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FROM THE COVER

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Evolution of the visual sensory system in cichlid fishes from crater lake Barombi Mbo in Cameroon

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Abstract

In deep-water animals, the visual sensory system is often challenged by the dim-light environment. Here, we focus on the molecular mechanisms involved in rapid deep-water adaptations. We examined visual system evolution in a small-scale yet phenotypically and ecologically diverse adaptive radiation, the species flock of cichlid fishes in deep crater lake Barombi Mbo in Cameroon, West Africa. We show that rapid adaptations of the visual system to the novel deep-water habitat primarily occurred at the level of gene expression changes rather than through nucleotide mutations, which is compatible with the young age of the radiation. Based on retinal bulk RNA sequencing of all eleven species, we found that the opsin gene expression pattern was substantially different for the deep-water species. The nine shallow-water species feature an opsin palette dominated by the red-sensitive (LWS) opsin, whereas the two unrelated deep-water species lack expression of LWS and the violet-sensitive (SWS2B) opsin, thereby shifting the cone sensitivity to the centre of the light spectrum. Deep-water species further predominantly express the green-sensitive RH2Aα over RH2Aβ. We identified one amino acid substitution in the RH2Aα opsin specific to the deep-water species. We finally performed a comparative gene expression analysis in retinal tissue of deep- vs. shallow-water species. We thus identified 46 differentially expressed genes, many of which are associated with functions in vision, hypoxia management or circadian clock regulation, with some of them being associated with human eye diseases.

KEYWORDS
adaptation, cichlids, colour vision, gene duplication, opsin genes, photoreceptor

1 | INTRODUCTION

Vision in vertebrates is accomplished by two basic types of photoreceptor cells in the retina: rod cells responsible for scotopic (dim-light) vision and cone cells responsible for colour vision during photopic conditions with a sufficient light intensity (Ebrey & Koutalos, 2001). At the molecular level, photoreception is initiated by a photon-induced conformational change of a light-sensitive visual pigment composed of the chromophore covalently linked to opsin protein (Lamb, Collin, & Pugh, 2007). Different opsin proteins produce visual pigments that vary in the maximum spectral sensitivity (λ_{max}) within the light spectrum with each visual pigment being most sensitive to a

particular part of the spectrum (Yokoyama, 2008). While most vertebrates possess only one rod opsin protein (RH1; but see Musilova et al., 2019) characteristic for the rod cells, there are, depending on the taxon, up to four groups of cone opsins in vertebrates, short-wavelength sensitive (SWS1, SWS2), middle-wavelength sensitive (RH2) and long-wavelength sensitive (LWS). Birds, for example, have four cone opsin genes in their genome, while mammals have only two (with the exception of some primates, including human, with three cone opsins sensitive to the blue, green and red part of the light spectrum; Ebrey & Koutalos, 2001). Teleost fishes, on the other hand, have increased their opsin gene repertoire compared to other vertebrates by repeated gene duplication events (Cortesi et al., 2015; Rennison, Owens, & Taylor, 2012), resulting in a median number of seven cone opsin genes in their genomes (Musilova et al., 2019).

Cichlid fishes are one of the most diverse teleost families and well-known for their ability to rapidly diversify into various ecological niches through adaptations to a wide range habitats and with respect to different depth ranges, food sources and/or feeding modes (Brawand et al., 2014; Salzburger, 2018). Cichlid fishes, and in particular the exceptionally species-rich cichlid assemblages in the East African Great Lakes Victoria, Malawi and Tanganyika, are also well-known as classic models for adaptive radiation (Malinsky et al., 2018; Muschick, Indermaur, & Salzburger, 2012; Seehausen, 2006; Verheyen, Salzburger, Snoeks, & Meyer, 2003). Less species-rich cichlid assemblages have been reported from various water bodies, for example from small crater lakes in Africa (Malinsky et al., 2015; Schliewen, Tautz, & Paabo, 1994) or Nicaragua (Barluenga, Stölting, Salzburger, Muschick, & Meyer, 2006; Kautt, Elmer, & Meyer, 2012), permitting important insights into the early phases of incipient speciation.

Adaptations in the visual system, be they through mutations in key spectral tuning sites in opsin genes shifting λ_{max} or via differential opsin gene expression, have previously been implicated with cichlid diversification (reviewed in Carleton, Dalton, Escobar-Camacho, & Nandamuri, 2016), for example in speciation via sensory drive in Lake Victoria cichlids (Seehausen et al., 2008) or in trophic diversification in Lake Malawi cichlids (Hofmann et al., 2009). Similarly, in crater lake Massoko (Africa), two recently diverged cichlid ecomorphs possess different alleles of the rod opsin (RH1) gene correlating with the different depths at which they occur (Malinsky et al., 2015). On a larger phylogenetic scale, RH1 shows similar amino acid substitutions in deeper living cichlid species in lakes Malawi and Tanganyika, functionally shifting the rod opsin protein to be more sensitive in the wavelength prevalent in the deep-water habitat (Nagai et al., 2011; Sugawara et al., 2005). Furthermore, it has been shown in Lake Malawi cichlids that different opsin gene expression profiles (also called alternative visual palettes) originate from similar gene contents in different species, representing in some cases adaptations of the visual system to a particular trophic niche, such as zooplankton feeding or algae scraping (Hofmann et al., 2009). Differences in opsin gene expression therefore allow the differential tuning of the visual system, even if the DNA sequences of the genes are very similar or identical between species.

With eight different visual opsin genes in their genomes (one rod and seven cone opsins; Carleton et al., 2016), cichlids are known to have a pretty good colour perception system and to be sensitive across a broad range of the light spectrum, ranging from UV (using the short-wavelength-sensitive opsin 1 gene; SWS1) through violet and blue (SWS2A and SWS2B), the middle-range (RH2Aα, RH2Aβ and RH2B) to red light (long-wavelength-sensitive opsin gene; LWS) (Carleton et al., 2016). Typically, not all of these visual opsin genes are expressed at the same time in the cichlids' retina (Carleton et al., 2016; Dalton, Loew, Cronin, & Carleton, 2014). Furthermore, it has been shown that the cones in the retina of cichlids are organized in the form of a mosaic with four double cones (each composed of two joint cone cells) surrounding one single cone (Fernald, 1981). Longer-sensitive opsin genes (RH2B, RH2Aα, RH2Aβ, LWS) are typically expressed in the double cones, while shorter-sensitive opsin genes (SWS1, SWS2B, SWS2A) are expressed exclusively in single cones (Dalton et al., 2014).

Here, we examine molecular adaptations in the opsin gene repertoire of the cichlid species assemblage from crater Lake Barombi Mbo in Cameroon (West Africa) in relation to the environment and the trophic ecology of the species, and with a particular focus on adaptations towards deep-water habitats with limited light penetration. Crater lake Barombi Mbo has a diameter of only 2.5 km but is more than 110 m deep (Trewavas, Green, & Corbet, 1972; Figure 1a) and hosts eleven endemic species of cichlid fishes (*Konia dikume*, *K. eisentrauti*, *Myaka myaka*, *Pungu maclareni*, *Sarotherodon steinbachi*, *S. lohbergeri*, *S. linnellii*, *S. caroli*, *Stomatepia mariae*, *St. mongo* and *St. pindu*; Figure 1c), which probably arose in situ following a colonization event no earlier than 1 million years ago (Cornen, Bande, Giresse, & Maley, 1992; Richards, Poelstra, & Martin, 2018; Schliewen & Klee, 2004). During this time frame, the species have diversified with respect to ecology, as documented by their trophic niche specialization (Baldo et al., 2017), as well as with respect to their water depth preference (Trewavas et al., 1972). While nine out of the eleven Barombi Mbo cichlid species are commonly and syntopically found in the shallow littoral zone of the crater lake, two species have colonized the deep-water zone: (a) *K. dikume* is an obligatory deep-water specialist that inhabits the zone at around 20 m of depth (Trewavas et al., 1972); and (b) *M. myaka* is a seasonal deep-water species that occurs in the deep-water habitat during the dry season (November–April), yet migrates into the shallow littoral zone for spawning during the peak of the rainy season (June–August) (Trewavas et al., 1972). The species living in the deep-water environment in Lake Barombi Mbo have to cope with lower light intensity and a narrower light spectrum (lacking the red and UV/violet range), they face a higher water pressure, and they have to deal with an oxycline and anoxic conditions below 15–25 m (Figure 1b; Trewavas et al., 1972).

Here, we focused on molecular adaptations to the deep-water habitat and we interpret them in the context of general patterns in the teleost fishes including the deep-sea fish. We combined a comparative transcriptomic analysis of retinal tissue with a candidate gene approach, that is, we inspected retina expression profiles

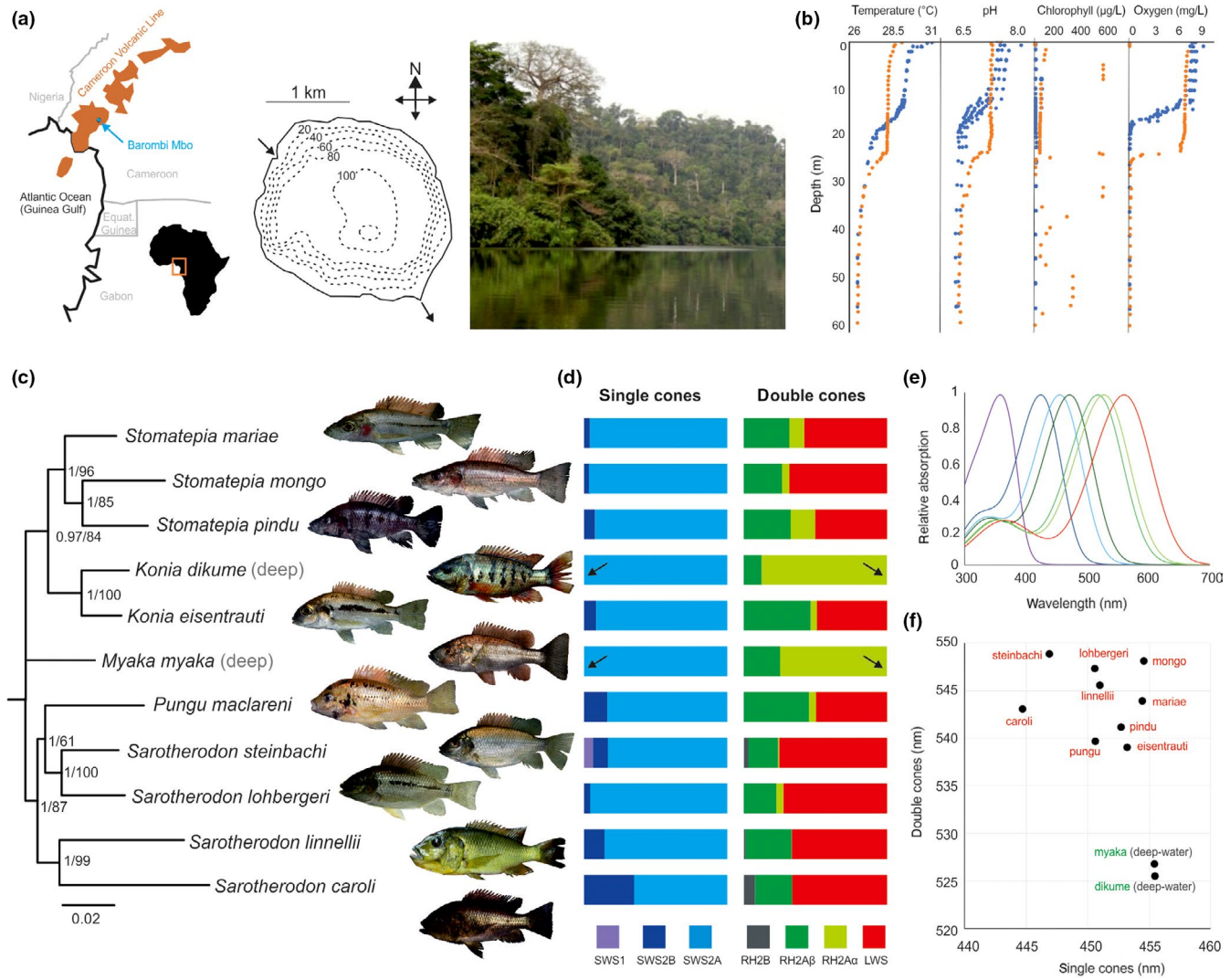

FIGURE 1 The visual system of the cichlid fishes from the crater lake Barombi Mbo. (a) Crater lake Barombi Mbo is located in South-West Cameroon in West Africa. The simplified topographic map is redrawn after Schliewen et al. (1994). (b) Limnological parameters in different depths of the crater lake measured with YSI 6600v2 sonde in the dry season (blue dots, 22 February 2017, three measurements), and the rainy season (orange; 27 July 2017; one measurement) show the presence of an oxy- and thermocline around 15–25 m of depth, the habitat of *Konia dikume*. (c) Phylogenetic hypothesis based on retinal transcriptome sequences of all 11 cichlid species from Barombi Mbo using Nile tilapia as an outgroup (based on 3,543 nuclear transcripts with 41,602 nonambiguous sites). The depicted Bayesian inference phylogeny was reconstructed with MRBAYES (Bayesian posterior probabilities and RAXML bootstrapping are shown for each branch). (d) Opsin gene expression profiles of all 11 Barombi Mbo cichlid species based on the retina transcriptomes and qPCR. For each species, the cone opsin expression levels are shown per gene and as proportions of the total cone opsin expression per species, separated into single and double cones. In the double cones of both deep-water species, *Konia dikume* and *Myaka myaka*, the LWS opsin is virtually not expressed and RH2Aα is more abundant than RH2Aβ. In the single cones, the deep-water species lack SWS2B expression and only show expression of SWS2A. Contrarily, in all shallow-water species more than three cone opsin genes are expressed. Interestingly, in the genera *Konia*, *Pungu*, *Stomatepia* and *Myaka*, both copies of the RH2A (i.e., RH2Aα and RH2Aβ) are expressed, while the genus *Sarotherodon* generally uses only one copy, RH2Aβ. SWS1 is only expressed in *S. steinbachi*. For further details on the interindividual variation see Figure S1 and for the exact values of the proportional opsin gene expression see Table S2. (e) Maximum spectral sensitivities of the seven cone opsins in the Nile tilapia (λ_{max} values taken from Spady et al., 2006, using equations of Govardovskii et al., 2000), a close relative of the Barombi Mbo cichlids. (f) Function plot of the visual system of the Barombi Mbo species calculated from the proportion of the double and single cone opsin gene expression following Carleton et al. (2016) and using the λ_{max} spectral sensitivity values of Nile tilapia opsin genes as reference (Spady et al., 2006)

and opsin gene evolution between shallow- and deep-water cichlid species. We further examined the possibility for UV vision in Barombi Mbo cichlids and its possible link to different trophic source. Integrating various approaches such as molecular evolutionary analyses, transcriptomics and whole-mount FISH (fluorescent in situ hybridization) of retina, we present a scenario describing the functional modifications of the visual system in the Barombi Mbo cichlid adaptive radiation, a textbook example of sympatric speciation (Coyne & Orr, 2004; Musilova et al., 2014; Richards et al., 2018). We further report a differential utilization of two green-sensitive

TABLE 1 List of individuals used for the retina transcriptome analysis and opsin gene sequencing

Species	Transcriptome sequencing	Transcriptome code	Sex	Genbank accession numbers	Individuals analyzed by quantitative RT-PCR	Individuals sequenced by Sanger (8 opsin genes)
Konia eisentrauti (shallow-water species)	3	42_02D7	Male	SAMN12385063	8	8
		43_02C6	Male	SAMN12385064		
		44_01I3	Female	SAMN12385065		
Konia dikume (deep-water species)	3	39_02A1	Female	SAMN12385066	12	8
		40_02A2	Male	SAMN12385067		
		41_02A4	Male	SAMN12385068		
Myaka myaka (deep-water species)	3	59_01G6	Male	SAMN12385069	13	7
		60_01G7	Male	SAMN12385070		
		64_13A5	Female	SAMN12385071		
Stomatepia mariae (shallow-water species)	3	46_01H3	Male	SAMN10473299	10	7
		47_01H2	Female	SAMN10473300		
		57_01H7	Male	SAMN10473301		
Stomatepia pindu	1	pind01A1	Male	SAMN12385075	9	8
Stomatepia mongo	1	mong16F1	Male	SAMN12385076	7	8
Pungu maclareni	1	pung02F6	Male	SAMN12385077	6	8
Sarotherodon caroli	1	caro16A1	Male	SAMN12385078	10	7
Sarotherodon linnellii	1	linn16E3	Male	SAMN12385079	12	8
Sarotherodon steinbachi	1	stei09B7	Male	SAMN12385080	5	8
Sarotherodon lohbergeri	1	lohb06A3	Male	SAMN12385081	6	8

opsin gene copies, RH2Aα and RH2Aβ, dominantly expressed in the deep-water or shallow-water species, respectively. Lastly, we reconstructed the phylogeny of the eleven Barombi Mbo species based on the retina transcriptomes.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Tissue samples were collected in crater lake Barombi Mbo (coordinate: 9°22′E, 4°38′N; Trewavas et al., 1972), South-West Cameroon, between 2013 and 2018 (research permit numbers: 0000047,49/MINRESI/B00/C00/C10/nye, 0000116,117/MINRESI/B00/C00/C10/C14, 000002-3/MINRESI/B00/C00/C10/C11, 0000032,48-50/MINRESI/B00/C00/C10/C12). Between 8 and 16 individuals per each of all 11 cichlid species were collected using gill nets and selective capturing by snorkelling in the shallow-water zone, and with gill nets and minnow traps for the deeper zone. Fin clips were taken from all specimens and stored in 96% EtOH for subsequent molecular analyses. Only adult individuals were selected for the analyses. In addition, we dissected retinas from the enucleated eyes from freshly euthanized specimens. Retinal tissue or the entire eye ball was fixed in RNAlater and stored at room temperature during the field work and then transferred to −80°C upon arrival to the laboratory. See Table 1 for the sample details.

2.2 | Sanger sequencing, haplotype network construction and amino acid diversity

DNA was extracted by the DNeasy Blood & Tissue kit (www.qiagen.com) following the manufacturer's protocol. The seven cone- and one rod opsin genes were Sanger-sequenced for 7–8 individuals per species. The opsin genes were amplified as two (RH2Aα, RH2Aβ), three (SWS1), four (SWS2A) or five (SWS2B, RH2B, LWS) PCR fragments, depending on their length (see Table S1 for a list of primers used and for PCR conditions; several primers used from Carleton, Hárosi, & Kocher, 2000; Carleton & Kocher, 2001; Chen, Bonillo, & Lecointre, 2003). For the rod opsin RH1, only a fragment of ca 500 bp was amplified as the gene is quite uniform. PCR reactions contained 1 μl of DNA, 5 μl of Master Mix Polymerase (PPP Master Mix, Top-Bio s.r.o.), 0.25 μl of each primer (forward and reverse), 0.25 μl of MgCl and 3.25 μl water to 10 μl volume. The PCR conditions were initial denaturation: 94°C for 300 s, 35 cycles of: denaturation: 94°C for 40 s, annealing between 56°C and 64°C depending on the pair of primers, for 30 s, extension: 72°C for 60 s and after 35 cycles final extension in 72°C for 300 s. PCR products were then purified with ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems) and sequenced on an ABI Prism 3100*xl* Genetic Analyzer (Applied Biosystems) applying the BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Applied Biosystems), or service-sequenced in Macrogen Inc. (https://dna.macrogen.com/eng/), South Korea.

The sequences were quality-checked and trimmed, and aligned to the annotated opsin genes of *Oreochromis niloticus* (GenBank, accession number version MKQE00000000.2) with Geneious 9.1.4 (https://www.geneious.com). Intron sequences were removed for the subsequent analyses. For LWS, the first exon was omitted from direct Sanger sequencing, as it is short and showed no variability between species based on the full-length gene data from the transcriptomics. Individual alleles were reconstructed using the PHASE algorithm in DNASP version 6 (Rozas et al., 2017). To visualize the DNA sequence diversity of the opsin genes, we created haplotype networks for each gene with PopART (Bandelt, Forster, & Röhl, 1999; Leigh & Bryant, 2015) using the minimum spanning network method (ε = 0) and phased sequences from 7 to 8 individuals per species. We translated the opsin gene sequences into the protein sequence, specifically focusing on amino acid substitutions in the key spectral tuning amino acid sites previously identified in vertebrates (Yokoyama, 2008) in general, and in teleost fishes in particular (Musilova et al., 2019).

2.3 | Transcriptome sequencing and analyses

We sequenced the whole transcriptomes from retinal tissue for 1–3 individuals per species, with a particular focus on the obligatory deep-water species, *K. dikume* and the seasonally deep-water species *M. myaka*, and two shallow-water benthopelagic dwellers, *K. eisentrauti* (the sister species to *K. dikume*) and *St. mariae* (distantly related; Figure 1c). RNA was extracted directly from the retina using the RNeasy Micro Kit (www.qiagen.com). Libraries of three individuals of *K. dikume*, *K. eisentrauti*, *M. myaka* and *St. mariae* were subsequently prepared with the TruSeq Stranded mRNA Library Preparation Kit (Illumina) and sequenced on the Illumina NextSeq 500 platform using paired-end reads of 81 bp at D-BSSE in Basel, Switzerland and one library per the remaining seven species were sequenced on the Illumina HiSeq 2000 in EMBL GeneCore centre in Heidelberg, Germany.

2.4 | Quantitative PCR of cone opsin genes

RNA was extracted directly from the retina using the RNeasy Micro Kit (www.qiagen.com) or using TRIzol (Invitrogen, USA; extraction protocol followed Santos et al., 2016). RNA was reverse transcribed into cDNA with high-capacity RNA-to-cDNA kit (Applied Biosystems). In a first step, we designed specific primers for quantitative RT-PCR to be used for the Barombi Mbo cichlids and to target all seven cone opsin genes including the two RH2A copies, RH2Aα and RH2Aβ (see Table S1 for details). At least one of the primers has been designed in the exon-exon boundary within the gene to avoid genomic contamination. For qPCR, an equimolar pool of the PCR products of the opsin genes cDNA was used as a positive control for the successful performance of the real-time PCR run. Primers used for the reference pool cDNA amplification are listed in Table S1. We used the SYBR Select Master Mix (Applied Biosystem) to prepare the reactions in 96-well plates for subsequent analyses on a StepOnePlus Real-Time PCR System (Applied Biosystems). Each mixture contained 10 µl of the Master Mix, 0.4 µl of the forward and reverse primer (each), and 2 µl of the cDNA (approximately 10 ng of the total amount). The annealing temperature of the amplification program was set to 61°C. The results were analyzed in the associated STEPONEPLUS Software version 2.3. We calculated C_T values by setting the same threshold (0.5) to all genes within the same plate. The observed C_T value was corrected for primer efficiency and the proportional expression of each cone opsin gene was then calculated (using ΔC_T) from the sum of all cone opsin genes expressed within the same individual (e.g., as described in Carleton et al., 2008). Such proportional calculations were performed separately for the double and single cones. A threshold of 3% of total single or double cone opsin expression was used to consider a gene present in the visual palette. The results from the qPCR approach and the transcriptomic analysis were highly consistent with respect to the relative expression levels per gene.

2.5 | Visual plot of single vs. double cone cells

Based on the observed values of the proportional expression, we reconstructed a visual plot of the Barombi Mbo cichlid fishes (Figure 1f) in which gene expression across genes is represented as a weighted average of all opsin gene types within the single and the double cones, following the equations in Carleton et al. (2016). We used the experimentally measured values of λ_{max} for all cone opsins in Nile tilapia, i.e., SWS1 (360 nm), SWS2B (425 nm), SWS2A (456 nm), RH2B (472 nm), RH2Aβ (518 nm), RH2Aα (528 nm), and LWS (561 nm) (Figure 1e; Spady et al., 2006). We assumed that the sensitivity of the different opsin proteins was similar as there is no substitution in the known key spectral tuning amino acid sites between the Barombi Mbo cichlids and the Nile tilapia (a distant relative to the Barombi Mbo species-flock).

2.6 | Comparative transcriptomics and differential gene expression analysis

To identify genes differentially expressed between deep- and shallow-water species from the entire retinal transcriptomes, we performed a map-to-reference approach. We compared the transcriptomes of four species from Barombi Mbo, two deep-water (*K. dikume* and *M. myaka*) and two shallow-water species (*K. eisentrauti* and *St. mariae*). We performed all pairwise deep- vs. shallow-water species comparisons (i.e., four combinations in total). We applied the TopHat-Cufflinks pipeline (Trapnell et al., 2012) to identify differentially expressed genes (*N* = 3 individuals per species). The quality of transcriptome sequences was controlled with FastQC (Andrews, 2010), and the reads were subsequently trimmed with TRIM GALORE version 0.4.4 (available at https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Filtered and trimmed sequences from each species were mapped to the genome assembly of Nile tilapia (version MKQE00000000.1). Reads were aligned to the annotated Nile tilapia genome with TOPHAT2 version 2.1.1 (Kim et al., 2013). After individual transcript assembly with CUFFLINKS version

2.2.2 (Trapnell et al., 2012), we calculated gene expression levels and tested for statistical significance of each observed change in expression employing Cuffdiff from the CUFFLINKS package. We then constructed a Venn diagram based on the differentially expressed genes using Vennt (available online at http://drpowell.github.io/vennt/), setting the False Discovery Rate threshold at 0.05.

2.7 | Whole-mount fluorescent in situ hybridization

In order to determine the spatial distribution of the expressed opsin genes in cone cells, we performed whole-mount FISH (fluorescent in situ hybridization) experiments on retinal tissue of the seasonally deep-water species *M. myaka*, and two shallow-water benthopelagic species, *K. eisentrauti* and *St. mariae*, following the protocol of Dalton et al. (2014). In each retina, a combination of two opsin genes was localised by employing RNA probes consisting of a template conjugated to fluorescein (FL) or digoxigenin (DIG), where FL was applied to the lower-expressed gene. We successfully localized the following opsin genes in retinas: the red-sensitive LWS, the green-sensitive RH2A (RH2Aα and RH2Aβ undistinguished) and the blue-sensitive SWS2A opsin. As a first step, cichlid RNA was transferred into cDNA using the Mint-2 cDNA synthesis kit (Evrogen). For probe amplification, we designed primers within the investigated opsin genes (including the T3 RNA polymerase binding motif). The template was then extracted from an electrophoresis gel and purified by MinElute Gel Extraction Kit (Qiagen). The probes were synthetized using the T3 RNA polymerase from the template together with FL and DIG labelling mixtures. Retinas were dissected and briefly fixed in 4% PFA followed by several PBS washing steps, and then stored in RNAlater solution in the field. In the laboratory, retinas were subsequently transferred from fixatives into methanol, cleared in xylene and subsequently rehydrated, permeabilised by proteinase *K* and refixed in paraformaldehyde. The samples were hybridized with pre-prepared probes overnight after prehybridization. Consequently, the retinas went through several SSCT washing steps and a blocking phase. Retinae were then incubated with anti-FL antibodies overnight. After washing away unbound antibodies in maleate solution, the fluorescein signal was amplified using the TSA Alexa Fluor 488 kit (Invitrogen). A subsequent overnight incubation in the anti-DIG antibodies was followed by maleate solution washes and amplification using the TSA Alexa Fluor 594 kit (Invitrogen). Finally, the retinas were cleared in 70% glycerol and mounted in the same solution on a slide. Confocal fluorescent microscopy was performed on the dyed retinas using a scanning confocal microscope (Zeiss LSM 880).

2.8 | Phylogeny reconstruction of the Barombi Mbo cichlid species flock

We used the sequenced retinal transcriptomes of the eleven Barombi Mbo species to reconstruct phylogenetic relationship between the species. To obtain a reference, we first used the raw reads of a retinal transcriptome of an outgroup species, the Nile tilapia (*Oreochromis niloticus*; GenBank accession number SRX095621). We then assembled the transcripts using the Trimmomatic option (Bolger, Lohse, & Usadel, 2014) to trim the raw reads and the Trinity assembler (Grabherr et al., 2011). We filtered out all contigs with less than 200 reads and we used the remaining 4,583 tilapia transcripts as a reference for subsequent mapping. We then mapped the eleven retinal transcriptomes (one per species) to the reference using bowtie2 (Langmead & Salzberg, 2012). For every species, we filtered out the transcripts with <50 reads, as well as mitochondrial transcripts. We then exported species-specific consensus sequences and filtered out all transcripts which have been missing in at least one of the species. In total, we used 3,543 nuclear transcripts found in all eleven species and in Nile tilapia, and performed alignments for each of the transcripts using MAFFT 7.407 (Katoh & Standley, 2013). We subsequently concatenated the alignments and considered only variable SNPs with no missing data and no ambiguities (in total 41,602 SNPs including tilapia, of which 11,319 were variable within the Barombi Mbo species flock). We reconstructed a phylogenetic tree using Bayesian Inference in MRBAYES 3.2.6 (Ronquist & Huelsenbeck, 2003), running the analysis for 10 million generations and a burnin phase of 25% on the CIPRES portal (Miller, Pfeiffer, & Schwartz, 2010), and maximum likelihood in RAXML (Stamatakis, 2014) with automatically determined sufficient number of bootstrap replicates. The tree topologies from both analyses were identical.

3 | RESULTS

3.1 | Nucleotide variation in opsin gene sequences

Among the 11 species of cichlid fishes from crater lake Barombi Mbo (7–8 specimens per species), we found only minor DNA sequence differences in the single rod opsin (RH1) and in the seven cone opsin genes (SWS1, SWS2B, SWS2A, RH2B, RH2Aβ, RH2Aα and LWS; ordered by their sensitivity). The haplotype sequences were deposited in the GenBank database (accession numbers MN258381–MN258511). The most variable genes at the nucleotide level were SWS1 and RH2Aα with 15 haplotypes each across the entire data set of 85 specimens from the 11 species, while the most conserved gene was LWS with only two haplotypes present (Figure 2). At the functional (amino acid) level, we detected three (SWS1, RH2Aα and SWS2B), two (RH2Aβ, RH2B), one (LWS) or no (SWS2A) amino acid substitution, respectively (Table 2). Among the 14 amino acid substitutions found in the cone opsin genes of the Barombi Mbo cichlids, only one affected a known key tuning site (i.e., an amino acid position previously identified to change the protein function, as in Yokoyama, 2008), namely in SWS1 in *M. myaka*. Note, however, that even in this case the allele was only found in a heterozygous state (Table 2). Two haplotypes were found in the single rod opsin (RH1) gene corresponding to two amino acid substitution in *St. pindu* (Table 2). Interestingly, the two deep-water species, *M. myaka* and *K. dikume*, shared an amino acid substitution at position 214 (V214I) of RH2Aα (Table 2). The most dissimilar opsin protein compared to the Nile tilapia (*O. niloticus*) was LWS (Table 2).

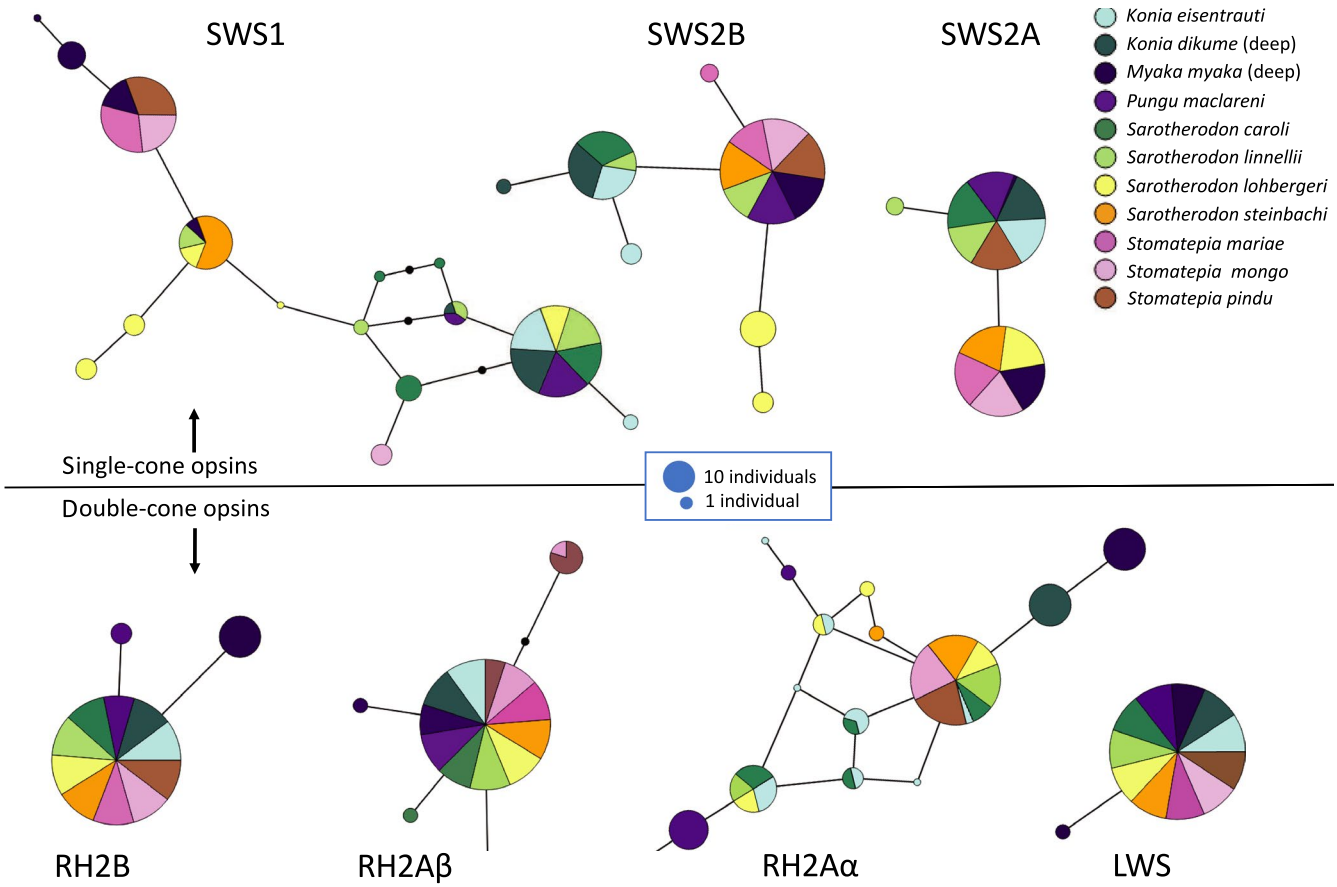

FIGURE 2 Haplotype network of the cone opsin gene repertoire in 11 cichlid species from Barombi Mbo. Each haplotype (covering virtually the entire coding region) is represented by a circle, the size of which indicates the number of individuals, in which this haplotype was found; the haplotypes are colour-coded according to species; lines between haplotypes represent mutational steps; black dots represent haplotypes not sampled in the 11 species and 7–8 specimens each

3.2 | Deep- vs. shallow-water opsin gene expression palette

In a second step, we focused on the cone opsin gene expression profiles of the 11 cichlid species from Barombi Mbo (Figure 1c) on the basis of RNA sequencing data from retinae (N = 1–3 per species; Table 1) in combination with quantitative real-time PCR experiments (N = 5–13 per species). The RNA-seq and real-time PCR results were congruent for the opsin gene expression patterns. Overall, we found strong differences in the opsin gene expression profiles between the two deep-water and the nine shallow-water species (Figures 1d and S1, Table S2). More precisely, there was a shift towards the middle-wavelength sensitive opsins in the cones (i.e., towards shorter wavelengths in the double cone cells, and towards longer wavelength in the single cones) in the deep-water species *K. dikume* and *M. myaka* (Figure 1f), as exemplified by a complete lack of expression of the red-sensitive opsin LWS and the blue/violet-sensitive SWS2B in the deep-water species (Figure 1f). Furthermore, in the retinal expression profiles of both deep-water species, the green-sensitive RH2Aα (showing a slightly longer-wavelength sensitivity compared to RH2Aβ) dominated over RH2Aβ. An opposite pattern was found in the nine remaining species inhabiting the shallow zones of crater lake Barombi Mbo, in which RH2Aβ was either dominant over RH2Aα (mostly genus *Stomatepia*) or even the only expressed green-sensitive opsin (Figures 1d and S1). The deep-water opsin gene expression palette in cichlid fishes from Barombi Mbo is therefore characterized by the exclusive expression of SWS2A in single cones, and RH2Aα in double cones (together with the low level of RH2Aβ expression), while the shallow-water palette is dominated by the expression of LWS and RH2Aβ in double cones and SWS2A together with SWS2B (or SWS1) in single cones (Figures 1d and S1).

3.3 | Differences in opsin gene expression within the shallow-water species

Our opsin gene expression experiments revealed that seven species living in the shallow waters of crater lake Barombi Mbo express five cone opsins (at a threshold of 3% of total single or double cone opsin expression), whereby six species seem to feature the same palette (*St. pindu*, *St. mongo*, *St. mariae*, *K. eisentrauti*, *P. maclareni*, *S. lohbergeri* – SWS2B, SWS2A, RH2Aα, RH2Aβ and LWS) and one of the species altering the green opsins (*S. caroli* – SWS2B, SWS2A, RH2B, RH2Aβ and LWS). One species (*S. linnellii*) has four expressed opsins

(SWS2B, SWS2A, RH2Aβ and LWS), which is also the lowest number of opsins identified in the shallow-water species as compared to three opsins expressed in the deep-water species (SWS2A, RH2Aα, RH2Aβ; see Figures 1d and S1, Table S2). Among the shallow-water species, only one species (*S. steinbachi*) was found to express the UV-sensitive SWS1. Interestingly, this species also shows the highest number of expressed opsins among all Barombi Mbo cichlids (Figures 1d and S1), with a total of six cone opsin genes being expressed: all three single cone opsins, SWS1, SWS2B and SWS2A, and three out of the four double cone opsins, RH2B, RH2Aβ, LWS (note that this species does not express the deep-water green-sensitive RH2Aα).

3.4 | Differential gene expression in the retina of deep- vs. shallow-water species

We applied a comparative transcriptomic approach on the basis of whole retinal transcriptomes between the two deep-water (*K. dikume* and *M. myaka*) and two shallow-water species (*K. eisentrauti* and *St. mariae*), testing all possible pairwise comparisons between deep- vs. shallow-water species ($N = 3$ individuals per species). Here, we were interested in genes that were differentially expressed between the two deep-water species when compared to two shallow-water species (Figure 3). We identified 26 upregulated, and 20 downregulated genes in both deep-water species compared to both shallow-water species (Figure 3, Tables 3 and S3). Among the differentially expressed genes, we identified genes known to be directly involved in colour vision, such as LWS and SWS2B. These two genes were found to basically lack expression in the deep-water species, thereby confirming our results from the qPCR approach. Another set of differentially expressed genes is known to be involved in vision, e.g., in photoreceptor development (*nr1d1*, *prdm1/blimp1*, *rorb*), eye development (*aldh1a1*), the phototransduction cascade (*cyng3*), or involved in vision in another way (*slc1a7*, *npas4*, *rbp1*, *tmem116*) (Table 3). Interestingly, four of the so identified genes have known functions in regulating the intraocular pressure, namely *slc4a5/NBC-1* and *g2e3* (both upregulated in the deep-water species), and *fhod1* and *paqr6* (both downregulated). Other differentially expressed genes are known to respond to hypoxia (such as the upregulated *hlf/epas1/HIF-1α-like* or the downregulated *dapk2*). Two of the so detected genes are known to be involved in primary haemostasis (*tbax2r*, *ptgis*), and nine differentially expressed genes are essential for circadian clock regulation (upregulated *per1*, two versions of *ciart*, *bhlhe41*, *nr1d1*, *nr1d2*; downregulated *nfil3*, *rorb*, *melanopsin*). Note that the red-sensitive LWS opsin was the most downregulated of all differentially expressed genes (Table 3).

3.5 | Spatial distribution of the opsin genes in the cone cells of retina

To further investigate functional adaptations of the visual system of the studied cichlid species, we specifically examined the distribution of the opsin genes in the cone cells of the retina, focusing on four genes with comparably high levels of expression according to the gene expression assays (LWS, RH2Aα, RH2Aβ and SWS2A; Figure 4). Overall, we confirmed the patterns generally known for cichlids, that is, LWS and RH2As are expressed exclusively in the double cones, while the expression of the SWS2A gene is located exclusively in the single cones. In our experiments, we did not find evidence for coexpression of two opsins in one cone cell (note, however, that we were not able to distinguish RH2Aα and RH2Aβ in the fluorescent in-situ hybridization (FISH) experiments and that we have only tested a subset of opsin genes). Most of the double cones of the shallow-water species are composed of two cells with different opsin genes expressed, mostly with the pair of LWS and RH2A cone cells. Interestingly, we further found several cases of the twin cones (i.e., double cones with the same opsin protein in both cells), with LWS being expressed in *K. eisentrauti* (Figure 4b). We have not found any case of twin cones with only RH2A (α or β) expression in the shallow-water species, whereas, in the deep-water species *M. myaka*, the RH2A twin cones was the only observed type of double cone cells (Figure 4h).

3.6 | Phylogeny of the Barombi Mbo species flock

We reconstructed phylogenetic relationships of the eleven species within the Barombi Mbo species flock based on the transcriptome data from retina. The phylogenetic analysis based on the 3,543 nuclear transcripts (11,319 variable SNPs; Figure 1c) revealed several strongly supported lineages (e.g., monophyletic genera *Konia* and *Stomatepia*) which are, however, grouped in weakly supported deeper relationships. Our phylogenetic reconstruction further suggests the presence of the deep-water species (i.e., *M. myaka* and *K. dikume*) in separate lineages.

4 | DISCUSSION

Vision is one of the most challenged sensory systems in deep-water habitats, which is mainly because of the limited availability of light (O'Carroll & Warrant, 2017). In this study, we investigated adaptations of the visual system in the adaptive radiation of cichlid fishes in crater lake Barombi Mbo with a particular focus on molecular adaptations of two species living in the deeper zone of this small and isolated water body. We found that, just like all other African cichlids studied so far, the Barombi Mbo cichlids have seven cone opsin genes and one rod opsin (Carleton et al., 2016). All of these opsin genes were expressed in at least one of the eleven Barombi Mbo cichlid species; however, no species showed expression of all of them (Figure 1). The visual system of the Barombi Mbo cichlid species flock shows the molecular mechanisms of adaptation at the level of DNA sequence (one exclusive deep-water amino acid substitution in RH2Aα), as well as in differential gene expression (alternative expression profiles, palettes). In general, the opsin genes are similar in sequence, while the expression patterns are considerably different between species suggesting that adaptations in the visual system

TABLE 2 Amino acid substitutions in the opsin genes of the Barombi Mbo cichlids. Variable positions within the coding region are shown for amino acid sites variable within the flock and between the flock and Nile tilapia. In case of RH2A, all positions variable within either copy or between copies are shown. Note that there is no amino acid change in SWS2A. Amino acid positions refer to the bovine rhodopsin, key tuning sites were defined after Yokoyama (2008)

	SWS1					SWS2B					RH2Aα								RH2Aβ	
Species	60	118	166	173	214	36	108	188	239	280	36	39	111	149	199	214	263	312	36	39
Nile tilapia (*Oreochromis niloticus*)	V	A	G	F	F	V	T	G	E	G	T	I	A	T	N	V	V	V/I	F	L
Konia dikume (deep)	L	.	.	.	I	I	I	S	.	.	I	V	.	S	.	I	.	V	.	.
Konia eisentrauti	L	.	.	F/L	I	I	I	S	E/V	.	I	V	.	S	.	.	.	V	.	.
Myaka myaka (deep)	L	A/T	G/S	.	I	I	I	.	.	.	I	V	G	S	.	I	.	V	.	.
Pungu maclareni	L	.	.	.	I	I	I	.	.	.	I	V	.	S	.	.	.	V/I	.	.
Sarotehrodon steinbachi	L	.	.	.	I	I	I	.	.	.	I	V	.	S	.	.	.	V	.	.
Sarotherodon caroli	L	.	.	.	I	I	I	S	.	.	I	V	.	S	.	.	.	V	.	.
Sarotherodon linnellii	L	.	.	.	I	I	I	G/S	.	.	I	V	.	S	.	.	.	V	.	.
Sarotherodon lohbergeri	L	.	.	.	I	I	I	.	E/V	G/E	I	V	.	S	.	.	.	V	.	.
Stomatepia mariae	L	.	S	.	I	I	I	.	.	.	I	V	.	T	.	.	.	V	.	.
Stomatepia mongo	L	.	G/S	.	I	I	I	.	.	.	I	V	.	S	.	.	.	V	.	.
Stomatepia pindu	L	.	S	.	I	I	I	.	.	.	I	V	.	S	.	.	.	V	.	.
only differentiated from tilapia	@				@	@	@				@	@								
variable within the species flock		@	@	@				@	@	@			@	@		@		@		
variable between α and β copy (RH2A only)											@	@					@	@	@	@
key tuning site		@																		

The "." symbol represents the amino acid variant identical to the Nile tilapia reference. The "@" symbol is used to highlight the substitution type.

happened mostly at the gene regulation level. Apart from the opsin genes, using comparative transcriptomics we identifieda set of 46 genes that are differentially expressed between deep- and shallow-water species.

4.1 | Evolution of the Barombi Mbo species flock

In order to interpret our findings of deep-water adaptations in an evolutionary context, we reconstructed phylogenetic relationships based on the retina transcriptome data. Our results are in accord with the previously published phylogenies (Richards et al., 2018; Schliewen & Klee, 2004; Schliewen et al., 1994) in the strong support for the genera monophyly (genera *Stomatepia*, *Konia*), paraphyly or polyphyly of the genus *Sarotherodon*, and uncertain position of *P. maclareni* and *M. myaka*. Contrarily, the relationships among the well-supported lineages are not clear in any previous hypotheses (including this study), or have very low support, which is probably due to the complexity at the onset of the species flock evolution. Our data further suggests that the two deep-water species have probably evolved its depth preference independently, or further events such as hybridization might have contributed to the evolution of these particular species. Future research on speciation genomics and the multiple individual approach will be required to fully enlighten the complexity of the Barombi Mbo species flock evolution.

4.2 | DNA mutations and amino acid substitutions in the opsin genes as a source of variability

The visual opsin genes of the Barombi Mbo cichlids show very little variation at the nucleotide level (Table 2), corresponding to the estimated geological age of current lake Barombi Mbo of less than 1 million years (Cornen et al., 1992) and the molecular dating of the onset of the Barombi Mbo radiation (around 1.7 million years ago ± 0.6 million years ago; Schedel, Musilova, & Schliewen, 2019). We observed between two and 15 haplotypes per gene (corresponding to 2–5 protein versions), whereby haplotypes are usually shared between species (Figure 1, Table 2). Interestingly, we detected one amino acid substitution in the green-sensitive RH2Aα that is exclusively present and fixed in the two deep-water species (V214I; Table 2). A polymorphism at the same position (but involving a different amino acid) has previously been reported in Nile tilapia (F214I; Spady et al., 2006). Amino acid position 214 of RH2Aα, which is not among the known key spectral tuning sites (Yokoyama, 2008), is located in the transmembrane region of the

						RH2B			LWS								RH1					
111	149	199	214	263	312	45	48	137	49	50	115	116	172	213	218	304	49	50	104	166	214	309
A	T	N	V	I	Q	V	V	I	I	A	Y	T	I	I	I	V	L	A	V	S	I	L
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	N/S	.	.	.	F	I	.	V	A/V	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	I/V	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	T/S	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
.	.	.	.	.	.	F	.	.	V	.	F	V	M	F	V	I	I	V	.	A	.	M
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						@			@		@	@	@	@	@	@	@	@		@		@
	@	@					@	@		@									@		@	
				@	@																	
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protein, but is not part of the chromophore-binding pocket (Spady et al., 2006). However, this position is located in close proximity of an amino acid site responsible for protein stability under higher pressure (position 213; Porter, Roberts, & Partridge, 2016), and a mutation at the same site (S214P) is known to cause tritanopia in human (i.e., malfunction of the blue cones; Weitz et al., 1992), suggesting that this position in the RH2Aα protein is of functional relevance.

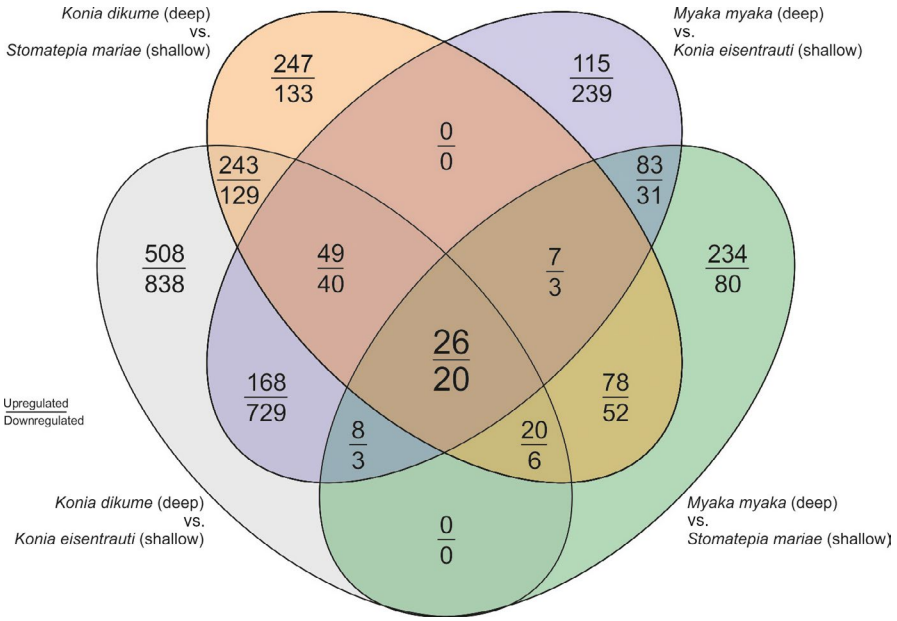

FIGURE 3 Venn diagram showing the number of the differentially expressed genes in the deep-water vs. shallow-water cross-species comparison. Retina transcriptomes of two deep-water species (*Konia dikume*, *Myaka myaka*) and two shallow-water species (*Konia eisentrauti*, *Stomatepia mariae*) were screened for differentially expressed genes applying a False Discovery Rate (FDR) of 0.05. A total of 46 genes presented in the centre of the diagram (26 upregulated and 20 downregulated genes) were identified as being differentially expressed. See Tables 3 and S3 for details about these genes

TABLE 3 Differentially regulated genes in the deep-water cichlids of crater Lake Barombi Mbo

Order in the list	Linkage group in Nile tilapia	Gene symbol	Mean log2FC deep/shallow-water (from all four comparisons)	log2FC – Konia_dikume (deep) vs. Konia_eisentrauti	log2FC – Konia_dikume (deep) vs. Stomatepia_mariae	log2FC – Myaka_myaka (deep) vs. Konia_eisentrauti
(a) Upregulated						
1	LG22	kmt5a[a]/SET8/Pr-Set7	6.00	7.23	7.69	4.31
2	LG22	ciart[a]	5.54	6.87	6.33	4.74
3	LG11	ciart[a]	3.85	5.04	5.18	2.53
4	LG11	hlf/epas1/HIF-1α-like	3.84	5.10	4.58	3.09
5	LG4	nr1d1/Rev-erbα	3.80	5.11	4.73	2.87
6	LG3b	per1	3.49	4.98	4.16	2.82
7	LG12	slc4a5[a]/NBC-1	3.45	4.90	3.62	3.28
8	LG11	clk2[a]	3.31	2.29	2.89	3.73
9	LG3	unchar.	3.26	4.20	4.52	1.99
10	Unplaced	g2e3[a]	3.04	3.68	3.85	2.23
11	LG7	bhlhe41	3.00	4.16	4.01	2.00
12	LG15	adcy8[a]	2.81	3.42	2.56	3.06
13	LG13	plaur[a]	2.75	3.53	3.15	2.35
14	LG15	slc1a[a]/EAAT5	2.57	3.66	2.53	2.61
15	LG9	cyng3/CNGB3	2.56	3.76	3.23	1.90
16	LG15	pnma1[a]	2.54	3.07	2.28	2.79
17	LG15	unchar.	2.28	2.81	2.26	2.30
18	LG3b	havcr2/TIM-3	2.26	2.61	1.88	2.63
19	LG15	unchar.	2.17	2.22	2.17	2.16
20	LG11	nr1d2	2.03	2.60	2.47	1.60
21	LG16	pde1a	1.99	2.42	2.27	1.71
22	Unplaced	unchar.	1.95	2.64	1.61	2.29
23	LG11	rbp1/crbp1	1.89	2.14	1.53	2.25

log2FC – Myaka_myaka (deep) vs. Stomatepia_mariae	Function possibly associated with deep-water environment?	Description of function (not exhaustive)	Reference
4.78		Transcription factor; epigenetics – lysine methyltransferase; monomethylates both histones and nonhistone proteins	UniProt, Corso-Díaz, Jaeger, Chaitankar, and Swaroop (2018)
4.20	Circadian clock	Transcription factor	UniProt
2.67	Circadian clock	Transcription factor	UniProt
2.57	Vision/hypoxia	Transcription factor, prevents retinopathy in hypoxia in mouse	Morita et al. (2003)
	Hypoxia	In response to hypoxia activates expression of erythropoietin in mammals; linked to hypoxia management in mummichogs	Wang and Semenza (1993), Townley et al. (2016)
		Immunity; function in immature blood cell formation	Wahlestedt et al. (2017)
2.49	Vision	Role in photoreceptor development. Known to prevent retina degeneration such as retinis pigmentosa and S-cone syndrome (dominance of S-cones over other cones and rods) in human	Mollema et al. (2011), Cruz et al. (2014)
	Circadian clock	Transcription factor	Huang, Zhang, Ye, and Wang (2016)
2.00	Circadian clock	Transcription factor	UniProt
	Hypoxia	Reported to show response to hypoxia in rats	Koltsova et al. (2014)
2.00	Vision	pH metabolism, maintain ocular homeostasis, irregularities can cause ocular abnormalities, such as glaucoma in human	Usui et al. (2001)
4.33		Splicing regulation	UniProt
2.32			
2.40	Vision	E3 ubiquitin-protein ligase. Essential in early embryonic development to prevent apoptotic death; reported to be linked with the intraocular pressure in mouse; linked to rod regeneration in zebrafish	UniProt, Panagis et al., 2011, Morris, Forbes-Osborne, Pillai, & Fadool, 2011
1.85	Circadian clock	Transcription factor; cold temperature response and coexpression with PER in *Squalius*	UniProt, Moreno, Sousa, Jesus, & Coelho, 2019
2.19		Calcium-stimulable adenylyl cyclase	UniProt
1.97		Receptor for urokinase plasminogen activator	UniProt
1.48	Vision	Postsynaptic receptor, mediates light responses in depolarizing bipolar cells in retinae of teleosts	Dennis, Chung, & Wu, 2014, Nelson & Singla, 2009
1.37	Vision	Visual signal transduction cascade, depolarization of rod photoreceptors; in cone cells codes for one part of the cone photoreceptor cyclic nucleotide-gated (CNG) channel. Known to be linked to achromatopsia (cone dystrophy) in human	UniProt, Johnson et al., 2004
2.00		Neuron-specific protein; function miscellaneous	GeneCards, UniProt
1.75			
1.90		Immunity: immunoglobulin superfamily, regulates macrophage activation	UniProt
2.12			
1.47	Circadian clock	Transcription factor	UniProt
1.56		cAMP and cGMP based key regulator of many important physiological processes, such as neuronal plasticity	UniProt
1.25			
1.64	Vision	Rod visual cycle, retina pigment epithelium. Retinoid-binding protein, facilitates transport of retinoids between the cell bodies and apical membranes	Huang, Possin, & Saari, 2009

(Continues)

TABLE 3 (Continued)

Order in the list	Linkage group in Nile tilapia	Gene symbol	Mean log2FC deep/shallow-water (from all four comparisons)	log2FC – Konia_dikume (deep) vs. Konia_eisentrauti	log2FC – Konia_dikume (deep) vs. Stomatepia_mariae	log2FC – Myaka_myaka (deep) vs. Konia_eisentrauti
24	LG23	pip5k1c	1.73	1.89	1.21	2.24
25	LG15	znf395[a]	1.48	1.76	1.64	1.33
26	LG2	npas4	1.45	1.84	1.43	1.47
(b) Downregulated						
1	LG5	opn1lw[a]/LWS	−9.32	−10.08	−11.51	−7.12
2	LG7	glg1[a]/ESL-1	−9.30	−8.35	−10.55	−8.05
3	LG14	vwa5a[a]	−3.92	−3.72	−4.58	−3.26
4	LG11	tmem116	−3.35	−3.72	−2.54	−4.15
5	LG6	nfil3	−3.24	−4.45	−4.02	−2.47
6	LG5	opn1sw[a]/SWS2B	−3.19	−4.05	−3.48	−2.91
7	LG8	ifit5[a]	−2.99	−3.43	−3.36	−2.62
8	LG18	rorb[a]	−2.81	−4.03	−3.48	−2.13
9	LG11	nbeal2	−2.75	−3.20	−3.59	−1.90
10	LG5	ptgis[a]	−2.70	−2.73	−2.66	−2.74
11	LG22	prdm1[a]/blimp1	−2.47	−3.27	−3.05	−1.88
12	LG15	tbxa2r	−2.43	−2.71	−2.23	−2.63
13	LG1	fhod1[a]	−2.24	−2.20	−2.22	−2.26
14	LG12	aldh1a1	−2.11	−2.37	−1.97	−2.24
15	LG4	unchar.	−2.04	−2.15	−1.69	−2.39
16	LG19	plekhh1[a]	−2.01	−2.96	−2.51	−1.52
17	LG18	opn4[a]	−1.97	−2.02	−2.01	−1.93
18	LG11	paqr6[a]	−1.83	−1.98	−1.27	−2.38
19	LG16	stk17b	−1.78	−2.44	−1.76	−1.81
20	LG7	dapk2	−1.38	−1.77	−1.59	−1.18

Note: For the full list including the genome coordinates and gene locations, as well as the full gene names, please see Table S3.
[a]Refers to the gene abbreviations that have not been implemented in Tilapia reference but are accepted in human

log2FC – Myaka_myaka (deep) vs. Stomatepia_mariae	Function possibly associated with deep-water environment?	Description of function (not exhaustive)	Reference
1.57		Variety of cellular processes such as vesicle mediated transport, cell adhesion, cell polarization and cell migration	UniProt
1.21		Transcription factor	UniProt
1.05	Vision/neuronal plasticity	Plays a key role in the structural and functional plasticity of neurons. Candidate gene for the occurrence of plasticity in the adult visual system	Maya-Vetencourt et al. (2012)
−8.55	Vision	Long-wavelength sensitive photoreceptor (red)	UniProt
−10.25		Immunity, fibroblast growth factor binding	UniProt
−4.12		Putative tumor suppressor	UniProt
−2.97	Vision	Putative role in photoreceptor protection: identified as a response to light damage in mouse	Natoli et al. (2010)
−2.04	Circadian clock	Transcription factor; regulates per2	UniProt
−2.33	Vision	Short-wavelength sensitive photoreceptor (blue)	UniProt
−2.55		Immunity; important for RNA recognition specificity in antiviral defence	UniProt
−1.59	Vision	Photoreceptor development; directs rod development from cone precursor. Regulates expression of SWS opsin in mouse	Jia et al. (2009), Srinivas et al. (2006)
	Circadian clock	Transcription factor	UniProt
−2.30	Haematopoiesis	NBEAL2 expression is required for the development of thrombocytes in zebrafish	Albers et al. (2011)
−2.68	Haemostasis	Catalyzes the isomerization of prostaglandin H2 to prostacyclin (= prostaglandin I2), a potent vasodilator and inhibitor of platelet aggregation	UniProt
−1.66	Vision	Photoreceptor development: controls photoreceptor vs. bipolar cell fate choice during retinal development. Without prdm1/blimp1, nascent photoreceptors are respecified as bipolar cells	Brzezinski, Lamba, and Reh (2010), Brzezinski, Park, and Reh (2013)
−2.15	Heamostasis	Interacts with thromboxane A2 to induce platelet aggregation and regulate haemostasis. A mutation in this gene results in a bleeding disorder	UniProt
−2.28	Vision	Involved in regulation of the intraocular pressure	Luna et al. (2012)
−1.85	Vision	Protects inner ocular tissues from ultraviolet radiation and reactive oxygen-induced damage. Mediates retinoic acid signalling during the eye development	Chen et al. (2013)
−1.93			
−1.07	Vision	Associated with diabetic retinopathy in human	Han et al. (2012)
−1.92	Vision	Melanopsin system plays role in visual pathways (isomerizes retinal). Nonimage forming vision	Hughes et al. (2016)
	Circadian clock	Regulation	UniProt
−1.67	Vision	Involved in regulation of the intraocular pressure, found in glaucome human/chimpanzee patients	Kompass et al. (2008)
−1.13	Haematopoiesis/hypoxia	Acts as a positive regulator of apoptosis. Reported to show response to hypoxia in rats	UniProt, Koltsova et al. (2014)
−1.00	Hypoxia	Triggers cell survival, apoptosis, and autophagy; depletion leads to decreased rate of oxidative phosphorylation	UniProt, Schlegel et al. (2016)

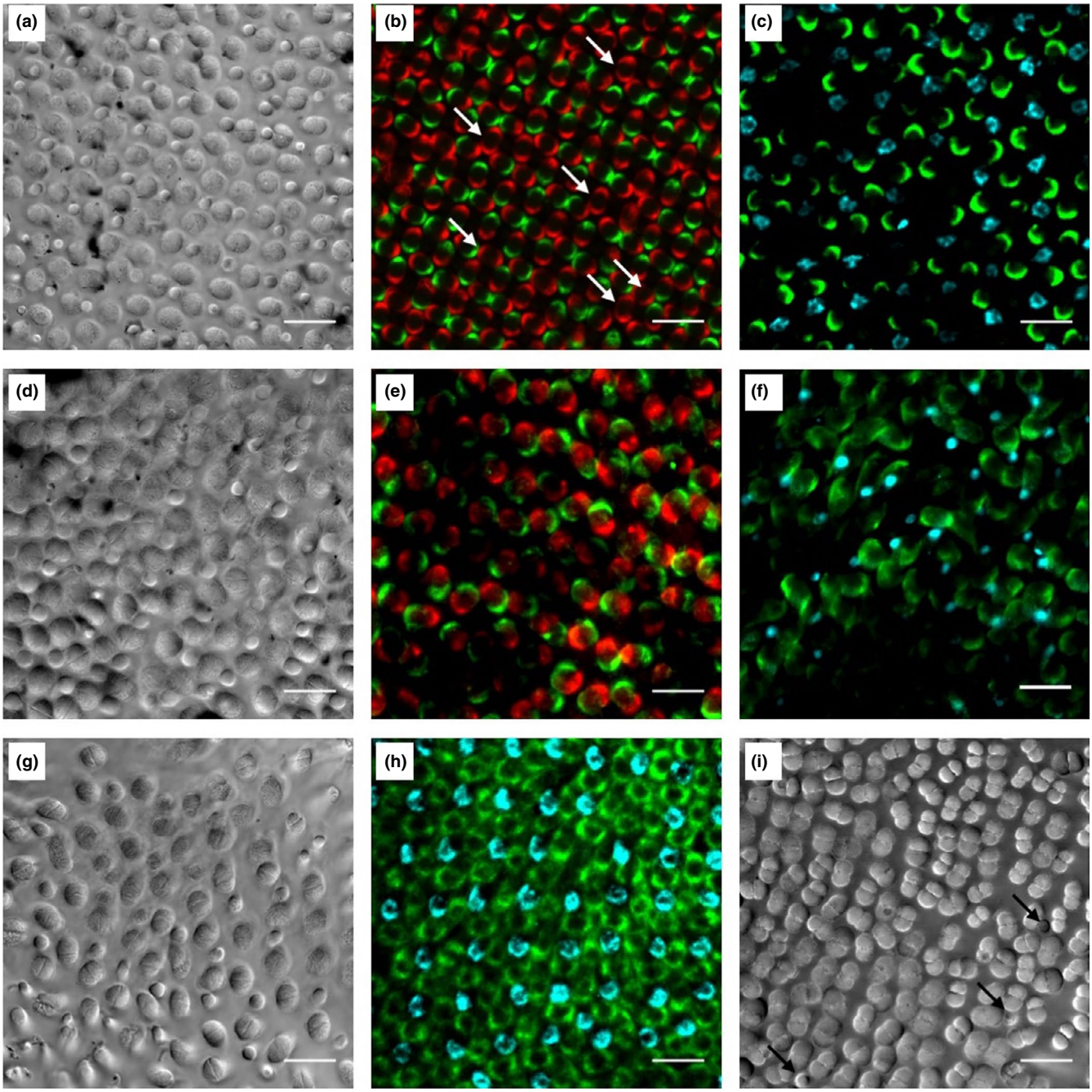

FIGURE 4 Cone cells in the retina of the Barombi Mbo cichlid fishes. (a, d, g, i) – Retinas with double and single cones in the bright field spectrum. (b, c, e, f, h) – Fluorescent in situ hybridization (FISH) using RNA probes of opsin genes expressed in the cone cells. (a) Shallow-water species *Konia eisentrauti*. (b) *K. eisentrauti* – red-sensitive (LWS) opsin in red, and green-sensitive RH2A (RH2Aα/RH2Aβ) in green. Note that most of the double cone cells express LWS in one cell and the RH2A gene in the other cell; however, some double cones express LWS in both cells (examples marked by the white arrow). (c) *K. eisentrauti* – green-sensitive RH2A (RH2Aα/RH2Aβ) in green and blue-sensitive (SWS2A) opsin gene in light blue. (d) shallow-water species *Stomatepia mariae*. (e) *S. mariae* – red-sensitive (LWS) opsin in red, and green-sensitive RH2A (RH2Aα/RH2Aβ) in green. (f) *S. mariae* – green-sensitive RH2A (RH2Aα/RH2Aβ) in green and blue-sensitive (SWS2A) opsin gene in light blue. (g) seasonally deep-water species *Myaka myaka*. (h) *M. myaka* – green-sensitive RH2A (RH2Aα/RH2Aβ) in green and blue-sensitive (SWS2A) opsin gene in light blue; note that the RH2A gene is expressed in both cells of the double cones. (i) Deep-water species *Konia dikume* with the black arrows highlighting the single cones. Scale = 20 μm

4.3 | Adaptation of the visual system by alternating gene expression

Our RNA-sequencing and quantitative PCR experiments revealed that all seven cone opsin genes are expressed in Barombi Mbo cichlids, however in varying constellations, whereby there is no species that expresses all of them. The nine shallow-water species feature a visual palette in which the double cone cells are dominated by the red photoreceptor (LWS) complemented by the shallow-water version of the green-sensitive opsin (RH2Aβ), and the single cone cells are dominated by the blue-sensitive photoreceptor (SWS2A) complemented by a low expression levels of the violet-sensitive opsin (SWS2B). The visual palette of the two deep-water species is characterized by comparably high expression levels of the second copy of the green-sensitive opsin (RH2Aα) and a lack of expression of LWS in the double cones, as well as virtually no expression of SWS2B in the single cones (Figure 1d). The observed alterations in the visual palette between shallow- and deep-water species, that is, the absence of red and UV/violet photosensitivity, is consistent with the narrower ambient light spectrum present in deeper zones of clear waters due to the attenuation of the red and UV/violet edges of the light spectrum with increasing depth (Carleton et al., 2016; O'Quin, Hofmann, Hofmann, & Carleton, 2010; Smith, van Staaden, & Carleton, 2012) (note that with a visibility of several meters, the conditions in oligotrophic crater lake Barombi Mbo are similar to the two East African Rift Valley lakes Malawi and Tanganyika; Carleton et al., 2016). Despite the comparable lacustrine environment, the alteration of the visual palette in Barombi Mbo involves a somewhat different set of genes compared to lakes Malawi and Tanganyika: Lake Malawi cichlids primarily differ in the expression of single cone opsins such as the UV- (SWS1) and violet/blue- (SWS2A and SWS2B) sensitive opsins (Hofmann et al., 2009; Parry et al., 2005), whereas in Barombi Mbo cichlids it is primarily the double cone genes that differ (see Figure 1e). Similar to what we found in Barombi Mbo cichlids, a trend of replacing the red-sensitive (LWS) opsin gene with the green opsins in the double cones was reported for deep-water Tanganyikan cichlids (O'Quin et al., 2010). Interestingly, the Barombi deep-water combination of SWS2A and RH2A opsin genes is quite unusual among cichlids and has so far been observed only in one Tanganyikan species (O'Quin et al., 2010). None of these previous studies in cichlids had the resolution to distinguish between RH2Aα and RH2Aβ, preventing a full comparison to the pattern of alternative usage of RH2A copies observed in Barombi Mbo cichlids.

4.4 | Functional differentiation of two copies of green-sensitive opsins, RH2Aα and RH2Aβ

In this study, we show for the first time that the expression of the two copies of RH2A is associated with water depth, irrespective of phylogenetic position. The nine shallow-water species use RH2Aβ as a green photoreceptor, while the two deep-water species use RH2Aα. The RH2Aα protein is functionally shifted towards longer wavelengths compared to RH2Aβ, and could thus be used to partially compensate the lack of sensitivity within the longer wavelength range caused by the absence of LWS in the deep-water species. The expected difference in λ_{max} between RH2Aα and RH2Aβ is ca. 10 nm in Nile tilapia (Spady et al., 2006). It remains to be tested if this difference could have any effect on the visual performance of Barombi Mbo cichlids. RH2Aα and RH2Aβ are the most similar opsin genes within the cichlid genome, which is either due to their more recent divergence via gene duplication or due to gene conversion (Carleton et al., 2016). Interestingly, differential selection has been detected between the two RH2A copies in some African cichlids (Weadick & Chang, 2012). In the light of our results, this might be due to adaptation to different water depths as well.

Little is known about subfunctionalization of RH2Aα and RH2Aβ in African cichlids, on their expression profiles during ontogeny (Carleton et al., 2008), or their coexpression with other opsins (Dalton et al., 2014). However, adult Nile tilapia were shown to express RH2Aβ at lower levels than RH2Aα, with both RH2A copies being present at quite low proportions (Spady et al., 2006) as compared to the Barombi Mbo cichlids. In general, the total proportion of all green-sensitive genes (RH2B, RH2Aα and RH2Aβ; compared to LWS) is much higher in the Barombi Mbo shallow-water species (average of the nine species: green/red = 49.4%/50.6%) than in the riverine Nile tilapia (green/red = 11.65%/88.65%; SRX095621). This suggests that the green opsins are important in the adaptation to the clear-water lacustrine environment. Similar visual system modifications were also observed between cichlids from clear crater lakes and turbid larger lakes in Nicaragua (Torres-Dowdall et al., 2017).

4.5 | Independent adaptation to the deep-water habitat

The visual system of the two deep-water cichlids in Barombi Mbo is functionally adapted to have the highest sensitivity in the centre of the light spectrum (Figure 1f). The Barombi Mbo cichlid species flock is probably derived from a riverine ancestor (genus *Sarotherodon*, Oreochromine lineage; Dunz & Schliewen, 2013) that putatively had a long-wavelength visual palette, as exemplified by the well-studied Nile tilapia (Spady et al., 2006). Within Barombi Mbo, the shorter-wavelength-shifted deep-water palette has most likely evolved twice from this ancestral, LWS-dominated palette. The two deep-water species, *K. dikume* and *M. myaka*, are neither closely related in our phylogenetic reconstruction (Figure 1c), nor in any of the alternative phylogenetic hypotheses (Richards et al., 2018; Schliewen & Klee, 2004; Schliewen et al., 1994). Such an independent adaptation of the visual system is comparable to the situation in lakes Malawi and Tanganyika, where the riverine ancestor of the respective cichlid species flocks most likely also featured a long-wavelength palette (Carleton et al., 2016; O'Quin et al., 2010) and different species have convergently evolved towards short- and medium-sensitive palettes (O'Quin et al., 2010).

The emergence of different palettes in lacustrine conditions has previously been hypothesized to partially occur due to a neotenic

shift via conserving or postponing the larval gene expression patterns into adulthood (O'Quin, Smith, Sharma, & Carleton, 2011). Interestingly, the double cone expression pattern observed in the Barombi Mbo deep-water species mimics to a certain degree that of larval tilapia (Spady et al., 2006), where LWS expression is lowered (even though still high) and RH2Aα is the dominant green-sensitive visual opsin. Contrarily, the single cones of the Barombi Mbo deep-water cichlids mirror the adult SWS2A-dominated expression profile of tilapia. This is different to what has been found in Lake Malawi cichlids where the single cone gene expression patterns are similar to the neotenic pattern of riverine fish, while the double cones remain unchanged (O'Quin et al., 2011).

4.6 | Opsin expression in shallow-water species and its link to feeding ecology

We also observed differences in gene expression profiles among the shallow-water species (Figure 1d). Three species from the genus *Sarotherodon* lack the expression of the deep-water green opsin (RH2Aα). Morphologically derived species, on the other hand, such as predatory and macroinvertebrate feeders *Stomatepia* (three species), and *K. eisentrauti*, the sponge specialist *P. maclareni*, and *S. lohbergeri* express both copies of RH2A. The expression profiles of the probably most recent species pair in Lake Barombi Mbo – *S. caroli* and *S. linnellii*, which are rather similar morphologically and virtually undistinguishable as juveniles – differ mainly by their usage of single cone opsins. *S. caroli* is the species in Barombi Mbo with the highest expression of the violet/blue-sensitive SWS2B (over 30% of the total single cone opsins; Figures 1d and S1, Table S2). We also detected low levels of RH2B expression in *S. caroli and S. steinbachi*, but not in *S. linnellii*. This gene is otherwise known to be used exclusively during the larval period in Nile tilapia (Spady et al., 2006). Similarly, we detected expression of the UV-sensitive SWS1 opsin (expressed in larvae of Nile tilapia; Spady et al., 2006) in one species, *S. steinbachi*, an algae/debris feeder and the most herbivorous species from the lake (Baldo et al., 2017; Trewavas et al., 1972; and personal observation of higher plant feeding). The link between herbivory and SWS1 expression is in agreement with the observations of O'Quin et al. (2010) in Malawi cichlids, where algae scrapers (together with zooplanktivores) have increased expression levels of SWS1. None of the species in Barombi Mbo has, however, the SWS1-dominated expression profile of the single cones as observed in Malawi (Hofmann et al., 2009).

4.7 | Spatial distribution of the cone cells in the retina and function of the visual system

Cichlids from the African lakes have previously been found to rely upon a trichromatic visual system (Carleton, 2009), but many species express more than three cone opsin genes, which can be explained by the coexpression of more than one gene per cell (Hofmann et al., 2009). In the Barombi Mbo cichlids, one species expresses six different cone opsin genes (all three single cone and three double cone opsins), seven species express five opsins and one other species expresses four different opsin genes. However, it remains open whether there is any coexpression of genes in a single cell and how many types of photoreceptor cells are present in the retina. Even in the most intensively studied cichlids with respect to their vision, the ones of Lake Malawi, it still remains unclear if the different cone classes are distinct enough to be more than trichromatic (Carleton et al., 2016). On the other hand, the Barombi Mbo deep-water species are clearly missing the red channel, and are probably dichromatic with strong dominance of RH2Aα in the double cones and SWS2A in the single cones. However, without functional measurements (such as microspectrophotometry) or behavioural experiments, any conclusion about their chromatic level is to a certain degree speculative. Other factors, for example the A1/A2 chromophore switch and ratio could also contribute to shifts in spectral sensitivity. The simultaneous usage of both chromophores has been reported previously e.g., in Victoria cichlids (Miyagi et al., 2012) or the Nile tilapia (Carleton et al., 2008), while Malawi cichlids seem to exclusively use the A1 chromophore (Carleton et al., 2016). Based on our data, we have no information about the chromophore type used in the Barombi Mbo cichlids.

We confirmed, using FISH, that LWS and RH2A (both copies) are expressed in the double cones and SWS2A is expressed in the single cones. This is in accordance with Dalton et al. (2014) who found that LWS, RH2B, RH2Aα and RH2Aβ always occurred in double cones, but never in single cones in Malawi cichlids. We have further identified the presence of the most common double cone cells, with one half (one cell) expressing LWS and the second cell expressing RH2A. In one species, *K. eisentrauti*, we also identified twin cones (double cones with both cone cells expressing the LWS opsin gene), while we have not detected such twin cones in the second shallow-water species, *St. mariae* (Figure 4f). Such twin cones with both cells expressing the identical opsin protein (LWS) have been previously observed in Neotropical cichlids, where they represent the dominant type of cone cells (Torres-Dowdall et al., 2017). Contrarily, in the deep-water species *M. myaka* we found that the twin cones express RH2A, putatively RH2Aα (note that our FISH probes were however not able to distinguish between RH2Aα and RH2Aβ). In three species – two shallow- (*St. mariae*, *K. eisentrauti*) and one seasonally deep-water (*M. myaka*) specialist – we found a typical retinal mosaic composed of the four double cones surrounding one single cone cell (Fernald, 1981), whereas the obligatory deep-water species, *K. dikume*, seems to have lost the regular retina mosaic and has predominantly double cones with less regular organization (Figure 4i).

4.8 | Comparative transcriptomics to study retina adaptation to the deep-water environment

In addition to the candidate gene approach focusing on opsins, we performed a comparative gene expression analysis between the retinal transcriptomes of two deep-water (*K. dikume* and *M. myaka*) and two shallow-water species (*K. eisentrauti* and *St. mariae*). While each

of the pairwise comparisons revealed hundreds of differentially expressed genes, only a subset of these genes is shared among comparisons (Figure 3). We thus identified 46 genes differentially expressed along the depth axis (Table 3), of which about half could be linked to a function relevant for living in the deep water, namely vision, hypoxia management, haemostasis and circadian clock. Interestingly, several of the differentially expressed genes are known to be associated with eye diseases in human, such as glaucoma, retinopathy or the S-cone syndrome.

The most differentially expressed gene was LWS (downregulated in the deep-water species). Apart from the opsin genes LWS and SWS2B we observed downregulation in *Tmem116* known to be involved in eye development and in the protection from light damage in mouse (Natoli et al., 2010) and *aldh1a1* known to mediate protection from UV radiation in the mammalian cornea (Chen, Thompson, Koppaka, Jester, & Vasiliou, 2013), suggesting possibly a reduced light protection system in the deep-water species. Interestingly, the *aldh1a1* has been previously found as missing in many teleosts (Braasch et al., 2016; Pittlik, Domingues, Meyer, & Begemann, 2008) but not in cichlid genomes (e.g., annotation of genomes in Brawand et al., 2014), and therefore, further interpretation about its exact function would require additional evidence.

The set of differentially expressed genes further contained several genes involved in photoreceptor cell development, including *nr1d1* (highly upregulated in deep-water species), which is also known (apart of other functions; Mollema et al., 2011) to protect the retina from the so-called S-cone syndrome, that is, the dominance of S-cones over other photoreceptor cell types, in humans (Cruz et al., 2014). Interestingly, we had observed a trend to loosen regularity in the retina mosaic (Figure 4i) and an obvious lack of single cones (equivalent to the S-cones in human) in the deep-water specialist *K. dikume*, which might be related to *nr1d1* overexpression. Finally, we found that the expression of *rorb* was highly correlated with SWS2B (downregulated in deep-water species). It has previously been shown in mouse that *rorb* regulates the expression of the short wavelength-sensitive opsin gene (Srinivas, Ng, Liu, Jia, & Forrest, 2006), and it is possible that a similar mechanism applies to cichlids.

Four of the differentially expressed genes are known to be involved in the regulation of the intraocular pressure: *slc4a5* (also known as NBC-1), responsible for maintaining the pH in the eye and known to cause glaucoma when mutated in humans (Usui et al., 2001); *g2e3*, associated with changes in the intraocular pressure in mouse (Panagis et al., 2011); *fhod1*, which has been shown to respond to changes in the intraocular pressure in rats (Luna et al., 2012); and *paqr6*, which has been associated with glaucoma in human and chimp (Kompass et al., 2008). It is worth pointing out that deep-water species live in higher pressure than their shallow-water relatives, with the pressure increasing each 10 m of depth by about one atmosphere. However, the exact mechanism of the regulation of the intraocular pressure in cichlids and its potential link to deep-water adaptation remains unknown.

Another set of differentially expressed genes between deep- and shallow-water species can be associated to hypoxia, a condition likely to be experienced by the deep-water cichlids in Barombi Mbo (Figure 1b). The obligatory deep-water specialist *K. dikume*, for example, is known to live in a depth of around 20 m and potentially even "dives" below the oxycline (detected between 15 and 25 m depending of season in Barombi Mbo; Figure 1b) into the anoxic zone to feed on *Chaoborus* larvae (Green, Corbet, & Betney, 1973). This species also shows several physiological adaptations towards a greater oxygen storage ability, such as a higher haemoglobin concentration, a higher red blood cell count, and possibly also a higher overall blood volume (Green et al., 1973). We found strong (in *K. dikume*) and moderate (in *M. myaka*) upregulation of *HIF-1α-like* (also known as *hlf* or *epas1*), which is known to activate the expression of erythropoietin in mammals (Wang & Semenza, 1993) and to prevent retinopathy (retina dystrophy) in mouse during hypoxia (Morita et al., 2003). Two genes involved in haemostasis, *ptgis* and *tbxa2r*, were also among the differentially expressed genes.

Interestingly, nine out of the 46 differentially expressed genes have known functions in the regulation of the circadian clock (Table 3), which is in turn regulated and calibrated by the daylight rhythm and, hence, is expected to underlie different constraints in the light-deprived deep-water habitat as compared to the shallow areas. Future studies are needed to address the circadian-clock regulation in more detail.

Taken together, we identified 46 genes that are differentially expressed in the retina between deep- and shallow water cichlids in crater Lake Barombi Mbo, many of which have known functions related to the visual system. It remains unclear, however, if what we detect in the comparative gene expression assays is a plastic response to the environmental conditions, or, alternatively, if modifications of the expression of any of these genes might have contributed to deep-water adaptations or even to speciation, for example by increasing tolerance or capacity to cope with the extreme environment allowing the ancestor to colonize the deep-water habitat in this crater lake.

In conclusion, our study unveils a complex scenario regarding the evolution of the visual system in a small-scale adaptive radiation, the monophyletic species flock of cichlid fishes in crater lake Barombi Mbo. We found associations in the visual palette with respect to adaptations to the deep-water habitat (one specific amino acid substitution, lack of red and violet sensitivity, alternative usage of RH2A copies), as well as to trophic specializations (UV-sensitivity). Our study describes for the first time the case of alternative involvement of the RH2Aα and RH2Aβ copies in the association with the deep-water environment. Overall, our results suggest that rapid adaptations of the visual system of cichlids to the novel deep-water habitat in Barombi Mbo primarily occurred at the level of gene expression changes rather than through mutations in opsin gene sequences. We further identified a set of 46 differentially expressed genes between deep- and shallow-water species, some of which can be associated with potential adaptation to the deep-water conditions, such as modified vision, hypoxia management, haemostasis or circadian clock. This demonstrates that adaptations in the visual system not only involve opsin genes.

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AUTHOR CONTRIBUTIONS

Z.M., and W.S. designed research; Z.M., A.I., A.R.B.-N., D.O., M.K., L.A., and K.R. performed the research; Z.M., D.O., M.K., L.A., and K.R. analyzed data; Z.M., and W.S. wrote the paper. All coauthors contributed their comments to the manuscript.

DATA AVAILABILITY STATEMENT

Molecular data is provided on GenBank database. DNA sequences of the opsin genes: Genbank accessions numbers MN258381–MN258511. Transcriptome data: GenBank accession accession numbers PRJNA556940 and PRJNA421052. See details on individual accession numbers in Table 1.

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SUPPORTING INFORMATION

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