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Diversity in visual sensitivity across Neotropical cichlid fishes via differential expression and intraretinal variation of opsin genes

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Abstract

The visual system of vertebrates has greatly contributed to our understanding of how different molecular mechanisms shape adaptive phenotypic diversity. Extensive work on African cichlid fishes has shown how variation in opsin gene expression mediates diversification as well as convergent evolution in colour vision. This trait has received less attention in Neotropical cichlids, the sister lineage to African cichlids, but the work done so far led to the conclusion that colour vision is much less variable in Neotropical species. However, as only few taxa have been investigated and as recent work found contradicting patterns, the diversity in meotropical cichlids might be greatly underestimated. Here, we survey patterns of opsin gene expression in 35 representative species of Neotropical cichlids, revealing much more variation than previously known. This diversity can be attributed to two main mechanisms: (i) differential expression of the blue-sensitive *sws2a*, the green-sensitive *rh2a*, and the red-sensitive *lws* opsin genes, and (ii) simultaneous expression of up to five opsin genes, instead of only three as commonly found, in a striking dorsoventral pattern across the retina. This intraretinal variation in opsin genes expression results in steep gradients in visual sensitivity that may represent a convergent adaptation to clear waters with broad light environments. These results highlight the role and flexibility of gene expression in generating adaptive phenotypic diversification.

KEYWORDS
convergent evolution, Neotropical cichlids, opsin coexpression, opsin expression, visual pigments

1 | INTRODUCTION

Understanding how phenotypic diversity originates and why it is maintained remains one of the major unsolved questions in evolutionary biology. One of the biggest challenges has been to link adaptive phenotypic divergence (either genetic or plastic) with the underlying molecular mechanisms, although the advances of modern molecular techniques recently permitted important progress in this area (e.g., Barrett et al., 2019; Kratochwil et al., 2018). The visual system of vertebrates provides a well-suited model to address this challenge for several reasons (Carleton et al., 2020; Owens & Rennison, 2017; Yokoyama, 2000). It is highly variable within and across species (Bowmaker, 2008; Carleton et al., 2016; Carleton & Yourick, 2020; Cortesi et al., 2015; Fuller et al., 2004) allowing for comparisons at different phylogenetic scales that can be linked to the advanced knowledge of the physics and biochemistry of vision

(Rodieck, 1998; Johnsen, 2012). Furthermore, extensive work on visual ecology and visual system evolution (Cronin et al., 2014; Lythgoe, 1979) has permitted a more detailed understanding of the genotype-phenotype associations, the mechanisms involved in tuning visual sensitivities, and the adaptive value of particular mutations and molecular changes (Bowmaker, 2008; Cheng & Flamarique, 2004; Novales Flamarique, 2016). Visual sensitivity is particularly variable in teleost fishes and this variability appears to have evolved in response to the greatly diverse photic conditions of aquatic environments (Carleton et al., 2020; Lythgoe, 1984; Musilova et al., 2018; Partridge & Cummings, 1999; Rennison et al., 2012; Warrant & Johnsen, 2013). Among teleosts, cichlid fishes have emerged as a particularly interesting system to study diversification in visual sensitivity as they use multiple molecular mechanisms to tune their vision and they have done it convergently across different lineages (O'Quin et al., 2010; reviewed in Carleton et al., 2016; Carleton & Yourick, 2020). Variation in the pattern of opsin gene expression is one of the main mechanisms responsible for much of the visual diversity seen across cichlid fishes.

Opsin proteins bound to photosensitive chromophores constitute the visual pigments that mediate vision in the photoreceptors of the retina (Wald, 1968; Yokoyama, 2000). Colour discrimination is possible due to specialized cone photoreceptors that express different opsin genes resulting in visual pigments that have maximum absorbance at varying parts of the light spectrum. The number of genes that code for cone opsin proteins involved in colour vision varies among organisms from only two up to 16 (Cronin et al., 2014). Cichlid fish genomes generally contain seven cone opsin genes resulting in visual pigments with different maximum sensitivities (UV: *sws1*, violet: *sws2b*, blue: *sws2a*, blue-green: *rh2b*, short-green: *rh2aβ*, long-green: *rh2aα*, and yellow/red: *lws*; reviewed in Carleton & Yourick, 2020). However, adult cichlid fish commonly express only a subset of three opsin genes at any given time, one in single cone photoreceptors (either *sws1*, *sws2b*, or *sws2a*) and two in double cone photoreceptors (either *rh2b*, *rh2aβ*, *rh2aα*, or *lws*; Carleton & Yourick, 2020). African cichlids have diversified in adult visual sensitivity to an impressive degree by using different combinations of typically three out of the seven cone opsin genes encoded in their genomes (Carleton & Kocher, 2001; Parry et al., 2005). Three different cone opsin palettes have repeatedly and independently evolved in cichlids from two clear water African Great Lakes (Malawi and Tanganyika; Carleton & Kocher, 2001; Hofmann et al., 2009; O'Quin et al., 2010); a short (*sws1*, *rh2b* and one of the *rh2a*), a medium (*sws2b*, *rh2b* and one of the *rh2a*), and a long palette (*sws2a*, one of the *rh2a*, and *lws*). Thus, the visual system of African cichlid fish provides a compelling example of how changes in gene expression may underlie major phenotypic differences across species (Carleton et al., 2016; Carleton & Yourick, 2020) and of how convergent phenotypic evolution is due to similar or the same molecular mechanisms (Carleton et al., 2016; O'Quin et al., 2010).

Neotropical cichlids have long diverged from their African sister lineage (98.9–143.9 mya based on Irisarri et al., 2018; but only 40.9–54.9 Ma based on Friedman et al., 2013; Matschiner et al., 2017; Vences et al., 2001). These two sister lineages, with all their diversity arising in different continents and in distinct ecosystems, provide a great opportunity to study the evolution of their visual systems in a comparative framework (Carleton et al., 2020; Carleton & Yourick, 2020). Recently, it has been suggested that Neotropical cichlids might have reduced phenotypic variation in visual sensitivity compared to their African relatives (Carleton et al., 2020; Schneider et al., 2020; Weadick et al., 2012). Most studies so far found limited variation in the patterns of opsin expression, showing that adult Neotropical cichlids mainly rely on the "long opsin palette" (i.e., *sws2a*, one of the two *rh2a* paralogues, and *lws*; e.g., Escobar-Camacho et al., 2017, Escobar-Camacho, Pierotti, et al., 2019; Schneider et al., 2020; Weadick et al., 2012). This might be related to the murkier, long wavelength shifted light spectra that characterize many aquatic environments in the Neotropics (Costa et al., 2013; Escobar-Camacho et al., 2017; Torres-Dowdall et al., 2017). However, early work using microspectrophotometry identified more variation in overall spectral sensitivity among Neotropical cichlids (Loew & Lythgoe, 1978; Lythgoe, 1984; Muntz, 1973). Recent work in *Amphilophus astorquii* and *Amatitlania siquia*, two Amphilophine cichlids inhabiting Nicaraguan clear water crater lakes with broad light spectra enriched in short wavelengths, found that adult fish of these species also express opsin genes characteristic of a "medium opsin palette" (*sws2b* and *rh2b* in single and double cones, respectively) in addition to opsin genes common to the long palette (Härer et al., 2018; Torres-Dowdall et al., 2017). Photoceptors expressing these extra opsin genes are not randomly distributed across the retina but follow a pattern: receptors expressing more long wavelength sensitive opsins are dominant in the ventral retina and those expressing more short wavelength sensitive opsins are limited to the dorsal retina (Torres-Dowdall et al., 2017). To date, it remains unclear whether this patterning occurs in other Neotropical cichlids, or if it only evolved in Amphilophine cichlids inhabiting clear water crater lakes. Several other Neotropical cichlid lineages have colonized clear water environments in which shifting sensitivities toward shorter wavelengths might be adaptive. Hence, it could be expected that patterns of opsin expression convergent to those seen in Amphilophines have evolved in other lineages, as suggested by early microspectrophotometry studies (Muntz, 1973).

Here, we investigated the visual system of Neotropical cichlids on a broad phylogenetic scale to determine the phenotypic diversity that results from variation in opsin gene expression patterns and ask whether their visual system is, as previously suggested, less diverse compared to that of African cichlids. Based on results from previous (e.g., Muntz, 1973) and new (e.g.,Härer et al., 2018; Torres-Dowdall et al., 2017) studies, we predicted that Neotropical cichlids have a diverse visual system, both in terms of variation across species as well as intraretinal variation (e.g., Torres-Dowdall et al., 2017). To address this question, we surveyed opsin gene expression in 35 species of Neotropical cichlids representing five of the seven tribes including the three most speciose groups (i.e., Geophagini, Cichlasomatini and Heroini) by combining retinal transcriptomes, quantitative real-time PCR data

and published information. Specifically, we examined if (i) the long opsin palette is indeed the norm across Neotropical cichlids, and (ii) if the simultaneous expression of four or five opsin genes is restricted to a few species of Amphilophines. Finally, using triple fluorescent in situ hybridization (FISH), (iii) we assessed if the expression of more than three opsin genes at a time is associated with a specific patterning of the retina.

2 | MATERIALS AND METHODS

2.1 | Specimens sampled for this study

A total of 63 individuals from 26 species were used to survey cone opsin gene expression across the Neotropical cichlid phylogeny (see Figure 1 and Table S1 for details). Additionally, data on opsin gene expression were obtained from the literature for eight more species and a second population of *Amatitlania siquia*. Data form 15 of the 26 species were obtained from transcriptomic analyses. For most of these species, only a single adult individual was sampled, with the exception of *Geophagus brasiliensis* (*n* = 2), *Hypsophrys nematopus* (*n* = 6), *Laetacara curviceps* (*n* = 2) and *Parachromis managuensis* (*n* = 6) (Figure 1, Table S1). Eleven additional species were surveyed for cone opsin gene expression using quantitative real-time PCR (qPCR). Again, one single adult individual was included, except for *Dicrossus maculata* (*n* = 2), *D. filamentosus* (*n* = 6), *Acarichthys heckelii* (*n* = 7), *Apistogramma baenschi* (*n* = 6), and *Geophagus abalios* (*n* = 6). Finally, we performed triple fluorescent in situ hybridization (FISH) in retinas of *A. baenschi* (*n* = 2) as a representative of a species expressing the long-wavelength palette (i.e., *sws2a*, *rh2a*, and *lws*) and for *A. siquia* (*n* = 5), *D. filamentosus* (*n* = 6), and *A. heckelii* (*n* = 4) (all these specimens were the same used for qPCR) as representatives of species expressing more than three cone opsin genes at significant levels (Figure 1).

To minimize diurnal variation in opsin expression, all specimens were euthanized with an overdose of MS-222 (400 mg/l) between 14:00 and 17:00 (Yourick et al., 2019). Retinas were immediately dissected and the right one was stored in RNAlater (Sigma-Aldrich) until RNA extraction, whereas the left one was fixed in 4% paraformaldehyde in phosphate buffered saline at 4°C overnight and subsequently stored in methanol at −20°C for conducting in situ hybridization. Specimens from Nicaragua were collected during field trips in 2013 and 2015 under MARENA permits DGPN/DB-IC-004-2013 & DGPN/DB-IC-015-2015. Other cichlid species were obtained from the pet market (Figure 1) and euthanized in accordance with the rules of the Animal Research Facilities of University of Konstanz and the animal protection authorities of the State of Baden-Württemberg (permits T16/13TFA and T19/03TFA).

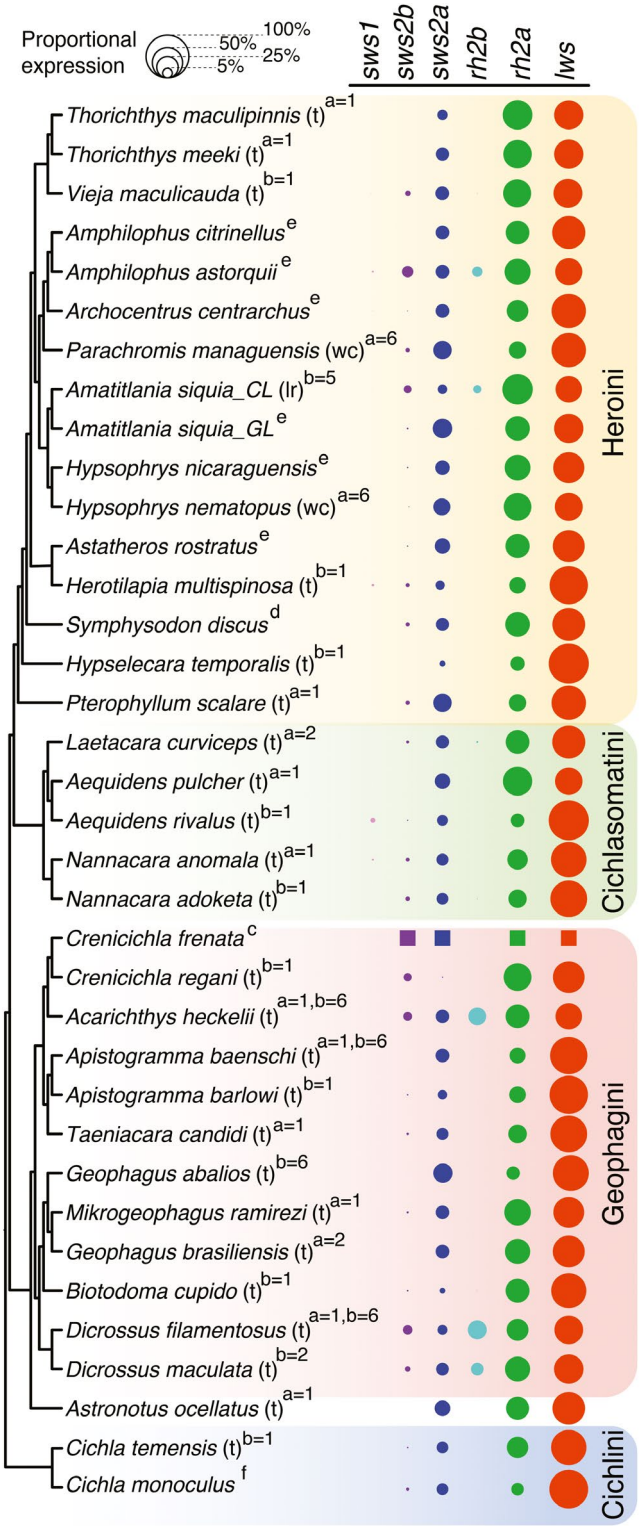

FIGURE 1 Variation in opsin expression patterns mapped onto a Neotropical cichlid phylogeny (modified from Ilves et al., (2018); branch lengths convey no information). The size of circles represent the proportional expression of each cone opsin gene. Letters within parentheses indicate the origin of samples used in this study (t, aquarium trade; lr, laboratory reared; wc, wild caught). Superscript letters indicate origin of data (a, transcriptomic data from this study, b, qPCR data from this study, c, data from Weadick et al., (2012); d, data from Escobar-Camacho et al., (2017); e, data from Härer et al., (2018); f, data from Escobar-Camacho, Pierotti, et al., (2019). Superscript numbers indicate sample size used in this study. Squares in *Crenicichla frenata* denote presence-absence in RNA, but no quantification is available (Weadick et al., 2012).

2.2 | Transcriptome analysis

To survey opsin gene expression in Neotropical cichlids, RNA was extracted from retinas using the commercial RNeasy Mini Kit (Qiagen). Concentrations were measured on a Qubit Fluorometer (Thermo Fisher Scientific). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were generated as described in (Härer et al., 2018), with the TruSeq Stranded mRNA HT sample preparation kit (Illumina) with an input of 30 ng of total RNA. The final library was amplified using 15 PCR cycles, and quantification and quality assessment were performed using the Agilent Bioanalyzer system. Individually labeled samples were pooled in equimolar aliquots and paired-end sequenced (2 × 150 bp) in one lane on an Illumina HiSeq2500 platform at TUCF Genomics (Tufts University, Massachusetts). As sequencing was done as part of a larger experiment, a total of 93 samples were pooled and information on total reads per sample is included in Table S1. Adapters were removed and reads were trimmed with Trimmomatic v0.36 (Bolger et al., 2014). For opsin expression analyses, trimmed reads were mapped against *A. citrinellus* reference sequences of 50 bp of the 5′ UTR and the first 150 bp of the coding sequence (CDS) for all cone opsins with bowtie 2.3.0 (Langmead & Salzberg, 2012) using default parameters. This approach follows Härer et al., (2018) and was preferred because *rh2aα* and *rh2aβ* underwent gene conversion (Torres-Dowdall et al., 2017). Only the 5′ UTR and the first exon show sequence variation whereas the rest of the CDS is identical, hence, reads could not be unambiguously assigned to either of the two paralogues for exons 2–5. Overall, mapping against the UTR and the first exon revealed similar results as when mapping against the full CDS (results not shown).

2.3 | Quantitative Real-Time PCR

Eleven additional species were surveyed for cone opsin gene expression using qPCR (Figure 1). For four selected species, qPCR was conducted in a larger number of specimens (Figure 2). These species were selected because in our survey (see results below and Figure 1) their opsin expression pattern departed from the norm of expressing only three opsin genes. RNA was extracted as described above and 500 ng to 1 µg of total RNA were reverse transcribed with a first-strand cDNA synthesis kit (GoScript Reverse Transcription System; Promega). qPCR was performed to quantify proportional expression levels for six cone opsin genes (*sws1*, *sws2b*, *sws2a*, *rh2b*, *rh2a* and *lws*) using specifically designed primers (Table S2; Härer et al., 2017; Torres-Dowdall et al., 2017). As for transcriptomic data, *rh2aα* and *rh2aβ* were not differentiated for qPCR analyses given that designing primers specific for each of the two paralogous is challenging due to gene conversion (Escobar-Camacho et al., 2017; Torres-Dowdall et al., 2017). Note that for some species it was not possible to estimate the efficiencies for primers designed to amplify *sws1*. Given that little to no sequence variation was seen in the binding site of the *sws1* primers (Table S3), most probably the lack of *sws1* signal is because this opsin does not seem to be expressed in adults of Neotropical cichlids. Furthermore, *sws1* pseudogenized in many Neotropical cichlid species (Escobar-Camacho et al., 2017; Hauser et al., 2021; Weadick et al., 2012). Proportional opsin expression was determined for each specimen by calculating the proportion of each opsin (T_i) relative to the total opsin expression (T_{all}), after Fuller et al., (2004):

$$\frac{T_i}{T_{all}} = \frac{\left(1/\left((1+E_i)^{Ct_i}\right)\right)}{\sum\left(1/\left((1+E_i)^{Ct_i}\right)\right)}$$

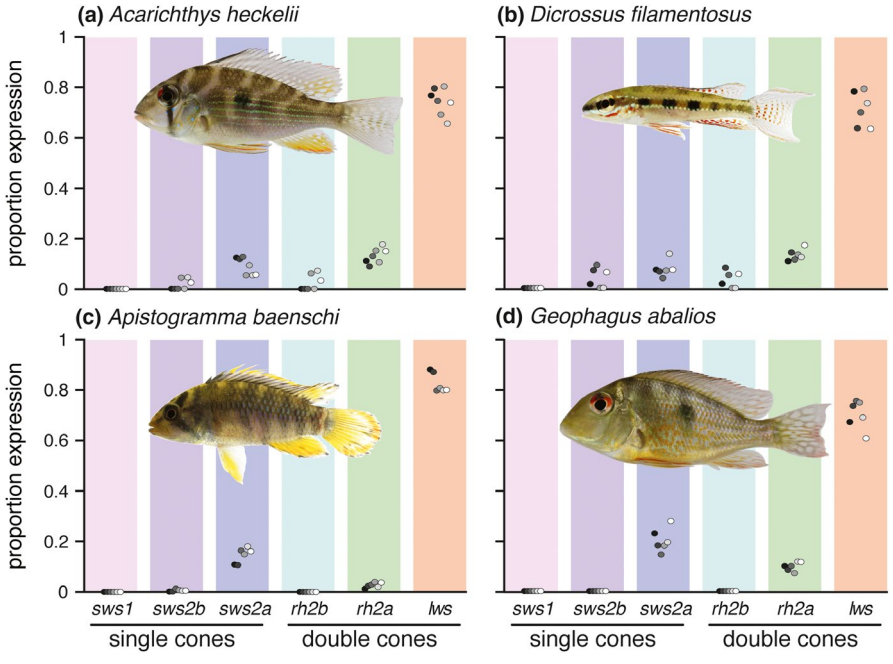

FIGURE 2 Pattern of opsin gene expression in four Geophagini species, with some species expressing up to five opsin genes at a time (a, b) and other related species expressing only three (c, d). Similar patterns were already observed in species of the tribe Heroini (Figure S1; Härer et al., 2018; Torres-Dowdall et al., 2017). Different symbol shades indicate different individuals that were used in this analysis. Six individuals per species were analysed, except for *A. heckelii* (n = 7).

where E_i represents the primer efficiency (Table S2) for primer *i* and Ct_i is the critical cycle number for gene *i* (the proportional expression values of the six opsins add up to 1 for each specimen).

2.4 | Weighted opsin gene expression as an estimation of predicted visual sensitivities

We estimated the weighted opsin gene expression (Carleton et al., 2016) using transcriptomic as well as qPCR data. Taking advantage of single cones expressing only *sws* genes and double cones expressing *rh2* and *lws* genes, weighted gene expression was calculated for single (X_{SC}) and double cones (X_{DC}) separately as:

$$X_{SC} = \frac{f_{sws1}X_{sws1} + f_{sws2b}X_{sws2b} + f_{sws2a}X_{sws2a}}{f_{sws1} + f_{sws2b} + f_{sws2a}}$$

$$X_{DC} = \frac{f_{rh2b}X_{rh2b} + f_{rh2a}X_{rh2a} + f_{lws}X_{lws}}{f_{rh2b} + f_{rh2a} + f_{lws}}$$

where f_i is the proportional expression of each opsin gene and X_i is a rank order of the opsin gene based on the spectral sensitivity of each of the opsins as in Carleton et al., (2016). This approach has the advantage that it allows to represent the diversity in visual sensitivity in a two-dimensional space and that it does not require assumptions about the peak of maximum absorption of each opsin gene for all the species included. Hence, weighted gene expression values could be interpreted as relative predicted sensitivity; individuals with lower values have predicted sensitivities at shorter wavelengths compared to individuals with higher values. Among others, differential opsin gene expression is a major factor in tuning visual sensitivities in cichlid fish (Carleton & Yourick, 2020). Thus, our value of predicted sensitivity can only be considered as an approximation to the visual sensitivity of the studied species.

2.5 | In situ hybridization

We performed triple fluorescent in situ hybridization (FISH) as previously described (Torres-Dowdall et al., 2017; Woltering et al., 2009). Retinas were either probed for all single cone opsin genes (*sws1*, *sws2b*, *sws2a*) or double cone opsin genes (*rh2b*, *rh2a*, *lws*). Fluorescence and differential interference contrast (DIC) images were obtained using a Leica DM6B microscope. The whole retina was photographed by sequentially taking images from different areas and later those photographs were stitched together using TrackEM2 in Image J (Schindelin et al., 2012; Tropea et al., 2017).

Photoreceptor identity maps were created by superimposing an eight by eight grid over whole retina compound images and counting between 100 and 200 photoreceptors at each of the 64 vertices of the grid (Figure S1). The count data was transformed to proportion of single cones that expressed *sws1*, *sws2b* or *sws2a* and proportion of double cones that expressed *rh2b*, *rh2a* or *lws*. In cases where transitions in cone photoreceptor identity were visually recognized, additional locations were counted to better capture these gradients. Topographic maps of retinas were constructed using the R-script by Garza-Gisholt et al., (2014) using the above-described photoreceptor identity maps as input data. A thin plate spline interpolation method with a smoothing parameter set at half the degree of freedom was used to construct the retinal maps (Garza-Gisholt et al., 2014). Given the steepness of the gradients seen in the analysed retinas, changing the smoothing parameter had little effect, therefore we present the results of using an intermediate smoothing degree.

3 | RESULTS

3.1 | Opsin gene expression across the Neotropical cichlid phylogeny

We conducted a phylogeny-wide survey on opsin gene expression patterns in 32 species of Neotropical cichlids by combining transcriptomic and quantitative-PCR data (n = 1–6 per species, Table S1). Additionally, opsin gene expression data for three more Neotropical cichlid species were extracted from the literature. All 35 species expressed the blue sensitive *sws2a*, one of the green sensitive *rh2a* paralogues, and the red sensitive *lws* (Figure 1), which corresponds to the long wavelength palette as described for African cichlids. However, there was a large degree of variation in the proportional expression of these three opsin genes. For example, *lws* represented more than 75% of total cone opsin transcripts in *Apistogramma baenschi*, whereas it constituted on average only 19% of the cone opsin transcripts in *Amatitlania siquia*. Similar variation was observed in the proportional expression of *sws2a*, it represented less than 6% of the cone opsin transcripts in *Thorichthys maculipinnis*, but above 25% in *Parachromis managuensis* (Figure 1; Table S1).

Several species of Neotropical cichlids expressed other opsins in addition to, but not in replacement of, *sws2a*, *rh2a* and *lws*. As previously described (Härer et al., 2018; Torres-Dowdall et al., 2017), two Amphilophine species inhabiting clear water crater lakes in Nicaragua (*Amphilophus astorquii* from Crater Lake Apoyo and *Amatitlania siquia* from Crater Lake Xiloá) expressed the violet sensitive *sws2b* and the blue-green sensitive *rh2b* opsins, but closely related species, or even populations as in *A. siquia*, inhabiting turbid lakes did not express these opsins (Figure S2). The same expression pattern was observed in three Geophagini species, *Acarichthys heckelii*, *Dicrossus filamentosus*, and *D. maculata* (Figure 1). qPCR analyses for multiple individuals of two of these Geophagini species validated the results but showed that there is intraspecific variation in cone opsin expression patterns (Figure 2). In these two species, only some of the individuals expressed *sws2b* and *rh2b* in addition to *sws2a*, *rh2a* and *lws*. qPCR was also performed in two other Geophagini species (*Apistogramma baenschi*, n = 6 and *Geophagus abalios*, n = 6) to determine if the intraspecific variation observed in *A. heckelii* and *D. filamentosus* was common to other species in this tribe. None of the analysed individuals of *A. baenschi* and *G. abalios* expressed *sws2b* or *rh2b* at

significant levels (i.e., above 1%, Figure 2). Across the Neotropical cichlid phylogeny, a few species beside those already mentioned also express *sws2b* at low levels but not *rh2b* (Figure 1).

3.2 | Relative cone opsin expression in Neotropical cichlids compared to African lineages

To summarize the diversity in the visual system of Neotropical cichlid fishes, due to variation in expression, we estimated the weighted opsin gene expression following Carleton et al., (2016). Neotropical cichlids occupy a large part of the weighted opsin gene expression space, expanding into the medium opsin expression palette (sensu Carleton et al., 2016; Figure 3). This is mostly due to the few species expressing *sws2b* in single cones and *rh2b* in double cones in addition to *sws2a*, *rh2a* and *lws*. None of the Neotropical species had single cone weighted expression values below 2, which reflects the fact that *sws1* was not expressed at substantial levels by any of the studied species as adults.

3.3 | Retinal patterning of opsin expression

To explore patterns of photoreceptor arrangement associated with variation in number of opsin genes expressed, we performed triple fluorescent in situ hybridization (FISH) in representative species expressing either three or five opsin genes simultaneously. We used retinas of *Apistogramma baenschi* (*n* = 2), as representative of a species expressing three opsins, and of *Amatitlania siquia* (*n* = 5), *Acarichthys heckelii* (*n* = 4), and *Dicrossus filamentosus* (*n* = 6), as representatives of species expressing five opsins. Retinas were either probed for single (*sws1*, *sws2b*, *sws2a*) or double cone opsin genes (*rh2b*, *rh2a*, *lws*). Due to technical difficulties only images from single cones were obtained from *A. heckelii* (Figure S3).

FISH confirmed the patterns of expression seen in the transcriptomic analysis and the qPCRs. In *A. baenschi*, a species expressing the long-wavelength palette (i.e., *sws2a*, *rh2a*, and *lws*) with *lws* contributing more than 75% of total cone opsin expression (Figure 2), FISH showed that this is translated into most double cones being twin cones with *lws* expressed in both members of the photoreceptor. In parts of the retina of *A. baenschi*, almost 90% of the double cones expressed *lws* (Figure 4a, Figure S4).

Individuals of *A. siquia* and *D. filamentosus* that expressed more than three opsin genes in their retina showed a clear dorsoventral patterning, with *sws2b* and *rh2b* being expressed only in the dorsal retina (Figure 4). In these species, all single cones expressed *sws2b* in the dorsal retina and *sws2a* in the ventral retina, but the transition from *sws2b* to *sws2a* single cones differed among species. In *A. siquia* the transition was gradual, with areas of the retina expressing the two single cone opsin genes in interspersed photoreceptors (Figure 4b, Figure S5). The transition was abrupt in *D. filamentosus*, with *sws2a* abruptly replacing *sws2b* in single cones, almost forming a hard discontinuity (Figure 4c; Figure S6). The same abrupt patterning was observed in single cones of *A. heckelii* (Figure S3). Intraretinal variation was also seen in double-cone photoreceptors. Around 50% of the double cones expressed *rh2b* in the dorsal retina of *A. siquia*

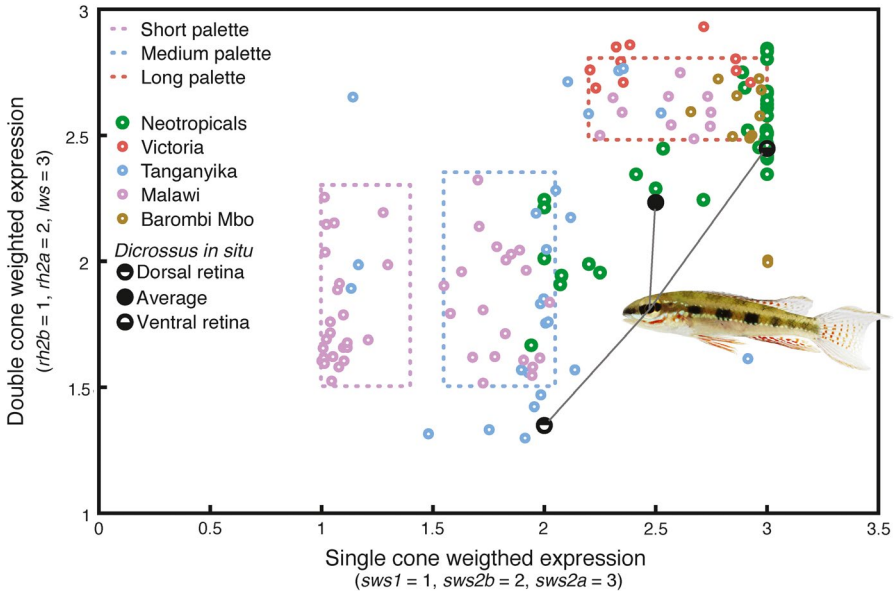

FIGURE 3 Weighted opsin gene expression in single versus double cones for African and Neotropical cichlids (modified from Carleton et al., 2016). Dotted line areas represent the phenotypic space of the three common patterns of opsin gene expression in African cichlids (short, medium and long palettes). The weighted opsin expression pattern of *Dicrossus filamentous* is depicted in black. The full black circle is the weighted expression averaged over the whole retina, the other two represent the weighted expression in the dorsal and the ventral retina. Original data of Malawi cichlids from (Hofmann et al., 2009), of Tanganyika and Victoria cichlids from (O'Quin et al., 2010), of Barombi Mbo cichlids from (Musilova et al., 2019), and of Neotropical cichlids from this study and Escobar-Camacho et al., (2017), Escobar-Camacho, Pierotti, et al., (2019) and Härer et al., (2018).

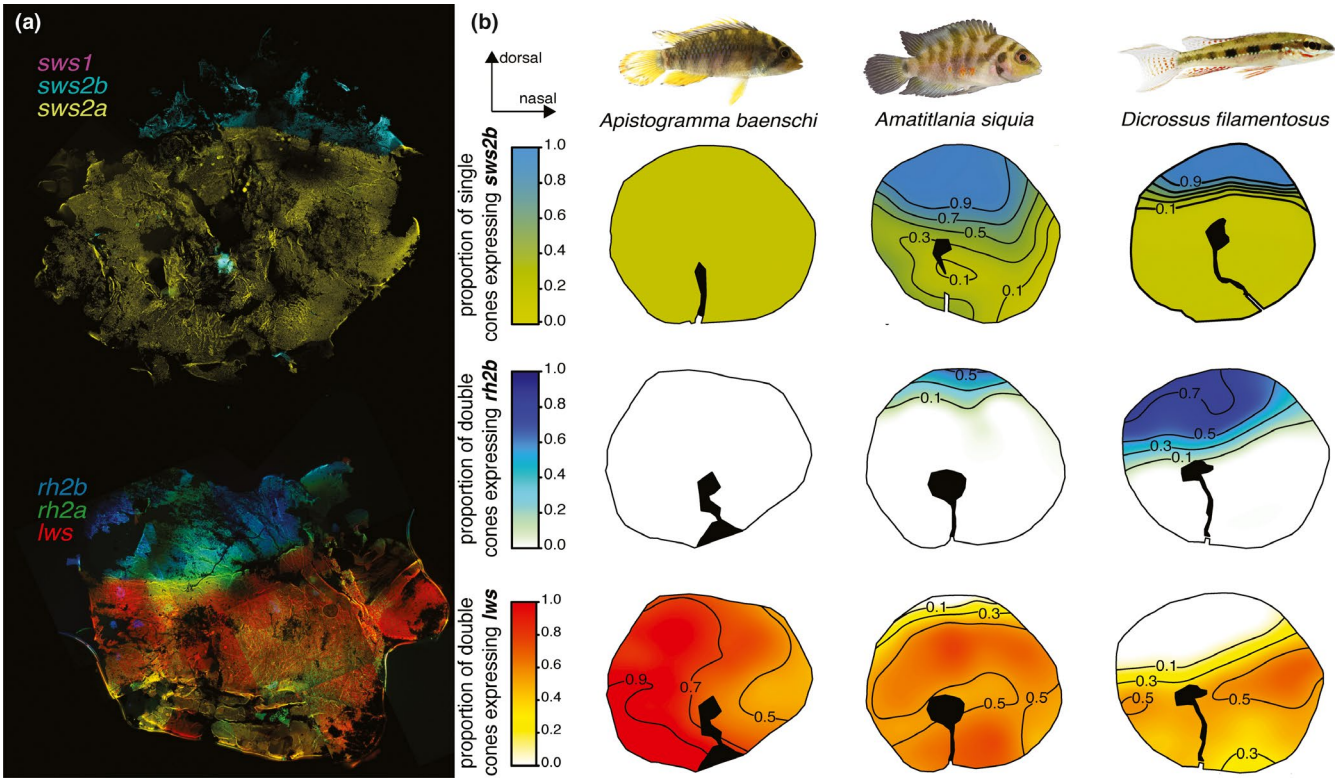

FIGURE 4 (a) Example of in situ hybridization on full retinas of *Dicrossus filamentosus* showing the spatial distribution of photoreceptors expressing different opsin genes. The retina in the top has been stained for the three opsin genes expressed in single cones (notice that *sws1* was not expressed in these adult specimens). The retina at the bottom has been stained for the three opsin genes expressed in double cones. (b) Retinal maps of cone photoreceptor identity (based on the opsin gene expressed) show distinct dorsoventral patterning in some Neotropical cichlid species. The first row of retinas shows the proportion of single cones expressing *sws2b* (as *sws1* was not expressed in any of these adult specimens, *sws2a* expression pattern can be inferred as the inverse of *sws2b*). The second and third rows of retinas show the proportion of double cones expressing *rh2b* and *lws*, respectively, across the retina (the pattern of *rh2a* could be inferred from the other two). The shape of the retinal maps was simplified to be approximately circular to aid visualization. Raw data for *D. filamentosus* (n = 6), *A. siquia* (n = 5), and *A. baenschi* (n = 2) stained retinas is presented in Figures S4–S6.

and up to 70% in *D. filamentosus* (Figure 4), but again the gradient differed between species. In the dorsal retina of *A. siquia*, *rh2b* was expressed in one member of double cones with the other member expressing *rh2a* or *lws*, but the latter was less common in the most dorsal part (Figure 4b). In *D. filamentosus*, most double cones expressed the *rh2a-rh2b* or *rh2a-lws* combinations and the transition between these two was also abrupt (Figure 4b; Figure S6).

4 | DISCUSSION

For many animals the visual system is the primary sensory system conveying information from the environment to the organism. As such, the visual system is under constant selection and in cichlid fishes it has been shown to have accumulated ample adaptive phenotypic divergence (Härer et al., 2018; Hofmann et al., 2009; Musilova et al., 2019; O'Quin et al., 2010; Torres-Dowdall et al., 2017; reviewed in Carleton & Yourick, 2020). We surveyed 35 species of Neotropical cichlids, a lineage that has been understudied compared to its African sister lineage in terms of its visual system, revealing substantial phenotypic variation resulting from different patterns of opsin gene expression (Figure 3). Like few other African cichlid species, some Neotropical species express four or five opsin genes simultaneously, departing from the common trichromatic pattern (reviewed in Carleton et al., 2016; Carleton & Yourick, 2020). Unlike other cichlid lineages, the expression of these "extra" opsins results in patterned retinas suggesting spatial subfunctionalization of the dorsal and ventral parts of the retina.

4.1 | Neotropical cichlids show substantial variation within their characteristic long wavelength sensitive cone opsin palette

The three visual opsin palettes characteristic of African cichlids (Carleton et al., 2016) are not well represented in Neotropical cichlids, as most species in our studied expressed *sws2a* in single cones and *rh2a* and *lws* in double cones (e.g., the long palette; Figure 1). One plausible explanation for the consistency of the long visual palette is that the largest part of the cichlid radiation in the Neotropics occurred in rivers (Ilves et al., 2018; Lopez-Fernandez et al., 2010), contrary to Africa where most of the diversity is found in the Rift

Valley Great Lakes (Brawand et al., 2014). Many of the riverine environments and some important lacustrine systems in the Neotropics have stained waters or carry significant amounts of suspended particles that tend to filter out short wavelength light resulting in a characteristic long wavelength shifted photic environment (Costa et al., 2013; Escobar-Camacho et al., 2017; Torres-Dowdall et al., 2017). Thus, it was expected that Neotropical cichlids generally have retinas most sensitive toward longer wavelengths (Carleton et al., 2020; Hauser et al., 2021; Muntz, 1973; Weadick et al., 2012). In fact, the longest wavelength sensitive opsin, *lws*, commonly contributed the largest proportion to the total cone opsin expression resulting in retinas with many twin double cones (i.e., double cones expressing the same opsin in both photoreceptor members), as exemplified in *A. baenschi* (Figure 4). While this general pattern holds, there is considerable variation among Neotropical cichlid species in the relative proportions at which the three different opsin genes of the long palette are expressed (Figure 1). Divergence in proportional expression is understood as an adaptive mechanism, as it frequently underlies predictable phenotypic variation across populations that occupy different photic environments (e.g., Escobar-Camacho, Pierotti, et al., 2019; Härer et al., 2018) or different microhabitats within environments (e.g., Musilova et al., 2019). Despite the importance of variation in proportional expression of the three opsins characteristic of the long palette, the strongest degree of variation among Neotropical species, both in terms of relative sensitivity predicted from opsin genes expression (Figure 3) and their patterns of intraretinal variation (Figure 4), is the result of expressing additional opsin genes (i.e., *rh2b* and *sws2b*).

4.2 | Multiple lineages of Neotropical cichlids express five cone opsin genes as adults

Five of the studied species were found to express five different opsin genes at substantial proportions (Figures 1 and 4; see also Härer et al., 2018; Torres-Dowdall et al., 2017). These species are spread out across the Neotropical cichlid phylogeny (Ilves et al., 2018) and close relatives to all these species expressed only the three opsin genes of the long wavelength palette (i.e., *lws*, *rh2a* and *sws2a*; Figures 1 and 2). This could be interpreted as convergent evolution of opsin expression patterns among Neotropical cichlids, potentially associated with the colonization of clear waters (Härer et al., 2018; Torres-Dowdall et al., 2017). Alternatively, unaccounted environmental effects during development could also explain phenotypic differences among these closely related species. In the case of *A. astorquii* and *A. siquia*, there is convincing evidence that shorter wavelength sensitivity evolved as an adaptation to clear water as phenotypic differences are maintained under common garden conditions (Härer et al., 2018; Torres-Dowdall et al., 2017). However, we have no information on the specific photic environments for the other species, and it would be interesting to incorporate this information in future studies to disentangle the contribution of adaptive evolution and phenotypic plasticity on visual phenotypes. Early reports on the spectral sensitivity of Neotropical cichlids found *A. heckelii* to inhabit relatively clear water environments and to have retinas with overall short wavelength sensitivity compared to other cichlid species (Muntz, 1973). *Dicrossus* spp. are form the Orinoco and Amazon drainages (Kullander, 2011), which have very variable photic conditions (Costa et al., 2013). The Tapajós River is the origin of *D. maculatus* and it has clear waters with a short wavelength shifted light spectrum (Costa et al., 2013). *D. filamentosus* is more widely distributed occurring in streams and rivers in the Amazon and Orinoco drainages (Kullander, 2011), but in this case, some of these rivers have light environments with long wavelength shifted spectra, like Río Negro (Costa et al., 2013). In any case, the simultaneous expression of five opsin genes resulted in retinas with overall predicted sensitivities that overlap with cichlids from the clear African Lakes Malawi and Tanganyika expressing a medium wavelength palette (Figure 3). Extensive work on opsin gene expression in African cichlids has shown that the medium (and short) sensitive palette evolved convergently in fish from Lakes Malawi and Tanganyika (reviewed in Carleton et al., 2016; Hofmann et al., 2009; O'Quin et al., 2010). Based on the results presented here, it could be argued that medium wavelength sensitivity evolved as well in the Neotropical cichlid lineage, potentially multiple times independently. Yet, this phenotypic convergence across African and Neotropical cichlids does not seem to be produced by convergent changes in the pattern of opsin gene expression. In fact, the attempt to categorize the visual system of Neotropical cichlids into the three visual palettes originally defined for African cichlids from Lake Malawi does not work effectively, as variation in the Neotropical lineage seems to be continuous rather than discrete (Figure 3). None of the Neotropical species studied here expressed only –or mostly– the three opsin genes that characterize the medium palette (*sws2b*, *rh2a*, *rh2b* sensu Carleton et al., 2016). Many species from Malawi and Tanganyika also depart from this simple trichromatic pattern by expressing four and occasionally five opsin genes simultaneously (Hofmann et al., 2009; O'Quin et al., 2010; Sabbah et al., 2010). In situ hybridization analyses of the African cichlid *Maylandia zebra* from Lake Malawi show that these extra opsins are not expressed individually in cones but are rather coexpressed with one of the predominantly expressed opsins within one photoreceptor (Dalton et al., 2014, 2017). In the Neotropical species studied here, there is some coexpression, but by far the most common fate of *sws2b* and *rh2b* is to be individually expressed in single cones and one member of double cones, respectively. This suggests that convergent changes in the overall predicted sensitivity of retinas of African and Neotropical cichlids could be based on nonconvergent patterns of gene expression at the individual photoreceptor cell level.

4.3 | Convergent patterns of intraretinal variation in cone opsin expression in Neotropical cichlids

The spatial arrangement of photoreceptors expressing different opsin genes is not random but highly patterned in Neotropical

cichlids, with *rh2b* and *sws2b* mostly being expressed in the dorsal retina and *lws* and *sws2a* in the middle and ventral retina (Figure 4, Figures S5 & S6). Patterned retinas have been reported for other fish species (reviewed in Temple, 2011). For example, a strong dorsoventral pattern of variation in opsin expression is seen in the archerfish (*Toxotes chatareus*; Temple et al., 2010) and the four-eyed fish (*Anableps anableps*; Owens et al., 2011), two fish species that live and perform ecologically relevant visual tasks at the interface between air and water (Owens et al., 2011; Temple et al., 2010). Intraretinal variation has also been described for cichlid fish (e.g., Dalton et al., 2014, 2017; Levine et al.,1979; Torres-Dowdall et al., 2017). Changes in the proportion of double cones expressing two different opsins in each member or just the same opsin in both (i.e., twin cones) have been found in Neotropical cichlids before (Levine et al., 1979), which is exemplified in our study by *A. baenschi* that presents a large proportion of twin *lws* cones in its nasal retina (Figure 4). In African cichlids, intraretinal variation results from the pattern of coexpression, with single cones coexpressing opsins all across the retina but in the foveal area, and double cones having a dorsoventral pattern of coexpression (Dalton et al., 2014, 2017). Intraretinal variation has also been documented for a Neotropical cichlid species (*Amphilophus astorquii*, Torres-Dowdall et al., 2017). However, the intraretinal changes in cone photoreceptor identity, exemplified in its most extreme form by *D. filamentosus*, has not been reported in cichlids before to the best of our knowledge. Actually, such an extreme dorsoventral pattern even rivals that seen in fish that live at the interface of air and water, such as the four eyed fish (*A. anableps*, Owens et al., 2011).

The intraretinal variation observed in some Neotropical cichlids is intriguing and it would be important to determine its biological relevance. The functional importance of patterned retinas has been extensively discussed, with some authors suggesting an adaptive value to it (e.g., Dalton et al., 2014; Temple et al., 2010) and others claiming that it is the outcome of nonadaptive developmental constraints (e.g., Ahnelt et al., 1995; Gaillard et al., 2009; reviewed in Temple, 2011). The main limitation to reaching a consensus is the still poor understanding of the visual tasks performed in nature, but support for an adaptive value of patterned retinas is slowly accumulating (Carleton et al., 2020). For example, electrophysiological analyses in northern anchovy (*Engraulis mordax*) demonstrated that intraretinal variation in opsin gene expression results in differences in visual sensitivity across the retina (Savelli & Flamarique, 2018). Moreover, intraretinal variation in opsin gene expression in the guppy (*Poecilia reticulata*, Rennison et al., 2011) seems to affect performance as individuals showed differential behavioral responses to light stimuli coming from the top versus the bottom in a wavelength specific manner (Sibeaux et al., 2019). In Neotropical cichlids, there are certain lines of evidence that would suggest an adaptive value to the intraretinal variation in cone identity. Patterned retinas have presumably convergently evolved in different lineages, suggesting a functional importance of this phenotype. However, developmental constraints could also produce a similar result at the phylogenetic level if selection to shift sensitivities toward shorter wavelengths produces patterned retinas as a byproduct (Szél et al.,1996). This could be the case if ontogenetic changes occur gradually across the retina. Although such information is not available, plastic changes in opsin expression do appear to follow a dorsoventral pattern, with changes occurring earlier in the ventral versus the dorsal retina (Härer et al., 2019). Nevertheless, the observation that this phenotype is associated with clear water environments (in at least four of the five species, see above) also suggests a functional role. Patterned retinas with short wavelength sensitivity in the dorsal retina, as seen in these Neotropical cichlid species, would be adaptive for fish foraging in the shallow of clear waters as this "blue-sensitive dorsal retina will *see* the background as dark, but suspended objects will look relatively brighter" (Lythgoe, 1984). For a long time, a goal of visual ecology has been to collect more pertinent behavioral data that allows to better evaluate the importance of intraretinal variation (Carleton et al., 2020; Levine et al., 1979; Temple, 2011), and among the species presented here, especially *D. filamentosus*, provides a promising system to advance knowledge in this area. Interesting behavioural assays to determine visual sensitivity were already implemented in cichlids (Escobar-Camacho, Taylor, et al., 2019) and other fish species (Sibeaux et al., 2019).

4.4 | Conclusions

The answer to the main question of this study – is the visual system of Neotropical cichlids less diverse than that of African cichlids? – is a loud and clear "it depends". Here, substantial quantitative and qualitative variation in opsin gene expression was identified for Neotropical cichlids, resulting in high interspecific differences in predicted visual sensitivity. Still, this variation falls short from that seen in cichlids from Lakes Malawi and Tanganyika. But in other African cichlids outside of those two very clear lakes, the diversity of visual sensitivities accounted by changes in opsin gene expression is relatively low as well (Carleton et al., 2005; Carleton et al., 2008; Fernald & Liebman, 1980; Hofmann et al., 2009; but see Musilova et al., 2019). Moreover, some Neotropical cichlid species show an overall shift in visual sensitivity toward shorter wavelength, which is convergent with that in African cichlids with medium sensitive opsin palettes. However, Neotropical cichlids might achieve this through nonconvergent underlying molecular mechanisms compared to African cichlids, as the drastically different dorsal and ventral sensitivities of these Neotropical cichlids have not been described before. Overall, these results highlight the astonishing flexibility of the visual system and the important role of changes in gene expression patterns in generating phenotypic diversity.

AUTHOR CONTRIBUTIONS

All authors developed the project. Andreas Härer, Nidal Karagic and Julián Torres-Dowdall collected and analysed the data. Julián Torres-Dowdall wrote the manuscript with revisions from all authors.

DATA AVAILABILITY STATEMENT

The short-read data and associated information have been archived in NCBI SRA database under the Bioproject accession number PRJNA701195 (SAMN15041046-SAMN15041151). A list of samples used in this study, including the origin of each specimen, is presented in Table S1. The sequences derived from this study are deposit in Genbank accession numbers MW588216-MW588262, MW591504-MW591516, and MW625376-MW625378 (Table S3).

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SUPPORTING INFORMATION

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

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