Supplementary Material for

Testing the sensory tradeoff hypothesis in New World bats

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Supplementary background

About 2000 years ago, Aristotle identified the five basic senses in humans including sight (vision), hearing (audition), taste (gustation), smell (olfaction), and touch (somatosensation) [S1]. Beyond these five commonly recognized senses, additional sensory modalities may be used to detect environmental stimuli including temperature (thermoception), pain (nociception), and balance (equilibrioception). Depending on the species, animals may rely on distinct sets of sensory modalities, with some species lacking one or more basic senses while others developing novel senses [S2-4]. The absence or reduction of a sense may have been shaped by positive selection [S5], convergent evolution [S6], and sensory tradeoffs [S7]. The sensory tradeoff hypothesis has been proposed frequently in cases in which an enhancement of one sensory modality has apparently resulted in the loss or reduction of other sensory modalities, possibly due to energy limitations [S8]. For example, the loss of vomeronasal olfaction coincided with the acquisition of trichromatic color vision in primates [S9]; similarly, the origin of a novel sensory modality – high-duty-cycle (HDC) echolocation – may have resulted in the loss of color vision in some lineages of bats [S7]. Such tradeoffs have been long observed in vertebrate brain regions, with an enlargement of one region being coupled with a reduction of another [S10]. Given the finite energy budgets in animals, sensory tradeoffs have been thought to result from the high energetic demands incurred by neural processing [S8].

Among the five basic senses, the visual system is particularly prone to be lost or reduced in nocturnal, cave-dwelling, and subterranean animals [S11]. In mammals, members of at least five orders (Carnivora, Cetacea, Chiroptera, Primates, and Rodentia) have independently lost color vision [S11]. Such widespread losses have been suggested to be driven by nocturnality, spectral tuning of opsins, adaptive gene loss, and taxon-specific reasons (each taxon may have a unique scenario underlying the gene loss) [S11]. It is not surprising that taxon-specific reasons underlie the widespread losses of color vision across animals, because they have evolved

taxon-specific adaptive characteristics that have allowed them to successfully radiate in diverse ecological niches.

One group of animals that show lineage-specific loss of color vision is the mammalian order Chiroptera: the bats [S7]. We found two sister clades of Old World HDC bats (Rhinolophidae and Hipposideridae) have lost the ability to distinguish colors, while the majority of LDC bats have dichromatic color vision in our previous study [S7]. Thus, a sensory tradeoff hypothesis between the loss of color vision and the origin of the HDC echolocation in bats was proposed [S7]. However, this hypothesis between the innovation of HDC echolocation and loss of color vision may be just a mere coincidence, and additional data and analyses are needed to justify this hypothesis. One powerful approach for addressing the issue of sensory tradeoffs is to examine replicated evolution of sensory modalities in phylogenetically divergent lineages. So we turn to the New World bats, which include the moustached bats (*Pteronotus parnellii*) and vampire bats. The former have independently evolved the HDC echolocation that is similar to the hipposiderid and rhinolophid lineages [S12, 13], while the latter have evolved a unique sensory modality among bats – the infrared sense [S3, 14]. The infrared sensing ability in the common vampire bat (*Desmodus rotundus*) was first discovered by the thermography experiment [S3]. Specifically, the three pits surrounding the central nose were found to always keep a lower temperature than other areas, which can be used to detect thermal radiation of their endothermic prey and locate optimal bite sites [S3]. An additional study identified a specific nucleus on the brainstem of the common vampire bat, which may be a part of the infrared processing system of vampire bats as it resembles with the features of the infrared nucleus in infrared-sensitive snakes [S15]. Despite the infrared processing system has not yet been investigated in the other two vampire bats (*Diphylla ecaudata* and *Diaemus youngi*), similar structures were expected to be present [S14, 16].

Supplementary methods

Critical spectral tuning sites and sliding window analysis

Previous studies have identified at least 11 amino acid sites involved in the spectral tuning of SWS1 pigments [S17] and 5 amino acid sites involved in the spectral tuning of M/LWS pigments, respectively [S18]. To examine the critical spectral tuning sites of *SWS1* and *M/LWS* genes in the New World bats, we deduced the SWS1 protein sequences of the New World bats and aligned them with the house mouse (*Mus musculus*) (GenBank accession: NM_007538), and the deduced M/LWS protein sequences of the common vampire bat *Desmodus rotundus* was aligned with those of other bats published previously [S7] and that of *Homo sapiens* (GenBank accession: NM_020061). Both alignments were performed using the BioEdit program [S19].

To visualize the average rates of nonsynonymous (d_N) and synonymous (d_S) substitutions per site for sequences with ORF-disrupting indels, the Nei and Gojobori method [S20] implemented in the program SWAAP 1.0.2 [S21] was applied. The SWAAP program estimates changes of evolutionary rates with a sliding window approach. All estimations were performed using a window size of 90 nt and a step size of 9 nt, except the white-winged vampire bat *Diaemus youngi* that was estimated with a window size of 30 nt and a step size of 6 nt.

Ancestral sequence reconstruction and evolutionary analysis

Ancestral sequences were inferred with a combination of the likelihood-based method [S22] implemented in the baseml program in PAML 4.8a [S23] and maximum parsimony [S24]. Maximum parsimony was employed primarily to infer the indel regions of the ancestral sequence because the Bayesian method could not deal with the indels. To examine whether a particular lineage of bats had undergone differential selective pressures as compared to other bats, we estimated the ratio (termed ω) of nonsynonymous (d_N) to synonymous (d_S) substitution rates using branch models in codeml program implemented in PAML 4.8a [S23]. The value of ω is an indicator of natural selection, with an ω value being equal to 1, less than 1, more than 1 indicating neutral evolution, purifying selection, and positive selection, respectively [S25].

A total of six selection tests using the codeml program were undertaken in our study. First, we tested whether the intact *SWS1* genes in the 15 bats (Dataset I in **table S3**) were subjected to purifying selection (model A0 versus model B1 in **table S3**). Second, we tested whether there is a difference in ω between the ancestral branch of vampire bats and other branches (model C0 and model D2 in **table S3**). Third, we tested whether the signal of relaxed selection has occurred in *Desmodus rotundus* (model E0 and model F2 in **table S3**). Fourth, we tested whether the signal of relaxed selection has occurred in *Diphylla ecaudata* (model G0 and model H2 in **table S3**). Fifth, we tested whether the signal of relaxed selection has occurred in *Diaemus youngi* (model I0 and model J2 in **table S3**). Finally, we conducted the relaxed selection test towards the null allele of *Pteronotus mesoamericanus* (model K0 and model L2 in **table S3**).

In addition, we detected whether relaxed selection occurred using the RELAX program [S26] implemented in HyPhy [S27]. The RELAX program detects relaxed selection based on codon levels. During calculation, the selection intensity parameter (*k*) will be introduced to compare the test branches with the reference branches. Similar to those models used in codeml, the RELAX program compares a null model assuming k equal to 1 for all branches with an alternative model assuming a different k value in test branches. If the alternative model fits the datasets better, selective strength will be considered as relaxed ($k < 1$) or intensified ($k > 1$) in the test branches relative to reference branches. In our study, the ancestral lineage of vampire bats, *Desmodus rotundus*, *Diphylla ecaudata*, *Diaemus youngi* and *Pteronotus mesoamericanus* were used as the test branches, respectively, while the remaining branches in each case were used as reference branches. Therefore, a total of five selection tests were undertaken using RELAX in our study (**table S3**).

Dating the pseudogenization events of *SWS1*

Pseudogenization events were found in four *SWS1* sequences, including three in vampire bats, and one in the null allele of *Pteronotus mesoamericanus*, and we investigated when the functional constraint on *SWS1* became relaxed in each sequence. We assumed that the functional relaxation on *SWS1* started at *t* million years ago (Ma), and the pseudogenization timing was estimated based on two different methods described in Meredith et al. [S28] and Zhao et al. [S29], respectively. The first method, known as the "synonymous substitution rate method", is based on changes in ω values (the ratio of non-synonymous to synonymous substitution rates). Specifically, the branch model in codeml [S23] was used to estimate ω for three different branch categories: functional branch, mixed branch and pseudogenic branch. The functional branches were the branches considered as background branches (those associated with the 15 New World bats with intact *SWS1*), and the mixed branch was the branch on which the relaxation of the functional constraint started. The pseudogenic branches were the branches on which the *SWS1* would be expected to evolve neutrally, thus the ω was fixed to 1 on those branches (the branches of the 4 bats possess *SWS1* pseudogenes). Except for the estimation of ω values for three branch categories, the divergence time was also needed for the estimation of *t*. Approximate divergence dates were taken from the following resources: (1) 27.0 Ma for the split between vampire bats (Desmodontinae) and New World fruit bats (Phyllostominae) [S30]; (2) 13.5 Ma for the divergence between *Pteronotus mesoamericanus* and *Pteronotus davyi* [S30]. The starting time (*t*) of functional relaxation was then obtained under the assumption of "a single rate of synonymous substitution on functional and pseudogenic branches" and "two rates of synonymous substitution on functional and pseudogenic branches", respectively. The mean of the two values of *t* obtained under the two assumptions was calculated and used in our subsequent analysis. Moreover, we modified this method by bootstrapping the codons of *SWS1* 10000 times, and estimated a posterior probability distribution of *t*.

In the second method, *t* was estimated based on the number of existing ORF-disrupting mutations [S29]. This method assumes that if the observation number

of ORF-disrupting substitutions in a gene is n, the starting time (t) of functional relaxation of this gene should be between t_n and t_{n+1} by estimating the waiting times for n (t_n) and n+1(t_{n+1}) ORF-disrupting substitutions. In our study, the observation number of ORF-disrupting substitutions in *SWS1* in the *Desmodus rotundus*, *Diphylla ecaudata*, *Diaemus youngi*, and *Pteronotus mesoamericanus* (the null allele) is 2, 2, 1, and 1, respectively. Waiting times were estimated using the modified program PSEUDOGENE [S9]. This program requires neutral rates of point mutations and indel mutations as input, we took both parameters from our previous study [S31]. Using the *SWS1* sequence of *Artibeus jamaicensis* as the input for the estimation of time *t* for the three vampire bats and the *SWS1* sequence of *Pteronotus davyi* as the input for the estimation of *t* for *Pteronotus mesoamericanus*, the pseudogenization process was simulated 10000 times and we acquired 10000 *t* for each of four species. Thus, another posterior probability distribution of *t* was estimated. Since independent information for the estimation of posterior probability distribution of *t* was used in the two methods, we combined them to obtain a final posterior probability distribution of *t*.

Supplementary results

Conservation of *SWS1* in most New World bats

The *SWS1* gene in mammals contains five exons and approximately 1000 nucleotides, which encode a seven-transmembrane-domain protein [S32]. By sequencing the *SWS1* gene in 16 species of New World bats, we newly obtained *SWS1* sequences spanning exon 1 to exon 4 in these species. In addition, we retrieved three additional *SWS1* sequences of New World bats from our previous study [S7]. Our dataset of *SWS1* contained all three species of vampire bats and other major lineages of the New World leaf-nosed bats (Phyllostomidae) as well as one species of New World HDC echolocating bat (Mormoopidae) **(figure 1 and table S1)**. After aligning *SWS1* sequences, we found 15 of the 19 bats to have a putatively functional *SWS1* gene characterized by an intact open reading frames (ORFs) (**figure 1**). The deduced protein sequences of these intact genes were subsequently aligned and used to

examine whether the 11 critical sites responsible for the spectral tuning are conserved in these species. The alignment (**figure S6**) showed that these functionally critical sites in each species are identical to those in the mouse (*Mus musculus*), which is known to have an ultraviolet (UV) pigment conferred by the SWS1 opsin [S33]. Of note, all the 15 bats with an intact and putative functional SWS1 opsin are low-duty-cycle (LDC) echolocators [S12]. We note that an intact *SWS1* can not predict dichromatic color vision in a bat, as functional loss of a gene may have occurred at transcriptional or translational stage.

To examine whether these 15 intact *SWS1* genes are under purifying selection and functional constraint, we undertook a selection test on bats by estimating the ratio (termed ω) of nonsynonymous (d_N) to synonymous (d_S) substitution rates. In this test, we assumed a uniform ω across all branches (model A0 in **table S3**), and the value of ω was estimated to be 0.16. The model A was next compared with the model B1, which assumed all branches have a same ω = 1 (**table S3**). This comparison revealed a significant difference between the two models, suggesting the overall ω of all intact genes is significantly smaller than 1 (ω = 0.16, *P* =1.70E-51, **table S3**). Thus, the *SWS1* gene is under overall strong purifying selection and functional constraint in these bats. We additionally examined overall variation of *SWS1* across all 15 bats with intact ORFs by comparing the gene tree with species tree. Both maximum-likelihood and Bayesian approaches recovered a same tree topology **(figure S7)**, which is not significantly different from the species tree [S30, 34, 35] (Kishino–Hasegawa test, *P* = 0.181; Shimodaria–Hasgawa test, *P* = 0.169; **table S4**). This comparison suggests that the overall variation of the intact *SWS1* sequences across these bats is low, confirming the conservation of *SWS1* in these species.

Supplementary discussion

In this work, we used New World bats to test the sensory tradeoff hypothesis between a loss of color vision and an origin of high-duty-cycle (HDC) echolocation [S7]. Through sequencing the short-wavelength opsin gene (*SWS1*) in 16 species (29

individuals) of New World bats, we found losses of color vision in the New World HDC echolocators, the same sensory tradeoff in Old World bats was replicated in New World bats. In addition, *SWS1* was found to be pseudogenized in all three vampire bats, suggesting another sensory tradeoff between the gain of the infrared sense in vampire bats and the loss of color vision.

In contrast to the pseudogenization of *SWS1* gene in the four New World bats (one HDC echolocator and three vampire bats), the *SWS1* opsin gene appears to be evolutionarily conserved in most New World bats examined **(figures 1 and S6a)**. The 11 critical sites implicated in spectral tuning are identical in all species with an intact *SWS1* gene **(figure S6a)**, which is predicted to encode a UV sensitive pigment [S17]. Indeed, behavioral studies have showed that eight species of New World bats are able to perceive UV visual stimuli [S36, 37], including one species of New World leaf-nosed bat (*Glossophaga soricina*) that was examined in this study [S36] **(figure 1)**. Genetic evidence indicates that most bats, including New World and Old World species, possess UV color vision [S7], and a behavioral study confirmed the cone-based UV sensitivity in two Old World bats [S38]. Why is UV vision retained in most bats? Potential benefits of UV vision in bats and other mammals include the detection of UV-reflective foods (e.g. flowers, fruits, and insects) or urine marks, communication with potential mates, and entrainment of circadian rhythms [S7, 36, 39, 40]. Nonetheless, the selective forces underlying the retention of UV vision in bats remain unclear.

Color vision in eutherian mammals is conferred by M/LWS and SWS1 opsins [S11]. The *M/LWS* opsin gene displays strong conservation and no ORF-disruptive mutations are known in mammals with the exception of a few cetacean species [S7, 41, 42]. Even in the highly specialized vampire bats, we observed intact ORFs and highly conserved protein sequences, through sequencing all six exons (≈1300 bp) of the *M/LWS* gene in five individuals of the common vampire bat **(figure S6b)**. Thus, the disruption or pseudogenization of the *M/LWS* gene may represent a fitness damage

that most mammals cannot tolerate, suggesting an indispensable role in certain physiological needs such as the regulation of circadian rhythms [S41], although it remains to be tested whether the gene is functional at transcriptional or translational stage.

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Table S1. Species and individuals used in this study.

Classification		Common name	Scientific name	AMNH sample ID
Family	**Subfamily**			
Mormoopidae		Parnell's mustached bat	*Pteronotus mesoamericanus*	274564
				274565
				278269
				278117
				278393
				278658
		Davy's naked-backed bat	*Pteronotus davyi*	274571
				274572
				278293
				278290
				278397
Phyllostomatidae	Stenodermatinae	little yellow-shouldered bat	*Sturnira lilium*	278121
		Cuban fig-eating bat	*Phyllops falcatus*	103067
		tent-making bat	*Uroderma bilobatum*	278102
		MacConnell's bat	*Mesophylla macconnelli*	109688
	Glossophaginae	tailed tailless bat	*Anoura caudifer*	109578
		Pallas's long-tongued bat	*Glossophaga soricina*	278304
		Antillean fruit-eating bat	*Brachyphylla cavernarum*	121988
		Cuban flower bat	*Phyllonycteris obtusa*	138092
	Phyllostominae	Linnaeus's false vampire bat	Vampyrum spectrum	110477
		white-throated round-eared bat	*Lophostoma silvicolum*	109530
		fringe-lipped bat	*Trachops cirrhosus*	207853

	Desmodontinae	common vampire bat	*Desmodus rotundus*	278264
				278277
				278291
				278296
				278298
		white-winged vampire bat	*Diaemus youngi*	110377
		hairy-legged vampire bat	*Diphylla ecaudata*	109328

Table S2. Details of primers used in this study.

Species	Primer sequence	Product	Primer sequence	Product
Sturnira lilium	F: TGGGATGGGCCTCAGTACCACAT	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: GGCACGATGAAGCAGAAGATGAAGAGG	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Phyllops falcatus	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Uroderma bilobatum	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Mesophylla macconnelli	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Anoura caudifer	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Glossophaga soricina	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Brachyphylla cavernarum	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Phyllonycteris obtusa	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Vampyrum spectrum	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Lophostoma silvicolum	F: TGGGATGGGCCTCAGTACCACAT	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: GGCACGATGAAGCAGAAGATGAAGAGG	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Trachops cirrhosus	F: ATGTCAGRGGARGAGTTTTATCTGTTCAAG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
	R: TATAGKACTCGCTGCGRTAYTTGGTGCC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Desmodus rotundus	F: TCAGGGAACCCAAAAGTGGCTTTG	Exon1-Exon5 of *SWS1*		
	R: GGTAGGAAGGTAATAGGAAAACGG	(≈3200bp)		

Diaemus youngi			F: CCRACTGGTACACTGTGGGCACC	Exon3-Exon4 of *SWS1*
			R: TGGGGTTGTAGACACAAGCACTC	(≈800bp)
Diphylla ecaudata	F: TCAGGGAACCCAAAAGTGGCTTTG	Exon1-Exon3 of *SWS1*	F: CACCTGGTTCCTCTTCATCTTCT	Exon3-Exon4 of *SWS1*
	R: CAGCATAGGGCACGTAACAGAGA	(≈1200bp)	R: GGTAGGAAGGTAATAGGAAAACGG	(≈800bp)
Pteronotus davyi	F: GGTCCAGACTCTTTGAGCCC	Exon1-Exon3 of *SWS1*		
	R: TGGGGCCAACTTGGCTAGAAG	(≈3200bp)		
Pteronotus	F: TGAGCAARATGTCRGGGGAGGAGG	Exon1-Exon3 of *SWS1*	F: GCAGTGTTCCTGTGGYCCYGACTGGTAC	Exon3-Exon4 of *SWS1*
mesoamericanus	R: TGGTGCCCACRGTGTACCAGTC	(≈1200bp)	R: GGAACTGCTTATTCATGAAGCAGTAGATGATGGG	(≈800bp)
Desmodus rotundus	F: CTGGACAGTAGAAGGGAACG	Exon1 of *LWS1*	F: GGAGCCTGTCAATGGCACTA	Exon2 of *LWS1*
	R: GTGACCTAAGCCCTCTCTCG	(≈1000bp)	R: CCCAGGTCCCTCCTATCTTA	(≈1900bp)
Desmodus rotundus	F: GAATGTCCGCCACCTCTGTA	Exon3- Exon4 of *LWS1*	F: CTCCTTGTTGCCCCATACTC	Exon5 of *LWS1*
	G: GCAGGTTTCATTTGAGCGTC	(≈1900bp)	R: ATGGGCAGTTTGCTGTGGAG	(≈1100bp)
Desmodus rotundus	F: GCTGTTCCCTGGTTTGTAGA	Exon6 of *LWS1*		
	R: GCTCTCCGCTCTAAATCCTT	(≈900bp)		

Table S3. Tests of selective pressures on the *SWS1* gene in the New World bats.

Selective pressures tests using codeml						
Models	**ω (d_N/d_S)**	**lnL**	**Δnp**	**Models compared**	**2Δlnl**	***P* value**
Dataset I: 15 sequences (all bats with intact *SWS1*)						
A0. All branches have the same ω	ω = 0.16	-2629.38				
B1a. All branches have the same ω = 1	ω = 1	-2743.34	1	B vs. A	227.92	1.70E-51
Dataset II:16 sequences (Dataset I plus the ancestral sequence of vampire bats)						
C0. All branches have the same ω	ω = 0.16	-2591.66				
D2. Ancestral branch of vampire bats has ω_1 and other branches have ω_0	ω_0 = 0.16, ω_1 = 1.27	-2591.66	1	D vs. C	0	1
Dataset III:16 sequences (Dataset I plus the *D. rotundus*)						
E0. All branches have the same ω	ω = 0.18	-2924.35				
F2. *D. rotundus* has ω_1 and other branches have ω_0	ω_0 = 0.16, ω_1 = 0.61	-2918.85	1	F vs. E	11	0.00091
Dataset IV:16 sequences (Dataset I plus the *D. ecaudata*)						
G0. All branches have the same ω	ω = 0.16	-2889.69				
H2. *D. ecaudata* has ω_1 and other branches have ω_0	ω_0 = 0.15, ω_1 = 0.32	-2888.67	1	H vs. G	2.04	0.15
Dataset V:16 sequences (Dataset I plus the *D. youngi*)						
I0. All branches have the same ω	ω = 0.17	-2646.35				
J2. *D. youngi* has ω_1 and other branches have ω_0	ω_0 = 0.16, ω_1 = 1.10	-2641.64	1	I vs. J	9.42	0.0021
Dataset VI:16 sequences (Dataset I plus the null allele of *P. mesoamericanus*)						
K0. All branches have the same ω	ω = 0.16	-2652.26				
L2. *P. mesoamericanus* has ω_1 and other branches have ω_0	ω_0 = 0.15, ω_1 = 0.55	-2650.36	1	L vs. K	3.80	0.05
Selective pressures tests using RELAX						
Models	***k* (selection intensity)**	**lnL**	**Δnp**	**Models compared**	**2△lnL**	***P* value**
Dataset II:16 sequences (Dataset I plus the ancestral sequence of vampire bats)						
M. All branches have the same *k* = 1		-2545.94				

N. Ancestral branch of vampire bats has a different k value relative to reference branches	$k = 1.128$	-2545.94	1	N vs. M	0	1
Dataset III:16 sequences (Dataset I plus the *D. rotundus*)						
O. All branches have the same $k = 1$		-2872.23				
P. *D. rotundus* has a different *k* value relative to reference branches	$k = 0.197$	-2866.80	1	P vs. O	10.86	0.00098
Dataset IV:16 sequences (Dataset I plus the *D. ecaudata*)						
Q. All branches have the same $k = 1$		-2844.93				
R. *D. ecaudata* has a different *k* value relative to reference branches	$k = 0.545$	-2843.88	1	R vs. Q	2.10	0.147
Dataset V:16 sequences (Dataset I plus the *D. youngi*)						
S. All branches have the same $k = 1$		-2595.52				
T. *D. youngi* has a different k value relative to reference branches	$k = 0$	-2590.92	1	T vs. S	9.20	0.0024
Dataset VI:16 sequences (Dataset I plus the null allele of *P. mesoamericanus*)						
U. All branches have the same $k = 1$		-2627.51				
V. *P. mesoamericanus* has a different *k* value relative to reference branches	$k = 0.240$	-2625.75	1	U vs. V	3.52	0.060

Table S4. Gene vs. species tree for the *SWS1* gene in the New World bats.

Test	Log likelihood score	Difference	*P* value for KH test	*P* value for SH test
Gene tree	-3108.66			
Species tree	-3084.37	24.29	0.181	0.169

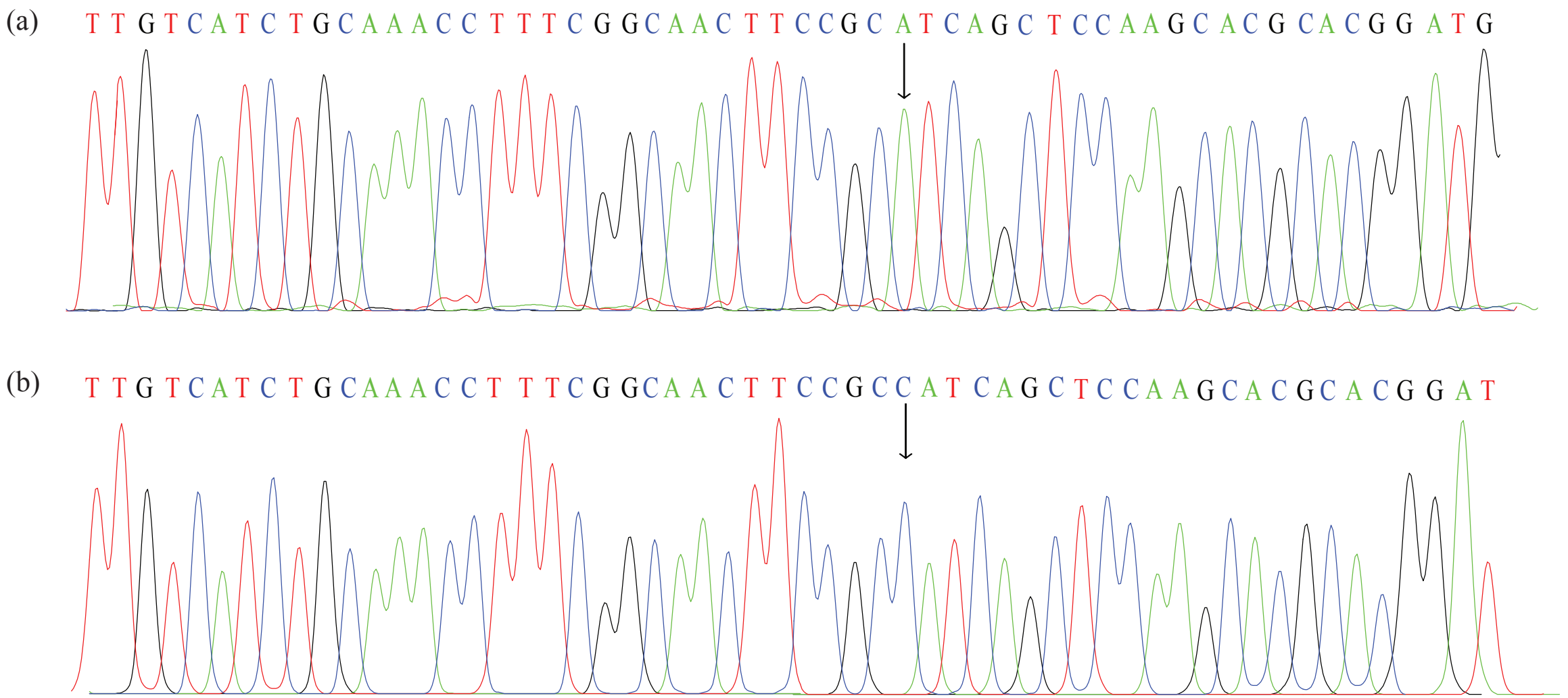

Figure S1. Sequencing chromatograms of the *SWS1* gene for *Pteronotus mesoamericanus*.
The arrow indicates the position where the 1-bp insertion occurs. (A) The intact allele cloned and sequenced; (B) The null-allele cloned and sequenced; (C) The 1-bp insertion in the heterozygous state confirmed by direct sequencing.

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                                                                         TM4
Homo sapiens                           GGCAACTTCC GC-TTCAGCT CCAAGCATGC ACTGACGGTG GTCCTGGCTA CCTGGACCAT TGGTATTGGC GTCTCCATCC CACCCTTCTT TGGCTGGAGC  519
Pteronotus davyi                       GGCAACTTCC GC-TTCAGCT CCAAGCACGC GCTGATGGTA GTCCTGGCCA CCTGGACCAT TGGTGTGGGC GTCTCCATCC CACCCTTCTT TGGCTGGAGC
P. mesoamericanus (intact allele)      GGCAACTTCC GC-ATCAGCT CCAAGCACGC ACGGATGGTA GTCCTGGCCA CCTGGACCAT TGGTATCGGC GTCTCCATCC CACCCTTCTT TGGCTGGAGC
P. mesoamericanus (null allele)        GGCAACTTCC GC[CAT]CAGCT CCAAGCACGC ACGGATGGTA GTCCTGGCCA CCTGGACCAT TGGTATCGGC GTCTCCATCC CACCCTTCTT TGGCTGGAGC

                                                                                                                      TM5
                                       CGGTTCATCC CTGAGGGCCT GCAGTGTTCC TGTGGCCCTG ACTGGTACAC CGTGGGCACC AAATACCGCA GCGAGTCCTA TACGTGGTTC CTCTTCATCT  619
                                       CGGTTCATCC CTGAAGGCCT GCAATGTTCC TGTGGCCCTG ACTGGTACAC CGTGGGCACC AAATACCGCA GCGAGTACTA TACCTGGTTC CTCTTCATCT
                                       CGGTTCATCC CCGAAGGCCT GCAATGTTCC TGTGGTCCTG ACTGGTACAC TGTGGGCACC AAATATCACA GCGAGTACTA TACCTGGTTC CTCTTCGTCT
                                       CGGTTCATCC CCGAAGGCCT GCAATGTTCC TGTGGTCC[TG A]CTGGTACAC CGTGGGCACC AAATATCACA GCGAGTACTA TACCTGGTTC CTCTTCGTCT

                                                                                                                                            719
                                       TCTGCTTCAT TGTGCCTCTC TCCCTCATCT GCTTCTCCTA CACTCAGCTG CTGAGGGCCC TGAAAGCTGT TGCAGCTCAG CAGCAGGAGT CAGCTACGAC
                                       TCTGCTTCAT CGTGCCTCTT TCCCTCATCT GCTTCTGCTA CTCTCAGCTG CTGCGGGCGC TCAGGGCTGT TGCAGCCCAG CAGCAGGAGT CAGCTTCCAC
                                       TCTGCTTCAT CGTGCCTCTT TCCCTCATCT GCTTCTGCTA CTCTCAGCTG CTGCGGGCGC TCAGGGCTGT TGCAGCCCAG CAGCAGGAGT CAGCTTCCAC
                                       TCTGCTTCAT CGTGCCTCTT TCCCTCATCT GCTTCTGCTA CTCTCAGCTG CTGCGGGCGC TCAGGGCTGT TGCAGCCCAG CAGCAGGAGT CAGCTTCCAC

                                                                                 TM6
                                       CCAGAAGGCT GAACGGGAGG TGAGCCGCAT GGTGGTTGTG ATGGTAGGAT CCTTCTGTGT CTGCTACGTG CCCTACGCGG CCTTCGCCAT GTACATGGTC  819
                                       CCAGAAGGCT GAGCGGGAGG TGAGCCGCGT GGTGGTGGTG ATGGTGGGCT CCTTCTGTCT CTGCTACGTG CCCTATGCTG CCCTGGCCAT GTATATGGTC
                                       CCAGAAGGCT GAGCGGGAGG TGAGCCGCAT GGTGGTGGCG ATGGTGGGAT CCTTCTGTCT CTGTTACGTG CCCTATGCTG CCCTGGCCAT GTATATGGTC
                                       CCAGAAGGC[T GA]GCGGGAGG TGAGCCGCAT GGTGGTGGCG ATGGTGGGAT CCTTCTGTCT CTGTTACGTG CCCTATGCTG CCCTGGCCAT GTATATGGTC

                                                                      TM7
                                       AACAACCGTA ACCATGGGCT GGACTTACGG CTTGTCACCA TTCCTTCATT CTTCTCCAAG AGTGCTTGCA TCTACAA  896
                                       AACCGCCGTA ACCACGGGCT GGACTTGCGG CTTGTCACCA TTCCTGCCTT CTTCTCCAAG AGCTCTTGCG TCTACAA
                                       AACAACCGTA ACCATGGGCT GGACTTGCAG CTCGTCACCA TTCCTGCCTT CTTCTCCAAG AGCTCTTGCG TCTACAA
                                       AACAACCG[TA A]CCATGGGCT GGACTTGCAG CTCGTCACCA TTCCTGCCTT CTTCTCCAAG AGCTCTTGCG TCTACAA
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Figure S2. Detailed alignment of *SWS1* sequences from *Pteronotus species*.
The 1-bp insertion and premature stop codons are boxed. Dashes are alignment gaps, question marks represent unamplified nucleotides, and numbers in the right indicate nucleotide positions following the reference sequence (*Homo sapiens*). Regions corresponding to transmembrane domains are indicated. Species indicated in bold font are high-duty-cycle echolocators.

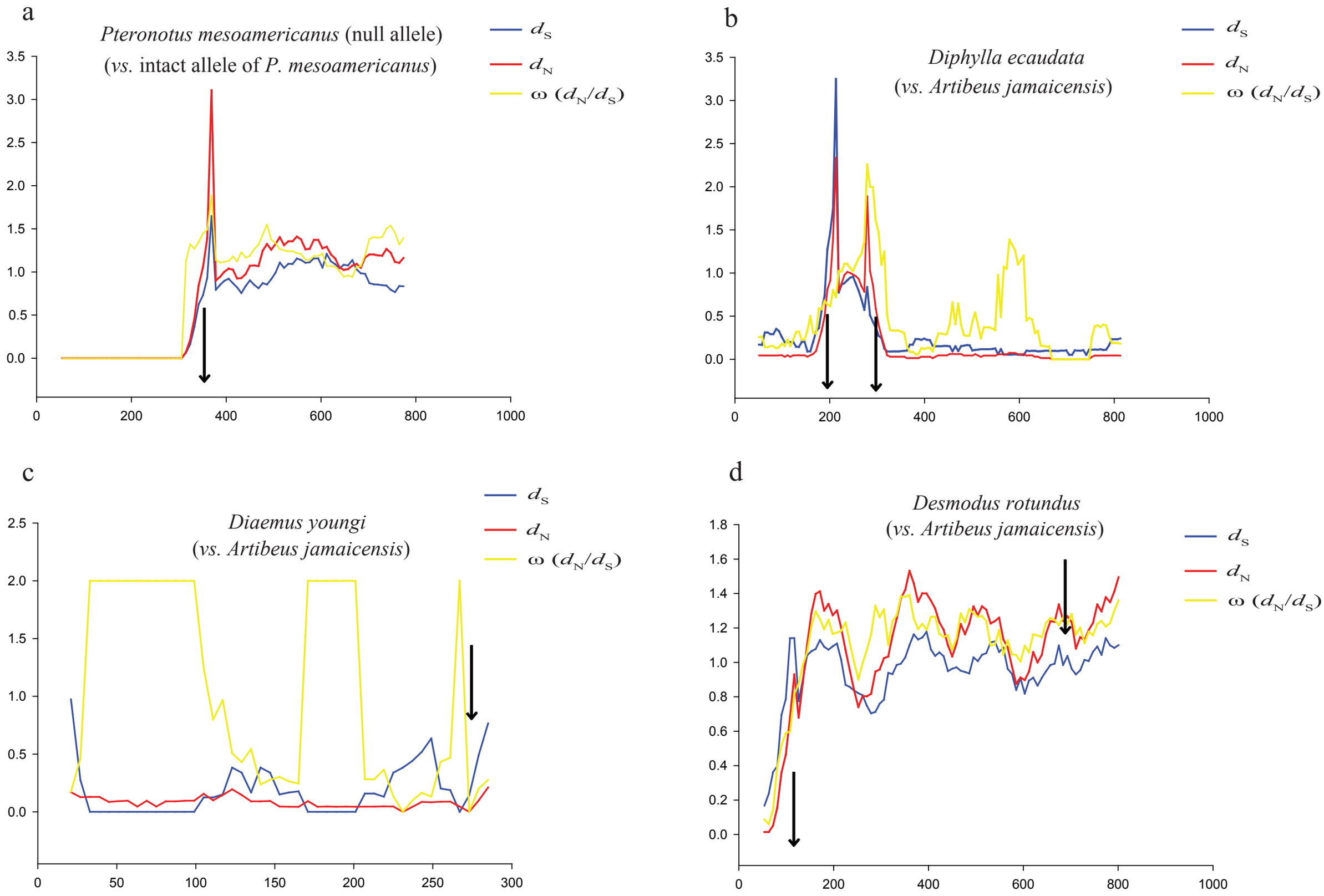

Figure S3. Sliding window analysis of evolutionary changes of the four *SWS1* sequences according to the alignments with their own reading frames.
This plot showed the *dS* (the synonymous substitution rate) in green line, the *dN* (nonsynonymous substitution rate) in red line, and the ω (the ratio of *dN*/*dS*) in yellow line.
The locations of frameshifting indels were indicated by an arrow.

Homo sapiens
Desmodus rotundus
Diaemus youngi
Diphylla ecaudata

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                                                                                                                    90
ATGAGAAAAA TGTCGGAGGA AGAGTTTTAT CTGTTCAAAA ATATCTCTTC AGTGGGGCCG TGGGATGGGC CTCAGTACCA CATTGCCCCT
ATG------T CAGGGGAGGA GGAGTTTTAT CTGTTTGAGA ACATCTCCTC GGTGGGACCA TGGGATGGGC CTCTGTACCA CATTGCCCCT
?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
ATG------T CAGGGGAGGA GGAGTTTTAT CTGTTCAAGA ACATCTCCTT GGTGGGACCG TGGGATGGGC CTCAGTACCA CATTGCCCTG
                                                TM1                                                 180
GTCTGGGCCT TCTACCTCCA GGCAGCTTTC ATGGGCACTG TCTTCCTTAT AGGGTTCCCA CTCAATGCCA TGGTGCTGGT GGCCACACTG
GTCTGGGCCT TCCACCTCCA GGCAGCCTTC ATGGGCTTTG TCTTCTTTGC AGGGATGCC- -TCAATGCCA TGGTGCTGGT GGCCA----
?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
GTCTGGGCCT TCCGCCTCCA GGCAGCCTTC ATGGGCTTTG TCTTCTTTGC AGGGACACCC CTCAATGCCA CGGTGCTGGT GGCCACACTG
                                                    TM2                                             269
CGCTACAAAA AGTTGCGGCA GCCCCTCAAC TACATTCTGG TCAACGTGTC CTTC-GGAGG CTTCCTCCTC TGCATCTTCT CTGTCTTCCC
------AAA AGCTGAGGCA GCCACTCAAC TACATTTTGA TCAGTGTGTC CCTG-GGGGG CTTCCTCTTC TGCATCTTCT CTGTCGCCAC
?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
CGCTACAGAA AGCTGAGGCG GCCACTCAAC TACATTTTGG TCAATGTGTC CCTGAGGGGG CTTCCTCTTT TGCATCTTCT CTGTCTCCAC
                                                                TM3                                 359
TGTCTTCGTC GCCAGCTGTA ACGGATACTT CGTCTTCGGT CGCCATGTTT GTGCTTTGGA GGGCTTCCTG GGCACTGTAG CAGGTCTGGT
TGTCTTCATT ACCAGTTGTC GGGGCTACTT CACCATCGGG CGCCGCATGT GTGCTTTGGA GGACTTCCTG GGCTCTACAG CAGGTCTGGT
?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
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                                                                                                    449
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CACAGGCTGG TCACTGGCCT TCCTGGCCTT TGAGCGATAC ATCGTCATCT GCAAACCCTT CGGCAGCTTC CGCTTCAGCT CCAGGCACGC
                                        TM4                                                         539
ACTGACGGTG GTCCTGGCTA CCTGGACCAT TGGTATTGGC GTCTCCATCC CACCCTTCTT TGGCTGGAGC CGGTTCATCC CTGAGGGCCT
ACTGTTGGTA GTCCTGGCCA CCTGGACCAT TGGCATCGGC GTCTCCATCC CACCCTTCTT TGGCTGGAGC CGGTTCATCC CCGAGGGCCT
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ACTGATGGTA GTCCTGACCA CCTGGACCAT TGGCATCGGC GTCTCCATCC CACTCTTCTT TGGCTGGAGC CGGTTCGTCC CCGAGGGCCT
                                                            TM5                                     629
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GCAATGTTCC TGTGGCCCCG ACTGGTACAC TGTGGGCACC AAATATCACA GCGAGTACCA CACCTGGTTC CTGTTCATCT TCTGCTTCAT
?????????? ?????????? ?????????? ?????????? AAATATCACA GTGAGTACTA CAGCTGGTTC CTCTTCATCT TCTGCTTCAT
GCAATGTTCC TGTGGCCCCG ACTGGTACAC CGTGGGCACC AAGTATCGCA GCGAGTACTA CACCTGGTTC CTCTTCATCT TCTGCTTCAT
                                                                                                    719
TGTGCCTCTC TCCCTCATCT GCTTCTCCTA CACTCAGCTG CTGAGGGCCC TGAAAGCTGT TGCAGCTCAG CAGCAGGAGT CAGCTACGAC
CGTGCATCTT TCCCTCATCT GCTTCTCCTA CTCTCAGCTG CTGGGGGCGC TCAGAGCTCT TGCAGCCCAG CAGCACGAGT CGGCTTCGAC
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CGTGCCTCTC TCCCTCATCT GCTTCTGCTA CTCTCAGCTG CTGGGGGCGC TCAGAGCTGT TGCAGCCCAG CAGCAGGAGT CAGCTTCGAC
                                                        TM6                                         809
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CCAGAAGGG- GAGCGGGAGG TGAGCCGCAT GGTGCTGGTG ATGGTGGGAT CCTTTTGTCT CTGTTACGTG CCCTATGCTG CCCTGGCCAT
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                                                                TM7                                 899
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GTATATGGTC AACCACCGTA GCCACGGGCT GGACTTCCAG CTGGTCACCA TTCCTGC--- CTTCTCCAAG AGTGCTTGTG TCTACAACCC
GTATATGGTC AACCACCGTA GCCACGGGCT GGACTTCCGG CTGGTCACCA T-CCTGCCTT CTTCTCCAA? ?????????? ??????????
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                                                                                                    989
CATCATCTAC TGCTTCATGA ATAAGCAGTT CCAAGCTTGC ATCATGAAGA TGGTGTGTGG GAAGGCCATG ACAGATGAAT CCGACACATG
CATCATCTAC TGCTTCATGA ATAAGCAGTT CTGGGCTTGC ATCACGGAGA TGGTGTGTGG GAAGTCCACG ACAGGTGAGT CTGACGTGTC
?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ?????????? ??????????
CATCATCTAC TGCTTCATGA ATAAGCAGTG CCAGGCTTGC ATCATGGAGA TGGTGTATGG GAAGTCCAGG ACAGAGGAGT CCGACGTGTC
                                                                                    1047
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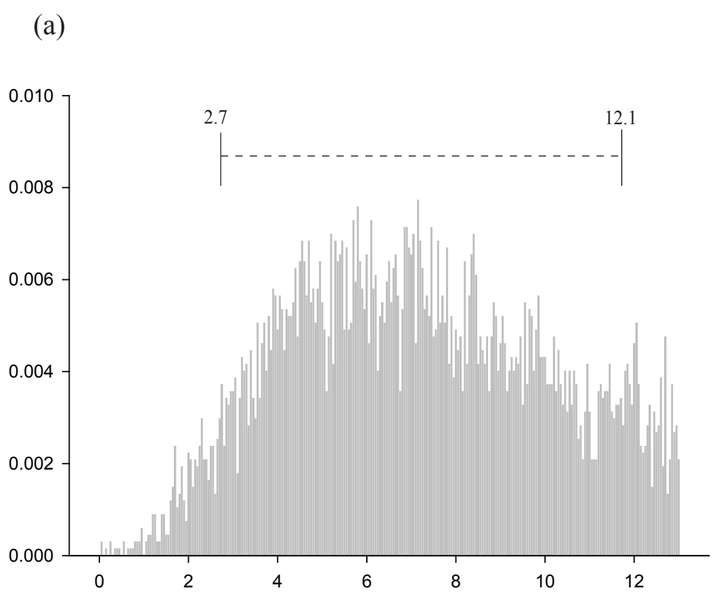
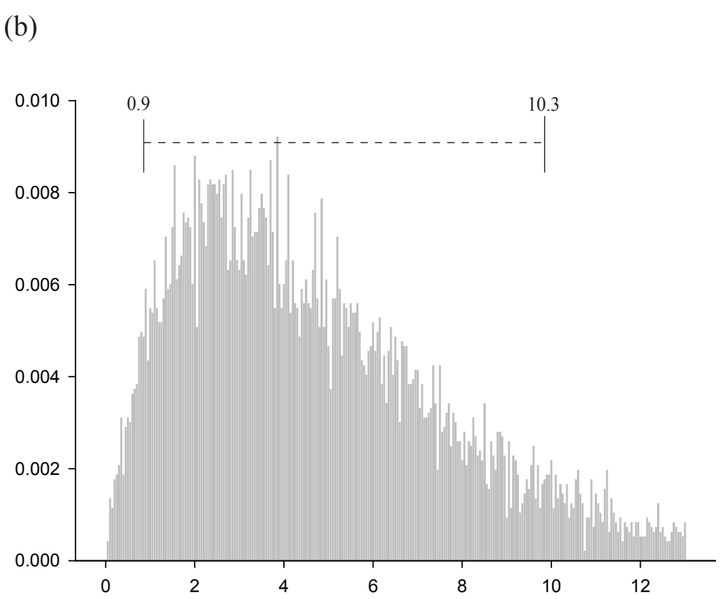
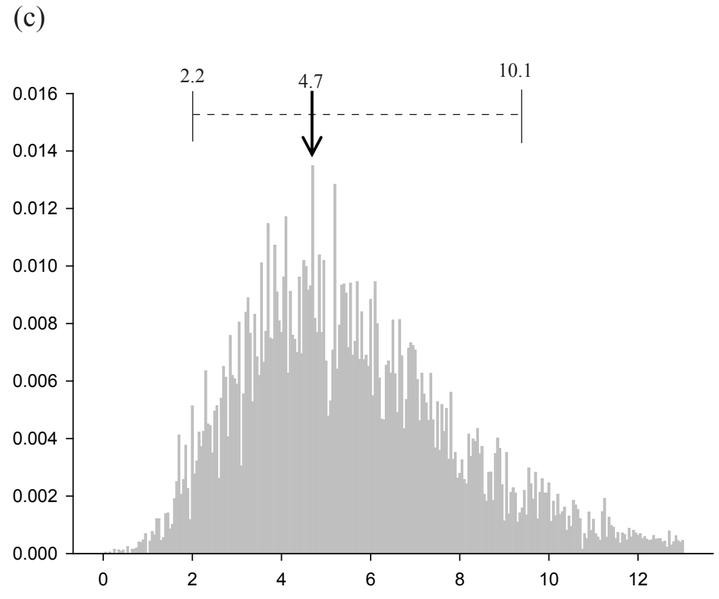
CAGTTCCCAG AGAACTGAAG TTTCTACTCT CTCTTCCAGC CAAGTTGGCC CCAGCTAA
?????????? ?????????? ?????????? ?????????? ?????????? ????????
CAGTTCCCAG AGAACTGAAG TTTCTACTCT CTC--CCAGC CAAGTTGGCC CCAGCTAA
```

Figure S4. Detailed alignment of *SWS1* sequences from vampire bats (Subfamily Desmodontinae). Insertions, deletions, and premature stop codons are boxed. Dashes are alignment gaps, question marks represent unamplified nucleotides, and numbers in the right indicate nucleotide positions following the reference sequence (*Homo sapiens*). All seven transmembrane domains are indicated.

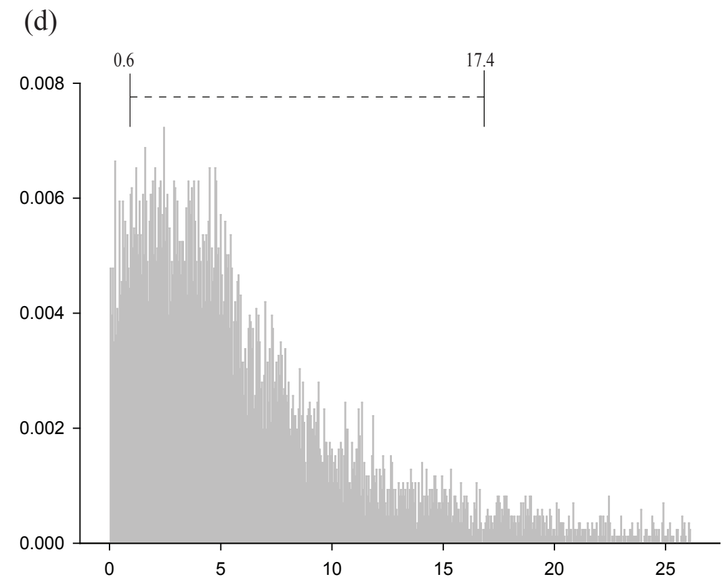
Desmodus rotundus

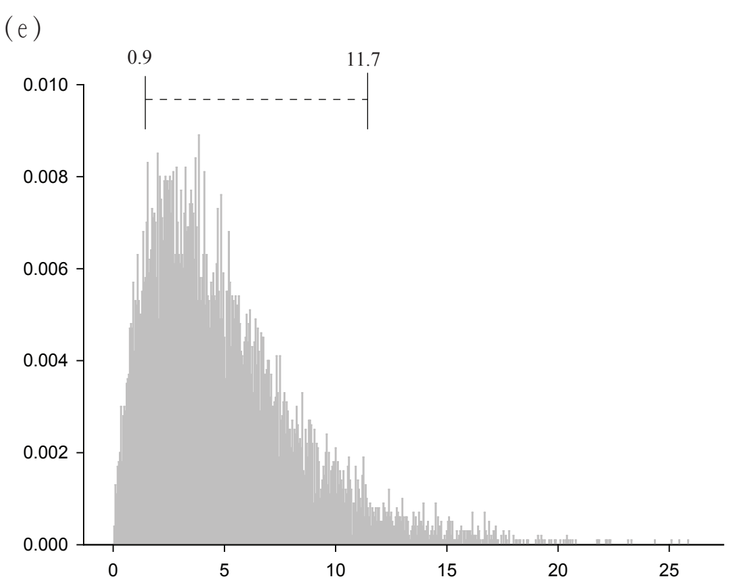


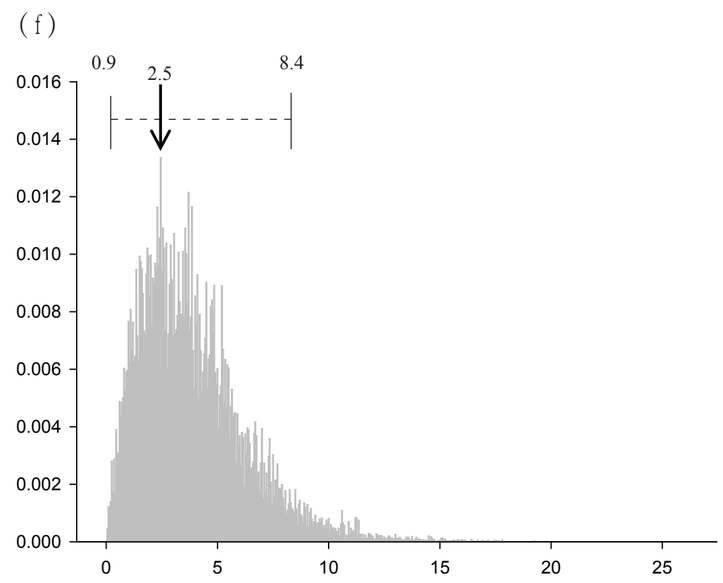




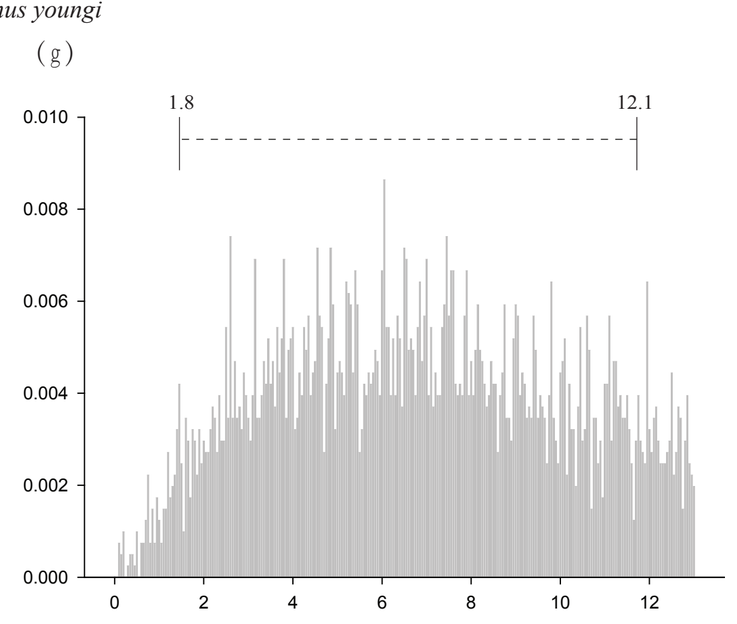
Diphylla ecaudata

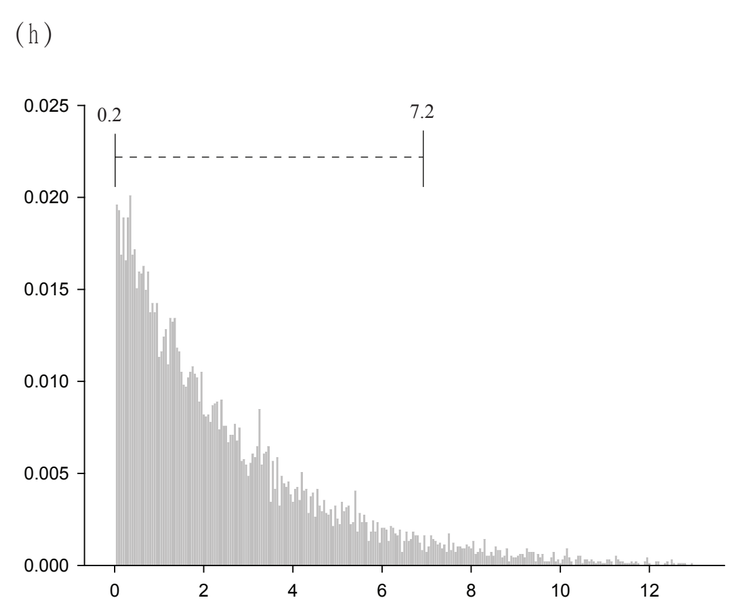


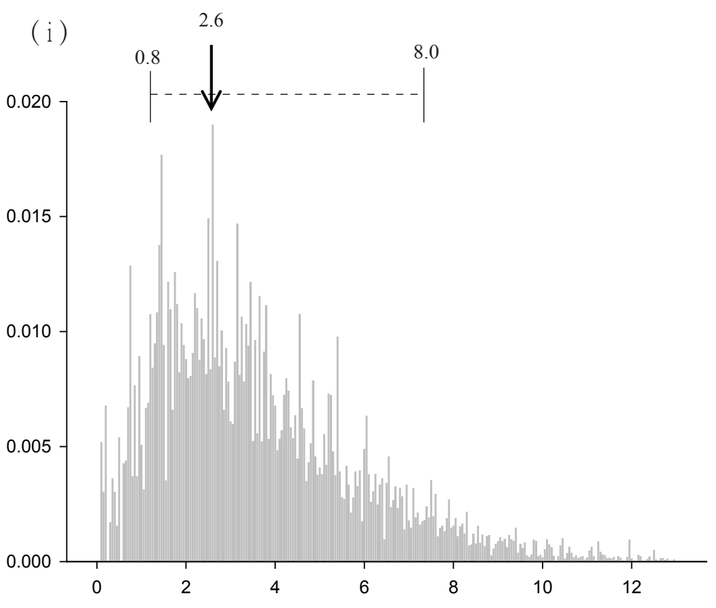




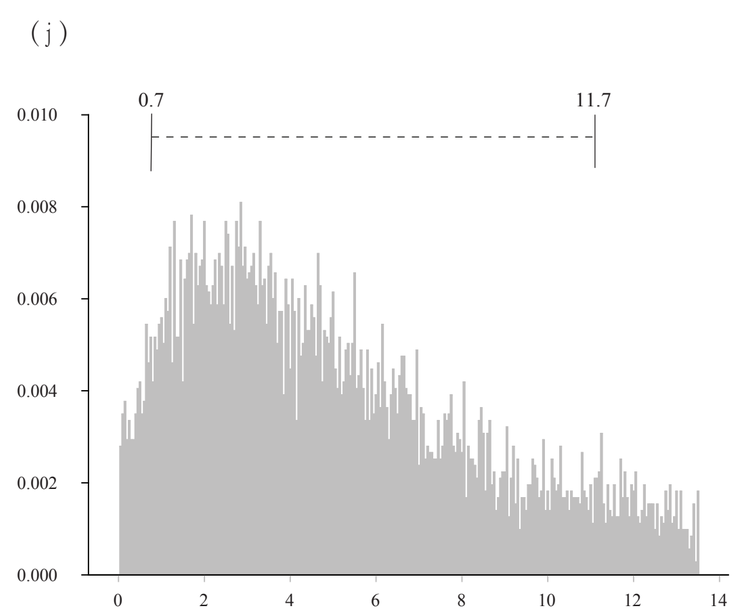
Diaemus youngi

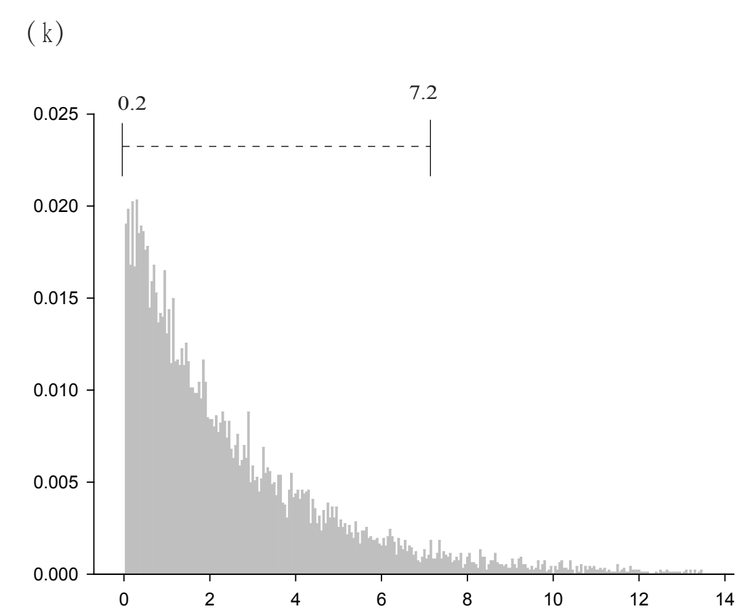






Pteronotus mesoamericanus（null allele）





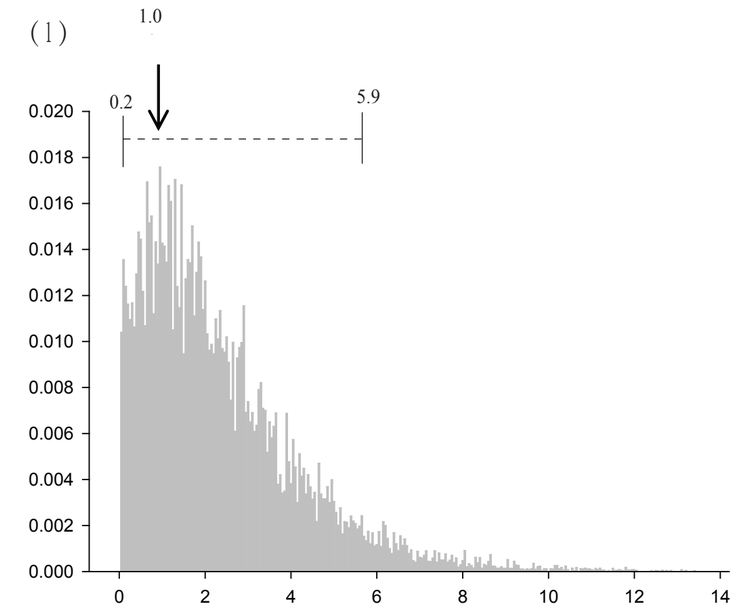


Figure S5. Posterior probability distributions of the time *t* when the relaxation of functional constraint on *SWS1* started. Posterior probability distribution of t based on the rate of synonymous substitution, the number of ORF-disrupting substitutions and both methods was shown for the common vampire bat (*Desmodus rotundus*) (a-c), the hairy-legged vampire bat (*Diphylla ecaudata*) (d-f), the white-winged vampire bat (*Diaemus youngi*) (g-i), and the *Pteronotus mesoamericanus* (j-l), respectively. The 95% confidence interval of t was indicated on the top of each panel, while the mode of *t* was shown only in four panels (c, f, i, and l).

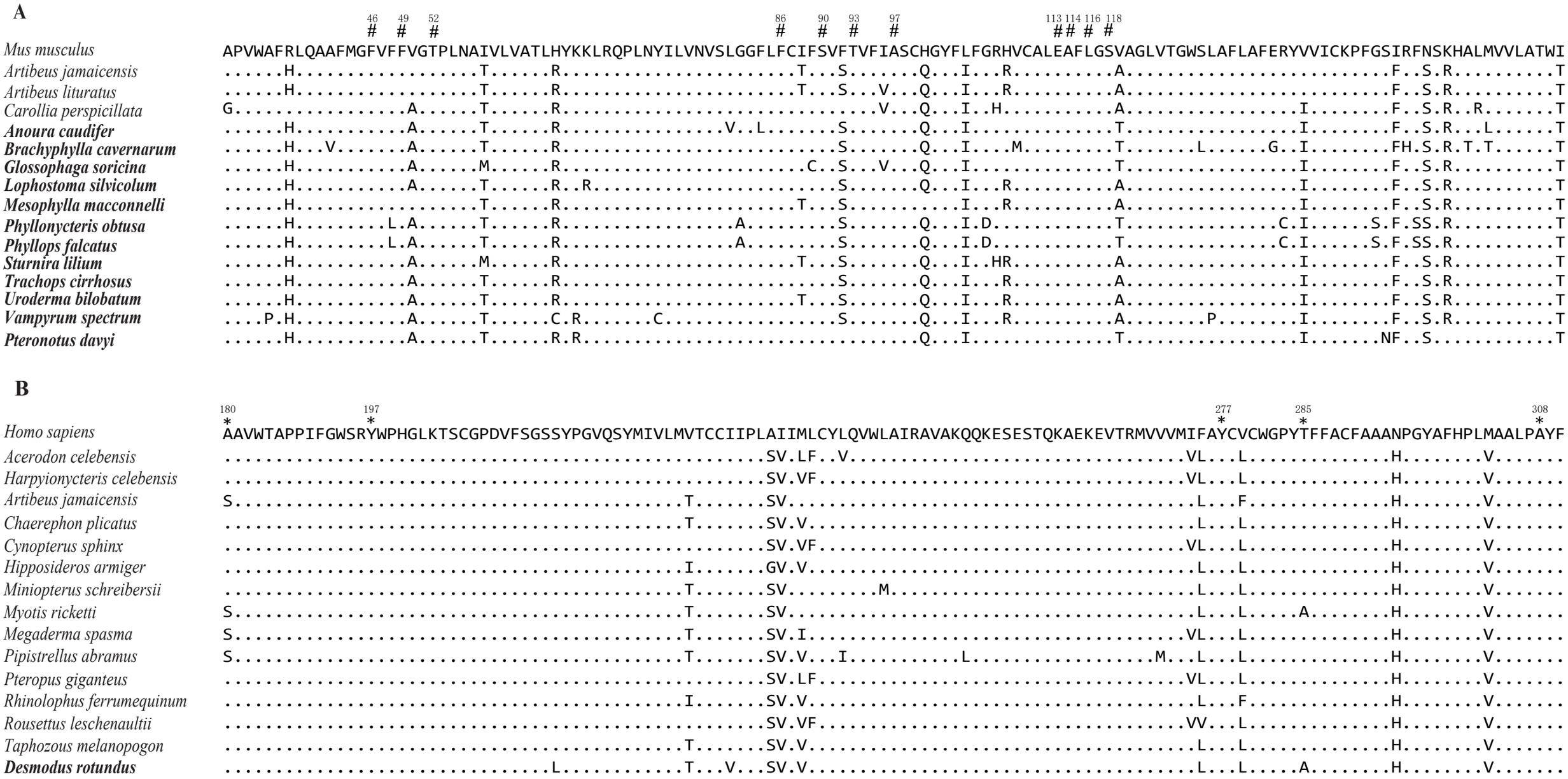


Figure S6. Alignments of amino acid sequences of cone opsins in the New World bats. Asterisks (*) indicate the 11 critical amino acid sites in the SWS1 opsin (a), and number signs (#) indicate the five key sites in the M/LWS opsin (b).

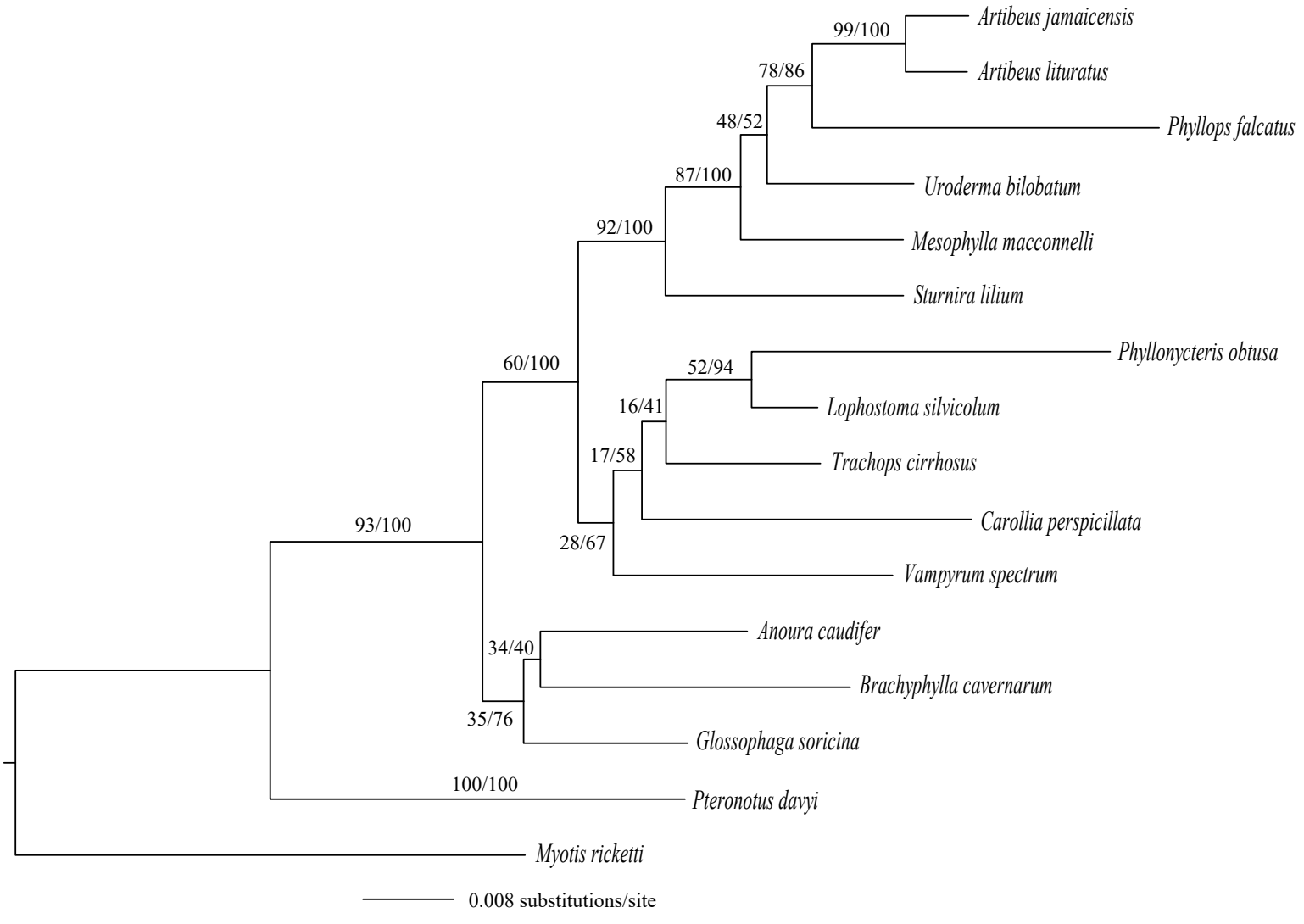


Figure S7. The maximum likelihood tree for the *SWS1* dataset of the New World bats. Numbers at the nodes are the ML bootstrap values/Bayesian posterior probabilities, shown as percentages.