Nocturnal Light Environments Influence Color Vision and Signatures of Selection on the *OPN1SW* Opsin Gene in Nocturnal Lemurs

Carrie C. Veilleux,*[,1] Edward E. Louis Jr,[2] and Deborah A. Bolnick[1,3]

[1]Department of Anthropology, University of Texas

[2]Center for Conservation and Research, Omaha's Henry Doorly Zoo and Aquarium, Omaha, Nebraska

[3]Population Research Center, University of Texas

***Corresponding author:** E-mail: carrie.veilleux@utexas.edu.

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Abstract

Although loss of short-wavelength-sensitive (SWS) cones and dichromatic color vision in mammals has traditionally been linked to a nocturnal lifestyle, recent studies have identified variation in selective pressure for the maintenance of the *OPN1SW* opsin gene (and thus, potentially dichromacy) among nocturnal mammalian lineages. These studies hypothesize that purifying selection to retain SWS cones may be associated with a selective advantage for nocturnal color vision under certain ecological conditions. In this study, we explore the effect of nocturnal light environment on *OPN1SW* opsin gene evolution in a diverse sample of nocturnal lemurs (106 individuals, 19 species, and 5 genera). Using both phylogenetic and population genetic approaches, we test whether species from closed canopy rainforests, which are impoverished in short-wavelength light, have experienced relaxed selection compared with species from open canopy forests. We identify clear signatures of differential selection on *OPN1SW* by habitat type. Our results suggest that open canopy species generally experience strong purifying selection to maintain SWS cones. In contrast, closed canopy species experience weaker purifying selection or a relaxation of selection on *OPN1SW*. We also found evidence of nonfunctional *OPN1SW* genes in all *Phaner* species and in *Cheirogaleus medius*, implying at least three independent losses of SWS cones in cheirogaleids. Our results suggest that the evolution of color vision in nocturnal lemurs has been influenced by nocturnal light environment.

Key words: **primate evolution, opsin genes, ecological genetics, selection.**

Article

Introduction

Although color vision has historically been considered superfluous for nocturnal animals (Walls 1942; Ahnelt and Kolb 2000), researchers have recently re-examined the significance of nocturnal color vision using behavioral, anatomical, molecular, and ecological techniques (Kelber et al. 2002; Kawamura and Kubotera 2004; Roth and Kelber 2004; Johnsen et al. 2006; Perry et al. 2007; Müller et al. 2009; Zhao, Rossiter, et al. 2009; Melin et al. 2012; Veilleux and Cummings 2012). Many of these studies have challenged the traditional view, suggesting that nocturnal color vision may be adaptive under certain conditions, even in mammals (Johnsen et al. 2006; Perry et al. 2007; Zhao, Rossiter, et al. 2009). These new findings have provoked spirited debate over the significance of nocturnality and color vision in the evolutionary origins and ecology of primates and other mammals (Tan et al. 2005; Perry et al. 2007; Ross et al. 2007; Ankel-Simons and Rasmussen 2008; Jacobs 2008).

Like most mammals, many primates possess short-wavelength-sensitive (SWS) cones and medium/long-wavelength-sensitive (MWS/LWS) cones that together facilitate dichromatic color vision (blues/violets vs. reds/yellows/greens) (Jacobs 2008). In several nocturnal primate and mammalian lineages, the *OPN1SW* opsin gene (coding for SWS visual pigments, sometimes referred to as the *SWS1* opsin gene) has accumulated deleterious mutations, resulting in loss of SWS cones and functional color vision (i.e., monochromacy) (Jacobs et al. 1996; Ahnelt and Kolb 2000; Kawamura and Kubotera 2004; Peichl 2005; Jacobs 2008, 2013). A recent analysis of *OPN1SW* in nocturnal strepsirrhine primates identified variation in the type of selection acting on this locus (Tan et al. 2005). Although Tan et al. (2005) found evidence of relaxed selection on *OPN1SW* in monochromatic lorises and galagos, most nocturnal lemurs exhibited lower lineage-specific substitution rates for nonsynonymous sites than for synonymous (presumed neutral) sites, reflecting a long-term signature of purifying selection to maintain gene function. Working from traditional assumptions that color vision is irrelevant for nocturnal species, Tan et al. (2005) argued that the functional *OPN1SW* genes and signatures of purifying selection indicate recent transitions to nocturnality. They proposed that the earliest primates were day-active, contrary to prevailing hypotheses that stress nocturnality as a critical factor in early primate evolution (Cartmill 1992; Sussman 1995; Ross et al. 2007).

However, others have suggested that signatures of purifying selection on the *OPN1SW* gene in nocturnal mammals

may indicate a selective advantage for nocturnal color vision rather than recent transitions to nocturnality (Perry et al. 2007; Zhao, Rossiter, et al. 2009; Zhao, Xu, et al. 2009). Perry et al. (2007), for example, identified recent purifying selection to maintain *OPN1SW* function in a population genetic study of nocturnal lemurs (aye-aye, *Daubentonia madagascariensis*), suggesting that dichromacy under nocturnal or twilight conditions may be adaptive in aye-ayes. Zhao, Rossiter, et al. (2009) drew a similar conclusion from their phylogenetic analysis of *OPN1SW* variation across 32 bat species. Contrary to the assumptions by Tan et al. (2005), many bats exhibit long evolutionary histories (>52 My) of both nocturnality and purifying selection to maintain dichromacy (Zhao, Rossiter, et al. 2009). Further, Zhao, Rossiter, et al. (2009) found that relaxed selection on *OPN1SW* and SWS cone loss in specific bat lineages is associated with changes in roosting behavior and the evolution of high-duty-cycle echolocation. These studies thus contribute to a growing body of evidence for the selective benefit of color vision under certain nocturnal conditions (Johnsen et al. 2006; Kelber and Roth 2006).

However, it is still unclear what ecological conditions drive differential selection on *OPN1SW* in nocturnal lemurs (Tan et al. 2005; Perry et al. 2007) and other nocturnal primates (Kawamura and Kubotera 2004). Cone loss in other vertebrates has sometimes been linked to differences in ambient light environment (Partridge and Cummings 1999; Peichl 2005). For example, some researchers hypothesize that the lack of short-wavelength (SW) light in coastal water habitats led to the convergent loss of SWS cones in pinniped and cetacean mammals (Peichl et al. 2001; Griebel and Peichl 2003; Peichl 2005). In this study, we hypothesize that a similar lack of SW light in certain nocturnal primate habitats may influence selection on *OPN1SW* for maintaining dichromacy. A recent analysis of nocturnal light in lemur habitats demonstrated that the nocturnal light environments in open and closed canopy forests differ substantially (Veilleux and Cummings 2012). Veilleux and Cummings found that in brighter lunar phases, SW light intensity (400–440 nm) in an open canopy forest was up to two orders of magnitude higher than in the understory of closed canopy rainforest. These findings suggest that nocturnal lemurs from open canopy and closed canopy forests encounter distinctly different visual environments during at least part of the lunar cycle. Because habitat differences in nocturnal light affect selection for dichromacy, we predict that lemur species from more open canopy forests will be exhibit signatures of purifying selection on *OPN1SW*. In contrast, species from closed canopy forests should exhibit either weaker purifying selection or a relaxation of functional constraint on *OPN1SW* because there is generally less SW light available to stimulate SWS cones.

To test these predictions, we analyzed selection on the *OPN1SW* opsin gene across nocturnal lemurs from differing habitat types and ecologies (106 individuals, 19 species, and 5 genera). Although all nocturnal, these lemur genera exhibit a range of body sizes, dietary preferences, and evolutionary histories (Hladik et al. 1980; Ganzhorn 1988; Gould et al. 2011). *Avahi* and *Lepilemur*, for example, have independently converged onto a similar medium-sized (0.5–1 kg, 0.7–1.2 kg, respectively) nocturnal folivorous niche (Ganzhorn 1988; Thalmann 2001), with *Avahi* hypothesized as having evolved from a day-active ancestor approximately 29 Ma (Roos et al. 2004). The three other genera (*Phaner*, *Cheirogaleus*, and *Microcebus*) are all members of the Cheirogaleidae family. *Phaner* (0.3 kg) is a specialized gummivore, whereas species of *Cheirogaleus* (0.07–0.4 kg) and *Microcebus* (0.05–0.09 kg) primarily consume fruit, flowers, and insects (Hladik et al. 1980; Ganzhorn 1988; Radespiel 2006; Gould et al. 2011). For each genus, we sampled individuals or populations from closed canopy rainforests and open canopy forests (including both spiny forest and seasonally open dry deciduous forest) to permit intrageneric comparisons of selection. Although Madagascar has experienced dramatic deforestation following human contact, all three of these habitat types have been present on the island since the late Eocene/early Oligocene (Wells 2003). Although the two *Cheirogaleus* species sampled were from different habitat types (rainforest *C. major*, dry deciduous forest *C. medius*), *C. medius* hibernates through the dry season and is only active in the rainy season (Fietz and Ganzhorn 1999; Fietz and Dausmann 2006). Because dry deciduous forests have dense foliage and canopies during the rainy season (Hladik 1980; Maass et al. 1995), both *Cheirogaleus* species likely encounter "closed canopy" nocturnal light regimes.

This study provides the first analysis of the *OPN1SW* gene to combine population genetic (Perry et al. 2007) and phylogenetic (Zhao, Rossiter, et al. 2009) approaches, making it possible to detect both recent and more ancient signatures of selection for dichromacy. For each species, we predicted SWS cone spectral tuning based on known *OPN1SW* spectral tuning sites. We also compared the predicted functional effects of nonsynonymous polymorphisms between species from different habitat types. At the population level, we estimated the type of recent selection (purifying vs. relaxed) acting on *OPN1SW* by comparing nucleotide diversity between functional classes of sites in the gene. At the phylogenetic level, we constructed a maximum likelihood (ML) tree of primate *OPN1SW* and used likelihood ratio tests (LRTs) to compare competing models of selection along evolutionary branches.

Results

Intraspecific and Interspecific Variation in *OPN1SW*

We sequenced the *OPN1SW* gene in 32 *Lepilemur* (6 species), 24 *Avahi* (4 species), 18 *Phaner* (3 species), 11 *Cheirogaleus* (2 species), and 21 *Microcebus* (4 species) individuals (table 1, collection locality and accession numbers in supplementary table S1, Supplementary Material online). Functionally important amino acid residues (Sakmar et al. 1989; Hunt et al. 1995; Kawamura and Kubotera 2004) are conserved in almost all species, suggesting that most nocturnal lemurs retain functional SWS cones. Three species (*Lepilemur edwardsi*, *C. major*, and *C. medius*) exhibit variation in the putative start codon (fig. 1). Among *L. edwardsi* individuals, there is a nonsynonymous polymorphism at the first position of the

Table 1. Summary of Population Genetic Results.

Species[a]	Forest Type	Functional Class[b]	Total bp	SNPs	θ_π (%)[c]	θ_W (%)[d]	Tajima's *D*
Lepilemur edwardsi (*n* = 8)	Open canopy	Silent	2,079	16	0.167	0.232	−1.115
		Synonymous	256	1	0.049	0.118	−1.162
		Intron	1,823	15	0.183	0.248	−1.029
		Nonsynonymous	782	1	0.03	0.039	−0.448
L. hubbardorum (*n* = 7)	Open canopy	Silent	2,081.46	4	0.067	0.060	0.332
		Synonymous	255.46	1	0.104	0.123	−0.341
		Intron	1,826	3	0.061	0.052	0.576
		Nonsynonymous	791.54	1	0.018	0.04	−1.155
L. leucopus (*n* = 7)	Open canopy	Silent	2,068.83	6	0.076	0.091	−0.60
		Synonymous	256.83	0	0	0	NA
		Intron	1,812	6	0.087	0.104	−0.60
		Nonsynonymous	790.17	1	0.033	0.04	−0.341
L. petteri (*n* = 3)	Open canopy	Silent	2,068.44	1	0.026	0.021	0.851
		Synonymous	256.44	0	0	0	NA
		Intron	1,812	1	0.029	0.024	0.851
		Nonsynonymous	790.56	1	0.068	0.055	0.851
L. mustelinus (*n* = 6)	Closed canopy	Silent	2,062.51	7	0.063	0.112	−1.713
		Synonymous	256.51	4	0.314	0.516	−1.403
		Intron	1,806	3	0.028	0.055	−1.629
		Nonsynonymous	790.56	6	0.175	0.251	−1.167
L. microdon (*n* = 1)	Closed canopy	Silent	2,070.92	2		NA	
		Nonsynonymous	790	4			
Avahi laniger (*n* = 8)	Closed canopy	Silent	2,041.27	12	0.146	0.177	−0.683
		Synonymous	255.27	3	0.269	0.354	−0.708
		Intron	1,786	9	0.128	0.152	−0.581
		Nonsynonymous	791.73	2	0.071	0.076	−0.189
A. peyrierasi (*n* = 3)	Closed canopy	Silent	2,039.28	4	0.065	0.086	−1.295
		Synonymous	255.28	0	0	0	0
		Intron	1,784	4	0.075	0.098	−1.295
		Nonsynonymous	791.72	2	0.084	0.11	−1.132
A. cleesei (*n* = 5)	Open canopy	Silent	2,042.10	15	0.199	0.26	−1.073
		Synonymous	255.10	4	0.042	0.055	−0.943
		Intron	1,787	11	0.168	0.218	−1.023
		Nonsynonymous	791.9	0	0	0	NA
A. occidentalis (*n* = 8)	Open canopy	Silent	2,046.17	4	0.030	0.059	−1.550
		Synonymous	255.17	1	0.092	0.118	−0.448
		Intron	1,791	3	0.021	0.050	−1.697
		Nonsynonymous	791.83	0	0	0	NA
Microcebus ravelobensis (*n* = 6)	Open canopy	Silent	2,302.5	16	0.272	0.230	0.769
		Synonymous	255.5	4	0.536	0.518	0.104
		Intron	2,047	12	0.239	0.194	0.957
		Nonsynonymous	791.5	1	0.052	0.042	0.541
M. murinus (*n* = 6)	Open canopy	Silent	1,960.61	23	0.390	0.388	0.003
		Synonymous	256.61	4	0.403	0.516	−0.781
		Intron	1,704	19	0.388	0.369	0.208
		Nonsynonymous	790.39	2	0.042	0.084	−1.451
M. griseorufus (*n* = 8)	Open canopy	Silent	1,850.67	17	0.369	0.277	1.311
		Synonymous	256.67	3	0.611	0.352	2.098*
		Intron	1,594	14	0.331	0.265	0.958
		Nonsynonymous	790.33	0	0	0	NA
M. simmonsi (*n* = 1)	Closed canopy	Silent	1,968.33	2		NA	
		Nonsynonymous	790.67	0			
Cheirogaleus major (*n* = 3)	Closed canopy	Silent	1,972.19	15	0.278	0.333	−1.035
		Synonymous	252.19	6	0.879	1.042	−0.931
		Intron	1,720	9	0.190	0.229	−1.020
		Nonsynonymous	794.81	3	0.126	0.165	−1.233

(continued)

Table 1. Continued

Species[a]	Forest Type	Functional Class[b]	Total bp	SNPs	θ_π (%)[c]	θ_W (%)[d]	Tajima's *D*
C. medius all ($n = 8$)	Closed canopy	Silent	1,975.27	23	0.271	0.351	−0.933
		Synonymous	254.27	2	0.141	0.237	−1.038
		Intron	1,721	21	0.290	0.368	−0.858
		Nonsynonymous	789.73	18	0.610	0.687	−0.465
C. medius normal allele (5 alleles) (half of gene)		Silent	1,108.53	8	0.38	0.347	0.661
		Synonymous	192.53	2	0.522	0.499	0.790
		Intron	916	6	0.350	0.314	0.764
		Nonsynonymous	566.47	7	0.675	0.593	0.913
C. medius 54bp-insert allele (10 alleles) (half of gene)		Silent	1,124.47	6	0.284	0.189	−0.106
		Synonymous	206.47	0	0	0	NA
		Intron	918	6	0.226	0.231	−0.106
		Nonsynonymous	609.53	2	0.109	0.116	−0.184
Phaner pallescens (Z, $n = 7$)	Open canopy	Noncoding	2,764	5	0.094	0.057	2.273*
P. pallescens (K, $n = 4$)	Open canopy	Noncoding	2,764	6	0.080	0.084	−0.201
P. parienti ($n = 3$)	Closed canopy	Noncoding	2,764	6	0.109	0.095	0.811
P. electromontis ($n = 4$)	Closed canopy	Noncoding	2,764	3	0.054	0.042	1.220

NOTE.—For *C. medius* normal and 54-bp insert alleles, data are from cloned sequences and only cover 1,734 bp. NA, not applicable.

[a]Sample size represents number of individuals examined.

[b]Silent class includes introns and synonymous sites, excluding 3′-untranslated region sites.

[c]Nucleotide diversity based on average pairwise differences per site.

[d]Nucleotide diversity based on the number of substitutions per site (Watterson 1975).

*For Tajima's *D* statistic, $P < 0.05$.

start codon (A/CTG, heterozygous in five individuals). In the two *Cheirogaleus* species, there is a fixed substitution in the third position (ATT), as previously reported (Tan et al. 2005). These start codon mutations likely do not affect gene function because a second start is found at the fourth codon position. Similar substitutions in the first start are found in tarsiers and rodents, which have functional SWS cones (Chiu et al. 1994; Kawamura and Kubotera 2004).

Species in all genera except *Phaner* exhibit indel polymorphisms in intron regions (supplementary table S2, Supplementary Material online). In *Lepilemur*, these polymorphisms are found in 4 of 5 populations, as well as in the single individual representing *L. microdon*. Interestingly, two closely related species of *Lepilemur* (*L. petteri* and *L. leucopus*) share an indel polymorphism in intron 4. Half of *Avahi* populations, both *Cheirogaleus* species and all three populations of *Microcebus* also exhibit intronic indel polymorphisms. Additionally, all *M. ravelobensis* individuals sequenced share an approximately 350 + bp insertion in intron 4 compared with other species.

In contrast to the high frequency of indel polymorphisms in introns, indel polymorphisms in exons were very rare. We identified one 3-bp indel polymorphism in exon 5 in *L. microdon* (present in the one individual sequenced). The deleted 3 bp results in the loss of one amino acid and a nonsynonymous change in the preceding amino acid (cysteine to tryptophan). We also identified two larger insertion polymorphisms in *C. medius* (discussed later).

Evidence for Loss of *OPN1SW* Functionality

Of the nocturnal lemur populations examined, only two groups (*Phaner* and *C. medius*) exhibit evidence for loss of functionality. All *Phaner* populations share a stop codon in the first exon (amino acid residue 33, fig. 1). Although a second potential start codon is found at residue 42 and there are no frameshift indels or other premature stops in the remaining sequence, the TATA box promotor is not found within 100 bp of the second start codon (fig. 1). Because this promotor is generally found in approximately 30-bp upstream of the *OPN1SW* transcription start site in humans and other mammals (Nathans et al. 1986; Srinivas et al. 2006), it is unlikely that the second start codon in *Phaner* would lead a functional SWS opsin protein. Further, truncating the opsin protein by the first 41 amino acids (including the entire N terminus region) would likely be highly deleterious (Doi et al. 1990).

In contrast to the uniform loss in *Phaner*, *C. medius* appears to exhibit intraspecific variation in functionality, suggesting that polymorphic dichromacy may exist in at least some populations. We identified three alleles in the population of *C. medius* (fig. 2). One allele appears functional ("normal allele") and is heterozygous in five individuals. The two other alleles exhibit duplications beginning at the same position in exon 2 (fig. 2*A*). In one insert allele (present in 1 heterozygote), there is a 4-bp duplication causing a frame-shift and stop codons in the coding region (fig. 2*B*), implying this allele is nonfunctional. The second insert allele has a 54-bp duplication, covering the last 11 bp of intron 1 and the first 43 bp of exon 2 (fig. 2*A*). Surprisingly, this 54-bp insert allele is present in all sampled individuals, either in homozygous ($n = 2$) or heterozygous state ($n = 6$). Although this larger insertion does not cause a frame-shift and the allele retains functionally important residues, it should add 18 amino acids to the protein (fig. 2*B*), which would likely have functional implications. The 54-bp insert allele may also influence splicing during translation because it contains part of the intron. Finally,

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Homo sapiens          GGTGGGAGGATCACCTATAAGAGGACTCAGAGGGGGGTGTGGGGCATCC-ATGAGAAAAATGTCGGAGGAA [ 71]
Lepilemur edwardsi    GGTGGAAGGATAATCTATAAGAGGAATCCAAAGGGG-TGTGGGGCATCCCMTGCATAAGATGTCAGGGGAA [ 71]
Avahi occidentalis    GGTGGAAGGATAATCTATAAGAGGAATCCAAGGGGG-TGTGGGGCATCCCATGCGAAAGATGTCAGGGGAA [ 71]
Microcebus simmonsi   -----AAGGATAATCTATAAGAGGAATCCAAGGCAG-TGTGGGGCATCCCATGAGTAAGATGTCAGGGGAA [ 71]
Cheirogaleus medius   ------AGGATAATCTATAAGAGGAATCCAAGGGGG-TGCAGGGCATCCTATTCATAAGATGTCAGGGGAA [ 71]
Cheirogaleus_major    --------------CTATAAGAGGAATCCAAGGGGG-TGCGAGGCATCCCATTCATAAGATGTCAGGGGAA [ 71]
Phaner pallescens     ------AGGATAATCTATAAGAGGAATCCAAGGGGG-TGTGGGGCATCCCATGCATAAGATGTCAGGGGAA [ 71]

Homo sapiens          GAG---TTTTATCTGTTCAAAAATATCTCTTCAGTGGGGCCGTGGGATGGGCCTCAGTACCACATTGCCCC [142]
Lepilemur edwardsi    GAGGAGTTTTATCTGTTCAAGAACCTCTCCTCGGTGGGGCCGTGGGATGGGCCTCAGTACCACATTGCCCC [142]
Avahi occidentalis    GAGGAGTTTTATCTGTTCAAGAATCTCTCCTCGGTGGGGCCGTGGGATGGGCCTCAGTACCACATTGCCCC [142]
Microcebus simmonsi   GAGGAGTTTTATCTATTCAAGAACCTCTCCTCAGTGGGGCCGTGGGATGGGCCTCAGTACCACATTGCCCC [142]
Cheirogaleus medius   GAGGAGTTTTATCTGTTCAAGAACCTCTCCTCGGTGGGGCCGTGGGATGGGCCTCAATACCACATTGCCCC [142]
Cheirogaleus major    GAGGAATCTTATCTGTTCAAGAACCTCTCCTCGGTGGGGCCGTGGGATGGGCCTCAATACCACAATGCTCC [142]
Phaner pallescens     GAGGAGTTTTATCTGTTCAAGAACCTCTCTTCTCTGGGRCCGTGGGATGGGCCTCAGTACCACATTGCCCC [142]

Homo sapiens          TGTCTGGGCCTTCTACCTCCAGGCAGCTTTCATGGGCACTGTCTTCCTTATAGGGTTCCCACTCAATGCCA [213]
Lepilemur edwardsi    TGTCTGGACCTTCTATCTCCAGGCAGCTTTCATGGGCTTTGTCTTCTTTGCAGGGACACCACTCAATGTCA [213]
Avahi occidentalis    TGTCTGGGCCTTCTACCTCCAGGCAGCTTTCATGGGCTTTGTCTTCTTTGTAGGGACACCACTCAATGTCA [213]
Microcebus simmonsi   TGTCTGGACCTTCTATCTCCAGGCAGCTTTCATGGGCTTTGTCTTCTTTGCAGGGACACCACTCAATGTCA [213]
Cheirogaleus medius   TGTCTGGACCTTCTATCTCCAGGCAGCTTTCATGGGCTTTGTCTTCTTTGCAGGGACACCACTCAATGTCA [213]
Cheirogaleus major    TGTCTGGACCTTCTATCTCCAGGCAGCTTTCATGGGCTTTGTCTTCTTTGCAGGGACACCACTCAATGTCA [213]
Phaner pallescens     TGTCTAGACCTTCTATCTCCAGGCAGCTTTCATGGGCTTTGTCTTCTTTGCAGGGACACCACTCAATGTCA [213]

Homo sapiens          TGGTGCTGGTGGCCACACTGCGCTACAAAAAGTTGCGGCAGCCCCTCAACTACATTCTGGTCAACGTGTCC [284]
Lepilemur edwardsi    TGGTGCTGGTGGCCACACTGCGCTACAAAAAGTTGCGGCAGCCACTCAACTACATTCTGGTCAATCTGTCC [284]
Avahi occidentalis    CGGTGCTGGTGGCCACACTGCGCTACAAAAGGTTGCGACAGCCACTCAACTACATTCTGGTCAATCTGTCC [284]
Microcebus simmonsi   TGGTGCTGGTGGCCACACTGCGCTACAAGAAGTTGCGGCAGCCACTCAACTACATTCTGGTCAATCTGTCT [284]
Cheirogaleus medius   TGGTGCTGGTGGCCACACTGCGCTACAAGAAGTTGCGGCAGCCACTCAACTACATTCTGGTCAATCTGTCC [284]
Cheirogaleus major    TGGTGCTGGTGGCCACACTGTGCCACAAGAAGTTGCAGCAGCCACTCAACTACATTCTGGTCAATCTGTCC [284]
Phaner pallescens     TGGTGCTGGTGGCCACGCTGCGCTACAAAAAGTTGCGGCAGCCACTCAACTACATTCTGGTCAATCTGTCC [284]
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Fig. 1. Aligned comparison of partial 5′-untranslated region and partial exon 1 for nocturnal lemur genera and humans. In the human sequence, the boxed nucleotides represent the TATA box promotor motif, "*" depicts the translation start site (Nathans et al. 1986). Potential start codons are represented by underline. The double-underlined nucleotides in the *Phaner* sequence represent the premature stop codon.

Tan et al. (2005) identified an additional *C. medius* allele that exhibits five nonsynonymous and six silent substitutions compared with the population studied here. One of these substitutions results in a premature stop codon at residue 317, whereas another changes a functional motif (the E/DRY motif, fig. 2*B*) that is highly conserved across G-protein-coupled receptors (Palczewski et al. 2000; Abdulaev and Ridge 2005). Thus, function of the Tan et al. *C. medius* allele may also be very impaired or lost (Sakmar et al. 1989; Wilbanks et al. 2002).

We also observed variation at splice sites at the exon–intron boundaries. Although most nocturnal lemurs retain the conserved mammalian GT/AG splice sites (Burset et al. 2000), both *C. major* and *C. medius* exhibit deleterious splice mutations, as previously reported by Tan et al. (2005). All *C. major* individuals share a 2-bp deletion of the donor splice GT at the beginning of intron 3. A second donor GT is found 4-bp downstream, but it would introduce a frameshift and multiple stop codons. *Cheirogaleus major* could possibly utilize a noncanonical splice pair (GA/AG). Although very rare (<0.02%), GA/AG is a normal splicing variant that can result in functional transcripts (Burset et al. 2000; Bradley et al. 2005). In contrast, the *C. medius* splice substitution (intron 4 acceptor site: AG to AA) has not been reported as potentially functional in studies of mammalian splice sites (Burset et al. 2000). Instead, it may result in the loss of exon 5 during translation in *C. medius*, as is seen with a GT–AA pair in a variant of the human leukocyte antigen-F gene (He et al. 2004).

Predicted Spectral Tuning of SWS Opsin Protein

We did not identify intrageneric variation in the amino acids present at 10 putatively important residues for SWS opsin spectral tuning (Fasick et al. 2002; Shi and Yokoyama 2003; Carvalho et al. 2012). In fact, the only instance of intraspecific variation was in *C. medius* with the nonfunctional 4-bp insert allele. However, we did identify variation between nocturnal lemur genera (table 2). In a recent study, Carvalho et al. (2012) expressed *OPN1SW* sequences for three lemurs (*Eulemur fulvus*, *Mir. coquereli*, and *D. madagascariensis*) to estimate the peak spectral sensitivity (λ_{max}) of the SWS visual pigment, and performed site-directed mutagenesis to explore the effect of changes at residue 86 (Cys to Val and Phe to Ser) on spectral tuning. Drawing on the results by Carvalho et al.

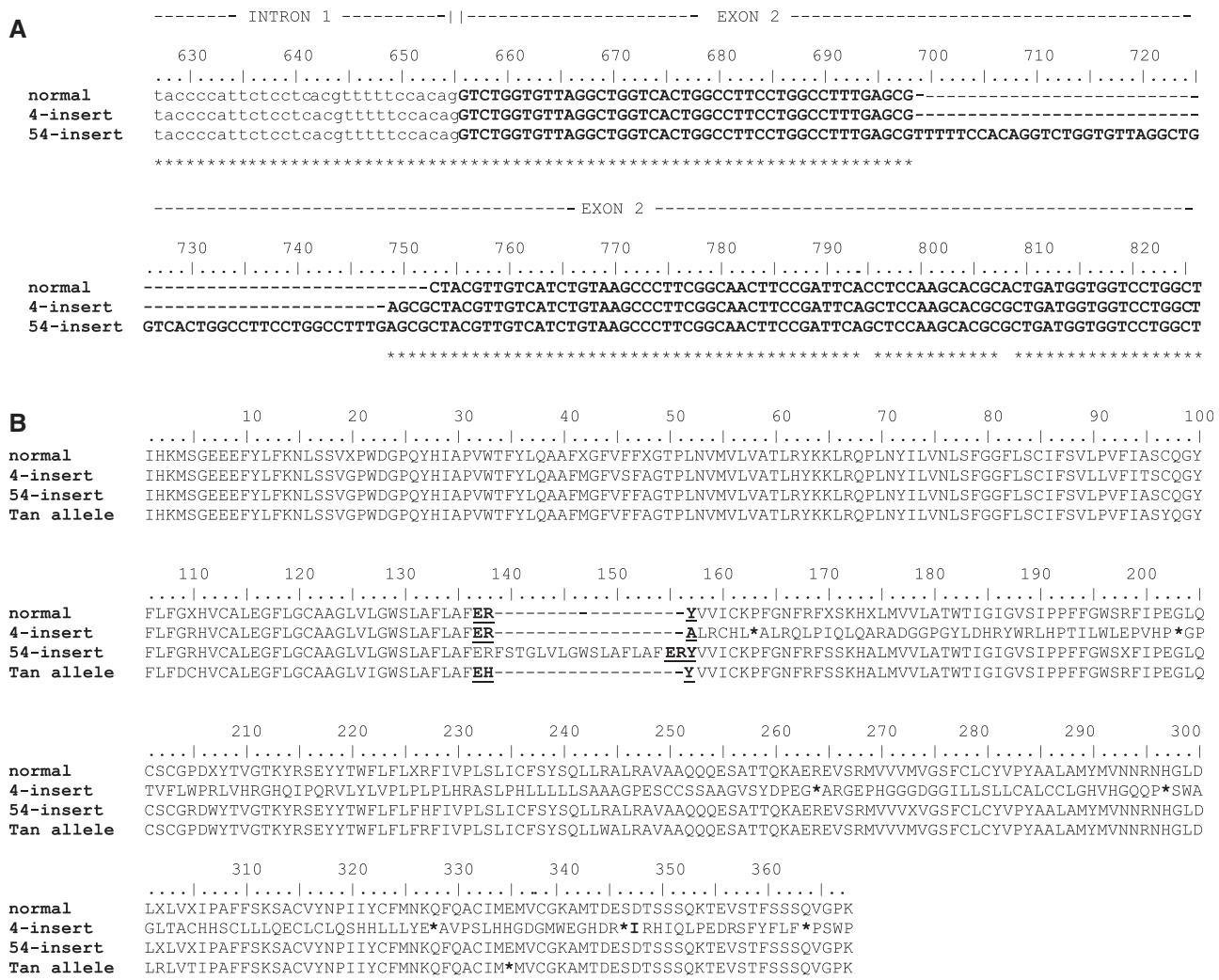

Fig. 2. Nucleotide and amino acid sequences for *Cheirogaleus medius* alleles. Alleles from our population include normal allele, 54- and 4-bp insert allele. (*A*) Nucleotide sequences depicting insertions in 4- and 54-bp insert alleles. Exons are bolded and in capital letters, introns are in lower case. (*B*) Amino acid sequences for the three alleles in our population and the published *C. medius* allele from Tan et al. (2005). Stop codons represented by asterisk (*), and functionally significant E/DRY motif region are bolded in each sequence.

(2012), we predicted λ_{max} for the genera in our study (table 2). *Microcebus*, *C. major*, and *C. medius* (normal and 54-bp insert alleles) share all suspected tuning residues with *Mirza*, suggesting that cheirogaleids share λ_{max} at 409 nm. *Lepilemur* residues are identical to the *Daubentonia* Phe86Ser mutant Carvalho et al. (2012) analyzed, suggesting λ_{max} at 416 nm. *Avahi* differs from *Lepilemur* only at residue 86. Because the presence of Val shifted λ_{max} to shorter wavelengths in the *Eulemur* Cys86Val mutant by 12 nm (Carvalho et al. 2012), we thus predict that *Avahi* has a more SW-shifted SWS pigment λ_{max} compared with *Lepilemur*.

Functional Predictions for Nonsynonymous Polymorphisms

We explored the potential effects of nonsynonymous polymorphisms (both single nucleotide polymorphisms [SNPs] and indels) on protein function using PROVEAN Protein (Choi et al. 2012). This program predicts whether amino acid substitutions and indels will have a neutral or deleterious effect on protein function based on comparisons with homologous amino acid sequences. Of 16 species (excluding *Phaner*), 12 exhibit at least one nonsynonymous polymorphism (fig. 3). The four species lacking nonsynonymous polymorphisms include three open canopy species (*A. cleesei*, *A. occidentalis*, and *M. griseorufus*) and one closed canopy species (*M. simmonsi*). However, it is important to note that *M. simmonsi* is represented by one individual. Using a generalized linear model assuming a Poisson distribution of substitutions, we tested whether the number of substitutions depended on habitat type, the functional effect of the substitutions (neutral or deleterious nonsynonymous), and an interaction between habitat and functional effect. Overall, we found significant effects of habitat, functionality, and a habitat-functionality interaction. Specifically, closed canopy species exhibit more substitutions overall ($P = 0.0006$), and deleterious substitutions exceed neutral substitutions ($P = 0.027$). Most importantly, the interaction ($P = 0.024$) indicates that the excess of deleterious substitutions is stronger in closed habitats and absent in open canopy habitats.

Table 2. Amino Acid Residues at *OPN1SW* Opsin Gene Spectral Tuning Sites and Predicted Peak Spectral Sensitivity (λ_{max}).

Species	46	49	52	81	86	90	93	114	116	118	λ_{max}
Carvalho et al. (2012)											
Daubentonia	F	F	T	F	F	S	P	G	L	S	406
Eulemur fulvus	L	F	A	F	C	S	P	G	L	S	413
Mirza coquereli	F	F	T	F	S	S	P	G	L	C	409
Mutants											
Eulemur Cys86Val	L	F	A	F	V	S	P	G	L	S	401
Daubentonia Phe86Ser	F	F	T	F	S	S	P	G	L	S	416
This study											
Lepilemur	F	F	T	F	S	S	P	G	L	S	416
Avahi	F	F	T	F	V	S	P	G	L	S	<416?
Microcebus	F	F	T	F	S	S	P	G	L	C	409
*Phaner**	F	F	T	F	S	S	P	G	L	S	
Cheirogaleus major	F	F	T	F	S	S	P	G	L	C	409
C. medius	F	F	T	F	S	S	P/L*	G	L	C	409

NOTE.—Species with asterisk suggested to have lost functionality due to premature stop codons. For *C. medius*, the allele with L at amino acid residue 114 is the 4-bp insert allele, which has multiple premature stop codons. λ_{max} predictions are based on similarities between residues at tuning sites for taxa in this study with those used in the visual pigment expression analyses in Carvalho et al. (2012). Note that residue numbering is based on the bovine rhodopsin (Shi and Yokoyama 2003). See text for an explanation of the ambiguity in predicted spectral tuning in *Avahi*.

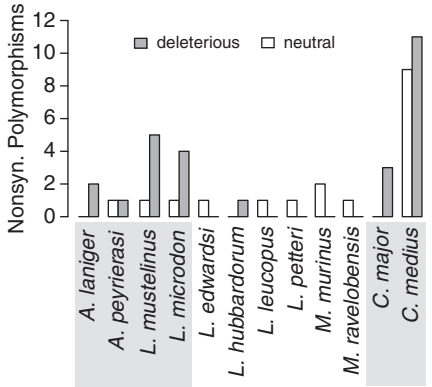

FIG. 3. Predicted effect of nonsynonymous polymorphisms. The number of deleterious (gray) and neutral (white) nonsynonymous polymorphisms (SNPs and indels) predicted for each species by PROVEAN Protein. Gray-shaded species represent closed canopy taxa. Four species are not present due to 0 nonsynonymous polymorphisms: *Microcebus simmonsi* (closed canopy), *M. griseorufus* (open canopy), *Avahi cleesei* (open canopy), and *A. occidentalis* (open canopy).

Looking just within closed habitats, there is a significantly higher rate of deleterious than neutral substitutions ($P = 0.027$), but a nonsignificant trend in the opposite direction in open canopy habitats ($P = 0.098$). This result is consistent with predictions of a habitat effect on differential selection for functional SWS cones. In this analysis, the 3-bp deletion polymorphism and the subsequent amino acid substitution in *L. microdon* are both predicted to be deleterious. Similarly, the 54-bp insertion polymorphism in *C. medius* is predicted to be deleterious.

Selection: Population Genetic Analyses

To test whether differences in nocturnal SW light between open and closed canopy forests influence differential selection on the *OPN1SW* gene, we examined signatures of recent selection at the population level in nocturnal lemurs (table 1 and fig. 4). We compared nucleotide diversity (θ_W: Watterson 1975) between silent (S) and nonsynonymous (N) site classes using two measures (coalescent tests and θ_{WN}/θ_{WS} ratios) following the analysis by Perry et al. (2007) of *OPN1SW* selection in *Daubentonia*. Because small sample sizes may not be representative of the variability present in a population, we restricted these analyses to populations represented by five or more individuals, excluding *L. petteri*, *A. peyrierasi*, and *C. major*.

Coalescent Tests

We used coalescent modeling to simulate "neutral" distributions of θ_W for each population based on the observed intron θ_W, and then tested whether the observed nucleotide diversity at nonsynonymous sites (θ_{WN}) is lower than expected under neutral evolution. Under purifying selection, θ_{WN} should be significantly lower than the simulated distributions, reflecting selection to remove nonsynonymous mutations (Perry et al. 2007). Following a sequential Bonferroni correction (Rice 1989), we found five species exhibiting significantly lower θ_{WN} than expected under neutrality, which suggests strong purifying selection for retaining *OPN1SW* functionality (fig. 4). All five populations are from open canopy forests, consistent with the hypothesis that the increased availability of SW light in open canopy forests leads to selection for SWS cone retention. These populations include the following: *L. edwardsi* ($P = 0.0003$), *A. cleesei* ($P = 0.0014$), *M. griseorufus* ($P = 0.000001$), *M. murinus* ($P = 0.0002$), and *M. ravelobensis* ($P = 0.0044$). Three of these are *Microcebus* species, suggesting phylogeny may also influence selective constraint. Additionally, one closed canopy species (*A. laniger*) approaches significance in the sequential Bonferroni correction ($P = 0.0098$,

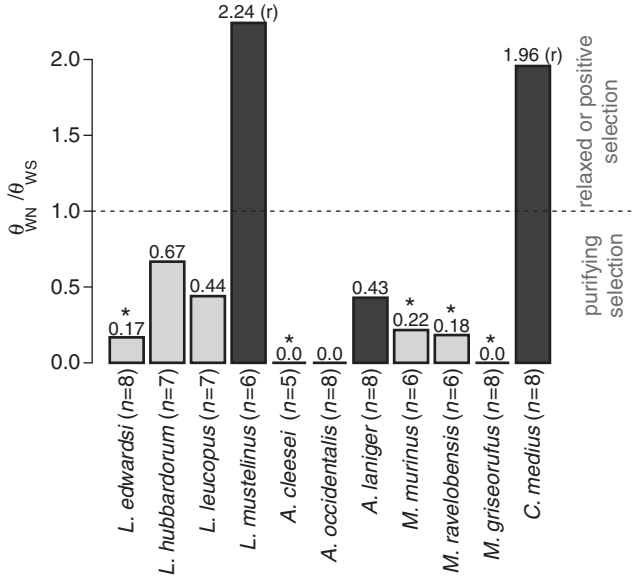

Fig. 4. θ_{WN}/θ_{WS} across nocturnal lemur populations. Bar colors represent habitat type: open canopy forest (light gray) and closed canopy forest (dark gray). Dashed line ($\theta_{WN}/\theta_{WS} = 1$) reflects the boundary between ratios suggestive of purifying selection (<1) and relaxed/positive selection (≥ 1). Asterisk (*) indicates species with θ_{WN} significantly lower than neutral simulations in Bonferroni-corrected coalescent tests. For species with $\theta_{WN}/\theta_{WS} > 1$, proposed selective regime listed in parentheses: relaxed selection (*r*) or positive selection (*p*), based on comparisons of site frequency spectra (supplementary fig. S2, Supplementary Material online). For species where bar is not visible: *Avahi cleesei* (open canopy), *A. occidentalis* (open canopy), and *Microcebus griseorufus* (open canopy).

Bonferroni cutoff at $P = 0.0083$), contrary to our stated hypothesis. Observed *P* values for the other five species tested were not close to the cutoff (>0.04 difference).

θ_{WN}/θ_{WS} Ratios

We also compared θ_W for nonsynonymous and silent sites using the θ_{WN}/θ_{WS} ratio as a measure of selection (Perry et al. 2007). Although less robust than the coalescent approach, this ratio estimates the relative variation at nonsynonymous and silent sites and suggest general patterns of selective regime. Ratios less than 1 suggest purifying selection to reduce nonsynonymous variation, whereas those ≥ 1 suggest either relaxed or positive selection (Perry et al. 2007). When θ_{WN}/θ_{WS} more than 1, we examined patterns of site frequency spectra (SFS) for nonsynonymous and silent sites (supplementary fig. S1, Supplementary Material online) to help distinguish between signals of relaxed and positive selection. Comparisons of θ_{WN}/θ_{WS} within and between genera suggest that habitat type and phylogeny have both influenced selection on *OPN1SW*. In general, species from open canopy forests have lower θ_{WN}/θ_{WS} than closed canopy species, particularly between congeners (fig. 4). However, the influence of habitat type differs between genera. In *Lepilemur*, the open canopy forest populations exhibit ratios suggestive of purifying selection, whereas the closed canopy population (*L. mustelinus*) exhibits a ratio and SFS suggestive of relaxed selection (fig. 4 and supplementary fig. S1, Supplementary Material online). By contrast, in *Avahi*, all three populations exhibit ratios suggestive of purifying selection, but the selective constraint appears stronger in the open canopy populations ($\theta_{WN}/\theta_{WS} = 0$). For *Microcebus* and *Cheirogaleus*, populations were only available for a single habitat type (open canopy *Microcebus* and closed canopy *Cheirogaleus*), preventing comparisons between congeners from different habitats. Still, the results from these two cheirogaleid genera are consistent with an effect of nocturnal light on selection for dichromacy. All three *Microcebus* populations exhibit signatures of purifying selection, whereas θ_{WN}/θ_{WS} and SFS for *C. medius* is suggestive of relaxed selection (fig. 4 and supplementary fig. S1, Supplementary Material online).

Selection: Phylogenetic Analyses

Bootstrap ML and Bayesian phylogenies for the primate *OPN1SW* gene are identical (fig. 5) and generally consistent with most lemuriform species trees (Roos et al. 2004; Yoder and Yang 2004; Poux et al. 2005; Andriaholinirina et al. 2006; Andriantompohavana et al. 2006, 2007; Louis et al. 2006, 2008; Zaramody et al. 2006; Fabre et al. 2009). Although some phylogenetic analyses have not been able to resolve the Indriidae-Cheirogaleidae-Lepilemuridae trichotomy (Roos et al. 2004; Berry and Semple 2006), our *OPN1SW* gene tree supports other analyses identifying Indriidae as an outgroup to the Cheirogaleidae-Lepilemuridae clade (Yoder and Yang 2004; Fabre et al. 2009). The *OPN1SW* gene tree differs only slightly from published mitochondrial DNA phylogenies in the placement of *M. ravelobensis* and *M. simmonsi* within *Microcebus* (Andriantompohavana et al. 2006; Louis et al. 2008) and *L. hubbardorum* and *L. microdon* within *Lepilemur* (Andriaholinirina et al. 2006; Louis et al. 2006).

We examined the type and strength of selection acting on *OPN1SW* across nocturnal lemur lineages by estimating the ratio of nonsynonymous and synonymous substitutions (ω) under different models of evolution and comparing these models with LRTs (Yang 2007). For these selection analyses, each species is represented by all individuals with unique coding sequences (supplementary fig. S3, Supplementary Material online). Because the 18 amino acid insertion polymorphism in *C. medius* may have significant (but currently unclear) functional effects and this insertion is present in all individuals, we excluded the insert alleles from these analyses. We employed three types of analyses to test for differences in selection: branch models, branch-site models, and site models (Yang 2007). Branch and branch-site models compare selection along certain branches of the tree ("foreground" branches) with all other branches ("background" branches), thus permitting us to directly test for differences in selection between open canopy and closed canopy nocturnal species. For these two types of models, we designated either closed canopy or open canopy lineages as foreground (supplementary fig. S3, Supplementary Material online). Although the branch models assume a single ω across codon sites within a lineage, the branch-site models permit ω to vary between sites and offer a test for codon sites under positive selection in

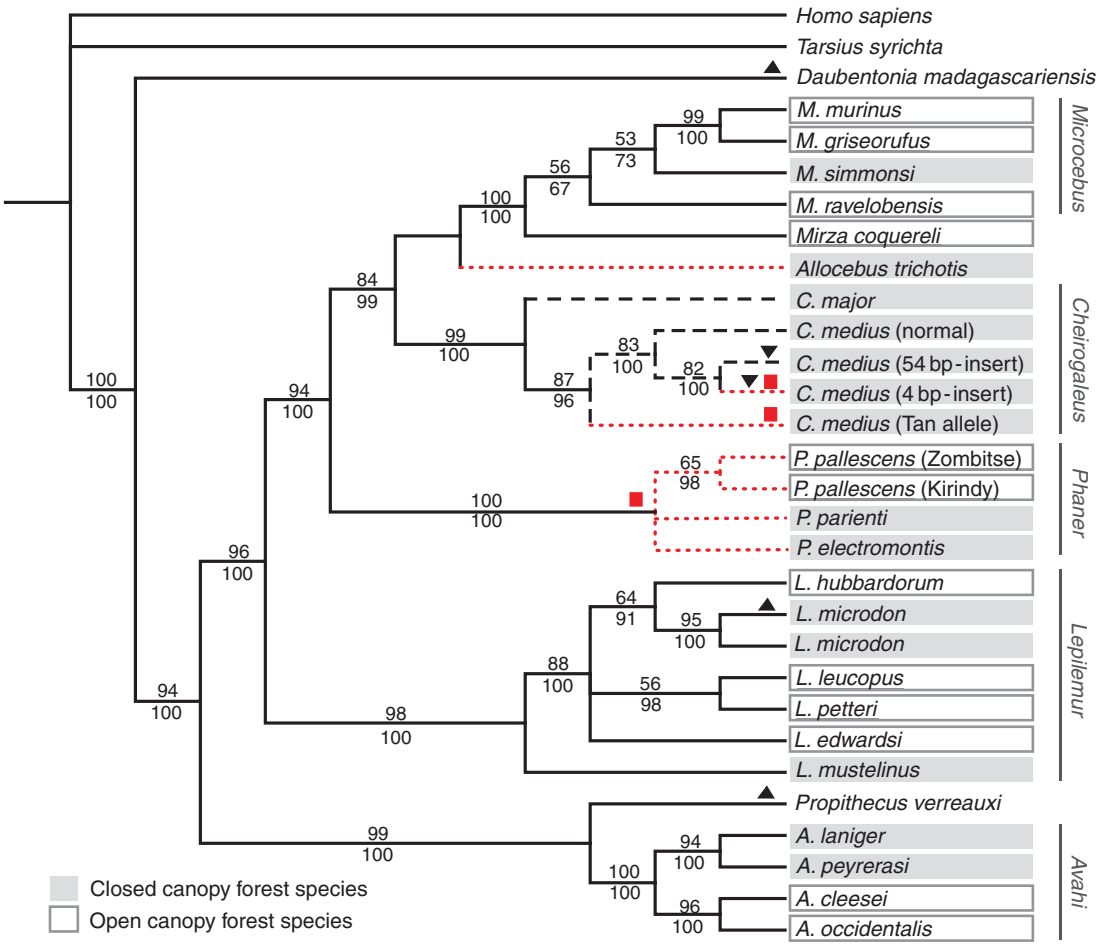

Fig. 5. Primate *OPN1SW* gene tree and signatures of selection. ML (−ln *L* = 3,506.981) for *OPN1SW* opsin gene under HKY + G model of sequence evolution. Numbers above and below each branch are the ML bootstrap values and Bayesian posterior probabilities, respectively. Dotted branches indicate confirmed or suspected SWS cone loss. Dashed lines reflect uncertainty due to mutations in splice sites. Premature stop codons, insertions, and deletions within Lemuriformes are represented by squares, downward oriented triangles, and upwarded oriented triangles, respectively. Sequence data are not available for *Allocebus trichotis*, but an anatomical study identified SWS cone loss (Peichl et al. 2004). Position of *Allocebus* within Cheirogaleidae from Roos et al. (2004).

foreground lineages (Yang 2007). In contrast to branch-based models, site models test for codon sites under positive selection across all branches of the tree.

Results of the branch model tests suggest that habitat type has had a significant effect on *OPN1SW* selection in nocturnal lemur lineages (tables 3 and 4). Under the two ratio closed canopy model, ω is higher for nocturnal lineages from closed canopy habitats ($\omega_1 = 0.550$) than for other branches in the tree ($\omega_0 = 0.203$). This model fits the data significantly better than the one ratio null model, which assumes a single ω for all branches ($P = 0.0031$). The two ratio open canopy model ($\omega_0 = 0.259$, $\omega_1 = 0.103$) also fits the data significantly better than the null model ($P = 0.0298$), suggesting that nocturnal lineages from open canopy forests have lower ω than other branches of the tree. Given that some of our previous results suggest *C. medius* may be in the process of losing *OPN1SW* functionality, we also compared the null model with a three ratio model that designated *C. medius* (noninsert alleles) and other closed canopy species as different groups of foreground branches (tables 3 and 4). For this model, ω was permitted to vary between background branches ($\omega_0 = 0.203$), other closed canopy species ($\omega_1 = 0.449$), and *C. medius* ($\omega_2 = 0.696$). Although the three ratio model fits the data significantly better than the null model ($P = 0.00079$), it does not significantly differ from the two ratio closed canopy model ($P = 0.57$), suggesting that *C. medius* ω does not substantially differ from that of other closed canopy branches. Together, these results support our predictions: lineages from open canopy forests have experienced strong levels of purifying selection to maintain *OPN1SW* function. In contrast, lineages from closed canopy forests have experienced weaker purifying selection for functional dichromacy.

Neither of the branch-site models designating open or closed canopy lineages as foreground fit the data significantly better than the null branch site models (table 4). These findings are consistent with the results of the branch model tests suggesting that most nocturnal lemur lineages are under some degree of purifying selection. Thus, habitat type does not appear to influence positive selection on sites in the *OPN1SW* gene.

Table 3. Summary of Phylogenetic Evolutionary Model Parameters.

Model	Ln *L*	*P*	Parameter Estimates
Branch models			
One ratio, ω estimated	−3,395.106	99	$\omega = 0.241$
Two ratio (closed canopy)	−3,389.475	100	$\omega_0 = 0.203$, $\omega_1 = 0.550$
Three ratio (closed canopy, *Cheirogaleus medius*)	−3,389.315	101	$\omega_0 = 0.203$, $\omega_1 = 0.449$, $\omega_2 = 0.696$
Two ratio (open canopy)	−3,392.745	100	$\omega_0 = 0.259$, $\omega_1 = 0.103$
Branch-site models			
Model A (closed canopy)	−3,354.959	102	$\omega_0 = 0.082$ ($p_0 = 0.76$), $\omega_1 = 1.0$ ($p_1 = 0.12$); $\omega_{2a} = 2.517$ ($p_{2a} = 0.10$), $\omega_{2b} = 2.517$ ($p_{2b} = 0.02$)
Model A (open canopy)	−3,361.321	102	$\omega_0 = 0.098$ ($p_0 = 0.84$), $\omega_1 = 1.00$ ($p_1 = 0.16$); $\omega_{2a} = 1.00$ ($p_{2a} = 0$), $\omega_{2b} = 1.00$ ($p_{2b} = 0$)
Site models			
M1a (nearly neutral)	−3,361.321	100	$\omega_0 = 0.098$ ($p_0 = 0.842$), $\omega_1 = 1.00$ ($p_1 = 0.158$)
M2a (positive selection)	−3,350.994	102	$\omega_0 = 0.113$ ($p_0 = 0.855$), $\omega_1 = 1.00$ ($p_1 = 0.130$), $\omega_2 = 7.939$ ($p_2 = 0.015$)
M7 (beta distribution)	−3,365.717	100	$p = 0.236$, $q = 0.704$
M8 (beta + $\omega > 1$)	−3,351.760	102	$p_0 = 0.983$, $p = 0.473$, $q = 1.522$ ($p_1 = 0.017$, $\omega = 7.106$)

Table 4. Summary of Phylogenetic LRTs.

Comparisons	2ΔLn *L*	*df*	*P* Value	Positive Sites
Branch models				
One ratio vs. two ratio (closed canopy)	11.262	1	0.0008	
One ratio vs. three ratio	11.261	2	0.0031	
One ratio vs. two ratio (open canopy)	4.721	1	0.0298	
Two-ratio (closed canopy) vs. three ratio	0.320	1	0.571	
Branch-site models				
Model A (Closed canopy) vs. null[a]	2.176	1	0.14	
Model A (Open canopy) vs. null[a]	<0.001	1	0.994	
Site models				
Model 1a vs. Model 2a	20.656	2	<0.00001	2**, 48**, 98**
Model 7 vs. Model 8	27.913	2	<0.00001	2**, 48**, 98**, 122*, 226*

NOTE.—Underlined model fits the data significantly better. Positive sites detected in BEB analysis. Site position aligned to *Propithecus coquereli* and includes the start codon as site 1.

[a]Null models in branch-site model tests are the same models (Model 2, NSSites 2), but with ω_2 fixed at 1 in the foreground branches (Yang 2007).

*$P < 0.05$.

**$P < 0.01$.

Interestingly, results of the site-specific models suggest that several codon sites in *OPN1SW* are under positive selection across the primates in our analysis (tables 3 and 4). We employed two pairs of site models to test for codon sites under positive selection: M1a versus M2a and M7 versus M8 (Yang 2007). In both comparisons, the models incorporating positive selection (M2a and M8) fit the data significantly better ($P < 0.00001$) than the alternative models (M1a and M7). With M2a, three sites were identified as under positive selection using the Bayes Empirical Bayes (BEB) analysis, whereas five sites were identified in M8 (table 4). None of the selected sites have previously been identified as involved in primate SWS cone spectral tuning or as functionally important. However, one site identified in both positive selection models (site 48) is located immediately after a putative spectral tuning site (site 49 in table 2, which is at position 47 in the nocturnal lemur *OPN1SW* alignment) and in close proximity to two other spectral tuning sites (sites 46 and 52 in table 2, sites 44 and 50 in the *OPN1SW* alignment).

Discussion

Differential Selection on *OPN1SW*

Although traditional assumptions of visual function and evolution tend to discount color vision at night, recent analyses of opsin genes and species ecology suggest an adaptive benefit for retaining color vision in certain nocturnal conditions (Kawamura and Kubotera 2004; Johnsen et al. 2006; Perry et al. 2007; Zhao, Rossiter, et al. 2009; Zhao, Xu, et al. 2009; Melin et al. 2012; Veilleux and Cummings 2012). The widespread variation across nocturnal mammals in color vision, including parallel loss of SWS cones in multiple mammalian clades, raises questions about which ecological factors influence selection to maintain dichromacy in nocturnal mammals (Jacobs et al. 1996; Ahnelt and Kolb 2000; Kawamura and Kubotera 2004; Peichl 2005; Jacobs 2008, 2013). Zhao, Rossiter, et al. (2009) hypothesized that changes in sensory ecology led to differential selection for dichromacy in bats. In this study, results from both population and phylogenetic analyses

suggest that habitat preference has influenced differential selection for color vision among nocturnal lemurs.

Consistent with our predictions, our results suggest that nocturnal lemurs experience stronger selection to retain dichromatic color vision in open canopy forests and weaker selective constraint in closed canopy forests. Closed canopy species were 1) more likely to have deleterious nonsynonymous polymorphisms, 2) did not exhibit significantly lower θ_{WN} than neutral expectations in coalescent tests, 3) exhibited higher population θ_{WN}/θ_{WS} ratios, particularly than their open canopy congeners, and 4) exhibited higher ω in lineage analyses. In contrast, only 1 of 9 open canopy species had a deleterious nonsynonymous polymorphism, 5 species had significantly lower θ_{WN} than expected under neutrality (1 of 9 was excluded from population analyses due to low sample sizes), and open canopy lineages exhibited lower ω than background branches. Moreover, the relatively low sample sizes and nucleotide diversity characterizing many of our populations, particularly from open canopy forests, may have contributed to a lack of statistical power. For example, in one open canopy species (*A. occidentalis*), coalescent simulations were not significant despite there being 0 nonsynonymous SNPs in the population, likely because intron θ_W was so low. In contrast, nucleotide diversity was generally higher in closed canopy populations compared with open canopy congeners (table 1).

These findings support our hypothesis that nocturnal light environment plays an important role in driving differential selection for SWS cone retention. Although some researchers propose that SWS cones are retained for activity at twilight or occasionally during the day (Perry et al. 2007; Müller et al. 2009; Melin et al. 2012), our results are not consistent with either explanation. Although *Avahi* is occasionally active during the day (unlike the other genera), this behavior has been seen in both habitat types (Ganzhorn et al. 1985; Warren and Crompton 1997). Diurnal habitats vary in light intensity and spectral quality, but are several orders of magnitude brighter than nocturnal environments (Pariente 1980; Endler 1993; Warrant 2004). Light levels are thus high enough in all diurnal environments that selection for dichromacy should not vary by habitat in species that exhibit occasional diurnal activity. Twilight environments are rich in SW light across habitats (Endler 1993) and the intensity of SW light at twilight is thought to be sufficient for mammalian color vision even in the rainforest understory (Melin et al. 2012). Thus, activity at twilight should also not lead to a habitat difference in selection for retaining SWS cones. Instead, we hypothesize that cone-based target detection at nocturnal light levels is driving the observed habitat difference in *OPN1SW* selection among nocturnal lemurs. Nocturnal light intensity, particularly SW light, is much greater in open canopy forests than in the understory of closed canopy forests (Veilleux and Cummings 2012). Consequently, open canopy species are more likely to encounter nocturnal light environments bright enough for SWS cone function (especially at higher moonlight intensities) and experience selection to maintain SWS cones. Lemurs from closed canopy forests are less likely to encounter nocturnal light levels sufficient for SWS cone function, particularly at lower canopy levels.

Although our results suggest that SWS cones are retained for vision at nocturnal light levels, it is unclear whether nocturnal lemurs utilize chromatic (color based) or achromatic (luminance based) target detection at night. Although SWS cones primarily contribute to blue–yellow color discrimination (Ahnelt and Kolb 2000; Silveira et al. 2005), some evidence suggests that they may also contribute to luminance vision (Chatterjee and Callaway 2002; Li and DeVries 2006). SWS cone retention thus could be advantageous for either type of nocturnal target detection. Species or genera may even vary in the type of target detection that is selectively important, as in seen surfperch (Cummings 2004).

Although we identified nocturnal light environment as an important factor in differential *OPN1SW* selection, our results suggest that phylogeny has also influenced selection for dichromacy. Among nocturnal lemurs with presumed functional *OPN1SW* genes, genera varied in the strength of purifying selection (as measured by coalescent tests and ratios) and the patterns differential selection between habitat types. The coalescent tests yielded significant results for all three *Microcebus* open canopy forest populations, compared with one population each of *Lepilemur* and *Avahi*. Some aspect of *Microcebus* ecology, such as their insectivorous–frugivorous diet, may make members of this genus more likely to retain functional SWS cones. Our study unfortunately lacks a closed canopy *Microcebus* population for comparison, but it would be interesting to compare coalescent and ratio results for closed canopy *Microcebus* populations with that seen in open canopy forests to see if this phylogenetic effect holds.

The two folivorous genera (*Avahi* and *Lepilemur*) vary in the patterns of differential selection between habitat types, also indicating a potential phylogenetic effect. Although closed canopy *Lepilemur* species exhibit signatures of relaxed selection on *OPN1SW*, closed canopy *Avahi* appears to be experiencing purifying selection, albeit weaker purifying selection than open canopy congeners. These results suggest that some aspect of evolutionary history or ecology shared by all *Avahi* species may result in selection to maintain dichromacy even in closed canopy habitats. We propose that dietary differences between the two genera may be responsible for the different patterns of selection. Aspects of leaf physiology important to folivores, such as protein, phenol, and tannin contents (Ganzhorn 1988; Norscia et al. 2012), are significantly correlated with chromatic differences in the blue–yellow channel (Dominy and Lucas 2004). Thus, for lemur folivores in nocturnal light environments that permit SWS cone function, dichromacy could be selectively important in foraging tasks. Although often sympatric, *Avahi* and *Lepilemur* in both habitats differ in the types of leaves they prefer and the vertical heights at which they forage (Ganzhorn 1988, 1989; Thalmann 2001). In particular, closed canopy *Avahi* often prefers leaves with high extractable protein content and forages in higher (i.e., brighter) canopy levels than sympatric *Lepilemur* (Ganzhorn et al. 1985; Ganzhorn 1989), which is consistent with differential selection for dichromacy between the genera. Future research exploring whether blue–yellow

color cues and nocturnal light environments differentially influence foraging in these genera would offer an excellent test of our hypotheses.

Suspected SWS Cone Loss in Cheirogaleids

In addition to identifying signatures of differential selection among nocturnal lemurs with presumed functional *OPN1SW* genes, we discovered evidence of possible SWS cone loss in the entire *Phaner* genus and in *C. medius*. The suspected loss of *OPN1SW* function in *Phaner* suggests that phylogeny has also played a role in patterns of SWS cone loss. The stop codon is shared by all *Phaner* species in our study, indicating that it likely predates their divergence. Because fossil evidence of lemur evolution in Madagascar prior to the late Pleistocene is lacking (Godfrey and Jungers 2003), it is not possible to explore habitat effects on SWS cone loss in this genus. One alternative ecological factor that may have influenced SWS cone loss in *Phaner* is their specialized gummivorous diet (Hladik et al. 1980; Génin et al. 2010). Color may be less important when foraging for exudate resources, as a recent study found that monochromacy in galagos was sufficient for detecting gums in nocturnal conditions (Moritz and Dominy 2010). Gummivory is also the major component of the diet of *Allocebus trichotis*, another cheirogaleid from closed canopy rainforests that has lost SWS cones (Peichl et al. 2004; Génin et al. 2010). Interestingly, shared deleterious *OPN1SW* mutations between lorisiform primates suggest that SWS cones were lost in the last common ancestor of lorises and galagos by approximately 37 Ma (Seiffert et al. 2003; Kawamura and Kubotera 2004), and dental morphology suggests that gums may have been a major component of the diet in the late Eocene stem galagid *Wadilemur elegans* (Kirk and Simons 2001). However, the strength of the link between gummivory and SWS cone loss is not clear, as gums can be an important component of the diet of *M. griseorufus* (78.6% of foraging time) and other *Microcebus* species (Génin et al. 2010), which exhibit signatures of strong purifying selection on *OPN1SW* in this study.

Several lines of evidence suggest that *C. medius* is experiencing relaxed selection on *OPN1SW*, including the presence of the nonfunctional 4-bp insert allele and the high number of deleterious polymorphisms in the population in this study, the additional deleterious mutations in the *C. medius* in the study by Tan et al. (2005), the splice site substitution in intron 4, and the signatures of relaxed selection found in both population and phylogenetic analyses. Together, these findings suggest *C. medius* is in the process of losing functional SWS cones. Relaxed selection and loss of SWS cones in closed canopy *C. medius* is consistent with a role of habitat type and nocturnal light environment in selection for dichromacy. Interestingly, *C. major*, the closed canopy congener of *C. medius*, exhibits signatures of purifying selection on *OPN1SW*. Although *C. major* has a 2-bp deletion in the intron 3 donor splice site, it is possible that the noncanonical GA–AG splice pair is functional in this species, as is seen occasionally in other genes (Burset et al. 2000; Bradley et al. 2005). We excluded *C. major* from the population analyses due to low sample size (three individuals), but we did identify substantial nucleotide diversity in the population (table 1: 15 silent SNPs, three nonsynonymous SNPs) compared with the other two populations with three individuals. The θ_{WN}/θ_{WS} for the three *C. major* individuals is suggestive of purifying selection (0.50), but this result needs to be confirmed in future studies with larger sample sizes. If future work confirms the presence of SWS cones and purifying selection in *C. major*, it would be interesting to explore what ecological factors drive differential selection for dichromacy between the *Cheirogaleus* congeners. One possible avenue of exploration is the role of folivory in the diet. Some evidence suggests that unlike *C. medius*, *C. major* consumes young leaves and buds in addition to fruits and insects (Ganzhorn 1988).

An additional aspect of the *OPN1SW* gene in *C. medius* that deserves future research is the 54-bp insertion found in all individuals sampled in this study. This insertion was not identified in the *C. medius* from Tan et al. (2005), raising the question of how frequent is this allele in other populations? The functional implications of this insertion would also benefit from experimental in vitro expression studies of the protein to determine whether it results in a recognizable opsin protein. It is possible that the gene is no longer involved in SWS cone production and may instead have a novel function.

Predicted SWS Cone Spectral Tuning

We did not identify differences between congeneric species in predicted SWS cone spectral tuning. Although our predictions were based on Carvalho et al.'s (2012) in vitro expression analyses of lemur SWS opsins, future studies should directly explore SWS λ_{max} for the species in this study. It is possible that other, currently unknown, amino acid sites also influence SWS spectral tuning in primates. Of particular interest may be the codon sites found to be under positive selection in our BEB analyses. For example, as mentioned previously, one site is immediately adjacent to known spectral tuning sites (site 48 in BEB analyses), and residues at this site varied between *Lepilemur* species.

A lack of variation in λ_{max} between congeneric species from different habitat types is not necessarily surprising. A recent study found that habitat type (closed canopy forest vs. open canopy forest/woodland) was not a significant factor in the spectral tuning of SWS and MWS/LWS cones in nocturnal mammals (Veilleux and Cummings 2012). However, Veilleux and Cummings (2012) did identify diet (fruit or flower consumption) as a significant influence on SWS cone spectral tuning in nocturnal mammals. Thus, the intergeneric variation we identified at predicted spectral tuning sites may indicate visual adaptations for detecting different food resources among nocturnal lemur genera. Alternatively, however, SWS cone λ_{max} could reflect phylogenetic inertia. All cheirogaleids (*Mirza*, *Microcebus*, *Cheirogaleus*, and even *Phaner*), for example, share residues at *OPN1SW* tuning sites.

Significance for Primate Evolution

Because population analyses reflect recent selective pressures while phylogenetic analyses reflect older or more long-term

pressures (Perry et al. 2007), the combination of population and phylogenetic analyses in this study enables us to examine the history of selection on the *OPN1SW* gene at different time scales and address recent controversies regarding primate evolutionary origins (i.e., Tan et al. 2005; Ross et al. 2007; Ankel-Simons and Rasmussen 2008). When Tan et al. (2005) argued for a diurnal/cathemeral primate ancestor, they interpreted signatures of purifying selection in nocturnal lemurs as evidence for recent transitions to nocturnality, suggesting that species became nocturnal so recently that a signal of relaxed selection is not yet present in phylogenetic analyses. However, we found both recent and more ancient signatures of strong purifying selection on *OPN1SW* in several populations of nocturnal lemurs. Because all nocturnal lemurs exhibit visual morphology characteristic of nocturnal adaptation (Kirk 2004; Perry and Pickrell 2010), the preponderance of genetic and morphological data makes it very unlikely that nocturnality is a recent phenomenon in lemurs. Rather than being restricted to an ecologically and morphologically derived species like *Daubentonia* (Perry et al. 2007), our results suggest that selection for nocturnal dichromacy is fairly common across nocturnal lemur families. In fact, SWS cone loss appears limited to certain lineages in Cheirogaleidae, where our results suggest up to three independent losses (*Phaner*, *Allocebus*, and possibly *C. medius*), despite strong purifying selection in other lineages. Thus, contrary to Tan et al. (2005), the retention of SWS cones and dichromacy is not incompatible with nocturnality in the last common ancestor of living primates. We instead suggest that the evolution of SWS cone loss in nocturnal lemurs, and possibly other nocturnal primates and nonvolant mammals, has been influenced by changes in nocturnal light environments.

Materials and Methods

Population Sampling

We obtained tissue or blood samples from 106 nocturnal lemurs from 20 populations (19 species) and five genera (*Avahi*, *Lepilemur*, *Phaner*, *Cheirogaleus*, and *Microcebus*). Samples were collected from wild individuals across Madagascar by E.E. Louis and colleagues and stored at Omaha's Henry Doorly Zoo and Aquarium (Omaha, NE). Collection locality, sample size, habitat type, and GenBank accession numbers for each population are provided in supplementary table S1, Supplementary Material online. For all genera, we sampled at least one individual endemic to each of two habitats (closed canopy rainforest and open canopy dry deciduous forest), and usually 8–16 individuals per habitat. For *Lepilemur* and *Microcebus*, we also sampled at least eight individuals endemic to open canopy spiny forest habitats (other nocturnal genera are not present in those habitat types). *Phaner pallescens* is represented by two populations (Kirindy Forest and Zombitse-Vohibasia National Park). Because these populations are separated by 300 + km of fragmented forests and large rivers, and exhibit average home range sizes less than 200 m (Schülke and Kappeler 2003), we assumed gene flow is limited and report results that treat the populations separately. We also obtained *OPN1SW* exon sequences for five other primates (*Propithecus verreauxi*, *Mir. coquereli*, *D. madagascariensis*, *Tarsius syrichta*, and *Homo sapiens*) from GenBank.

Amplification and Sequencing of Genomic DNA, and Nucleotide Sequence Alignment

Genomic DNA was obtained from whole genome amplifications of digested tissue using the TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences) at the Molecular Genetics Laboratory at Henry Doorly Zoo and Aquarium (Omaha, NE). The *OPN1SW* gene was amplified in two ~1.7 kb fragments using lemur-, genus-, or species-specific polymerase chain reactions (PCR) primers (available upon request) designed in Primer 3Plus (Untergasser et al. 2007). PCR were carried out using High Fidelity Platinum Taq Polymerase, 10× High Fidelity Buffer, and $MgSO_4$ (Invitrogen), MasterAmp 10× PCR Enhancer (Epicentre), 10 mM GeneAmp dNTPs (Applied Biosystems), and 20 μM primers in 25 or 50 μL reactions. The PCR conditions were 1) an initial 2 min hold at 94 °C; 2) 35–60 cycles of 30 s at 94 °C, 30 s at the annealing temperature for that primer pair, and 3 min at 68 °C; 3) a final hold for 10 min at 68 °C. PCR products were purified using either the QIAquick PCR Purification Kit (Qiagen) or magnetic beads (Thermo Scientific). PCR products were sequenced in 300–800 bp fragments using genus- and species-specific sequencing primers (available upon request). Sequencing was performed on a 3730 DNA Analyzer (Applied Biosystems) at the University of Texas at Austin DNA Sequencing Facility. We sequenced every base in each individual at least two times. Nucleotide sequences were aligned in BioEdit 7.0.9.0 (Hall 1999) and Sequencher 4.9 (Gene Codes) using the *Eulemur OPN1SW* sequence (Kawamura and Kubotera 2004) to identify exon–intron boundaries. Sequencing results for several populations indicated the presence of indel polymorphisms in some individuals. For these individuals, we used cloning to confirm the presence of these polymorphisms and to identify the correct sequence of each allele. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen), and multiple clones (3–15) were sequenced for each PCR product. Each indel was sequenced at least twice per individual to confirm the sequence of each allele.

Functional Predictions for the SWS Opsin Protein

To explore how *OPN1SW* gene variation within and between populations could influence SWS cone functionality, we aligned all exon sequences in the open reading frame and translated codons into amino acids. We examined variation at 10 critical sites in the *OPN1SW* opsin protein for spectral tuning (Fasick et al. 2002; Shi and Yokoyama 2003; Carvalho et al. 2012) and at functionally important residues (Sakmar et al. 1989; Hunt et al. 1995; Palczewski et al. 2000; Kawamura and Kubotera 2004; Santillo et al. 2006). These functionally important residues include two cysteine residues for the disulfide bond (residue 108 and 185), glutamate for the Schiff-base counter-ion (residue 111), lysine for the Schiff-base linkage to the chromosphore (residue 294), and the

glutamic acid–arginine–tyrosine triplet (132–133–134) that forms the E/DRY motif.

We also examined the potential effects of nonsynonymous polymorphisms (SNPs and indels) within nocturnal species on protein function using the PROVEAN Protein v.1.1 (Choi et al. 2012) online server (http://provean.jcvi.org/, last accessed January 15, 2013). PROVEAN uses BLAST sequences related to a query sequence to classify variant versions of the query sequence as either "neutral" or "deleterious" (Choi et al. 2012). For our analyses, we used the *Propithecus coquereli OPN1SW* amino acid sequence (Tan et al. 2005) as the query sequence and tested polymorphisms found in each nocturnal lemur species as variants. We chose *Propithecus* as the query sequence because experimental evidence confirms they have functional SWS cones (Jacobs et al. 2002). We used a general linear model implemented in R version 2.15.2 (R Core Team 2012). We assumed a Poisson distribution of substitutions and tested whether the number of nonsynonymous substitutions depended on habitat type, the functional effect of the substitutions (deleterious or neutral), and an interaction between habitat and functional effect. *Phaner* was excluded from these analyses.

Population Genetic Analyses

For each population, we estimated nucleotide diversity (θ_W) for nonsynonymous and silent sites (synonymous sites + introns) using the number of substitutions per site (Watterson 1975). All analyses assume that silent θ_W reflects neutral evolution. Although selection may act on synonymous mutations in some genes (Chamary and Hurst 2005), Perry et al. (2007) found that silent site diversity in the *OPN1SW* gene in *Daubentonia* was comparable with diversity across 15 autosomal intergenic regions, suggesting neutral evolution at silent sites in lemur *OPN1SW* genes and supporting this assumption for our study. We restricted analyses to species represented by five or more individuals to better reflect the nucleotide diversity present in the population. We excluded *Phaner* because the evidence suggests loss of *OPN1SW* functionality. We performed coalescent simulations using estimates of nucleotide diversity (θ_W) from intronic regions as a measure of neutral evolution in each population. This method was adapted from an analysis by Perry et al. (2007) of *OPN1SW* in a population of *Daubentonia*, which used intergenic regions for similar coalescent simulations. We simulated 10,000 genealogies with no recombination to test how often the observed nonsynonymous θ_W fit simulated distributions under neutrality. We used a sequential Bonferroni correction (Rice 1989) to adjust *P* values for multiple tests. Perry et al. (2007) used θ_π (the average number of pairwise differences per site) in their coalescent simulations; however, we employed θ_W to account for potential sampling effects in our study. Because θ_π incorporates information about the frequency of alleles in a population (Perry et al. 2007), it may be subject to sampling effects that can skew allele frequencies (small sample sizes or samples collected from closely related individuals, i.e. from a single sleeping hole). θ_W is more conservative because it excludes frequency information (Kreitman 2000; Perry et al. 2007), but it may be more appropriate here because it is more robust to sampling effects (Ewens 1983; Eshleman et al. 2011). Although we report only the results of the tests using θ_W, we repeated all analyses using θ_π and found the same pattern of results as for θ_W (see supplementary note, Supplementary Material online).

We also compared nucleotide diversity for nonsynonymous (θ_{WN}) and silent sites (θ_{WS}) using the θ_{WN}/θ_{WS} ratio. This ratio was adapted from the analysis by Perry et al. (2007) using θ_π. A ratio less than 1 suggests purifying selection, whereas a ratio ≥ 1 suggests either positive selection or a relaxation of functional constraint. To distinguish between relaxed and positive selection when θ_{WN}/θ_{WS} more than >1, we compared the distribution of alleles (site frequency spectrum or SFS) between nonsynonymous and silent site functional classes (supplementary fig. S1, Supplementary Material online). Under neutral evolution, the expected proportion of SNPs for each allele frequency is proportional to the population size. Purifying selection results in an increased proportion of SNPs segregating at low frequencies in the population, whereas positive selection increases the proportion of SNPs at higher frequencies (Nielsen 2005). Additionally, we computed Tajima's *D* statistic (Tajima 1989) to test for differences in the SFS at nonsynonymous and silent sites (supplementary table S2, Supplementary Material online). A significantly negative Tajima's *D* represents an excess of rare alleles and is often indicative of purifying selection, whereas a significantly positive *D* statistic indicates an excess of intermediate frequency alleles and balancing selection. However, tests using the SFS may also be influenced by demographic history and population structure, which can mimic signatures of selection (Kreitman 2000; Nielsen 2005). Because, nonsynonymous and silent functional classes should be similarly affected by population history (Akashi 1999), we computed Tajima's *D* separately for nonsynonymous and silent classes to control for the effects of demography and population structure. We also conducted McDonald–Kreitman tests (McDonald and Kreitman 1991) between congeneric populations, however, the results of these tests were not informative (see supplementary note, Supplementary Material online). All analyses were calculated in DnaSP v.5.10.01 (Librado and Rozas 2009).

OPN1SW Opsin Gene Tree Reconstruction

After removing introns, we generated a consensus sequence for each nocturnal lemur species, representing population SNPs with ambiguous DNA codes (e.g., "r" and "y"). Three species were represented by multiple sequences: *P. pallescens* (separate Zombitse and Kirindy sequences), *C. medius* (normal allele, 4bp-insert allele, 54bp-insert allele, and Tan et al.'s [2005] allele), and *L. microdon* (one allele with 3-bp deletion in exon 5). We aligned nucleotide sequences using ClustalW implemented DAMBE (Xia 2001; Xia and Xie 2001). We constructed an ML tree of the *OPN1SW* sequences with *Tarsius* and *Homo* as outgroups, using PAUP* version 4.0 (Swofford 2003). We employed a heuristic search with tree bisection and reconnection (TBR) branch swapping, starting

from a neighbor-joining tree and used the parameters estimated by ModelTest 3.6 (Posada and Crandall 1998) using hierarchical LRTs. ModelTest selected the HKY + G model of sequence evolution, corresponding to base frequencies A = 0.1906, C = 0.2848, G = 0.2463, and T = 0.2784; Ti/tv ratio = 3.3592; proportion of invariant sites = 0; and Gamma distribution shape parameter = 0.4777. We then generated 100 bootstrap replicate ML trees using a heuristic search and TBR branch swapping. We also constructed a Bayesian tree using Markov Chain Monte Carlo runs for the *OPN1SW* gene using MrBayes v.3.2.1 (Ronquist et al. 2012). We ran four simultaneous chains (three hot, one cold), and sampled every 100 generations. We ran as many generations as needed to reach convergence, defined as an average standard deviation of split frequencies less than 0.01 (120,000 generations for our data).

Phylogenetic Tests of Selection

To examine selection acting on the *OPN1SW* gene at the lineage level, we employed a codon-based method using the codeml program in PAML version 4.5 (Yang 2007). We removed the sequences thought to be nonfunctional (e.g., with stop codons) from the data set (all *Phaner* species, *C. medius* 4-bp insert allele, and *C. medius* Tan et al. allele). Because the function of the *C. medius* 54-bp insert alleles is unclear, we also excluded those alleles. For these analyses, we included all individuals with unique coding sequences to represent each species. Sequences for these individuals included ambiguous DNA codes for SNPs. Codon sites with ambiguous DNA codes were included in the codeml analyses (clean data = 0). Because recombination can influence branch-site model results (Anisimova et al. 2007), we used DnaSP to reconstruct haplotypes from sequences with ambiguous coding (using PHASE) and test for recombination (Librado and Rozas 2009). For 11 of 14 species, the minimum number of recombination events (R_m) estimated for most lemur species was 0. For two species (*L. mustelinus* and *M. griseorufus*) R_m was estimated at 1 (of 12 sequences each), whereas for *C. medius* normal alleles R_m was estimated at 2 (of 8 sequences). These estimates appear within the range Anisimova et al. (2007) found to still be accurate in branch-site model LRTs (3 or fewer recombination events in 10 sequences). We realigned the nucleotides of the remaining sequences, and estimated the ratio of nonsynonymous (d_N) to synonymous substitutions (d_S) in the coding region for all external and internal branches of ML tree under different models of evolution (table 1). The d_N/d_S ratio (ω) indicates the type and magnitude of selection acting on the gene, where $\omega < 1$ reflects purifying selection, $\omega = 1$ reflects neutral evolution, and $\omega > 1$ reflects positive selection (Yang 1998, 2007).

In codeml, we used LRTs to compare competing models of *OPN1SW* gene evolution, as has been done in studies of opsin genes in bats (Zhao, Rossiter, et al. 2009; Zhao, Xu, et al. 2009), cichlids (Spady et al. 2005), and cavefish (Li and He 2009). The LRT statistic was computed as 2log likelihood difference between the two models and was tested against the χ^2 distribution, where the degrees of freedom equals the difference between the number of parameters in the two nested models (Yang 2007). We used branch model, branch-site model, and site model tests. Branch and branch-site model tests allow users to designate certain branches of interest in the trees as "foreground" and compare ω estimated for these branches with an ω estimate for all other branches of the tree ("background" branches: Yang 2007).

Branch models evaluate variation in ω between lineages within the tree (Yang 2007). For these tests, the null model assumes one ω for all branches of the tree (one ratio model). We tested for a habitat effect on specific lineages by comparing the null one ratio model with several two ratio and three ratio models. These alternative models estimate one ω value for foreground branches (ω_1 for two ratio models, ω_1 and ω_2 for three ratio) and another ω value (ω_0) for all background branches. We ran several models designating different sets of branches as foreground to test for a change in selection with habitat type (table 3, supplementary fig. S3, Supplementary Material online): 1) closed canopy species (two ratio), 2) open canopy species (two ratio), and 3) closed canopy species with *C. medius* designated as a separate foreground branch (three ratio). In these analyses, we classified the last common ancestor of the *M. griseorufus* and *M. murinus* clades as "open canopy." These sister species are suspected of diverging approximately 8.4 Ma from a southern open canopy ancestor before *M. murinus* expanded into the northern open canopy dry deciduous forests and southeastern littoral forests during the Pleistocene (Yoder and Yang 2004; Kappeler et al. 2005; Schneider et al. 2010; Hapke et al. 2013).

Unlike branch models, which assume a single ω across all codon sites in a sequence, branch-site models allow ω to vary among codon sites. Thus, branch-site models provide a test to detect potential codon sites under positive selection in designated foreground branches (Yang 2007). We utilized Model A (Model 2, NSSites 2) to determine whether open canopy or closed canopy lineages have experienced positive selection on any codon sites compared with background branches. Model A employs four classes of codon sites: 1) one class constrained to purifying selection for both background and foreground $0 \leq \omega_0 \leq 1$; 2) one class under neutral evolution for both background and foreground $\omega_1 = 1$; 3) one class permitted to be under positive selection $\omega_{2a} \leq 1$ in foreground branches but constrained to purifying selection in background; and 4) one class permitted to be under positive selection $\omega_{2b} \leq 1$ in foreground branches but constrained to neutral evolution for background branches (Yang 2007). For these branch-site models, the null models are the same Model A but with ω_2 fixed at 1 for foreground branches. If LRTs for branch-site models were significant for positive selection, we used the BEB method to calculate posterior probabilities for site classes and to identify amino acid sites under positive selection (Yang 2007).

Site-specific models also test for heterogeneous selection pressure across codon sites. However, in contrast to branch-site models, site models test across all branches of the tree rather than only the foreground branches. We compared two sets of site models (M1a vs. M2a; M7 vs. M8) to test for codon

sites under positive selection in the *OPN1SW* gene (Yang 2007). The nearly neutral M1a permits two ω values for codon sites ($\omega_0 < 1$, $\omega_1 = 1$), whereas M2a includes the possibility of sites under positive selection by permitting three ω values ($\omega_0 < 1$, $\omega_1 = 1$, $\omega_2 > 1$). M7 (beta) differs from M1a in permitting 10 ω classes with a more continuous variation following the β distribution, but restricts all $\omega < 1$. In contrast, M8 (beta and ω) allowed an 11th class ($\omega > 1$) to permit positive selection on sites (Yang et al. 2000). If LRTs for site-specific models were significant for positive selection, we used the BEB method to calculate posterior probabilities for site classes and to identify codon sites under positive selection (Yang 2007).

Supplementary Material

Supplementary notes, tables S1 and S2, and figures S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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