as a marker for species identification and assistance in phylogenetic studies should be encouraged.

Research involving opsin genes and species of Neotropical cichlids is just starting (Weadick et al. 2012, Schott et al. 2014) and seems to be highly promising. Further studies would contribute towards the understanding of the species' sensory system and their relationship with the dynamics of Neotropical environments.

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SUPPLEMENTARY MATERIAL

Table SI – Genetic distances according to JTT amino acid substitution model (under the diagonal) and K2P nucleotide substitution model (above the diagonal). with 1000 resampling of bootstrap. 1-10 = Neotropical cichlids; 11-28 = African cichlids (11 = lotic environment. Nile River; 12-15 = Lake Tanganyika; 16-17 = Lake Victoria; 18-28 = Lake Malawi).

Table SII – Amino acids variable sites (exons 2-4) and their respective position in *LWS* opsin protein structure, of the Neotropical cichlids and African cichlids. Obs.: the alignment was carried according with the positions of bovine rhodopsin. Yellow = possible convergent site.

Table SIII – Neotropical cichlids *LWS* codons under selection according to FUBAR model, with posterior probability >80%.

Table SIV – African cichlids *LWS* codons under selection according to FUBAR model, with posterior probability >80%.

Table SV – Neotropical cichlids *LWS* codons under selection according to REL model, with REV substitution model and posterior probability >80%.

Table SVI – African cichlids *LWS* codons under selection according to REL model, with REV substitution model and posterior probability >80%.