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Ancestral Loss of Short Wave-Sensitive Cone Visual Pigment in Lorisiform Prosimians, Contrasting with Its Strict Conservation in Other Prosimians

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Abstract. Mammals are basically dichromatic in color vision, possessing middle to long wave-sensitive (M/LWS) and the short wave-sensitive (SWS) cone opsins in the retina, whereas some nocturnal mammals lack functional SWS opsins. Prosimians, primitive primates consisting of three extant groups (Lorisiformes, Lemuriformes, and Tarsiiformes), include many nocturnal species. Among nocturnal prosimians, a species of lorisiforms, the greater galago (*Otolemur crassicaudatus*), is known to lack a functional SWS opsin gene, while lemuriforms and tarsiiforms appear to retain SWS opsins in the retina. It has not been established, however, whether the loss of SWS opsin is a universal phenomenon among lorisiforms and whether the functional SWS opsin genes of lemuriforms and tarsiiforms are under strict or relaxed selective constraint. To gain better insight into an association between nocturnality and loss of SWS function, we isolated and sequenced the SWS opsin genes from two species of lorisiforms, the slow loris (*Nycticebus coucang*; nocturnal) and the lesser galago (*Galago senegalensis*; nocturnal), and one species each of lemuriforms and tarsiiforms, the brown lemur (*Eulemur fulvus*; cathemeral) and the western tarsier (*Tarsius bancanus*; nocturnal), respectively. Our sequence analysis revealed that (1) the SWS opsin gene was disrupted in the common ancestor of galagids and lorisids and (2) the rate of nonsynonymous nucleotide substitution has been kept significantly lower than that of synonymous substitution in tarsier and lemur, demonstrating the presence of strict selective constraint on the SWS opsin genes in tarsiiforms and lemuriforms.

Key words: Blue cone pigment — Prosimian — Galago senegalensis — Nycticebus coucang — Tarsius bancanus — Nocturnal

Introduction

Visual pigments are photoreceptive molecules for vision and, in vertebrates, reside in rod and cone photoreceptor cells in the retina. They consist of a protein moiety, opsin, and a chromophore, either 11-*cis* retinal or 11-*cis* 3,4-dehydroretinal in vertebrates. The visual opsins of vertebrates are classified into five groups: rod opsin or the rhodopsin group (RH1), ultraviolet–blue or the short wave-sensitive type 1 cone-opsin group (SWS1), blue or the short wave-sensitive type 2 cone-opsin group (SWS2), green or the rod opsin-like cone-opsin group (RH2), and green–red or the middle to long wave-sensitive cone-opsin group (M/LWS) (Yokoyama 2000). Rod cells/opsins are specialized for dim light (scotopic) vision and cone for bright light and color (photopic) vision.

While many species of birds, reptiles, and fish retain the four types of the cone opsins and are potentially tetrachromatic in color vision (Ebrey and

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Koutalos 2001), mammals are considered to have lost SWS2 and RH2 opsins in the Mesozoic nocturnal ancestor (Ahnelt and Kolb 2000). Extant mammals are basically dichromatic, with only SWS1 (SWS hereafter) and M/LWS as cone opsins and with solely 11-*cis* retinal as a chromophore (Jacobs 1993). Higher primates are unique in attaining trichromatic vision by diversifying the M/LWS opsin gene through either gene duplication or allelic diversification (Jacobs 1999).

Some mammals, on the contrary, appear to have decreased the chromatic dimension even further by losing SWS opsins. The growing list of them includes nocturnal rodents and carnivores (Calderone and Jacobs 1999; Jacobs and Deegan 1992; Peichl and Moutairou 1998; Szel et al. 1996) and marine animals (Fasick et al. 1998; Levenson and Dizon 2003; Peichl et al. 2001; Peichl and Moutairou 1998). In primates, two nocturnal species, the owl monkey (*Aotus trivirgatus*) and the greater galago (*Otolemur crassicaudatus*), are documented as lacking functional SWS opsin gene by independent disruptive mutations (Jacobs et al. 1996). The owl monkey is the only nocturnal species among higher primates (simians) and the galago belongs to the primitive primate group (prosimians) which includes many nocturnal species. These observations imply some causal relation between nocturnality and loss of the SWS opsin in terrestrial mammals.

Prosimians comprise two strepsirhine groups, Lorisiformes and Lemuriformes, and one haplorhine group, Tarsiiformes (Fleagle 1999). While lorisiforms and tarsiiforms are all nocturnal, lemuriforms include not only nocturnal but also diurnal and cathemeral species. A diurnal and a cathemeral lemuriform species, the ringtail lemur (*Lemur catta*) and the brown lemur (*Eulemur fulvus*), respectively, have been shown to retain sensitivity to short-wavelength light in an electrophysiological study (Jacobs and Deegan 1993). Importantly, a nocturnal lemuriform species, the gray mouse lemur (*Microcebus murinus*), and a tarsiiform species, the eastern tarsier (*Tarsius spectrum*), are also shown to retain SWS opsins in the retina by immunohistochemistry (Dkhissi-Benyahya et al. 2001; Hendrickson et al. 2000). The absence of a SWS cone is suggested for a *Loris* species by an immunohistochemical study but the finding needs confirmation from further samples (Ahnelt and Kolb 2000). Among nocturnal mammals whose SWS opsin is known, or supposed, to be lost, so far only the owl monkey and the greater galago have been examined for the gene structure (Jacobs et al. 1996). Even for the two species, only partial nucleotide sequences of one coding exon (exon 1 in owl monkey and exon 4 in greater galago) are available, which has made difficult a phylogenetic analysis with any statistical significance.

To gain a better insight into an association between nocturnality and loss of SWS function in prosimian primates, we aimed here to clarify (1) whether the loss of SWS opsin is specific to the greater galago or occurs also in other galagid species and in lorisids, the sister group of galagids in lorisiform prosimians, and (2) whether the seemingly functional SWS opsins in tarsiiforms and lemuriforms are indeed under strict selective constraint. Toward this goal, we determined complete gene structures of the SWS opsins of two species of lorisiforms, the lesser galago (*Galago senegalensis*) and the slow loris (*Nycticebus coucang*), one lemuriform, the brown lemur (*Eulemur fulvus*), and one tarsiiform, the western tarsier (*Tarsius bancanus*), and examined their nucleotide sequences with special attention to the difference of silent (synonymous) and amino acid-altering (nonsynonymous) nucleotide substitutions.

Materials and Methods

Genomic Library Screening

Genomic DNA of a lesser galago (*Galago senegalensis*) and a western tarsier (*Tarsius bancanus*) was extracted from the fibroblast cell lines. Genomic DNA of a brown lemur (*Eulemur fulvus*) was extracted from a frozen tissue. That of a slow loris (*Nycticebus coucang*) was provided by Dr. O. Takenaka (Kyoto University). Genomic libraries of these primates were constructed using *Bam*HI-digested EMBL3 λ phage vector and *Sau*3AI-partially digested genomic DNA (12–20 kb). For the probe preparation, the cDNA encoding full coding region of the SWS opsin gene of a common marmoset (*Callithrix jacchus*) was isolated from its previously prepared ocular RNA (Kawamura et al. 2001) by reverse-transcription (RT) polymerase chain reaction (PCR) using the oligo nucleotide primers designed based on the published nucleotide sequence (GenBank accession No. L76201).

The cDNA probes were labeled with [α-^{32}P]dCTP using the random primer method. Plaque hybridization was carried out at 55°C in a solution consisting of 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 5 μg/ml *E. coli* DNA. The hybridized membranes were washed in 1× SSC/0.1% SDS at 55°C four times (20 min each), which allows approximately 30% mismatch (Sambrook and Russel 2001). One clone each from the four species was found to contain the entire exons of the SWS opsin gene (Fig. 1). After restriction mapping of these clones, the restriction fragments hybridized to the screening probes were subcloned into the pBluescript II (SK−) plasmid vector (Stratagene, La Jolla, CA). Sequencing of these subclones was carried out for both strands using the Thermo Sequenase Cycle Sequencing Kit (Amersham, Piscataway, NJ) with dye-labeled primers and the LI-COR 4200L-1 automated DNA sequencer.

DNA Sequence Analysis

Repetitive sequence elements were identified using Repeat Masker (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). Nucleotide sequences were aligned using CLUSTAL W (Thompson et al. 1994) and the alignment was refined visually. Subsequent phylogenetic analyses were conducted using the MEGA2 program version 2.1 (Kumar et al. 2001; Nei and Kumar 2000). The number of nucleotide substitutions per site (d) for two sequences was estimated by Tamura and Nei's (1993) method, which takes into

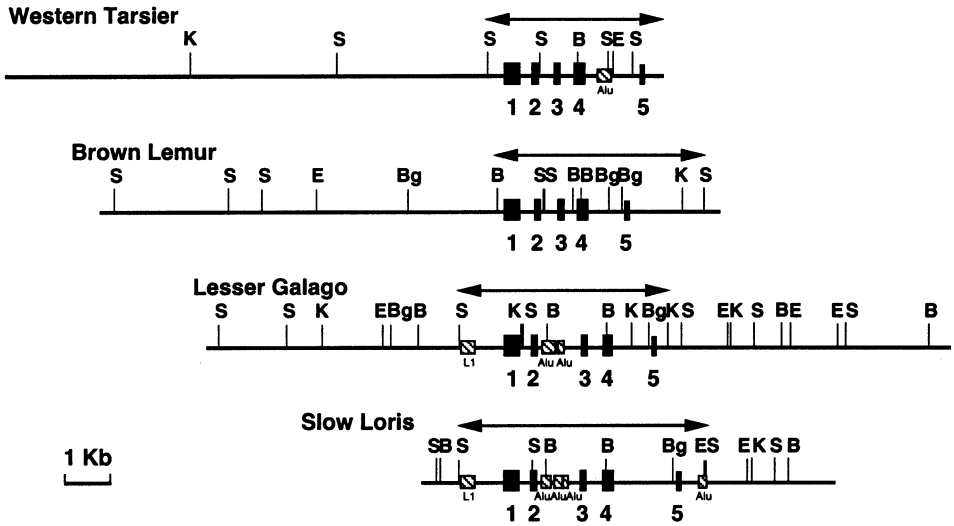

Fig. 1. Genomic structures of the SWS opsin genes of western tarsier, brown lemur, lesser galago, and slow loris. The five exons are indicated by black boxes. The Alu and L1 repetitive elements are depicted by hatched boxes. The nucleotide sequences were determined for the regions indicated by the arrowed lines. The DDBJ/EMBL/GenBank accession numbers of the sequence data are AB111463 (western tarsier), AB111464 (brown lemur), AB111465 (lesser galago), and AB111466 (slow loris). B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sac*I.

account substitutional rate differences between nucleotides and inequality of nucleotide frequencies and compensates for multiple substitutions. In the calculation, gap sites were removed from the alignment and only common sites among all sequences compared were considered. The number of substitutions per synonymous site (d_S) and per nonsynonymous site (d_N) was estimated by the Pamilo–Bianchi–Li method (Li 1993; Pamilo and Bianchi 1993). For calculation of d_S and d_N for the coding region, gap sites were excluded from the analysis, and when a gap exclusion disrupted the codon triplet, the remaining nucleotide sites of such codons were also excluded. When a sequence contained premature stop codons, such codon sites were also removed from the alignment. The first nine nucleotides shown in Fig. 2 were regarded as the coding region in this study despite the position shift of the initiation codon in the tarsier, loris, and galago. Phylogenetic trees were constructed by applying the neighbor-joining method (Saitou and Nei 1987). The reliability of the tree topology was evaluated by bootstrap analysis with 1000 replications.

Results

Genomic Structures of the SWS Opsin Genes of Prosimians

The genomic DNA clones containing the SWS opsin genes were isolated from genomic libraries of four prosimians, western tarsier, brown lemur, lesser galago, and slow loris (Fig. 1). The vertebrate SWS opsin genes consist of five exons and four introns (Yokoyama 2000). The same exon–intron structure was identified in the four species on the basis of the nucleotide sequence similarities to the exons of the human SWS opsin gene (Nathans et al. 1986). No other related gene was detected by Southern hybridization to the genomic DNA in the four species (data not shown). In the region where the nucleotide sequence was determined (Fig. 1, arrowed lines), one Alu repetitive element was detected in intron 4 of the tarsier SWS opsin gene. In the galago and loris genes, L1 and multiple Alu elements were detected in the upstream flanking region and in intron 2, respectively. An additional Alu was found in the downstream flanking region of the loris SWS opsin gene. An Alu and a mer13 element previously identified in intron 3 and intron 4, respectively, of the human and squirrel monkey SWS opsin genes (Shimmin et al. 1997) were not identified in the four prosimian species. Similarly, the Alu elements in the prosimian genes were not found in the human and squirrel monkey SWS opsin genes. These indicate multiple insertional events of repetitive elements around the SWS opsin gene during primate evolution.

Coding Disruptions in the SWS Opsin Genes of Lesser Galago and Slow Loris

In the coding region of the tarsier and lemur SWS opsin genes, no insertion/deletion (indel) or nonsense point mutation was found and functionary important residues are conserved. These residues include a lysine for the Schiff-base linkage to the chromophore (Wang et al. 1980), a glutamate residue for the Schiff-base counter ion (Sakmar et al. 1989; Zhukovsky and Oprian 1989), and two cysteine residues for the disulfide bond (Karnik et al. 1988) (Fig. 2). Multiple serines and threonines in the C-terminal region for the targets of opsin kinase (Ohguro et al. 1994) are also observed.

On the contrary, multiple out-frame indels and nonsense mutations creating premature stop codons were identified in the coding regions of the lesser

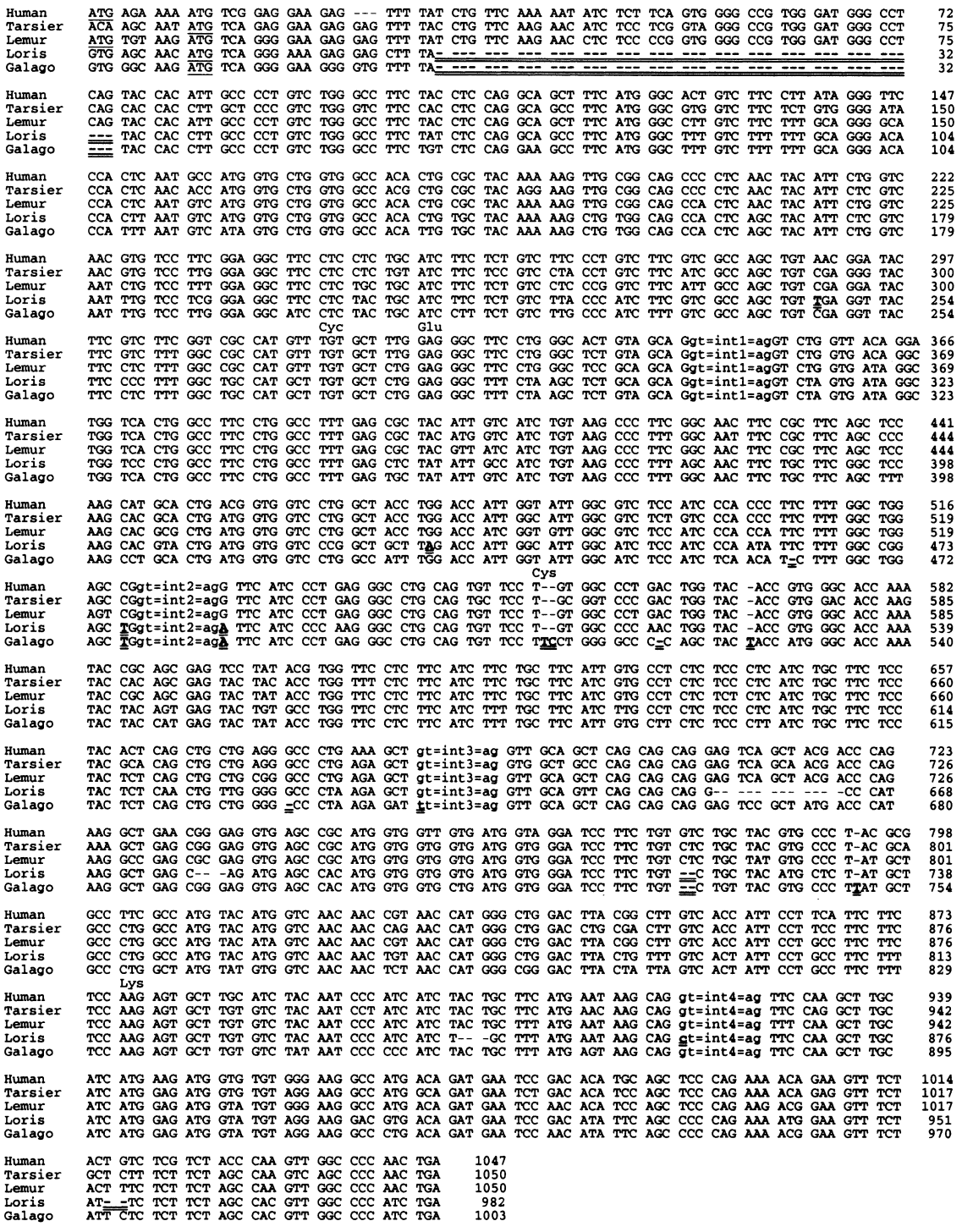

Fig. 2. Alignment of the nucleotide sequences of the prosimian SWS opsin genes to the coding region of the human SWS opsin gene (GenBank U53874). Positions of the four introns (int1–int4) are indicated with the splice donor (gt) and acceptor (ag) sequences. Gaps necessary to increase the sequence similarity are indicated by dashes. The putative initiation codons (ATG) are underlined. Point mutations creating premature stop codons, those disrupting splice donor sequences, and indels disrupting the reading frame are double-underlined. Positions of a Lys residue for the Schiff-base linkage to the chromophore, a Glu residue for the Schiff-base counter ion, and two Cys residues for the disulfide bond are indicated above the corresponding codons.

galago and slow loris SWS opsin genes (Fig. 2). Among them, two indels, 46- and 2-bp deletions in exons 1 and 4, respectively, and two nonsense mutations in the codon interrupted by intron 2 are shared between the two species (Fig. 2). The 2-bp deletion in exon 4 is also reported in the greater galago SWS opsin gene, where only exon 4 is sequenced (Jacobs et al. 1996). These shared mutations

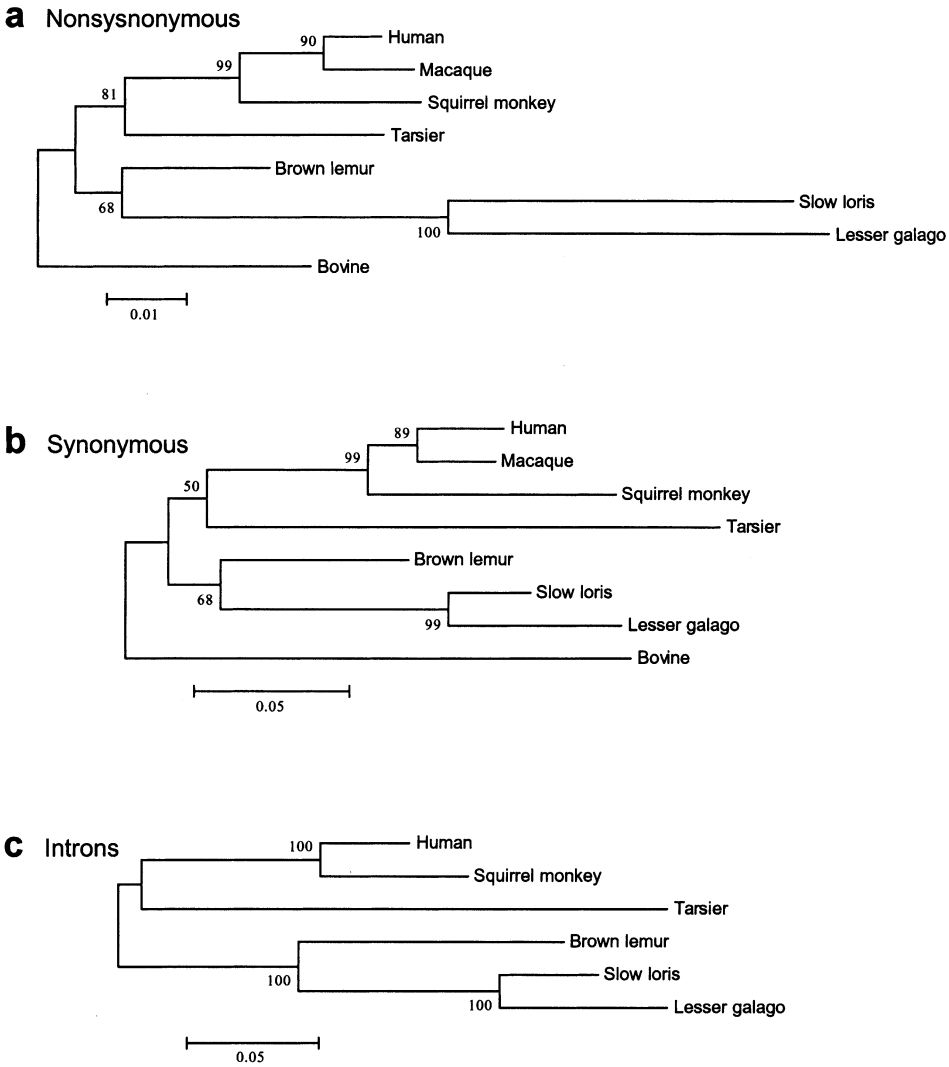

Fig. 3. Phylogenetic trees of primate SWS opsin genes constructed with (**a**) nonsynonymous sites, (**b**) synonymous sites, and (**c**) introns. For a and b, common 314 codons among the sequences are considered. For c, sequences of introns 1 to 4 are combined and the common 1145 bp is considered. The bootstrap probabilities are given for each node. Scale bar: number of nucleotide substitutions per site. Accession numbers: U53874 (human), AF158977 (macaque; *Macaca fascicularis*), U53875 (squirrel monkey; *Saimiri boliviensis*), and U92557 (bovine; *Bos taurus*).

likely occurred in the common ancestor of galagids and lorisids. Other mutations are species-unique and include the ones disrupting the splice donor sequence in intron 3 of galago and in intron 4 of loris and the one disrupting one of the cysteine residues for the disulfide bond in galago (Fig. 2). In these genes the putative initiation codon, "ATG," corresponds to the fourth codon of the human SWS opsin gene. This shift, however, also occurs in the tarsier as well as in the mouse and rat (Chiu et al. 1994; Zhao et al. 1997) and does not appear to be defective.

Selective Constraint of the SWS Opsin Genes in Prosimians

When we compare nonsynonymous substitutions (d_N) among primate SWS opsin genes and construct a phylogenetic tree, it becomes conspicuous that the slow loris and the lesser galago show the longest branch lengths (Fig. 3a). Consistently, when Tajima's (1993) relative rate test was applied for the first and second codon positions, the slow loris and the lesser galago were shown to have evolved significantly faster than the others. In contrast, the differences in the branch lengths are less evident when synonymous substitutions (d_S) are considered (Fig. 3b). The intron tree (Fig. 3c) is unrooted because no intron sequence has been determined for SWS opsin genes of nonprimate mammals. The difference between the branch length leading to the loris or the galago and that to the lemur is not statistically significant in the rate test.

When d_S and d_N values are compared for all pairs of the primates examined in Fig. 3, d_S values are significantly larger than d_N values in all but the galago–loris

Table 1. Differences between synonymous and nonsynonymous substitutions (d_S–d_N; below diagonal) and their standard errors (above diagonal) among primate SWS opsin genes[a]

	HSA	MFA	SBO	TBA	EFU	NCO	GSE
		0.016	0.026	0.039	0.032	0.040	0.042
MFA	0.035**		0.025	0.039	0.031	0.040	0.040
SBO	0.080***	0.079***		0.043	0.034	0.042	0.044
TBA	0.191***	0.175***	0.237***		0.040	0.042	0.048
EFU	0.120***	0.105***	0.128***	0.213***		0.031	0.033
NCO	0.106**	0.098**	0.117**	0.144***	0.054*		0.023
GSE	0.128***	0.106**	0.148***	0.204***	0.087**	−0.009	

[a] The common 314 codons are considered. Statistical significance is evaluated by the one-tail Z test (Nei and Kumar 2000). * Significant at the 5% level. ** Significant at the 1% level. *** Significant at the 0.1% level. HSA, *Homo sapiens*; *MFA*, *Macaca fascicularis*; SBO, *Saimiri boliviensis*; TBA, *Tarsius bancanus*; EFU, *Eulemur fulvus*; NCO, *Nycticebus coucang*; GSE, *Galago senegalensis*.

Table 2. Numbers of nucleotide substitutions per site between slow loris and lesser galago SWS opsin genes

Region	Length	Distance
5′-flanking	949 bp	0.075 ± 0.009
Introns	1928 bp	0.096 ± 0.007
3′-flanking	237 bp	0.072 ± 0.019
Coding	979 bp	0.090 ± 0.010
Synonymous	347 codons	0.082 ± 0.019
Nonsynonymous	347 codons	0.092 ± 0.012
Total	4093 bp	0.088 ± 0.005

comparisons (Table 1). There is no statistical difference between d_S and d_N values (two-tail Z test) in the galago–loris comparison. When only DNA sequences of the lesser galago and the slow loris are compared, the d value (0.09 ± 0.01) for the entire coding region, including both synonymous and nonsynonymous sites, is not statistically different form those for noncoding regions including the 5′-flanking region (0.075 ± 0.009), introns (0.096 ± 0.007), and the 3′-flanking region (0.072 ± 0.019) (Table 2). The d value for the entire DNA sequence (4093 bp) including both coding and noncoding regions is 0.088 ± 0.005, a value comparable to that (0.101) for the ε-globin noncoding sequences between greater galago (*Otolemur crassicaudatus*) and slow loris (Goodman et al. 1998). These results clearly show that the SWS opsin genes of lorisiforms are under no selective constraint and that, in contrast, a strict purifying selection is operating on those of the tarsier and the lemur as in the case of the higher primates (Shimmin et al. 1998).

Discussion

We showed here that the SWS opsin genes of the lesser galago (*Galago senegalensis*) and the slow loris (*Nycticebus coucang*) lost their function by common mutations between them. This indicates that the disruption of the SWS opsin gene previously reported for the greater galago (*Otolemur crassicaudatus*) (Jacobs et al. 1996) originates in the common ancestor of galagids and lorisids. We also showed that the SWS opsin genes of the western tarsier (*Tarsius bancanus*) and the brown lemur (*Eulemur fulvus*) are not only intact but also indeed under strict selective constraint. Although importance of the SWS opsin in the cathemeral blown lemur is conceivable, how can we reconcile the contrasting statuses of the SWS opsin genes between the two nocturnal groups, lorisiforms and tarsiiforms? The presence/absence status of SWS opsins among nocturnal mammals is likely to reflect a severity of nocturnality and other factors such as retinal anatomy and dependence on other sensory abilities including olfaction and audition.

Tarsiers are reported to be active several hours before sundown, relatively quiet in the middle of the night, and then active again just before and after dawn (Hendrickson et al. 2000). Their retinal structure is also comparable to that of diurnal higher primates, with a relatively high peak density of cones (50,000/mm^2, about one-fourth that of higher primates), presence of fovea, and absence of tapetum (Hendrickson et al. 2000). The fovea is a retinal region where the inner retinal layers migrate laterally to create a pit and the cones migrate centrally to increase the density for high visual acuity. The tapetum is a reflective retinal structure in back of the photoreceptor layer and serves as a mirror to allow the second passage of light in the photoreceptors, increasing visual sensitivity and potentially decreasing resolution due to scattering of reflected light. On the other hand, galagos and lorises have a well-developed tapetum (Fleagle 1999). The galago has been shown to lack a prominent foveal pit and its peak cone density is low (8500/mm^2) (Hendrickson et al. 2000; Wikler and Rakic 1990). Galagos and lorises feed on fruit and gums as well as insects. Galagos are known as prominent leapers and to have outstanding auditory/echolocating ability, with large and well developed ears, while lorises are slow climbers and have been described as specialized for olfactory foraging for stinking or poisonous insects which other animals tend to avoid (Macdonald 1984). These eco-

logical and anatomical differences between lorisiforms and tarsiiforms are likely related to varying degree of their dependence on vision.

Loss of SWS cones is always associated with disruptions of the SWS opsin gene in animals whose SWS opsin genes have been examined (Fasick et al. 1998; Jacobs et al. 1996; Levenson and Dizon 2003). The features of the cone-loss phenomena so far observed for mammals are (1) lost cones are always SWS cones and not M/LWS cones, (2) loss of the SWS cone is in nocturnal or marine animals, and (3) while the losses in marine animals are presumably universal, those in nocturnal animals are occasional (Calderone and Jacobs 1999; Fasick et al. 1998; Jacobs and Deegan 1992; Jacobs et al. 1996; Levenson and Dizon 2003; Peichl et al. 2001; Peichl and Moutairou 1998; Szel et al. 1996). The first feature can be explained by the minority status of SWS cones in the retina. SWS cones of mammals usually constitute only 2–10% of the entire cone population, are spaced irregularly (Dkhissi-Benyahya et al. 2001), and are considered to contribute little to the photopic sensitivity and spatial/temporal resolution and primarily to color vision (Jacobs et al. 1996). Therefore, loss of M/LWS cones would cause considerable deformation of retinal structures and photopic vision, while that of SWS cones would be much milder.

From the second and the third features, several authors have inferred a "convergent" adaptive evolution of the "pseudo" SWS opsin gene in nocturnal or marine animals (Jacobs et al. 1996; Levenson and Dizon 2003; Peichl et al. 2001). In this hypothesis, SWS function is regarded as not just unnecessary for the dim-light or aquatic turbid environment but its loss is somehow advantageous to the animals (see Peichl et al. [2001] and Levenson and Dizon [2003] for details of the discussion). However, unlike the usual cases of convergent evolution where genes "gain" specific functions by specific types of mutations, "loss" of function occurs by any one of the broad spectrum of disruptive mutations and hence is likely to happen without aid of positive selection in different evolutionary lineages wherever the selective constraint for the gene is absent. Therefore, "convergence" itself does not constitute a basis on which positive selection is argued for. Instead, if the loss of SWS opsin is advantageous and occurred relatively recently, such selection might be detected as a selective sweep which is manifested as homogeneity of the sequence within the species as suggested by Jacobs et al. (1996).

On the other hand, the importance of loss of function could be assessed by the importance of an intact SWS gene of nocturnal animals since many of them retain a functional SWS opsin gene. The d_S–d_N analysis applied in this study should provide a simple and straightforward evaluation. It is now apparent that the SWS opsin in tarsier is as important as in diurnal higher primates. But the situation could be different in other nocturnal animals with a different ecology and retinal anatomy. Relaxation of selective pressure on a seemingly intact gene has been reported for the ion channel TRPC2 of New World monkeys, which is involved in pheromone transduction (Liman and Innan 2003). Discovery of an intact but relaxed SWS opsin gene in nocturnal mammals would support the view that the loss of SWS opsin is a rather neutral event and not an advantageous one for them.

The peak cone density of gray mouse lemur (*Microcebus murinus*) is as low as that of galago (about 8000/mm^2) and the SWS cone represents less than 0.2% of the total cone population (Dkhissi-Benyahya et al. 2001). This extremely low number and irregular distribution of SWS cones precludes a significant role in color vision or image formation even during periods of dawn and dusk (Dkhissi-Benyahya et al. 2001). It would be of great interest to apply d_S–d_N analysis to the SWS opsin gene of the gray mouse lemur for further understanding of the relation between the fate of the SWS opsin gene and the ecology of the animal.

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