Molecular Evolution of Bat Color Vision Genes

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The two suborders of bats, Megachiroptera (megabats) and Microchiroptera (microbats), use different sensory modalities for perceiving their environment. Megabats are crepuscular and rely on a well-developed eyes and visual pathway, whereas microbats occupy a nocturnal niche and use acoustic orientation or echolocation more than vision as the major means of perceiving their environment. In view of the differences associated with their sensory systems, we decided to investigate the function and evolution of color vision (opsin genes) in these two suborders of bats. The middle/long wavelength (M/L) and short wavelength (S) opsin genes were sequenced from two frugivorous species of megabats, *Haplonycteris fischeri* and *Pteropus dasymallus formosus*, and one insectivorous species of microbat, *Myotis velifer*. Contrary to the situation in primates, where many nocturnal species have lost the functional S opsin gene, both crepuscular and strictly nocturnal species of bats that we examined have functional M/L and S opsin genes. Surprisingly, the S opsin in these bats may be sensitive to UV light, which is relatively more abundant at dawn and at dusk. The M/L opsin in these bats appears to be the L type, which is sensitive to red and may be helpful for identifying fruits among leaves or for other purposes. Most interestingly, *H. fischeri* has a recent duplication of the M/L opsin gene, representing to date the only known case of opsin gene duplication in non-primate mammals. Some of these observations are unexpected and may provide insights into the effect of nocturnal life on the evolution of opsin genes in mammals and the evolution of the life history traits of bats in general.

Introduction

Many terrestrial mammals have color vision based on two spectrally different visual pigments located in two types of retinal cone photoreceptors: cones with a long/middle wavelength (L/M or red/green) opsin and cones with a short wavelength (S or blue) opsin (Jacobs 1993; Ahnelt and Kolb 2000). For example, cats, dogs, and goats possess L and S opsins, while pigs, rabbits, and deer have M and S opsins (Sun, Macke, and Nathans 1997; Radlwimmer and Yokoyama 1998; Yokoyama and Radlwimmer 1999; Hendrickson and Hicks 2002). Some primates have undergone one or more duplications of the M/L opsin gene, thus becoming trichromatic (Nathans, Thomas, and Hogness 1986; Jacobs et al. 2002). Many nocturnal primates (e.g., the owl monkey and the bushbaby), carnivores (e.g., the common raccoon, the crab-eating raccoon, and the kinkajou), and rodents (e.g., the pygmy field mouse, Gairdner's shrew mouse, and the Syrian golden hamster) are monochromatic, lacking a functional S cone (Jacobs and Deegan 1992; Calderone and Jacobs 1999; Peichl and Pohl 2000). These observations suggest that a nocturnal lifestyle may be coincident with the eventual loss of a functional S opsin gene.

Bats of the suborder Microchiroptera occupy a nocturnal niche, and although they possess sight, members of this suborder use acoustic orientation (echolocation), rather than vision, as a major means of perceiving their environment (Bhatnagar 1975; Fuzessery et al. 1993; Heffner, Koay, and Heffner 2001). Thus, bats may represent the ideal model for studying the influence of a nocturnal life-style on the evolution of color vision genes.

Key words: bats, opsin genes, color vision, nocturnal life, opsin duplication.

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The monophyly of the order Chiroptera (bats) was questioned earlier (Pettigrew 1986) but has been supported by recent studies (Nikaido et al. 2000; Teeling et al. 2000, 2002). Chiroptera is divided into two suborders, Megachiroptera (megabats) and Microchiroptera (microbats). Megachiroptera has only one family, Pteropodidae, and megabat species are either frugivorous or nectarivorous, lacking laryngeal echolocation and relying on olfaction or vision to search food. The flying fox (family Pteropodidae, genus *Pteropus*) is known to possess a highly developed visual system (Kalko, Herre, and Handley 1996; Schnitzler and Kalko 1998; Phillips 2000). However, as seen in typically nocturnal mammals, *Pteropus giganteus* has a very low cone/rod ratio (1/250) (Jacobs 1993), and consequently, its S or M/L opsin may not be functional. The suborder Microchiroptera contains 17 families, and although families like the Phyllostomidae reveal a diverse array of feeding preferences, members of most families are predominantly insectivorous. In addition, microbats have the ability to produce ultrasounds via the larynx, and members of this suborder rely on echolocation rather than a nocturnal eye for exploiting their environment (Bhatnagar 1975; Fuzessery et al. 1993; Schnitzler and Kalko 1998; Phillips 2000). Although one might predict that microbats may have lost functional opsin genes, Joshi and Chandrashekaran (1985) in their study of *Hipposideros speoris* found that this species responds to visual spectra of 430 and 520 nm, suggesting the existence of functional M and S opsins.

To our knowledge, no previous molecular study has examined the function and evolution of color vision genes in bats. Therefore, we have sequenced the M/L and S opsin genes in two megabats (pigmy fruitbat and flying fox) and one microbat (little brown bat).

Materials and Methods

This study includes two species of megachiropteran, *Haplonycteris fischeri* (Pteropodidae; known as Philippine

Table 1
Primers Used in Amplifying the Bat S and M/L Opsin Genes

Gene	PCR Product	Primer Pair
S	Exon1-Exon4	5′ GGATGGGCCTCAGTACCAC 3′
		5′ YACCACCATGCGGCTCACCTC 3′
	Exon4-Exon5	5′ ATGTAYATGCTCAACAACCGTAACC 3′
		5′ AGAYTCATCTGTCATGARCTTCC 3′
M/L	Exon2-Exon4	5′ ACCCTCTGTGCGTTGTGGA 3′
		5′ GCTGAGCGGGATGATGC 3′
	Exon4-Exon6	5′ TGCATCATCCCGCTCAG 3′
		5′ CTGGAGGTGCTGGAGAGTTCA 3′

dwarf fruit bat) and *Pteropus dasymallus formosus* (Pteropodidae; known as flying fox), and one species of microchiropteran, *Myotis velifer* (Vespertilionidae). To investigate the level of polymorphism in opsin genes, genomic DNA was isolated from frozen tissues of 10 individuals of *H. fischeri* obtained from five islands in the Philippines (Catanduanes, Mindoro, Sibuyan, Negros, and Mindanao). Genomic DNA was isolated from a blood sample of *P. dasymallus formosus* (one individual) obtained from the Taipei zoo. Genomic DNA was isolated from cryo-frozen liver samples of *M. velifer* obtained from central Texas.

The polymerase chain reaction (PCR) was used to amplify M/L and S opsin genes. The PCR primers were designed using conserved regions of the opsin genes of primates, mice, and squirrels. As a result of variation in these genes, both PCR and sequencing primers were degenerate (table 1). Overlapping products of exons 2 (or 1) through 4 and exon 4 through exon 6 (or 5) of the M/L and S opsin genes were amplified and sequenced using these primers.

The PCR reactions contained 1 μl (100 ng/μl) genomic DNA, 5 μl X10 buffer, 1.5 μl (50 mM) MgCl, 1 μl (1 μM) of each primer and 0.04 U *Taq* DNA polymerase. Reactions were conducted on a PerkinElmer 9600 with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1–2 min for 35 cycles, and final extension at 72°C for 5 min. The PCR products were checked by agarose-gel electrophoresis and cloned for sequencing in the PCR-XL-TOPO vector with the use of the TOPO XL PCR cloning kits (Invitrogen, Inc.) according to the manufacturer's protocol.

All DNA fragments were cycle sequenced using BigDye sequencing kits (Applied Biosystems) and an ABI 377A automated DNA sequencer. Removal of excess fluorescent terminators was accomplished by Sephadex spin column. To avoid artifacts, multiple clones were sequenced. In *H. fischeri*, two copies of the M/L opsin were sequenced. To identify whether the two copies belong to one or two loci, 10 individuals were sequenced for polymorphism.

Sequence alignments were performed using Seqman (DNASTAR, Madison, Wis). We estimated the phylogeny of vertebrate opsins by a variety of methods. First, we determined the best-fit model of nucleotide evolution using hierarchical likelihood ratio tests (LRT) implemented in Modeltest (Posada and Crandall 1998). We also used LRT to compare a model that assumes that rate heterogeneity is

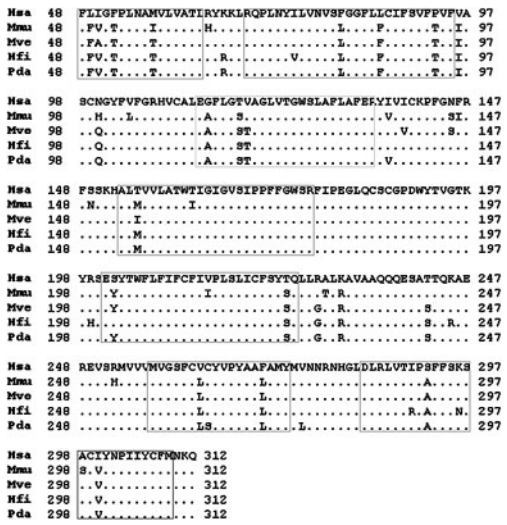

FIG. 1.—Alignment of partial protein sequences of S opsin genes from partial exon 1 to exon 4. The species included are *Homo sapiens* (Hsa), *Mus musculus* (Mmu), *Haplonycteris fischeri* (Hfi), *Myotis velifer* (Mve), and *Pteropus dasymallus formosus* (Pda). Residues identical to the human residues are indicated with a dot. Gray boxes indicate α helical regions.

partitioned among sites of codons, i.e. different rates are assumed for first, second, and third positions of codons. Next, we used the best-fit model to perform a heuristic search in PAUP*4.0 (Swofford 1999). We also performed bootstrap analysis with 1,000 pseudo-replicates using both the parsimony method and, separately, the Neighbor-Joining method employing ML distances from the best-fit model.

The number of nucleotide substitutions between sequences, the number of substitutions per nonsynonymous site (K_A), and the number of substitutions per synonymous site (K_S) were calculated by DnaSP (Rozas and Rozas 1999).

Results

S Opsin

The S opsin genes of *H. fischeri* (*Hfi*), *P. dasymallus formosus* (*Pda*), and *M. velifer* (*Mve*) were sequenced from partial exon 1 through exon 4, encoding 265 amino acids (fig. 1). In comparison with human and mouse S opsin genes, most of the sequenced regions were well conserved, especially for the functional critical sites, i.e., the disulfide linkage of Cys109 and Cys186 (Karnit and Khorana 1990), the Schiff's base counterion Glu113 (Nathans 1990), and the site of retinylidene Schiff's base formation Lys295 (Wang, McDowell, and Hargrave 1980; fig. 1). In addition, the interhelical cytoplasmic loops and luminal loops were more conserved than the transmembrane α helical regions. The three bat S opsin sequences are identical to those of mouse S opsin (P359), rat S opsin (P358), and chameleon S opsin (P358) at the five critical functional sites (T52, F86, T93, A114, and S118) (Yokoyama, Radlwimmer, and Kawamura 1998; Yokoyama and Yongsheng 2000), indicating that the three bat S opsins may be maximally sensitive to ultraviolet (UV) light.

Table 2
Non-Silent Differences and Silent Differences, the Number of Nonsynonymous Substitutions per 100 Sites (K_A), and the Number of Synonymous Substitutions per 100 Sites (K_S) Between Species in the S Opsin and M/L Opsin Genes

	Non-Silent Differences	K_A	Silent Differences	K_S	K_A/K_S
Short wavelength					
Hfi-Mve	12	0.020	53	0.327	0.061
Hfi-Pda	10	0.017	29	0.161	0.106
Hfi-Hsa	31.8	0.055	57.2	0.362	0.152
Hfi-Mmu	26	0.045	74	0.516	0.087
Pda-Mve	8	0.014	48	0.287	0.049
Pda-Hsa	29	0.050	44	0.260	0.192
Pda-Mmu	20	0.034	72	0.493	0.069
Mve-Hsa	29.3	0.051	50.7	0.309	0.165
Mve-Mmu	19.5	0.034	78.5	0.560	0.060
Hsa-Mmu	32	0.056	64	0.423	0.132
Middle/long wavelength					
Hfi-Mve	9	0.021	34	0.291	0.073
Hfi-Pda	3	0.007	16	0.123	0.057
Hfi-Hsa	15	0.035	42	0.388	0.091
Hfi-Mmu	13.5	0.032	47.5	0.449	0.071
Pda-Mve	10	0.024	27	0.222	0.106
Pda-Hsa	16	0.038	33	0.287	0.132
Pda-Mmu	14.5	0.034	42.5	0.388	0.088
Mve-Hsa	18	0.043	29	0.245	0.174
Mve-Mmu	18.5	0.044	36.5	0.319	0.138
Hsa-Mmu	23.5	0.056	36.5	0.325	0.173

NOTE.—Has, *Homo sapiens*; Mmu, *Mus musculus*; Hfi, *Haplonycteris fischeri*; Pda, *Pteropus dasymallus formosus*; and Mve, *Myotis velifer*. Total number of nucleotide sites compared. Short wavelength opsin gene: partial exon 1 to exon 4, 795 bp. Medium/long wavelength opsin gene: exon 3 to exon 5, 575 bp.

The K_A/K_S ratios are 0.165 for the comparison between *Mve-Hsa* (*Homo sapiens*, Hsa) and 0.192 for the comparison between *Pda-Hsa,* which are significantly higher than the comparisons between bats and mouse, e.g., 0.060 for *Mve-Mmu* (*Mus musculus*, Mmu) and 0.069 for *Pda-Mmu* (table 2, chi-square test, $P < 0.05$). Using likelihood ratio tests implemented by Modeltest, we found the best-fit model for vertebrate short wavelength opsin data (fig. 3*A*) to be the Transversion Model with invariant sites and Gamma distributed rate heterogeneity (TVM + I + Γ) (Posada and Crandall 1998). Parameter values were as follows: base composition A = 0.206, C = 0.300, and G = 0.253; substitution rates: A–C = 1.1434, A–G = 3.5525, A–T = 1.2413, C–T = 1.7495, and C–G = 3.5525; gamma shape = 0.9389; and proportion of invariant sites = 0.2070. A model partitioning rate heterogeneity among different codon positions did not describe the data better than the gamma distribution. The phylogenetic tree of vertebrate S opsins (fig. 3*A*) suggests that the SW1 (UV) opsin may represent the ancestral S opsin and have been preserved in the rodents and bats, though it has been shifted to a violet opsin in bovine by gaining three critical changes (F86Y, T93I, S118C) and shifted to a blue opsin in higher primates by gaining five critical changes (T52F, F86L, T93P, A114G, S118T).

M/L Opsin

The M/L opsin genes of *H. fischeri*, *P. dasymallus formosus*, and *M. velifer* were sequenced from exon 3 through exon 5, encoding 191 amino acids (fig. 2). The critical functional sites of the M/L opsin are at positions 180, 197, 277, 285, and 308 of the mature peptide region (Yokoyama and Radlwimmer 1999, 2001). *M. velifer* revealed an amino acid replacement at site 180, A180S (a change from A to S), and the amino acid replacements F277Y and A285T were found in all three bat species studied (fig. 2). According to Yokoyama and Radlwimmer's (2001) five-site rule and other functional studies (Neitz, Neitz, and Jabobs 1991; Asenjo, Rim, and Oprian 1994), the latter two substitutions suggest that the M/L opsin in the three bats is the L type. An additional replacement, V274I, was found in *M. velifer*, but site 274 is known to have a minor influence on spectral sensitivity (Yokoyama and Radlwimmer 1999, 2001). Note that the different chromophore (11-*cis*-retinal [vitamin A_1], 11-*cis*-3, 4-dehydroretinal [vitamin A_2]) or colored oil droplets cause shift of spectrum (Harosi 1994), but placental mammals use neither vitamin A_2 adlehyde nor colored oil droplets. Therefore, the red/green color vision might be determined by using the five-site rule. Besides the five-site rule, E129 and K312 form the C1-binding site, and loss of the C1-binding site was shown to shift spectral tuning by 30 nm toward red (Kleinschmidt and Harosi 1992). However, these two sites have been conserved in the M/L opsins of all vertebrates studied to date (Kleinschmidt and Harosi 1992; Sun, Macke, and Nathans 1997; Yokoyama and Radlwimmer 1999). Our sequence data include the K312 site (in exon 5) in all three bats studied and the E129 site (in exon 2) in the microbat species. Thus, it is likely that the C1-binding site has been conserved in the three bat species studied, so the five-site rule should apply to these bats, or at least to the microbat.

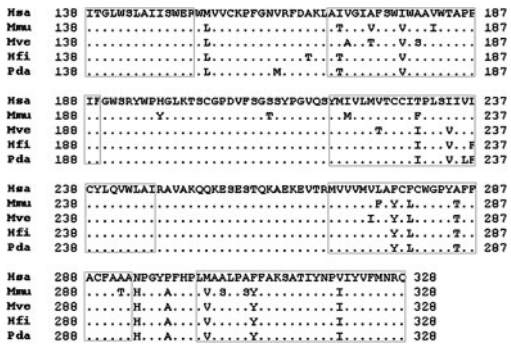

FIG. 2.—Alignment of partial protein sequences of M/L opsin genes from exon 3 to exon 5. The species included are *Homo sapiens* (Hsa), *Mus musculus* (Mmu), *Haplonycteris fischeri* (Hfi), *Myotis velifer* (Mve), and *Pteropus dasymallus formosus* (Pda). Residues identical to the human residues are indicated with a dot. Gray boxes indicate α helical regions.

Comparisons of substitution rates among *H. fischeri*, *P. dasymallus formosus*, and *M. velifer* revealed 43 changes in the *Hfi-Mve* comparison, and 37 changes in the *Pda-Mve* comparison, indicating a higher substitution rate in the Hfi branch (fig. 3*B*). However, the K_A/K_S ratio for *Hfi-Mve* (0.073) is lower than that for *Pda-Mve* (0.106). Table 2 shows the replacement and silent differences and nonsynonymous (K_A) and synonymous (K_S) substitutions per 100 sites in bat S opsin and M/L opsin genes. There is no significant difference in pairwise comparisons using a chi-square test ($P > 0.05$).

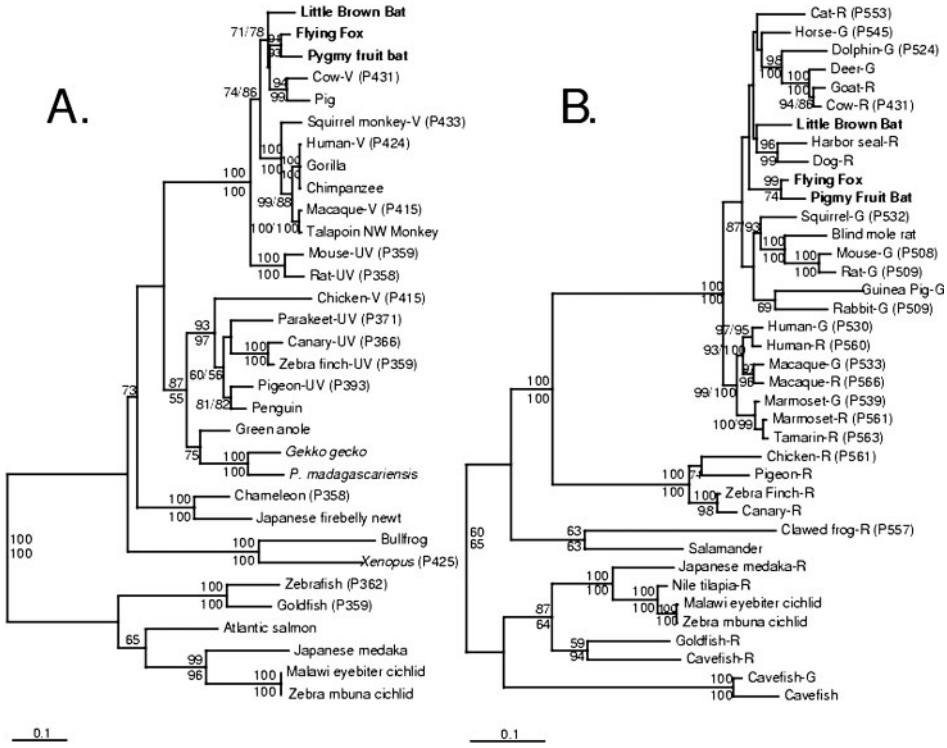

FIG. 3.—Phylogenetic analyses of vertebrate SWS (short wavelength spectroscopy)1 opsins (*A*) and LWS (long wavelength spectroscopy) opsins (*B*). Both trees are rooted by assuming that fishes form a monophyletic outgroup. The trees were found by heuristic searches using the maximum likelihood (ML) and assuming the best-fit model based on hierarchical likelihood ratio tests implemented with Modeltest (Posada and Crandall 1998). Numbers at nodes represent bootstrap proportions based on 1,000 pseudo-replicates. Above each node or slash are bootstrap values based on parsimony; below each node are bootstraps based on Neighbor-Joining using ML distances from the best-fit model. Absence of a number represents a bootstrap value below 50%. V = violet, UV = ultraviolet, G = green, and R = red. Each number in parentheses refers to the λ_{max}value. GenBank accession numbers: *SWS1 data*: chimpanzee AH005813; talapoin L76226; gorilla AH005811; cow U92557; pig AY091587; human NM_001708; macaque AF158976; squirrel monkey U53875; mouse NM_007538; rat AF051163; blind mole rat pseudogene AY099455; canary AJ277922; chicken M92039; pigeon AJ238856; penguin AJ277991; parakeet Y11787; zebra finch AF222331; bullfrog AB001983; gecko AY024356; *Phelsuma* AF074045; clawed frog U23463; chameleon AF134192; salamander AF038948; Japanese newt AB052889; zebrafish AF109373; goldfish D85863; Japanese medaka AB001605; cichlid1 AF191220; cichlid2 AF191219; and Atlantic salmon AY036959. *LWS data*: cat AF132040; dolphin AF055457; horse AF132043; human-G AH005296; macaque-G AF158975; human-R AH005298; macaque-R AF158968; marmoset-G AF051594; marmoset-R AF051582; mole rat-G AF139726; mouse-G AF011389; rat-G AH006946; squirrel-G AF132044; guinea pig-G AF132042; rabbit-G AH006945; chicken-R M62903; clawed frog-G U90895; deer AF132041; goat AH006594; cow-R AF280398; harbor seal AF110495; dog-R AF031533; frog-R U90895; tamarin AH012149; pigeon AH007800; zebra finch AF222333; canary AJ277925; tiger salamander AF038947; medaka-G AB001603; eyebiter cichlid AF247125; Mbuna cichlid AF247126; Nile tilapia AF247128; medaka-R AB001604; goldfish L11867; zebrafish grops1 NM_131253; zebrafish grops2 NM_131254; gecko-G M92036; *Phelsuma* AF074043; cavefish-G AH003046; cavefish-R AH003047; and cavefish opsin AH002422.

The best-fit model for middle/long wavelength vertebrate opsin data (fig. 3*B*) was Hasegawa-Kishino-Yano with Invariant sites and Gamma distributed rate heterogeneity (HKY85 + I + G). Parameter values were as follows: base composition A = 0.209, C = 0.314, and G = 0.244; transition/transversion ratio = 1.782; gamma shape = 1.290; proportion of invariant sites = 0.397. For neither data set did a model with rate heterogeneity partitioned among codon sites fit the data better than the gamma rate models. A model partitioning rate heterogeneity among different codon positions did not describe the data better than the gamma distribution. The resulting tree suggests that the L opsin may represent the ancestral state of the mammals and that it has been preserved in bats and cats but became an M (green) opsin in rodents, horses, and rabbits. In higher primates, a duplication of the L opsin occurred, and one of the two opsins became an M opsin. However, the bootstrap values in many of the nodes of the tree are weak, so the above scenario is uncertain and it is possible that the ancestral opsin of mammals was an M opsin and not an L opsin.

Duplication of the M/L Opsin Gene in *H. fischeri*

When we sequenced the region from exon 4 to exon 5 of the M/L opsin gene in an individual from *H. fischeri*, an additional copy was found. While exons 4 and 5 of the two copies were identical at nonsynonymous sites, the two copies of intron 4 differed by 20 nucleotides and 11 indels, including one large indel of ~750 bp and involving a total of 794 sites. These differences appeared to be too large to represent two alleles of the same locus, indicating the possibility of two duplicate genes. Indeed, the sequence data from intron 5 revealed three different sequences, denoted as 5–1, 5–2, and 5–3 (fig. 4). Whereas introns 5–1 and 5–2 may represent two polymorphic alleles from the same locus, intron 5–3 differs from intron 5–1 at 10 sites and should represent another locus. (Because a locus can

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Intron 5-1  1    TAAGTCACACCATTGCAGAGCAAACTTGAAATAGACTCCAAAGAGCCTCA 50
Intron 5-2  1    ..........................A..................... 50
Intron 5-3  1    ....................................G........G 50

Intron 5-1  51   TGATGGCTCCCCACCTGGAGTCACATCTGGCTCTGAGACAATAACCAGCAT 100
Intron 5-2  51   ..................................................... 100
Intron 5-3  51   ..................................................... 100

Intron 5-1  101  GTGACCCAAGTCCAGAAGCTCTCCCTCTGAATCCTTTCGTGATGTCTGAG 150
Intron 5-2  101  ..................................................... 150
Intron 5-3  101  ..........................G...........A..G........ 150

Intron 5-1  151  GGTAGGTGTTTCTTGCAGTGCCTGGGCACTGTGCACTCTTCCCTTCTGCT 200
Intron 5-2  151  ..................................................... 200
Intron 5-3  151  ......C.....................G...................... 200

Intron 5-1  201  GACCTGTGGGTTAGCCTAGAGCTCAAACACCTGAAGGTCACCTGAACAGG 250
Intron 5-2  201  ....................................A.............. 250
Intron 5-3  201  ..................................................... 250

Intron 5-1  251  GTTACATTTAGCCTAGACATACACTTCTTGCTTTTACAGGCGCAACTCTT 300
Intron 5-2  251  ..................................................... 300
Intron 5-3  251  ......................G.................T......... 300

Intron 5-1  301  CTCACAGCTTTCATCTTCCCACCTTCAGACTCTATCTCTAATGAGGACAC 350
Intron 5-2  301  ..........................A................A..... 350
Intron 5-3  301  ...G.................................................. 350

Intron 5-1  351  AAAGAGGCCCTTCTTGG 367
Intron 5-2  351  ................. 367
Intron 5-3  351  ................. 367
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FIG. 4.—Alignment of three partial intron 5 sequences from one individual of *H. fischeri*, indicating that the two M/L opsin copies are from different loci. Introns 5-1 and 5-2 were apparently two polymorphic alleles sequenced form one gel band, whereas intron 5-3 was sequenced from the other gel band and evidently represents a second locus.

have at most only two different sequences, the presence of three sequences should indicate a gene duplication.) When we saw the above sequence differences, we decided to perform PCR on the same region from 10 more individuals. We found that each of them had two 2-gel bands. Sequencing of the two bands revealed the same differences in intron 4 (including one large indel), a finding that supports the presence of two duplicate M/L loci. Furthermore, the sequencing of exon 5 also revealed two different copies of the exon, as shown in table 3; all the differences are synonymous.

Discussion

The present study indicates that both megabats and microbats have a functional L opsin and a functional S opsin. This finding does not support the common view that the megabats have overall better vision than microbats (Bhatnagar 1975; Pettigrew et al. 1988; Fuzessery et al. 1993; Kalko, Herre, and Handley 1996). However, this view may still be true because there are morphological (e.g., lens, cornea, vitreous body shape/transmittance) or neuroanatomical (the relative proportion of rods vs. cones) differences between the visual systems of megabats and microbats. Alternatively, it is possibile that the opsins may have a function unrelated to vision—e.g., circadian rhythm regulation (Nei, Zhang, and Yokoyama 1997). The fact that the S opsin has been highly conserved in both megabats and microbats implies that the S opsin of bats still performs an important function despite the suggestion that bats have been occupying a nocturnal niche for about 80 myr (Nikaido et al. 2000). Alternatively, it is highly possible that the assumption of a long history of nocturnal life is wrong.

The three S opsin sequences of bats show conservation at the functional critical sites—i.e., the disulphide linkage of Cys109 and Cys186, the Schiff's base counterion Glu113 and at the site of retinylidene Schiff's base formation Lys295. Their interhelical cytoplasmic loops and luminal loops are more conserved than those found in primates (Shimmin, Mai, and Li 1997). The conservative cytoplasmic loops are related to the function in binding of transduction and initiation of phototransduction (Hunt et al. 1995). However, in nocturnal animals the S opsin tends to lose its function (Jacobs 1993; Jacobs, Neitz, and Neitz 1996). We note that the owl monkey *Aotus* diverged from *Callithrix* approximately 13 MYA, but it has lost its S opsin. The Syrian golden hamster also lost the S opsin after separation from other members of the subfamily Cricetinae approximately 20–29 MYA (O'hUigin and Li 1992; Calderone and Jacobs 1999). In contrast, it has been suggested that bats have occupied a nocturnal niche for some 80 myr (Nikaido et al. 2000), yet both suborders still maintain a functional S opsin gene. Thus, it is possible that the two bat lineages have evolved the nocturnal life-style only relatively recently or have not been strongly nocturnal for a very long time; otherwise, they would have lost the S opsin gene.

In addition, we found that the S opsins of the three bats studied are actually sensitive to UV light according to Yokoyama and Yongsheng's (2000) five-site rules. Sites T52, F86, T93, A114, and S118 are also noted to be the ancestral amino acids of the UV pigment shared by chameleon, rat, and mouse (Yokoyama and Yongsheng 2000). One the one hand, UV vision may shift to blue vision by introducing a single change F86Y, or by accumulating any two of the critical amino acid changes (Yokoyama and Radlwimmer 2001; Cowing et al. 2002). On the other hand, fish, chameleon, mouse, and rat pigments have retained their UV sensitivities by accumulating no more than one of the five amino acid changes (Yokoyama and Yongsheng 2000). Bats may have preserved the UV pigment for an important purpose related to vision, even though the UV light is not available in the dark (Hut, Scheper, and Daan 2000). This hypothesis is supported by the evidence that visual pigments of vertebrates have usually been under strong selective pressure (Bowmaker 1991), and that most mammals have shifted to blue light. Alternatively, the preservation of UV light sensitivity in bats may suggest a short history of nocturnal life.

Table 3
Different Sites in Exon 5 of Duplicated L Opsin Genes from 10 Individuals of *H. Fischeri* Sampled from Catanduanes, Mt. Katanglad (Mindanao), Negros Oriental (Negros), Sibuyan, and Mindoro, Philippines

	First Copy of L Opsin Exon 5			Second Copy of L Opsin Exon 5		
Locations	446	482	479	446	482	497
Catanduanes	C	C	G	C	C	G
	C	C	G	C	C	G
Mindanao	C	T	G	C	C	T
	C	T	G	C	C	T
Negros	C	C	G	C	C	G
	C	C	G	C	C	G
Sibuyan	C	C	T	C	C	T
	C	C	T	C	C	T
Mindoro	C	C	T	G	C	T
	C	C	T	G	C	T

Two different evolutionary scenarios, flight-first or echolocation-first, have been proposed for the origin of acoustic orientation and true flight in bats (Arita and Fenton 1997; Simmons and Geisler 1998). The flight-first hypothesis suggests that ancestral bats possessed enhanced vision, followed by a reduction and simplification of the visual system as more emphasis was placed on laryngeal echolocation in microbats. In contrast, the echolocation-first hypothesis suggests that megabats lost laryngeal echolocation and developed better vision independently. Recent studies support the idea that flight evolved before echolocation (Arita and Fenton 1997; Simmons and Geisler 1998). However, vision in some microbats did not become less acute (Pettigrew et al. 1988). Some microbats possess similar numbers of retinal ganglion cells to those seen in rats and cats, and the visual acuity of microbats appears to be no less than that of megabats (Pettigrew et al. 1988; Heffner, Koay, and Heffner 2001). Vision may serve an important role in microbats that migrate long distances (Suthers and Wallis 1970), and some microbats even use vision in capturing prey (Bell 1985). Therefore, it is possible that microbats still retain multiple opsins for special occasions under light, or that the preservation of visual acuity (UV opsin) may enhance flight in the only nocturnal flying mammal. It is interesting to note that most microbats use vision quite well, and on moonlit nights they can avoid capture nets, not by using echolocation but by vision.

The critical functional sites of the M/L opsin include sites 180, 197, 277, 285, and 308, which had correctly predicted the in vitro-expressed pigment of many mammals studied to date (Sun, Macke, and Nathans 1997; Yokoyama and Radlwimmer 2001; Deeb et al. 2003). Compared to human M opsin (530 nm), *H. fischeri* and *P. dasymallus formosus* M/L opsins have two substitutions (F277Y, A285T), and *M. velifer* has three substitutions (A180S, F277Y, A285T), and these substitutions should shift their spectral sensitivity peaks toward red by ~23 and ~28 nm, respectively (Neitz, Neitz, and Jabobs 1991; Asenjo, Rim, and Oprian 1994; Yokoyama and Radlwimmer 2001). We have therefore proposed that these bats have the L-type opsin, although this proposal needs to be substantiated by a functional study. It seems that these species of bats have enjoyed the red vision, while some other mammals have shifted to the green vision (e.g., horse, dolphin, mouse, and rabbit), because it appears that the common ancestor of vertebrates had the L opsin (Yokoyama and Radlwimmer 2001). This finding is in agreement with the fact that *H. fischeri* and *P. dasymallus formosus* are fruigivorous, and thus may use color vision while foraging for ripe fruits. Interestingly, *M. velifer* (microbat) has one more substitution (A180S), which probably causes another 7-nm shift toward red. In these strong nocturnal insectivores with echolocation, the putative red shift suggests that either microbats have not been strongly nocturnal for a very long time or that the L opsin in micrbats may serve a purpose unrelated to vision.

It has been commonly believed that LW (long wavelength) and SW (short wavelength)/UV pigments are the consequence of the nocturnal life of ancestral mammals. However, recent phylogenetic evidence indicates that the UV pigment was the ancestral pigment of vertebrates and that blue pigments evolved from UV pigments in different vertebrate lineages (Hunt et al. 2001; Cowing et al. 2002). Some rodents (Jacobs 1992; Jacobs, Fenwick, and Williams 2001) and the bats in this study are the only UV-sensitive mammals known to date. We note that the lens of some diurnal rodents absorbs UV light and reduces the light that reaches the retina by 50% (Hut, Scheper, and Daan 2000), and some rodents live a strong nocturnal life without benefit of UV light except for circadian rhythms (Jacobs, Fenwick, and Williams 2001). It has been noted that a higher ratio of 360:520 nm light is emitted at dawn and at dusk (Hut, Scheper, and Daan 2000). Because the flying fox is known to be active at dusk (Kalko, Herre, and Handley 1996; Schnitzler and Kalko 1998; Phillips 2000), it might benefit from UV light. However, it is not clear whether microbats, which are strongly nocturnal, benefit from UV light.

Primates are thus far the only mammals known to possess duplicate genes for the M and L opsins. The present investigation shows that *H. fischeri* has a duplication of the L-opsin gene. The duplication may be fairly old, because the two copies have accumulated 20 nucleotide substitutions and 11 indels in intron 4, although exons 4 and 5 remain identical at nonsynonymous sites. The selective advantage of this duplication is unclear. Many nocturnal mammals (e.g., subterranean blind mole rats), cavefish, and crayfish have preserved the M/L opsin, and the reason was suspected to be related to circadian rhythm (Jacobs 1993; Yokoyama et al. 1995; Crandall and Hillis 1997; Shimmin, Mai, and Li 1997; David-Gray et al. 1999; Dkhissi-Benyahya et al. 2001). Thus, duplication of the L opsin may facilitate the regulation of circadian rhythm. However, the raccoon and the kinkajou have a single cone that responds to spectrum 550–560 nm, and the owl monkey and the thick-tailed bushbaby have a single cone sensitive to spectrum 543–545 nm (Jacobs and Deegan 1992; Jacobs, Neitz, and Neitz 1996). Therefore, if λ_{max} (the absorbance spectrum maximum) relates to circadian rhythm regulation, there appears to be less selective constraint on the visual spectra.

Theory suggests three alternative outcomes for duplicated genes: (1) one copy may become nonfunctional; (2) one copy may acquire a beneficial function and be preserved by natural selection; and (3) the functions of both copies may become suboptimal (Ohno 1970; Lynch and Conery 2000). The most common fate for duplicate genes has been thought to be loss of a functional copy (Ohno 1970). The duplication of the L opsin in *H. fischeri* has accumulated several mutations in the introns, but the exons have been conserved. It is likely that maintaining both copies is beneficial. Multiple M- and/or L-opsin expressions have been found in humans, with the expression level higher than that for a single copy (Sjoberg et al. 1998). Overlapping expression in a single cone cell was also found in invertebrates (a crab, *Hemigrapsus sanguineus*, and a butterfly, *Papilio xuthus*). These species express two similar opsins in a photoreceptor cell, and these two loci may serve to broaden the spectral sensitivity of the photoreceptors (Sakamoto et al. 1996; Kitamoto et al. 1998). *Pteropus giganteu*s (megabat) has a very low cone/rod ratio (1/250) (Jacobs 1993); therefore, it is

possible that the increase in mRNA may increase the expression level and facilitate the sensitivity of photoreceptor cell.

The duplication of the L opsin in *H. fischeri* is the first case of opsin duplication found in nonprimate eutherian mammals. However, a recent microspectrophotometry study suggested trichromacy in Australian marsupials (Arrese et al. 2002). These observations suggest that duplication of the M/L opsin gene may have a broader distribution among non-primate mammals than currently known.

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