

RESEARCH ARTICLE

Characterization of Opsin Gene Alleles Affecting Color Vision in a Wild Population of Titi Monkeys (*Callicebus brunneus*)

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The color vision of most platyrrhine primates is determined by alleles at the polymorphic X-linked locus coding for the opsin responsible for the middle- to long-wavelength (M/L) cone photopigment. Females who are heterozygous at the locus have trichromatic vision, whereas homozygous females and all males are dichromatic. This study characterized the opsin alleles in a wild population of the socially monogamous platyrrhine monkey *Callicebus brunneus* (the brown titi monkey), a primate that an earlier study suggests may possess an unusual number of alleles at this locus and thus may be a subject of special interest in the study of primate color vision. Direct sequencing of regions of the M/L opsin gene using feces-, blood-, and saliva-derived DNA obtained from 14 individuals yielded evidence for the presence of three functionally distinct alleles, corresponding to the most common M/L photopigment variants inferred from a physiological study of cone spectral sensitivity in captive *Callicebus*. *Am. J. Primatol.* 73:189–196, 2011. © 2010 Wiley-Liss, Inc.

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INTRODUCTION

The diversity of color perception within the order Primates is exceptional among mammals [Jacobs, 1993, 2009]. The two most common varieties of primate color vision are trichromacy, allowing animals to routinely distinguish among colors that appear to humans as green, yellow, orange, and red, and dichromacy, in which such colors are apt to be confused with each other [Jacobs, 2008; Sharpe et al., 1999]. Both of these varieties occur as polymorphisms in the majority of Neotropical monkeys (Platyrrhini) and in several lemurs (Strepsirrhini, Lemuroidea), resulting in a rich mixture of both dichromatic and trichromatic individuals in the same population of animals [Jacobs, 2007, 2008].

Polymorphic color vision results from an X-linked genetic polymorphism [Kawamura et al., 2001] of the M/L opsin locus. Usually two or three functionally distinct alleles code for M/L opsins that yield photopigments, contained in retinal M/L cone receptors, with maximal absorbance between 530 and 565 nm. Hemizygous males and homozygous females produce only a single variant of the M/L opsin, allowing (in combination with retinal S cones absorbing maximally at shorter wavelengths) for dichromacy. Heterozygous females produce two variants of M/L

opsin which segregate into different M/L cone cells in the retina and support trichromacy [reviewed in Jacobs, 2007, 2008; Surrige et al., 2003].

The relationship between the absorbance properties of platyrrhine M/L photopigments and the corresponding nucleotide changes in M/L opsin gene alleles has been well studied. In platyrrhines, crucial

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nonsynonymous changes at positions 180 in exon 3 and 277 and 285 in exon 5 of the M/L opsin gene cause the wavelength of peak photopigment light absorbance to vary over a range of about 30 nm [Hiramatsu et al., 2004; Neitz et al., 1991; Shyue et al., 1998]; several other sites may play minor roles (Table I).

The similarity and conservative maintenance of polymorphic color vision in nearly all extant platyrrhine genera suggest that it originated at least 20 million years ago in a basal platyrrhine [Boissinot et al., 1998; Hunt et al., 1998] and is under balancing selection [Surridge & Mundy, 2002]. The presumed selective advantages of trichromacy over dichromacy to wild primates are the subject of considerable debate. One hypothesis is that trichromacy facilitates the detection of yellow, orange, and red food items (e.g. edible fruits and leaves) against the green foliage background of tropical forests [Dominy & Lucas, 2001; Mollon, 1989; Sumner & Mollon, 2000a]. Others suggest an alternative or additional trichromat advantage in assessing the ripeness of fruits [Sumner & Mollon, 2000b], detecting and assessing conspecifics [Changizi et al., 2006; Fernandez & Morris, 2007; Sumner & Mollon, 2003], or detecting predators [Caine, 2002; Smith et al., 2005]. It may also be the case that frequency-dependent selection, group selection, and/or niche divergence have contributed to the maintenance of opsin polymorphisms in primates [Melin et al., 2008; Mollon et al., 1984] such that each color vision phenotype in a polymorphic primate population has an advantage for a particular ecological task [e.g. detecting certain fruits under differing forest light regimes: Melin et al., 2009; Regan et al., 2001; Yamashita et al., 2005]. Similarly, recent evidence suggests that dichromatic primates may have an advantage over trichromats while foraging for cryptically colored food items such as insects, and consequently, that a pure heterozygote (trichromat) advantage may be insufficient to explain the maintenance of the opsin polymorphism in some primate species [Caine et al., 2010; Melin et al., 2007, 2010].

In terms of polymorphic color vision, one of the most unusual platyrrhines is *Callicebus*, the titi monkey. In contrast to the two or three different M/L photopigments found in most other platyrrhine populations, five M/L variants were detected in a physiological study of captive *Callicebus*. By measuring the spectral sensitivities of M/L cones in the retinas of 82 *Callicebus* individuals [perhaps including representatives of *C. cupreus cupreus*, *C. cupreus ornatus*, and hybrids thereof: Bunce, 2009: Appendix A] at the California National Primate Research Center (CNPRC), Jacobs and Deegan [2005] found evidence for five distinct classes of M/L photopigment having peak absorbances at approximately 530, 536, 542, 551, and 562 nm, occurring at colony-wide frequencies of 0.068, 0.203, 0.085, 0.288, and

0.356, respectively. Photopigments corresponding (approximately) to each of these five M/L variants have been detected in other primate populations (Table I). However, aside from rare recombinants [Boissinot et al., 1998; Cropp et al., 2002], to date, no other primate population is known to contain all five M/L variants simultaneously [Jacobs & Deegan, 2005]. Examination of this unusual color vision of *Callicebus*, both in terms of the genetic basis for the five M/L photopigment classes, and the presumed adaptive basis for the maintenance (potentially over a very long period of evolutionary time) of such a large number of alleles, may yield important insights about the evolution of color vision, particularly when *Callicebus* is compared with other primates with different allele complements and ecologies.

This study provides a characterization of alleles of the polymorphic M/L opsin gene in *Callicebus* and constitutes the first step in a research program designed to examine the adaptive basis of polymorphic color vision in this primate genus.

## METHODS

### Sample Collection

Between August 2005 and October 2006, we noninvasively collected fecal samples (preserved with CaSO<sub>4</sub>-CoCl<sub>2</sub> indicating desiccant: W. A. Hammond Drierite Co. Ltd., Xenia, OH), and/or directly collected blood and saliva samples (on FTA<sup>®</sup> Classic Cards: Whatman plc., Brentford, Middlesex, UK) [Smith & Burgoyne, 2004] during animal capture, from 14 wild *Callicebus brunneus* individuals [brown titi monkeys: Hershkovitz, 1990] living in five socially monogamous groups, each group defending an exclusive territory in the vicinity of the Estación Biológica de Cocha Cashu, Manu National Park, Madre de Dios, Peru (11°53'S, 71°24'W) [see Terborgh, 1983 for a detailed site description]. Within groups, animals were individually recognizable by collars fit during this and previous investigations [Bossuyt, 2002; Bunce, 2009; Rodman & Bossuyt, 2007; A. Porter, personal communication]. The sample size was limited because of logistic constraints inherent in primate fieldwork, for example difficulty in locating, identifying, and habituating multiple small groups in a population covering a large area. All field methods complied with University of California, Davis IACUC protocol 05-11725 and research and sample exportation permits issued by the Peruvian National Institute of Natural Resources (INRENA).

### DNA Extraction

DNA was extracted in the Molecular Anthropology Laboratory of the University of California, Davis, (UCD) 5–36 months after sample collection. DNA from blood and saliva samples dried on FTA<sup>®</sup> Classic Cards was prepared using a manufacturer

**TABLE I. M/L Photopigment Peak Absorbance and Amino Acid Changes in the M/L Opsin Genes of Representative Primate Genera**

Genus	Approximate wavelength of peak light absorbance <sup>a</sup>						Absorbance tuning site and peak absorbance shift (nm) <sup>b</sup>								
							Exon 3			Exon 4			Exon 5		
	530 nm	535 nm	543 nm	550 nm	556 nm	562 nm	<i>180</i> A→S 6	<i>197</i> Y→H 28	<i>229</i> F→I 2	<i>230</i> I→T -2	<i>233</i> S→G 1	<i>277</i> F→Y 9	<i>285</i> A→T 15	<i>308</i> S→A 27	<i>309</i> Y→F -2
<i>Homo</i>						X	S	H	I	I	A	Y	T	A	Y
<i>Alouatta</i>						X	S	-	F	L	G	Y	T	A	Y
<i>Cebus</i>						X	S	H	F	L	G	Y	T	A	Y
<i>Ateles</i>						X	S	H	F	L	G	Y	T	A	Y
<i>Saguinus</i>						X	S	H	F	L	G	Y	T	A	Y
<b><i>Callicebus</i></b>						<b>X</b>	<b>S</b>	<b>H</b>	<b>F</b>	<b>L</b>	<b>G</b>	<b>Y</b>	<b>T</b>	<b>A</b>	<b>Y</b>
<i>Saguinus</i>					X		A	H	F	L	S	Y	T	A	Y
<i>Propithecus</i>					X		A	-	I	I	S	Y	T	A	Y
<i>Cebus</i>				X			A	H	I	L	S	F	T	A	Y
<i>Ateles</i> <sup>c</sup>				X			S	H	I	L	S	F	T	A	Y
<b><i>Callicebus</i></b>				<b>X</b>			<b>A</b>	<b>H</b>	<b>I</b>	<b>F</b>	<b>S</b>	<b>F</b>	<b>T</b>	<b>A</b>	<b>Y</b>
<i>Saguinus</i>			X				A	H	I	L	S	Y	A	A	Y
<i>Propithecus</i>			X				A	-	I	I	S	Y	A	A	Y
<i>Callicebus</i>			X				-	-	-	-	-	-	-	-	-
<i>Cebus</i>		X					A	H	I	L	S	F	A	A	Y
<b><i>Callicebus</i></b>		<b>X</b>					<b>A</b>	<b>H</b>	<b>I</b>	<b>F</b>	<b>S</b>	<b>F</b>	<b>A</b>	<b>A</b>	<b>Y</b>
<i>Homo</i>	X						A	H	I	T	S	F	A	A	F
<i>Alouatta</i> <sup>d</sup>	X						A	-	I	L	S	F	A	A	Y
<i>Callicebus</i>	X						-	-	-	-	-	-	-	-	-

Haplotypes described in this study are in bold.

<sup>a</sup>In vivo absorbance measures for *Homo*: Stockman and Sharpe [2000]; *Alouatta*: Jacobs et al. [1996]; *Cebus*: Jacobs and Deegan [2003]; *Ateles*: Jacobs and Deegan [2001]; *Saguinus*: Jacobs et al. [1987]; *Propithecus*: Jacobs et al. [2002]; *Callicebus*: Jacobs and Deegan [2005]. Note that in vivo absorbance measurements tend to yield peak estimates that are slightly longer than in vitro measurements of reconstituted photopigment [Jacobs, 2007].

<sup>b</sup>Translated haplotypes for *Homo*: Nathans et al. [1986] (GenBank NM000513, NM020061); *Alouatta*: Hunt et al. [1998]; *Cebus* and *Ateles*: Hiramatsu et al. [2005] and Hiramatsu et al. [2008] (GenBank AB193772-84, AB193790, AB193796); *Saguinus*: Surridge and Mundy [2002] (GenBank AY142399-410); *Propithecus*: Tan and Li [1999] (GenBank AF431738-43); *Callicebus*: this study (GenBank GU002165-8), haplotypes tentatively matched with absorbances. Dashes indicate un-reported sequences. The *Ateles* 535 nm opsin reported by Riba-Hernandez et al. [2004] is excluded pending description. Amino acid sites in exons 3, 4, and 5 determined to be important to photopigment tuning in primates (and mammals): 180, 277 and 285 (in italics): Neitz et al. [1991]; 197 and 308: Yokoyama and Radlwimmer [2001]; 229 and 223: Shyue et al. [1998]; 230 and 309: Asenjo et al. [1994]. Approximate shifts in peak absorbance corresponding to amino acid changes are taken from the above references and are shown directly below the site number. Absorbance shifts are approximately additive and reversible, except for sites 230 and 309, where reversing the direction of the amino acid change has a negligible effect on pigment absorbance [Asenjo et al., 1994]. Asenjo et al. [1994] report a non-reversible 3 nm shift for Y116S, but this site in exon 2 was not sequenced in the present study.

<sup>c</sup>Hiramatsu et al. [2008] suggest that this *Ateles* haplotype likely corresponds to the approximately 550 nm peak absorbance variant reported by Jacobs and Deegan [2001]. There is also evidence that this haplotype occurs in *Cebus* [Soares et al., 2010].

<sup>d</sup>It is currently unclear which sites differentiate the presumed *Alouatta* 530 nm gene from the *Cebus* 535 nm allele.

recommended Proteinase K digestion protocol, followed by an elution step entailing the addition of 50  $\mu\text{L}$  ddH<sub>2</sub>O to the sample-bearing card piece in a 1.5 mL micro-centrifuge tube and heating at 90°C for 30 min on a heat block.

DNA extraction from fecal samples was performed as follows. Between 40 and 90 mg of the outer layer of each desiccated fecal pellet was manually removed with a sterile razor blade [Wehausen et al., 2004]. The outer pellet material was placed in a 2 mL micro-centrifuge tube containing 1.6 mL of ASL Buffer (QIAmp<sup>®</sup> DNA Stool Mini Kit, Qiagen Inc., Valencia, CA), incubated at 10°C for 10–12 days, and manually mixed by tube inversion for 30 sec each day. After incubation, 400  $\mu\text{L}$  of fresh ASL Buffer was added to each tube, samples were vortexed for 1 min, and DNA was extracted using a QIAmp<sup>®</sup> DNA Stool Mini Kit (Qiagen Inc.) following the manufacturer's recommended protocol beginning after the ASL Buffer digestion and homogenization step.

Extracted sample concentrations were quantified by M.N. using a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). An A260 reading was taken according to the manufacturer's instructions using 1.5–2.0  $\mu\text{L}$  of sample. Blanks of H<sub>2</sub>O and TE DNA suspension buffer were used, and there was no measured difference between blanks. Total DNA concentrations ranged between 8.4 and 58.0 ng/ $\mu\text{L}$ , with a mean of 22.5 ng/ $\mu\text{L}$ .

### Amplification and Sequencing

Amplification and sequencing were performed independently for all *C. brunneus* individuals by J.A.B. at UCD, M.N., and D.B. at The Medical College of Wisconsin, USA (MCW), and A.K.S. at the University of East Anglia, UK (UEA). Methods are presented for work at UCD. Methods used at MCW and UEA are available upon request [Mancuso et al., 2006; Surridge et al., 2002]. Results from the three laboratories were not shared until all analyses were completed.

Regions of exons 3, 4, and 5 of the *C. brunneus* M/L opsin gene, encompassing sites implicated in the absorbance of light by M/L photopigments (Table I), were amplified separately using the following primers: Exon 3: 5'-CTCCAACCAAGATGGGCGG-3' and 5'-ATCACAGGTCTCTGGTCTCTG-3' [derived from Dulai et al., 1994]; Exon 4: 5'-GCCGGCCCTTCTCTCCAG-3' and 5'-TGATTCAGGGGAGAGAA-GCTTA-3', [derived from Cropp et al., 2002]; Exon 5: 5'-TCCACCCCCGACTCACTATCC-3' and 5'-ACGGTATTTTGAGTGGGATCTGCT-3', and also 5'-GAATCCACCAGAAGGCAGAG-3' and 5'-ACGGGTTGTAGATAGTGGCA-3' [derived from Dulai et al., 1994]. PCR reactions contained 2.5  $\mu\text{L}$  of DNA template, 0.5  $\mu\text{L}$  of 10  $\mu\text{M}$  dNTP mix (2.5  $\mu\text{M}$  each dNTP), 2.5  $\mu\text{L}$  of 10  $\times$  NH<sub>4</sub> Reaction Buffer without MgCl<sub>2</sub> (Bioline USA Inc., Taunton, MA), 1.7  $\mu\text{L}$

(1.5  $\mu\text{L}$  when amplifying the entire exon 5) of 25 mM MgSO<sub>4</sub>, 0.65  $\mu\text{L}$  of 0.1  $\mu\text{g}/\mu\text{L}$  BSA, 0.5  $\mu\text{L}$  of each (the forward and the reverse) primer at a concentration of 10  $\mu\text{M}$ , 0.12  $\mu\text{L}$  of 5 U/ $\mu\text{L}$  Platinum<sup>®</sup> Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA), and ddH<sub>2</sub>O to a final volume of 25  $\mu\text{L}$ . The "touch-down" thermocycling profile consisted of an initial denaturing at 94°C for 3 min, followed by 60 cycles of denaturing at 94°C for 15 sec, annealing at 64°C (exon 3), 62°C (exon 4), or 59°C (exon 5) for 30 sec (dropping the annealing temperature 0.1°C per cycle), and extension at 72°C for 20 sec. This was followed by a final extension at 72°C for 5 min. Each PCR was performed with a positive control consisting of human DNA and a negative control consisting of ddH<sub>2</sub>O instead of template DNA. Amplification of an appropriately sized fragment was confirmed by electrophoresis using a size ladder on a 1.3% agarose gel. PCR products were purified by adding 0.2  $\mu\text{L}$  of 20 U/mL Exonuclease 1 enzyme, 0.2  $\mu\text{L}$  of 10  $\times$  NEBuffer for Exo 1 (New England Biolabs, Ipswich, MA), and ddH<sub>2</sub>O to bring each PCR product to a final volume of 100  $\mu\text{L}$ , and then heated at 37°C for 90 min followed by 20 min at 80°C. Samples were filtered through MultiScreen<sup>®</sup> filter plates (Millipore Corporation, Billerica, MA) by pulling a vacuum of approximately 4.7 kPa until dry, and then re-hydrated with 25  $\mu\text{L}$  ddH<sub>2</sub>O. Sequencing (forward and reverse strands) was performed at the University of California DNA Sequencing Facility (Division of Biological Sciences, University of California, Davis, CA) with an ABI 3730 DNA Analyzer using BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA). Sequences were aligned with Sequencher<sup>™</sup> 4.7 DNA Sequence Assembly Software (Gene Codes Corporation, Ann Arbor, MI).

### Genotype Determination

Genotypes were assigned to all 14 individuals using site 180 in exon 3 and sites 277 and 285 in exon 5, as amino acid changes at these sites are known to cause significant shifts in the light absorbance peaks of primate M/L photopigments. Nearly all known platyrrhine M/L opsin variants can be discriminated by changes at these sites (Table I).

To minimize the risk of assigning incorrect genotypes as a consequence of allelic dropout (i.e. in a heterozygote, the stochastic failure of one of two alleles to amplify during PCR) and false alleles (i.e. PCR or sequencing errors, typically sporadic, suggesting heterozygosity in a homozygote or hemizygote), across the three laboratories we conducted a total of 4–16 independent PCRs for exons 3 and 5 (combined) per *C. brunneus* individual. Allelic dropout was diagnosed in putatively heterozygous females when sequences resulting from a PCR displayed no heterozygosity, while sequences from

at least six other independent amplifications of the same exon from that individual all displayed identical heterozygosity at known photopigment absorbance tuning sites. A nucleotide call at an isolated heterozygous site from an otherwise homozygous exon sequence fragment was deemed a false allele if it was not present in the complementary sequence fragment nor in the sequences from at least six (for females) or two (for males) other independent amplifications of the same exon from that individual.

For three putatively homozygous females (Groups 3, S, and 2: Table II) we performed 5, 11, and 16 independent PCRs, respectively, for exons 3 and 5 (combined) to rule out heterozygosity. The high degree of replication and concordance across three independent laboratories gives us confidence in our genotyping assignments.

### Sample Attribution

A challenge in the genotyping of wild primates from whom only noninvasively collected fecal samples have been obtained is assuring correct attribution of collected samples to sample-donating animals. One way to reduce the risk of misattribution is to check for matching genotypes derived from at least two independently collected fecal samples from the same putative feces-donating individual [e.g. Hiramatsu et al., 2005]. This procedure was not always possible in this study, as four *C. brunneus* individuals are each represented by a single fecal

sample (in the absence of an additional blood or saliva sample) (Table II). However, as a result of the monogamous social system and strict territoriality of *C. brunneus*, in combination with the genetic sex determination method of Di Fiore [2005] and genotypes derived from blood and saliva samples collected from captured animals, each of these four fecal samples could be unambiguously attributed to a single individual (given the reasonable exclusion of infants and extra-group individuals). Additionally, each of these four attributions matched the field attributions of the experienced observer (J.A.B.) who collected the samples.

## RESULTS

### Alleles and Genotypes

The results presented below have been combined for the three laboratories conducting blind parallel PCR and sequence analysis (UCD, MCW, and UEA). Assigned genotypes for all *C. brunneus* individuals corresponded exactly across laboratories.

Analysis of site 180 in exon 3 and sites 277 and 285 in exon 5 of the X-linked M/L opsin gene from homozygous and hemizygous *C. brunneus* individuals revealed the presence of three functionally distinct alleles (i.e., each of these individuals had one of three alleles). Sequences are available in GenBank (accession numbers GU002165-8). On the right-hand side of Table I, the three allele haplotypes are matched with haplotypes of other known platyrrhine

**TABLE II. Color Vision Genotyping Results for 14 wild *Callicebus brunneus* Individuals From Five Socially Monogamous Groups**

Group	Individual	Sample type <sup>a</sup>	Sex <sup>b</sup>	Exons sequenced <sup>c</sup>	Alleles <sup>d</sup>	Inferred vision
1	Adult female	Feces <sup>e</sup>	f	<b>3</b> (6, 4), <b>4</b> (2, 1), <b>5</b> (19, 10)	535+550	Trichromacy
1	Adult male	Feces 1, Feces 2	m	<b>3</b> (7, 5), <b>4</b> (2, 1), <b>5</b> (15, 9)	535	Dichromacy
1	Subadult female	Feces <sup>e</sup> , Saliva <sup>e</sup>	F	<b>3</b> (4, 2), <b>5</b> (16, 8)	535+550	Trichromacy
2	Adult female	Feces, Blood	F	<b>3</b> (8, 6), <b>5</b> (15, 10)	550	Dichromacy
2	Adult male	Feces 1, Feces 2	m	<b>3</b> (4, 2), <b>4</b> (1, 1), <b>5</b> (10, 5)	550	Dichromacy
3	Adult female	Feces, Blood <sup>e</sup>	F	<b>3</b> (6, 4), <b>4</b> (2, 1), <b>5</b> (13, 7)	550+562	Trichromacy
3	Adult male	Feces 1, Feces 2	–	<b>3</b> (5, 3), <b>4</b> (1, 1), <b>5</b> (10, 6)	562	Dichromacy
3	Subadult male	Blood	M	<b>3</b> (2, 1), <b>5</b> (6, 3)	562	Dichromacy
3	Juvenile female	Feces	f	<b>3</b> (2, 1), <b>5</b> (7, 4)	562	Dichromacy
S	Adult female	Feces	f	<b>3</b> (4, 3), <b>4</b> (2, 1), <b>5</b> (14, 8)	550	Dichromacy
S	Adult male	Feces 1, Feces 2	m	<b>3</b> (7, 4), <b>4</b> (3, 2), <b>5</b> (21, 11)	535	Dichromacy
W	Adult female	Feces, Saliva <sup>e</sup>	F	<b>3</b> (4, 3), <b>4</b> (2, 1), <b>5</b> (13, 8)	550+562	Trichromacy
W	Adult male 1	Saliva	M	<b>3</b> (2, 1), <b>5</b> (6, 3)	550	Dichromacy
W	Adult male 2 <sup>f</sup>	Feces	–	<b>3</b> (2, 1), <b>4</b> (1, 1), <b>5</b> (8, 4)	550	Dichromacy

<sup>a</sup>Blood and saliva samples collected from captured animals on FTA<sup>®</sup> DNA preservation cards. When two fecal samples were collected independently from the same animal, they are labeled with numbers.

<sup>b</sup>Upper case letters: blood and saliva samples directly collected from captured animals (M = male, F = female); lower case letters: fecal samples subjected to the genetic sex determination procedure of Di Fiore [2005] (m = male, f = female); dashes: fecal samples collected from individuals whose sex was inferred from behavior.

<sup>c</sup>Names of exons sequenced (3, 4, or 5) are in bold. In parentheses after each sequenced exon are: (1) the number of sequenced fragments (forward and reverse strands counted separately) spanning sites 180 (exon 3), sites 277 and 285 (exon 5), or any part of exon 4; and (2) the number of independent PCR amplifications yielding the sequenced fragments. The number of sequenced fragments is not always evenly divisible by the number of PCRs, because, in the UCD laboratory, occasionally only the forward or the reverse strands were successfully sequenced.

<sup>d</sup>Allele names correspond to approximate peak photopigment light absorbance in nanometers. See Table I.

<sup>e</sup>Sample from which one exon 5 PCR showed evidence of allelic dropout in one of the three laboratories.

<sup>f</sup>Adult Male 1 in Group W disappeared during the study and was replaced by Adult Male 2 [Bunce, 2009].

alleles based on these three sites. The *C. brunneus* haplotypes correspond most closely with platyrrhine alleles coding for M/L opsins with peak light absorbances at approximately 535, 550, and 562 nm (Table I) that, in turn, correspond with the three most common M/L pigment classes reported by Jacobs and Deegan [2005] for the captive *Callicebus* colony at the CNPRC (i.e., 536, 551, and 562 nm, respectively).

Table II presents the genotyping results for the 14 *C. brunneus* individuals in this study. In the four cases where both feces and blood or saliva were collected from the same individual, matching haplotypes were obtained from each sample type. This confirms the reliability of fecal DNA for this analysis. Three of the five adult females (Groups 1, 3, and W) and the subadult female (Group 1) were found to be heterozygous at the M/L opsin locus (Table II). In these females, the patterns of heterozygosity at sites known to affect peak light absorbance by photopigments are consistent with the interpretation that each female possessed two of the three M/L alleles found in homozygous and hemizygous individuals (above).

### Allelic Dropout and False Alleles

According to the criteria above (see Methods: Genotype Determination), no evidence of allelic dropout was observed in exon 3 sequences (examined at UCD and MCW). Exon 4, which was only sequenced at UCD, could not be amplified for several individuals. Additionally, presumed allelic dropout was observed in all exon 4 sequences from two (Groups 3 and W) of the three heterozygous adult females (i.e., no heterozygosity was observed in any exon 4 sequence for these individuals). For this reason, genotyping assignments were based solely on exons 3 and 5, and data from exon 4 are included in Tables I and II only for completeness. Across the three laboratories, five exon 5 PCRs showed evidence of allelic dropout (Table II). Two cases involved DNA extracted from feces, and three involved DNA extracted from blood or saliva. Allelic dropout for any particular *C. brunneus* individual never occurred in more than one of the three laboratories.

Our allelic dropout rate was relatively high [five of the 46 exon 3 and exon 5 PCRs from heterozygotes, compared with two of 27 PCRs for SurrIDGE et al., 2002], and it is unclear why this was the case. Allelic dropout occurred for PCRs of blood and saliva samples containing at least 28 ng of template DNA, well above the threshold of 201 pg of template DNA per reaction recommended by Morin et al. [2001] to rule out allelic dropout in putative homozygotes with 99% certainty after only two PCRs. This suggests that low DNA template amounts during PCR are perhaps less likely to be responsible for the observed rate of allelic dropout. It may be that mismatches between the primers used in this study (having

originally been designed for other species) and the target *C. brunneus* sequences contributed to allelic dropout. Drop-out occurred for all three alleles, so there is little evidence of allele-specific primer-target mismatches. However, future studies of *C. brunneus* may benefit from designing species-specific primers.

Evidence of false alleles was only encountered for exon 5 sequences and only at UCD. Twelve of the 13 cases of false alleles involved an adenine residue at site 277 on a forward strand amplified using a primer pair derived from Dulai et al. [1994] (above). Eight of the 13 cases occurred in males, who are hemizygous, and 12 cases involved fecal samples. It is unclear why our false allele rate was relatively high (13 of 136 total exon 3 and exon 5 PCRs). However, the consistency in the residues constituting the false alleles and the fact that false alleles were only observed in one of the three laboratories, perhaps suggests they are artifacts of the sequencing procedure at UCD.

### DISCUSSION

Here we have provided molecular genetic evidence that three M/L opsin alleles controlling polymorphic color vision in *Callicebus* occur at reasonably high frequencies in a wild population. These opsin alleles appear to correspond to the three most common M/L photopigment variants reported by Jacobs and Deegan [2005] in a captive *Callicebus* population, with peak absorbances at approximately 535, 550, and 562 nm (Table I). Among platyrrhines, three common photopigment variants with similar absorbance properties have also been reported in *Cebus* [capuchins: Jacobs & Deegan, 2003], *Saimiri* [squirrel monkeys: Cropp et al., 2002; Mollon et al., 1984], *Brachyteles* [muriquis: Talebi et al., 2006], and *Pithecia* [sakis: Boissinot et al., 1998; Jacobs & Deegan, 2003], whereas two of these three variants have been found so far in *Ateles* [spider monkeys: Hiramatsu et al., 2005; Jacobs & Deegan, 2001; but see Riba-Hernandez et al., 2004], and *Lagothrix* [woolly monkeys: Jacobs & Deegan, 2001]. This contrasts with the three M/L photopigments common in callitrichines (tamarins and marmosets), having peak absorbances at approximately 544, 556, and 563 nm [reviewed in Jacobs, 2007].

We did not find genetic evidence of the two least common M/L photopigment variants reported by Jacobs and Deegan [2005] for captive *Callicebus*. However, we suspect that their absence is most likely a result of the small sample size. Indeed, our results can be interpreted as fully consistent with the observed lower frequencies of these two photopigment variants in the earlier physiological study. Future studies augmenting the current genetic sample from a wild population are necessary to accurately estimate allele frequencies in *Callicebus*. However, our characterization of three M/L alleles in

this genus serves as an important starting point for investigation of the utility of polymorphic color vision in *Callicebus*.

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## REFERENCES

- Asenjo AB, Rim J, Oprian DD. 1994. Molecular determinants of human red/green color discrimination. *Neuron* 12: 1131–1138.
- Boissinot S, Tan Y, Shyue S-K, Schneider H, Sampaio I, Neiswanger K, Hewett-Emmett D, Li W-H. 1998. Origins and antiquity of X-linked triallelic color vision systems in New World monkeys. *Proceedings of the National Academy of Sciences USA* 95:13749–13754.
- Bossuyt F. 2002. Natal dispersal of titi monkeys (*Callicebus moloch*) at Cocha Cashu, Manu National Park, Peru. *American Journal of Physical Anthropology Supplement* 34:47.
- Bunce JA. 2009. Ecology and genetics of color vision in *Callicebus brunneus*, a Neotropical monkey, Ph.D. Dissertation, University of California Davis, Davis, CA. 185p. Available from: ProQuest Dissertations & Theses, Ann Arbor, MI; AAT 3375520.
- Caine NG. 2002. Seeing red: consequences of individual differences in color vision in callitrichid primates. In: Miller LE, editor. *Eat or be eaten: predator sensitive foraging among primates*. Cambridge: Cambridge University Press. p 58–73.
- Caine NG, Osorio D, Mundy NI. 2010. A foraging advantage for dichromatic marmosets (*Callithrix geoffroyi*) at low light intensity. *Biology Letters* 6:36–38.
- Changizi MA, Zhang Q, Shimojo S. 2006. Bare skin, blood and the evolution of primate colour vision. *Biology Letters* 2: 217–221.
- Cropp S, Boinski S, Li W-H. 2002. Allelic variation in the squirrel monkey X-linked color vision gene: biogeographical and behavioral correlates. *Journal of Molecular Evolution* 54:734–745.
- Di Fiore A. 2005. A rapid genetic method for sex assignment in non-human primates. *Conservation Genetics* 6:1053–1058.
- Dominy NJ, Lucas PW. 2001. Ecological importance of trichromatic vision to primates. *Nature* 410:363–366.
- Dulai KS, Bowmaker JK, Mollon JD, Hunt DM. 1994. Sequence divergence, polymorphism and evolution of the middle-wave and long-wave visual pigment genes of great apes and Old World monkeys. *Vision Research* 34: 2483–2491.
- Fernandez AA, Morris MR. 2007. Sexual selection and trichromatic color vision in primates: statistical support for the preexisting-bias hypothesis. *American Naturalist* 170:10–20.
- Hershkovitz P. 1990. Titis, New World monkeys of the genus *Callicebus* (Cebidae, Platyrrhini): a preliminary taxonomic review. *Fieldiana (Zoology)* n.s. 55:1–109.
- Hiramatsu C, Radlwimmer FB, Yokoyama S, Kawamura S. 2004. Mutagenesis and reconstitution of middle-to-long-wave-sensitive visual pigments of New World monkeys for testing the tuning effect of residues at sites 229 and 233. *Vision Research* 44:2225–2231.
- Hiramatsu C, Tsutsui T, Matsumoto Y, Aureli F, Fedigan LM, Kawamura S. 2005. Color vision polymorphism in wild capuchins (*Cebus capucinus*) and spider monkeys (*Ateles geoffroyi*) in Costa Rica. *American Journal of Primatology* 67:447–461.
- Hiramatsu C, Melin AD, Aureli F, Schaffner CM, Vorobyev M, Matsumoto Y, Kawamura S. 2008. Importance of achromatic contrast in short-range fruit foraging of primates. *Public Library of Science One* 3:e3356.
- Hunt DM, Dulai KS, Cowing JA, Julliot C, Mollon JD, Bowmaker JK, Li W-H, Hewett-Emmett D. 1998. Molecular evolution of trichromacy in primates. *Vision Research* 38: 3299–3306.
- Jacobs GH. 1993. The distribution and nature of colour vision among the mammals. *Biological Reviews of the Cambridge Philosophical Society* 68:413–471.
- Jacobs GH. 2007. New World monkeys and color. *International Journal of Primatology* 28:729–759.
- Jacobs GH. 2008. Primate color vision: a comparative perspective. *Visual Neuroscience* 25:619–633.
- Jacobs GH. 2009. Evolution of colour vision in mammals. *Philosophical Transactions of the Royal Society of London B* 364:2957–2967.
- Jacobs GH, Deegan II JF. 2001. Photopigments and colour vision in New World monkeys from the family Atelidae. *Proceedings of the Royal Society of London B* 268: 695–702.
- Jacobs GH, Deegan II JF. 2003. Cone pigment variations in four genera of New World monkeys. *Vision Research* 43: 227–236.
- Jacobs GH, Deegan II JF. 2005. Polymorphic New World monkeys with more than three M/L cone types. *Journal of the Optical Society of America A* 22:2072–2080.
- Jacobs GH, Neitz J, Crognale M. 1987. Color vision polymorphism and its photopigment basis in a callitrichid monkey (*Saguinus fuscicollis*). *Vision Research* 27:2089–2100.
- Jacobs GH, Neitz M, Deegan JF, Neitz J. 1996. Trichromatic colour vision in New World monkeys. *Nature* 382:156–158.
- Jacobs GH, Deegan II JF, Tan Y, Li W-H. 2002. Opsin gene and photopigment polymorphism in a prosimian primate. *Vision Research* 42:11–18.
- Kawamura S, Hirai M, Takenaka O, Radlwimmer FB, Yokoyama S. 2001. Genomic and spectral analyses of long

- to middle wavelength-sensitive visual pigments of common marmoset (*Callithrix jacchus*). *Gene* 269:45–51.
- Mancuso K, Neitz M, Neitz J. 2006. An adaptation of the Cambridge Colour Test for use with animals. *Visual Neuroscience* 23:695–701.
- Melin AD, Fedigan LM, Hiramatsu C, Sendall CL, Kawamura S. 2007. Effects of colour vision phenotype on insect capture by a free-ranging population of white-faced capuchins, *Cebus capucinus*. *Animal Behaviour* 73:205–214.
- Melin AD, Fedigan LM, Hiramatsu C, Kawamura S. 2008. Polymorphic color vision in white-faced capuchins (*Cebus capucinus*): is there foraging niche divergence among phenotypes? *Behavioral Ecology and Sociobiology* 62:659–670.
- Melin AD, Fedigan LM, Hiramatsu C, Hiwatashi T, Parr N, Kawamura S. 2009. Fig foraging by dichromatic and trichromatic *Cebus capucinus* in a tropical dry forest. *International Journal of Primatology* 30:753–775.
- Melin AD, Fedigan LM, Young HC, Kawamura S. 2010. Can color vision variation explain sex differences in invertebrate foraging by capuchin monkeys? *Current Zoology* 56:300–312.
- Mollon JD. 1989. “Tho’ she kneel’d in that place where they grew...”: the uses and origins of primate colour vision. *Journal of Experimental Biology* 146:21–38.
- Mollon JD, Bowmaker JK, Jacobs GH. 1984. Variations of colour vision in a New World primate can be explained by polymorphism of retinal photopigments. *Proceedings of the Royal Society of London B* 222:373–399.
- Morin PA, Chambers KE, Boesch C, Vigilant L. 2001. Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Molecular Ecology* 10:1835–1844.
- Nathans J, Thomas D, Hogness DS. 1986. Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. *Science* 232:193–202.
- Neitz J, Neitz M, Jacobs GH. 1991. Spectral tuning of pigments underlying red-green color vision. *Science* 252:971–974.
- Regan BC, Julliot C, Simmen B, Vienot F, Charles-Dominique P, Mollon JD. 2001. Fruits, foliage and the evolution of primate colour vision. *Philosophical Transactions of the Royal Society of London B* 356:229–283.
- Riba-Hernandez P, Stoner KE, Osorio D. 2004. Effect of polymorphic colour vision for fruit detection in the spider monkey *Ateles geoffroyi*, and its implications for the maintenance of polymorphic colour vision in platyrrhine monkeys. *Journal of Experimental Biology* 207:2465–2470.
- Rodman PS, Bossuyt FJ. 2007. Fathers and stepfathers: familial relations of old and new males within groups of *Callicebus brunneus* in southeastern Peru. *American Journal of Physical Anthropology* S44:201.
- Sharpe LT, Stockman A, Jagle H, Nathans J. 1999. Opsin genes, cone photopigments, color vision, and color blindness. In: Gegenfurtner KR, Sharpe LT, editors. *Color vision: from genes to perception*. Cambridge: Cambridge University Press. p 3–51.
- Shyue S-K, Boissinot S, Schneider H, Sampaio I, Schneider MP, Abee CR, Williams L, Hewett-Emmett D, Sperling HG, Cowing JA, Dulai KS, Hunt DM, Li W-H. 1998. Molecular genetics of spectral tuning in New World monkey color vision. *Journal of Molecular Evolution* 46:697–702.
- Smith LM, Burgoyne LA. 2004. Collecting, archiving, and processing DNA from wildlife samples using FTA databasing paper. *BMC Ecology* 4. DOI: 10.1186/1472-6785-4-4.
- Smith AC, Buchanan-Smith HM, Surrridge AK, Mundy NI. 2005. Factors affecting group spread within wild mixed-species troops of saddleback and mustached tamarins. *International Journal of Primatology* 26:337–355.
- Soares JGM, Fiorani M, Araujo EA, Zana Y, Bonci DMO, Neitz M, Ventura DF, Gattass R. 2010. Cone photopigment variations in *Cebus apella* monkeys evidenced by electroretinogram measurements and genetic analysis. *Vision Research* 50:99–106.
- Stockman A, Sharpe LT. 2000. The spectral sensitivities of the middle- and long-wavelength-sensitive cones derived from measurements in observers of known genotype. *Vision Research* 40:1711–1737.
- Sumner P, Mollon JD. 2000a. Catarrhine photopigments are optimized for detecting targets against a foliage background. *Journal of Experimental Biology* 203:1963–1986.
- Sumner P, Mollon JD. 2000b. Chromaticity as a signal of ripeness in fruits taken by primates. *Journal of Experimental Biology* 203:1987–2000.
- Sumner P, Mollon JD. 2003. Colors of primate pelage and skin: objective assessment of conspicuousness. *American Journal of Primatology* 59:67–91.
- Surrridge AK, Mundy NI. 2002. Trans-specific evolution of opsin alleles and the maintenance of trichromatic colour vision in Callitrichine primates. *Molecular Ecology* 11:2157–2169.
- Surrridge AK, Smith AC, Buchanan-Smith HM, Mundy NI. 2002. Single-copy nuclear DNA sequences obtained from noninvasively collected primate feces. *American Journal of Primatology* 56:185–190.
- Surrridge AK, Osorio D, Mundy NI. 2003. Evolution and selection of trichromatic vision in primates. *TRENDS in Ecology and Evolution* 18:198–205.
- Talebi MG, Pope R, Vogel ER, Neitz M, Dominy NJ. 2006. Polymorphism of visual pigment genes in the muriqui (Primates, Atelidae). *Molecular Ecology* 15:551–558.
- Tan Y, Li W-H. 1999. Trichromatic vision in prosimians. *Nature* 402:36.
- Terborgh J. 1983. Five New World primates: a study in comparative ecology. In: Krebs JR, Clutton-Brock T, editors. *Monographs in Behavior and Ecology*. Princeton: Princeton University Press. 260p.
- Wehausen JD, Ramey II RR, Epps CW. 2004. Experiments in DNA extraction and PCR amplification from Bighorn Sheep feces: the importance of DNA extraction method. *Journal of Heredity* 95:503–509.
- Yamashita N, Stoner KE, Riba-Hernandez P, Dominy NJ, Lucas PW. 2005. Light levels used during feeding by primate species with different color vision phenotypes. *Behavioral Ecology and Sociobiology* 58:618–629.
- Yokoyama S, Radlwimmer FB. 2001. The molecular genetics and evolution of red and green color vision in vertebrates. *Genetics* 158:1697–1710.