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Visual pigments of African cichlid fishes: evidence for ultraviolet vision from microspectrophotometry and DNA sequences

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Abstract

We have found evidence for ultraviolet visual capabilities in a Lake Malawi cichlid fish, *Metriaclima zebra*. Microspectrophotometry of single cones revealed a visual pigment with peak sensitivity at 368 ± 4 nm. *M. zebra* also expresses a putative ultraviolet opsin gene whose sequence is closely related to the SWS-1 opsin for other fishes. Several other African cichlids have a functional copy of this UV gene in their genomic DNA, but do not appear to express this gene as adults. These results suggest that ultraviolet vision is important for some cichlid fishes. UV wavelengths should therefore be included in future studies of cichlid vision, behavior and color patterns. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The cichlid fishes of the East African Rift lakes are one of the best known examples of rapid vertebrate radiations. In Lake Malawi, an estimated 500–1000 species have arisen in the last 1.5 million years (Fryer & Iles, 1972; Lewis, Reinthal & Trendall, 1986). Males display bright breeding coloration and this color pattern is a key distinction between closely related species, as has been demonstrated from taxonomic (Ribbink, Marsh, Marsh, Ribbink & Sharp, 1983), behavioral (McElroy & Kornfield, 1990), and genetic work (Albertson, Markert, Danley & Kocher, 1999). Speciation of these fishes may be driven by sexual selection for these male traits based on female preferences (Dominey, 1984).

Several lines of evidence suggest that vision is important in cichlid mate choice. Behavioral experiments show that females correctly choose conspecifics when visual cues are available (Kellogg, 1997; Seehausen & van Alphen, 1998), but choose non-assortatively when color differences are masked (Seehausen, van Alphen & Witte, 1997; Seehausen & van Alphen, 1998). Male color pattern is therefore an important character used by females to recognize and choose mates. Recent theories on sexual selection suggest that male color patterns may evolve to better stimulate female visual systems (Ryan, 1990; Kirkpatrick & Ryan, 1991; Endler, 1992). To understand this evolution, it is necessary to determine the spectral sensitivities of the cichlid visual system.

Several studies have examined the visual systems of a few African cichlid species including *Haplochromis burtoni* from Lake Tanganyika (Fernald & Liebman, 1980; Fernald, 1981, 1984) and several haplochromines from Lake Victoria (van der Meer & Bowmaker, 1995). These species have well defined retinal mosaics composed of double cones and one type of single cone. The absorption maxima for the cones from all of these species are similar. Single cones have a short wavelength sensitive pigment (455–465 nm) and double cones contain medium (520–540 nm) and long (560–600 nm) wavelength sensitive pigments.

The survey of fish visual pigments by Levine and MacNichol (1979) include two species from Lake

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Malawi: the rock dwelling *Pseudotropheus zebra* (now *Metriaclima zebra* (Stauffer, Bowers, Kellogg & McKaye, 1997)) and the sand dwelling predator *Haplochromis compressiceps* (now *Dimidiochromis compressiceps* (Eccles & Trewavas, 1989)). Absorbance data were taken only for double cones, but there were marked differences between the two species. The peak absorbances of *D. compressiceps* pigments were 536 and 569 nm, while those of *M. zebra* were 488 and 533 nm. The considerable blue shift of the cones of *M. zebra* relative to *D. compressiceps* suggested that *M. zebra* might have ultraviolet sensitive single cones.

A variety of fish species have ultraviolet sensitive cones (Hárosi & Hashimoto, 1983; Bowmaker, 1990, 1995; Hawryshyn & Hárosi, 1991, 1994) which are integrated into their color opponent system (Hárosi & Fukurotani, 1986; Hashimoto, Hárosi, Ueki & Fukurotani, 1988; Douglas & Hawryshyn, 1990; Jacobs, 1992; Tovee, 1995). The UV sensitive fishes studied to date typically have four visual pigments, one of which is UV sensitive and found in an additional corner cone in the retinal mosaic (Bowmaker, 1995). UV sensitivity may be lost (Bowmaker & Kunz, 1987) or even regained (Beaudet, Novales Flamarique & Hawryshyn, 1997) with age.

Visual pigments are composed of an opsin protein bound to a chromophore, where the chromophore is most commonly either 11-*cis* retinal (A_1) or 3-dehydroretinal (A_2) (Bowmaker, 1995). There are five primary classes of vertebrate opsins: the rod opsin, RH1, and four cone opsins: SWS-1 (ultraviolet sensitive), SWS-2 (short wave sensitive), RH2 (mid wavelength sensitive and similar to rod opsin), and LWS/MWS (mid to long wavelength sensitive) (Okano, Kojima, Fukada, Shichida & Yoshizawa, 1992; Hisatomi, Kayada, Aoki, Iwasa & Tokunaga, 1994; Chang, Crandall, Carulli & Hartl, 1995; Yokoyama 1994, 1995, 1997). Each of these classes has distinctly different amino acid sequences with certain key sites controlling the peak absorption (Chang et al., 1995; Yokoyama & Radlwimmer, 1998). Because each of the five opsin classes diverged prior to the evolution of jawed fish, the class to which a particular opsin belongs can be uniquely determined from short (200 bp) segments of DNA sequence obtained with the use of degenerate primers (Hisatomi et al., 1994).

Sequences for complete cone opsins are available for only a handful of fish species including a blind cavefish, *Astynax fasciatus* (Yokoyama & Yokoyama, 1990a,b, 1993; Register, Yokoyama & Yokoyama, 1994), a goldfish *Carassius auratus* (Johnson, Grant, Zankel, Boehm, Merbs, Nathans et al., 1993; Hisatomi, Satoh, Barthel, Stenkamp, Raymond & Tokunaga, 1996), a killifish *Oryzias latipes* (Hisatomi, Satoh & Tokunaga, 1997) and a zebrafish *Danio rerio* (Vihtelic, Doro & Hyde, 1999). Goldfish, killifish and zebrafish all have a gene from each of the four cone classes, including the SWS-1 ultraviolet class. In cavefish, genes for all but the SWS-1 class have been found.

We are interested in characterizing the visual system of Lake Malawi cichlids and understanding the role that vision plays in sexual selection and speciation. We are particularly interested in whether UV vision plays a role in any of their visual activities. Since the waters of Lake Malawi are some of the clearest in the world (Muntz, 1976), ultraviolet light should be efficiently transmitted (Loew & McFarland, 1990) and therefore could be an important part of cichlid visual signaling. This first study uses microspectrophotometry (MSP) to demonstrate the presence of UV sensitive visual pigments, and DNA sequencing of opsins to detect expression of a putative ultraviolet opsin gene.

2. Materials and methods

2.1. Cichlid taxa

We studied two Lake Malawi cichlid species previously analyzed by microspectrophotometry: *M. zebra* and *D. compressiceps* (Levine & MacNichol, 1979) as well as *Labeotropheus fuelleborni* and a tilapia, *Oreochromis niloticus*. *M. zebra* and *L. fuelleborni* are members of the rock dwelling mbuna found in Lake Malawi while *D. compressiceps* is a sand dwelling piscivore. The tilapia *O. niloticus* is widely distributed across Africa and is a sister group to the cichlids of the three major East African lakes (Streelman, Zardoya, Meyer & Karl, 1998). Individuals of *M. zebra* and *L. fuelleborni* were obtained in the field and shipped to our fish facility at the University of New Hampshire. The *M. zebra* individual used for DNA sequencing was a female orange blotch morph from Nkhata Bay. Three different male individuals of *M. zebra* from Nkhata Bay were used for the spectral determinations. The *L. fuelleborni* was from the southeast arm of the lake. *D. compressiceps* and *O. niloticus* were obtained from commercial fish breeders in the US. One individual each of *M. zebra* and *D. compressiceps* was used for the degenerate primer study. One individual from each of *M. zebra*, *D. compressiceps* and *O. niloticus*, was used for mRNA preparations and cDNA sequencing. Fin clips from these same individuals, as well as an individual of *L. fuelleborni*, were used for genomic DNA sequencing.

2.2. Spectral determination

Spectral determinations were carried out on several wild caught *M. zebra* and one individual of *O. niloticus*. Experimental specimens were dark adapted for at least 1 h prior to use and anaesthetized with MS-222. Fish were enucleated and the retina was dissected out under

dim red light. Retina were mounted between quartz cover slips and sealed with paraffin wax. The instrumental description as well as methods of data analysis have been previously described (Hárosi & MacNichol, 1974; Hárosi, 1982, 1987; Singarajah & Hárosi, 1992).

2.3. Genomic DNA sequences using degenerate primers

DNA sequence was obtained from a short segment of the cone opsins using degenerate primers for both *M. zebra* and *D. compressiceps* (Table 1). The primers are slight variations of those of Hisatomi et al. (1994). They were designed to amplify all cone opsins and matched two conserved regions (amino acid sequences S T Q K A E and Y N P I/V I/V Y) spanning TM 6 and 7 in exon 4 (Fig. 1). The forward and reverse primers have EcoRI and HindIII restriction sites, respectively, for directional cloning. Genomic DNA was extracted from 1 to 2 mm^2 fin clips using proteinase K digestion followed by phenol:chloroform extraction and ethanol precipitation. DNA was resuspended in TE/10 and 1 μl was used in a 50 μl PCR reaction for 35 cycles (94°C 1 min, 40°C 1 min, 72°C 1 min). To produce enough template for cloning, 1 μl of this reaction mix was used in a second PCR reaction following the same conditions. The 210 bp product was digested using EcoRI and HindIII, gel purified and ligated into pBluescript II SK (Stratagene). XL-1 Blue cells were electroporated with 1 μl of the ligation mixture and plated. The plasmid was extracted from an overnight culture using a Wizard miniprep kit (Promega) and sequenced on an ABI 373A automated sequencer using M13 reverse primer. Strands were sometimes sequenced in the complimentary direction with T7 primer. Sequences from three to eight clones were compared for each opsin class to determine the gene sequence.

Table 1
Primers used for UV opsin amplifications[a]

Application	Primer name	Primer sequence
Genomic degenerate PCR	OPF	GCGAATTCGCNTCNACNCARAARGCNGA
	OPR	CGAAGCTTACRTANATNAYNGGRTTRTA
5′ RACE	UV cR1	CGAAGCTTGGTGACAAGTCGATAGT
	polyCamp	GCGAATTCGCGGCCGCGGATC_{12}
	Camp	GCGAATTCGCGGCCGCGGAT
3′ RACE	UV cF1	GCAAGCTTGTGGGGTCCTTCGTCAC
	UV cF2	GCGCGGAATTCTGCTATGGCCCGTACGCC
	polyTamp	GCGAATTCGTCGACAAGGCT_{17}
	Tamp	GCGAATTCGTCGACAAGGC
Genomic UV opsin	UV F1	GCGCGGAATTCAAAGAGCTCAGGGTCACAATG
	UV R1	GCGCGCAAGCTTCCCATGAACCAGGTGAAGG
	UV F2	GCGCGGAATTCGTGACCGCCTGGTCTTTG
	UV R2	GCGCGCAAGCTTAGCAGCTGGGAGTAGCAGAA
	UV F3	GCGCGGAATTCACATCCCTGAAAGTCTGGGC
	UV R3	GCGCGCAAGCTTCTGTTTGTTCATGAAGACGTAGA
	UV F4	GCGCGGAATTCAGTCAGCCTCCACCCAGAAG
	UV R4	GCGCGCAAGCTTGCTCAGTCAACGCCCTCTTA
μsat amplification	usat F	AGCTGCTGGGTGCTCTGA
	usat R	dye-CTGCAACCTGCAGAGGAAAC

[a] Except for the microsatellite primers, all primers have either an EcoRI (GAATTC) or HindIII (AAGCTT) restriction site at the 5′ end for cloning.

2.4. cDNA sequences from retinal mRNA

Sequences for the putative UV opsin gene were obtained from messenger RNA expressed in retinas using rapid amplification of cDNA ends (RACE, Frohman, Dush & Martin, 1988; Ohara, Dorit & Gilbert, 1989; Hisatomi et al., 1996). Total RNA was extracted from the retinas of both eyes from a single fish using guanidine isothiocyanate (Trizol, Gibco), precipitated with isopropanol and resuspended in water. Messenger RNA was isolated using poly-T magnetic beads (Dynal, 1995). The poly-T on the beads was used as the reverse transcription primer with a M-MLV reverse transcriptase (Superscript II, Gibco) at 42°C for 1 h. The resulting first strand cDNA was poly-G tailed to create a priming site for second strand cDNA synthesis (Lambert & Williamson, 1993) as follows. Unprimed oligo dT was removed with T4 DNA polymerase. The mRNA template was removed by denaturing at 95°C and the remaining cDNA was poly G tailed with terminal transferase. Second strand cDNA was made using a polyC primer in a PCR mixture for one cycle of 94°C for 1 min, 40°C for 15 min and 72°C for 30 min. This primer contained unique sequence for further amplification and a unique restriction site for directional cloning. The double stranded DNA was then stored for RACE amplifications.

RACE was performed in both the 3′ and 5′ directions using gene specific primers (Table 1) designed from the

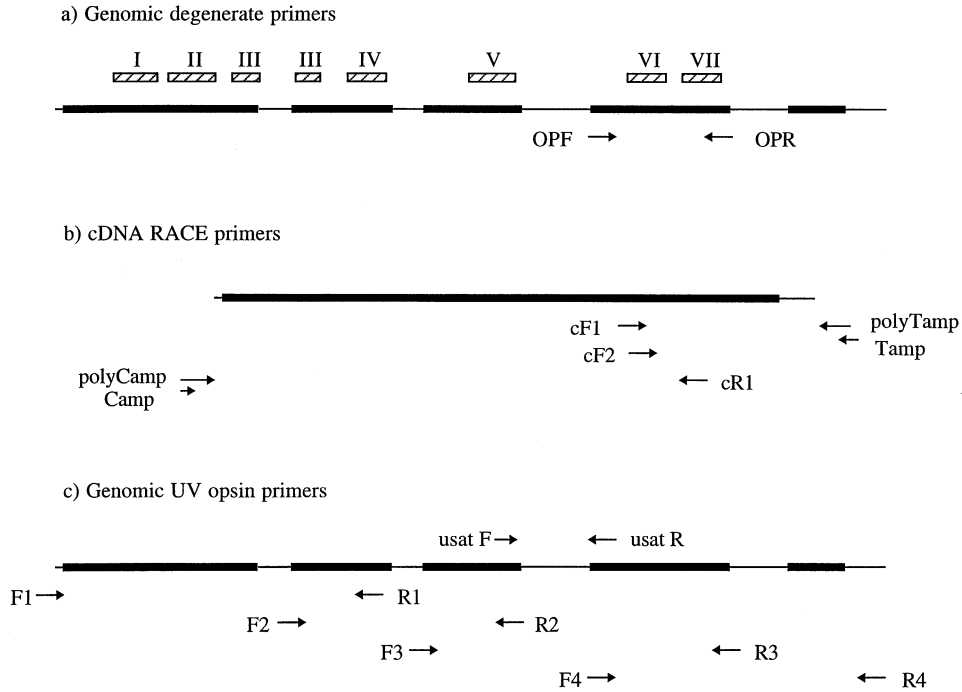

Fig. 1. Amplification strategies and primer locations used for degenerate primer, RACE and genomic opsin sequencing. (a) Degenerate primers used to amplify genomic DNA in TM VI and VII. Exons are shown as heavy lines and TM regions are identified by Roman numerals. (b) RACE primers used to amplify retinal cDNA. (c) Gene specific primers used to amplify the entire genomic UV gene and the microsatellite repeat in intron 3. Primer pairs used together are shown on the same line. Primer sequences are given in Table 1.

degenerate primer UV opsin sequence (Fig. 1). 3′ RACE used the first strand cDNA with primers cF1 (1 μM), polyTamp (50 nM) and Tamp (2 μM). 5′ RACE used the second strand cDNA with primers cR1 (1 μM), polyCamp (50 nM) and Camp (2 μM). 5′ RACE mix was added to the beads and heated to 95°C to denature the second strand into solution. 3′ RACE mix was then added to the beads which still held the first strand cDNA. Primer cF1 or cR1 was added and 10 cycles of single sided amplification were performed (94°C 20 s, 54°C 45 s, 72°C 2 min). The Tamp (3′ RACE) or Camp (5′ RACE) primers were added with a small quantity of polyTamp or poly Camp and the mix cycled for an additional 40 cycles. The resulting products were gel purified and then agar digested with β-agarase. The 3′ RACE product was reamplified using a nested primer (cF2) and T amp (93°C 30 s, 50°C 1 min, 72°C 2 min for 35 cycles) and directly sequenced. The 5′ RACE band was reamplified using the same 5′ RACE primer and C amp (93°C 30 s, 50°C 1 min, 72°C 2 min for 35 cycles). The product was restricted with EcoRI and HindIII and cloned into pBluescript II. Three clones were sequenced in both the forward and reverse directions using M13R and T7 primers.

2.5. Complete genomic opsin sequences

The cDNA sequence from the RACE procedure was used to design four pairs of primers to amplify the UV opsin gene from genomic DNA (Primer 3 software, Rozen & Skaletsky, 1998). These primers (Table 1) were placed in the exons to amplify across each of the four introns (Fig. 1). The entire UV opsin gene was then amplified using four separate PCR reactions for each of the four cichlid species. The reactions contained 1 μl of genomic DNA in a 50 μl reaction and were amplified for 35 cycles (93°C 30 s, 50°C 45 s, 72°C 1.5 min). The products were gel purified, agarased and cycle sequenced in both directions using an ABI 373A sequencer.

2.6. Amplification of a microsatellite repeat

The sequences for the UV opsin revealed a tetranucleotide repeat in intron 3. We designed primers to amplify this intron in order to look for variation in repeat length within an individual (alternate alleles) and between species. The PCR conditions were 2 μl of DNA in a 20 μl reaction, amplified for 30 cycles at an annealing temperature of 54°C. The products were run on the ABI 373A with size standards in the same lanes and analyzed with Genescan software.

2.7. Sequence analysis

The coding region for the nucleotide sequences were compared using MEGA (Molecular Evolutionary Genetics Analysis ver 1.0, Kumar, Tamura & Nei, 1993).

This program was used to calculate the divergence at synonymous and nonsynonymous sites between each pair of sequences. The inferred amino acid sequences for the cichlid UV opsins were compared with several other fish opsin amino acid sequences as well as those for chicken obtained from Genbank. The octopus rhodopsin gene was used as the outgroup. These sequences were aligned with Megalign in the Lasergene Navigator program (DNASTAR) using the Clustal V method (Higgins & Sharp, 1989). Phylogenetic relationships of the opsin amino acid sequences were estimated by the neighbor joining distance method using PAUP 3.1 (Phylogenetic Analysis Using Parsimony; Swofford, 1993). The significance of each node was assessed from 1000 bootstrap replicates.

3. Results

3.1. Spectral determination

We concentrated our survey of retinal photoreceptors primarily on single cones. From three individuals of *M. zebra*, we measured 12 single cones. From these, ten sets of data were further analyzed and seven of them were selected for averaging. The absorbance spectrum shown in Fig. 2 is the mean of 88 scans of seven different cells. The spectra were individually smoothed by Fourier filtering and then averaged to give a peak wavelength sensitivity (mean $\pm$ 1 S.D.) at 368 ± 4 nm ($n = 7$). Similarly, the individually processed dichroic ratios yielded 2.4 ± 0.5 ($n = 5$). The average spectrum (solid line) of Fig. 2 peaks at 369 nm and has a corresponding dichroic ratio of 2.1. The latter value compares favorably with dichroic ratios obtained from other fish cones (e.g. Hárosi & Hashimoto, 1983).

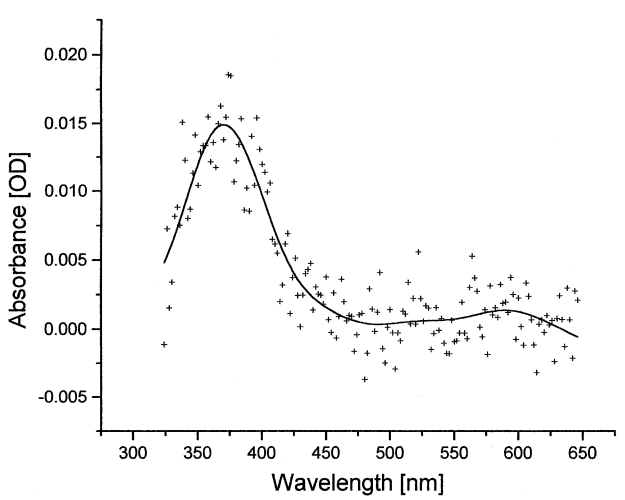

Fig. 2. MSP spectrum of single cones from Lake Malawi cichlid, *M. zebra*. Spectra is the average of seven cones taken from three different fish. Raw data and a Fourier smoothed curve are shown. The peak absorbance is 368 ± 4 nm. The minor irregularities in the spectrum at 470 and 570 nm are believed to be insignificant. Their small amplitude of $1–2 \times 10^{-3}$ OD correspond to the instrument's limit in amplitude discrimination.

We also measured five outer segments from three double cones. These were of the L/S type with peaks at 531 nm ($n = 1$) and 482 ± 1 nm ($n = 2$). On one occasion, both outer segments of a double cone were measured in superposition yielding a broader spectrum with two shoulders; this was consistent with merging the two peaks obtained from individual L and S members. Our double cone measurements essentially confirm the λ_{max} values obtained previously by Levine and MacNichol (1979).

The main peak's half bandwidth (HBW, which is also the full width at half maximum) was determined whenever possible. The UV-type spectrum's HBW was 4800 ± 800 cm^{-1} ($n = 2$), the L member of a double cone was 4000 cm^{-1} and the S member of double cones was 4600 ± 200 cm^{-1} ($n = 2$). These values are indicative of visual pigments in which the predominant chromophore type is vitamin A_1-derived (Hárosi, 1994).

Following spectral scanning, some of the measured cells were recorded on video tape under infrared illumination. Dimensions from three single cone outer segments were ascertained. The cells were 2.7–3.4 μm at the base, 4–7.6 μm tapered length and about 1.7 μm diameter at the tip. Based on these figures, the average pathlength in our measurements is estimated to be 2.5 μm. If we take the individually processed single cone spectra with their corresponding dichroic ratios, the mean transverse peak density is 0.02278 ± 0.0053 ($n = 5$) and the transverse specific density, Str = 0.009 μm^{-1}. The latter value is lower than specific densities determined for some photoreceptors. However, it is comparable with results obtained from other UV-type cones, such as that in rainbow trout (Hawryshyn & Hárosi, 1994).

A brief survey of the *O. niloticus* retina from a single fish showed no evidence for UV cones, as the peak absorbances were 450–470 nm for the few single cones examined.

3.2. Genomic DNA sequences using degenerate primers

Sequences from the degenerate primer amplification of both *D. compressiceps* and *M. zebra* yielded 160 bp of unique sequence. Sequenced clones were sorted into classes to determine the consensus sequence for each class. Based on these results, four classes of opsin genes were found for each of these two species. Phylogenetic comparisons of these sequences with those of the goldfish (Johnson et al., 1993; Hisatomi et al., 1996) identified them as the SWS-1, SWS-2, RH2 and MWS/LWS genes.

Table 2
Size of microsatellite bands using primers usat F and usat R (Table 1) for one individual from each of four cichlid species[a]

Species	Allele size (bp)		Repeat number	
	Allele 1	Allele 2	Allele 1	Allele 2
D. compressiceps	171	175	8	9
O. niloticus	171	179	10	12
M. zebra	171	179	8	10
L. fuelleborni	163	171	6	8

[a] Sizes and number of repeats (n) are given for each of two alleles for the $(TCAA)_n$ repeat.

3.3. UV opsin cDNA sequences from retinal mRNA

RACE amplifications of the UV gene were performed in both the 3′ and 5′ directions for all three species. There was no product from 5′ RACE amplification of *D. compressiceps* or *O. niloticus*. The 3′ RACE amplification of these two species produced three bands. All were found to be products arising from contaminating *E. coli* DNA.

For *M. zebra*, there was one unique band for the 5′ RACE (900 bp) and two positive bands for 3′ RACE (700 and 350 bp). When the larger 3′ RACE band was amplified with a nested primer, the product could be directly sequenced to yield the 3′ UV opsin sequence. The 5′ RACE band was cloned and positive clones were tested for size of insert. A range of insert sizes occurred since some inserts were the result of incomplete reverse transcription. A series of clones with varying insert sizes was chosen so that the entire 900 bp RACE product could be sequenced using M13R and T7 plasmid primers. The 3′ and 5′ sequences overlapped by 80 bp and were easily aligned to produce the sequence for the entire coding region (Genbank accession # AF191219). The 5′ untranslated region of the gene was only 18 bp long. This short UTR segment is similar to the 15 bp 5′ UTR observed for the UV cDNA from killifish (Hisatomi et al., 1996).

3.4. Complete genomic sequences of the UV opsin

Using the four sets of overlapping primer pairs, the UV opsin gene was amplified from genomic DNA of *M. zebra* (the same individual as used for the RACE protocol), *D. compressiceps*, *L. fuelleborni* and *O. niloticus*. These primers amplified the UV opsin from all four species equally well. The genomic sequences, including those from the introns, are deposited in Genbank (accession # AF191220-3). The genomic sequence from *M. zebra* matched the RACE cDNA sequence exactly in all coding regions. The sequences for *D. compressiceps* and *M. zebra* are identical for the entire sequence, except for the number of tetranucleotide repeats in intron 3. The sequences for *M. zebra* and *L. fuelleborni* were everywhere identical except for the number of tetranucleotide repeats and two bases of the non-repetitive sequence from intron 3.

3.5. Amplification of a microsatellite

The one region of the gene which showed significant variation among the Lake Malawi species is the $(TCAA)_n$ tetranucleotide repeat in intron 3. Amplifying across the intron with the microsatellite primers yielded a PCR fragment of approximately 170 bp. When the fragments were analyzed on a gel, each of the four species showed two alleles with different tetranucleotide repeat number (Table 2). The length of the non-repetitive sequence between the forward and reverse primers can be determined from the DNA sequence. Using these lengths and the size determined from the microsatellite gel, the number of repeats was determined (Table 2). The repeat number was confirmed based on the sequence data. It is interesting to note that, although the alleles sized on the gel are the same for tilapia and *M. zebra*, the number of repeats for these bands is actually different. This is the result of an 8 bp deletion in the tilapia intron sequence immediately following the repeat which offsets the increase by two repeat units (8 bp) of the repeat in tilapia relative to *M. zebra*.

3.6. Sequence analysis

Since the genomic DNA sequence is identical in the coding region for all three Lake Malawi species, the translated amino acid sequences are also identical. Although there are no differences among the Lake Malawi species, there are considerable differences at both the nucleotide and the amino acid level between the Lake Malawi species and tilapia. In the nucleotide coding regions, there are 33 synonymous changes (247 total synonymous sites) and 21 nonsynonymous changes (758 total nonsynonymous sites) which result in a difference of 18 amino acids. There are also 28 changes in the introns (373 total sites) for this gene.

The amino acid sequence for Lake Malawi cichlids is shown in Fig. 3 and compared to that of tilapia. The amino acid sequences for the three other known fish SWS-1 genes are also shown. The amino acid sequences from these UV genes can be compared phylogenetically to assess their relatedness and homology to other known vertebrate genes. We used the neighbor joining algorithm on the proportion of amino acid differences to compare the Lake Malawi and tilapia UV genes with all opsins from goldfish, killifish, zebrafish, blind cavefish and those from chicken. A subset of this comparison including the fish UV genes and the cone

opsins of goldfish and chicken are shown in Fig. 4. Bootstrap values are high, particularly for the UV opsin clade, indicating strong support for this grouping. This suggests that the UV genes are all homologous and originated as part of the SWS-1 class early in vertebrate evolution as has been previously shown (Yokoyama, 1994; Kawamura & Yokoyama, 1996; Hisatomi et al., 1996).

4. Discussion

4.1. Ultraviolet visual pigment

We have detected an ultraviolet visual pigment in *M. zebra* by MSP with peak sensitivity at 368 nm. This supports our hypothesis that the blue shifted visual pigments in *M. zebra* double cones do correlate with a UV sensitive visual pigment in single cones. It also strongly suggests that at least this Lake Malawi cichlid is sensitive to ultraviolet wavelengths of light.

The half bandwidths for the *M. zebra* spectral peaks suggest that this cichlid predominantly utilizes an A_1 chromophore, though a small admixture of A_2 chromophore can't be ruled out. Use of an A_1 based pigment has been observed previously in Lake Malawi species (Muntz, 1976) and is consistent with Lake Malawi's high water clarity and constant solar illumination resulting from the lake's near equatorial location.

For *M. zebra* to be capable of UV vision, UV light must be transmitted through the ocular media, includ-

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                                                <---------I--*---------
   Malawi  MGKHFHLYEN ISKISPFEGP QYYLAPVWAF YLQAAFMGFV FFAGTPLNFI 50
  Tilapia  ..Y.......  ...V......  ......T...  ..........  L........V
Killifish  ...Y.Y....  ...VG.YD..  ......T...  ..........  ..V......V
 Goldfish  .DAWTYQFG.  L.........  ..H...K...  ..........  ..V.....A.
Zebrafish  .DAWAVQFG.  A..V.....E  ..HI..K...  ..........  .IV...M.G.

           --->          <------------II-------------->
   Malawi  VLVATMKYKK LRVPLNFILV NISFSGFIFV TFSVSQVFLA SMRGYYFLGH 100
  Tilapia  ..........  ..........  ..........  ..........  ..........
Killifish  ..L..A....  ......Y...  ..T.A.....  ..........  .V.....F.Q
 Goldfish  ..FV......  ..Q...Y...  ...LG....D  ........FS  AL.....F.Y
Zebrafish  ..FV......  ..Q...Y...  ...LA....D  .......SVC  AA....S..Y

            <----*----*-III------------->                    <----
   Malawi  TLCALESAVG SVAGLVTAWS LAVLSFERYL VICKPFGAFK FGSNHALAAV 150
  Tilapia  ......A...  AI........  ..........  ..........  ..........
Killifish  ......A...  A......S..  ..........  ..........  ..........
 Goldfish  ....M.A.M.  .I.....G..  ....A....V  .......S..  ..QSQ..G..
Zebrafish  ...SM.A.M.  .I.....G..  ....A....V  .......S..  ..QGQ.VG..

           ---IV------------->                              <**--
   Malawi  AFTWFMGIGC ACPPFFGWSR YIPEGLGCSC GPDWYTHNEQ YNTTSYTHFL 200
  Tilapia  .......V..  ..........  ..........  .........E  ......IY..
Killifish  I......VVR  -.........  ..........  ......NC.E  FSCA..SK..
 Goldfish  .L..II....  .T...W....  .....I.TA.  ......K..E  ...E...Y..
Zebrafish  V...II.TA.  .T........  .......TA.  ......KS.E  ..SE...Y..

           *-----*----V----------->                         <----
   Malawi  MVTCFIIPLS IIIFCYSQLL GALRAVAAQQ AESASTQKAE KEVSRMIIVM 250
  Tilapia  LI....F..T  ..........  ..........  ..........  ..........
Killifish  L.....C.IT  ....S.....  ..........  ..........  ..........
Goldfish   L.S..MM.IM  ..T.S.....  ..........  ..........  ......VV..
Zebrafish  LI...MM.MT  ....S.....  ..........  ...E......  R.....VV..

           ---VI------------->          <-------VII---*---------
   Malawi  VGSFVTCYGP YALAALYFAY STDENKDYRL VTIPAFFSKS ACVYNPLIYV 300
  Tilapia  ..........  ..........  ..........  ..........  S.........
Killifish  .A........  ...T.Q.Y..  .Q........  ..........  S........A
 Goldfish  .....V....  ..IT....S.  AE.S......  .A..SL....  S........A
Zebrafish  .....L..A.  ..VT.M...N  .DEP......  .A........  SS.......A

           ->
   Malawi  FMNKQFNGCI MEMVFGKTMD ESSEVSTKTE VSTAS-                335
  Tilapia  ..........  .......K..  ..........  .....-
Killifish  ..........  .......K.E  .A....S...  ...D.-
 Goldfish  .......A..  ..T....KI.  ......S...  T.SV.A
Zebrafish  .......A..  ..T....KI.  ......S...  T.SV.A
```

Fig. 3. Amino acid sequence for UV opsin gene for Lake Malawi cichlids (all three species *M. zebra*, *L. fuelleborni*, and *D. compressiceps* have identical sequence) compared to tilapia, *O. niloticus*, killifish, *Oryzias latipes* (Hisatomi et al., 1997), goldfish, *Carassius auratus*, (Hisatomi et al., 1996), and zebrafish, *Danio rerio*, (Vihtelic et al., 1999). Amino acid number is indicated at the right. TM regions are delimited (← →) and marked by Roman numeral. Sites which differ in polarity between the Malawi cichlids and tilapia are marked within the TM region with a *.

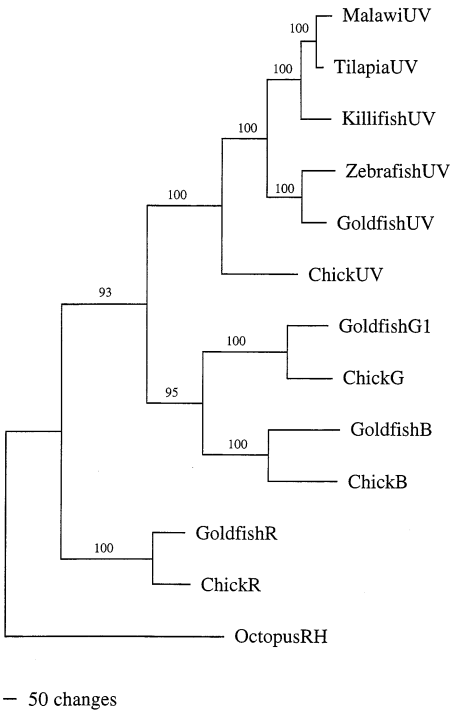

Fig. 4. Phylogenetic relationships obtained by neighbor joining of proportion of amino acid differences for the ultraviolet opsin genes of Lake Malawi cichlids, tilapia, killifish (# AB001605, Hisatomi et al., 1997), and zebrafish (# AF109373, Vihtelic et al., 1999) compared to all cone opsins of goldfish *Carassius auratus* (red # L11867, green # L11866, blue # L11864, Johnson et al., 1993; UV # D85863, Hisatomi et al., 1996), and chicken (red # M62903, green # M92038, blue # M92037, and violet # M92039, Okano et al., 1992). Octopus rhodopsin ((# X07797, Ovchinnikov, Abdulaev, Zolotarev, Artamonov, Bespalov, Dergachev et al., 1988) was used as the outgroup. Bootstrap values obtained from 1000 replicate samples are shown at each node. Genes are identified by species and followed by a letter to indicate the spectral sensitivity: R for red, G for green, B for blue, and UV for ultraviolet wavelengths.

ing the cornea, lens and vitreous humor. We have recently measured cornea and lens transmissions for *M. melabranchion* (closely related to *M. zebra*) and *D. compressiceps* and have found them both to be transmissive well into the UV (in prep). This agrees with UV transmission data for other African cichlids (Thorpe, Douglas & Truscott, 1993; Thorpe & Douglas, 1993).

Lens transmissions have been observed to change with fish age and size. Thorpe and Douglas (1993) found the 50% lens transmission of the tilapia, *O. niloticus*, varied from 340 to 400 nm as the lens diameter increased from 1 to 7 mm. Since tilapia grow to be quite large, their lenses are likely not UV transmissive for much of their adult life. In contrast, most of the mbuna are quite small (< 10 cm lengths) with lenses that are less than 3 mm in diameter (personal observation). These lenses may stay UV transmissive for most of their lifetime.

The 368 nm peak sensitivity that we observed for the *M. zebra* visual pigment is similar to that of many other fish having ultraviolet vision, where peak wavelength sensitivities vary from 350 to 390 nm (Hárosi 1994; Bowmaker, 1995). For many of these species, the ultraviolet cones function primarily in juveniles. However, studies on pomacentrids show that one species, *C. punctipinnis*, has ultraviolet visual capabilities as adults, but not as juveniles (McFarland & Loew, 1994). These studies demonstrate that ultraviolet visual pigments can be differentially expressed during the life cycle.

4.2. Putative ultraviolet opsin gene and its expression

We have found a putative ultraviolet opsin gene in the genomic DNA of three Lake Malawi cichlids and a tilapia. The UV genes appear to be fully functional and contain the amino acid sites characteristic of an opsin protein (Hisatomi et al., 1996). Comparison of the cDNA and genomic DNA sequences in *M. zebra* shows that the introns are delimited by the standard GT..AG splicing junctions in locations identical to other visual pigments (Yokoyama & Yokoyama, 1990a,b, 1993; Register et al., 1994). Because the *M. zebra* cDNA sequence had only 18 bp upstream of the start codon, the first primer designed to amplify the genomic DNA sequence included the first three bases of the start codon for the coding sequence, ATG. We can therefore not demonstrate conclusively that the start codon is present in all four cichlid species. However, it is unlikely that the primer would have amplified well if one of these bases were different. We therefore believe that all four species, *M. zebra*, *D. compressiceps*, *L. fuelleborni* and *O. niloticus* have a fully functional opsin coding region. Future efforts to express the opsin protein and determine the wavelength sensitivity of the corresponding visual pigment are required to prove that this putative SWS-1 gene encodes an ultraviolet sensitive visual pigment.

An SWS-1 mRNA was amplified from the retina of an adult *M. zebra*. The cDNA sequence was identical with the coding regions of the genomic sequence. In contrast, neither tilapia nor *D. compressiceps* expressed an mRNA in the retina corresponding to this gene. Comparisons of the RACE primer sequences with the genomic sequences showed them to be an exact match for all species. Therefore, the RACE procedure should amplify any mRNA transcripts present in the retina. The retinal RNA preparations for tilapia and *D. compressiceps* did produce RACE products for several other opsin genes (in prep) but not for the SWS-1 opsin gene. This suggests that this gene is not expressed in adult tilapia or *D. compressiceps*. Because the RACE procedure uses only one gene specific primer, it is not a definitive test for gene expression. Preliminary results from more sensitive RT-PCR (reverse transcription PCR) expression assays confirm that the UV gene is expressed in *M. zebra*, but not in *O. niloticus*. Further

RT-PCR studies are in progress to examine expression of all the opsin genes in a variety of cichlid species at several life stages. This will help determine if and when the SWS-1 gene might be expressed to make ultraviolet visual pigments.

4.3. Sequence analysis

The coding sequences for all three Malawi species are identical at both the nucleotide and amino acid level. This is consistent with the recent origins (< 1.5 million year) of the Lake Malawi species flocks. Comparisons of the tilapia and Malawi cichlid nucleotide sequences show 13.4% synonymous and 2.8% nonsynonymous differences. Assuming a 10 million year divergence time between tilapia and Lake Malawi cichlids, these correspond to divergence rates ($r = K/2T$) of 6.7×10^{-9} and 1.4×10^{-9} substitutions per site per year. These rates are consistent with those estimated for other nuclear genes (Li, 1998). The synonymous rate is significantly greater than the nonsynonymous rate, as expected for a protein coding region under purifying selection.

It has been proposed that changes in amino acid polarity at sites in the transmembrane regions are key to changes in peak wavelength sensitivity. Nonpolar to polar changes in TM III and TM VII are thought to cause shifts to shorter wavelengths (Chang et al., 1995). The sites which differ in polarity between the Malawi species and tilapia are marked (*) in Fig. 3. Of these, S107A and S111A (TM III), would contribute to shorter wavelength sensitivities in the Malawi species, but A291S (TM VII) would shift tilapia to shorter wavelengths. Future expression studies (Oprian, 1993; Kawamura & Yokoyama, 1998) or site directed mutagenesis are required to test for the importance of these sites in tuning visual pigment sensitivities.

The phylogenetic analysis suggests that the cichlid ultraviolet gene is a member of the ancestral SWS-1 family of opsin genes. This gene is found in all vertebrate groups including fish (Hisatomi et al., 1996, 1997), birds (Okano et al., 1992; Wilkie, Vissers, Das, DeGrip, Bowmaker & Hunt, 1998), reptiles (Kawamura & Yokoyama, 1996) and mammals (Chiu, Zack, Wang & Nathans, 1994). The cichlid gene is most similar to that of killifish, which agrees with current hypotheses of fish phylogenetic relationships. Within the Cichlidae, *O. niloticus* is basal to the haplochromine cichlids of the east African lakes. Since *O. niloticus* has an SWS-1 gene, it is likely to be found in cichlids from lakes Tanganyika and Victoria as well.

4.4. Possible function of ultraviolet vision

If *M. zebra* can detect ultraviolet light, what function would this visual capability serve? Several possible uses for ultraviolet vision have been proposed including foraging, navigation, and communication (Tovee, 1995). UV vision has been demonstrated to improve foraging for zooplanktivores (Browman, Novales-Flamarique & Hawryshyn, 1994). Many of the rock dwelling cichlids are predominantly herbivorous, feeding on algae growing on the rock substrate. However, they also feed on zooplankton (Reinthal, 1990) and are often observed going up into the water column to feed. Cichlids may therefore utilized UV vision to locate zooplankton.

Another possible explanation for the function of ultraviolet vision is its use in orientation and navigation (Tovee, 1995). Ultraviolet vision for long distance navigation or migration is unlikely to be important for the rock dwelling cichlids as males maintain fixed territories all year long. However, these fish do go up into the water column to feed and must then find their way back to the substrate. UV vision might enable them to orient on the lek and better find their particular territory within a larger assemblage of breeding males. Females may also use ultraviolet vision for physical orientation as they first sample males across a lek and then return to the location of their chosen mate.

It is also possible that ultraviolet vision is useful for communication between individuals. Male *M. zebra* are blue with black bars. This blue coloration has been shown to be highly reflective in the ultraviolet (Kornfield, 1991). Breeding sites are typically in high clarity, shallow waters (< 10 m depths). Attenuation coefficients for naturally clear waters (Smith & Baker, 1981) suggest that 70% of the solar ultraviolet illumination at 370 nm can penetrate to these depths. Since females survey the lek from distances of 5–10 m they should receive significant quantities of ultraviolet light reflected from the male color patterns. Studies with birds under UV+ and UV− conditions have shown that ultraviolet light is important in female starlings' ranking of mates (Bennett, Cuthill, Partridge & Lunau, 1997). Studies of cichlid mate choice under UV+ and UV− conditions are currently underway to assess whether UV signals play a significant role in cichlid mating (Jordan & Kellogg, personal communication).

4.5. Color usage by African cichlids

There has been considerable interest in African cichlids because of their bright coloration. Several studies have tried to determine what constraints are acting on color patterns and whether there is evidence for restrictive, adaptive color usage or more divergent sexually selected coloration (McElroy, Kornfield & Everett, 1991; Deutsch, 1997). These studies were based on computer analysis of color photographs which were reproduced in monographs on the Lake Malawi cichlids (Lewis et al., 1986; Ribbink et al., 1983). One possible limitation of this technique is the lack of

ultraviolet wavelengths in photographic representations of color patterns. Another complication results from color analysis based on hues defined for the human visual system which will skew the prevalence of particular 'colors'. Such analysis would best be done in terms of the color coding mechanisms of the animal doing the viewing (Chittka, 1992).

The conclusions that are drawn from these analyses suggest that cichlid color patterns are somewhat restricted, relying heavily on blues and yellows. They find little correlation between color usage and phylogeny, habitat (substrate, depth) or diet. Indeed variation in coloration was greater within species complexes rather than between them. From the lack of evidence for adaptive usage of color, and the large variation in color within species complexes, they conclude that color patterns are the result of sexual selection and female mate choice. To prove this conclusion, it would be ideal to repeat these analyses using live fish, with equipment which is sensitive over the spectral range of the cichlid visual system. We are working to define this spectral range for a number of cichlid species from Lake Malawi at different life stages. This will help define the color space being used by these fish and enable us to ask questions about the role of vision in the speciation of these fish.

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