Trehalase Gene as a Molecular Signature of Dietary Diversification in Mammals

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Abstract

Diet is a key factor in determining and structuring animal diversity and adaptive radiations. The mammalian fossil record preserves phenotypic evidence of many dietary shifts, whereas genetic changes followed by dietary diversification in mammals remain largely unknown. To test whether living mammals preserve molecular evidence of dietary shifts, we examined the trehalase gene (*Treh*), which encodes an enzyme capable of digesting trehalose from insect blood, in bats and other mammals with diverse diets. Bats represent the largest dietary radiation among all mammalian orders, with independent origins of frugivory, nectarivory, carnivory, omnivory, and even sanguivory in an otherwise insectivorous clade. We found that *Treh* has been inactivated in unrelated bat lineages that independently radiated into noninsectivorous niches. Consistently, purifying selection has been markedly relaxed in noninsectivorous bats compared with their insectivorous relatives. Enzymatic assays of intestinal trehalase in bats suggest that trehalase activity tends to be lost or markedly reduced in noninsectivorous bats compared with their insectivorous relatives. Furthermore, our survey of *Treh* in 119 mammal species, which represent a deeper evolutionary timeframe, additionally identified a number of other independent losses of *Treh* in noninsectivorous species, recapitulating the evolutionary pattern that we found in bats. These results document a molecular record of dietary diversification in mammals, and suggest that such molecular signatures of dietary shifts would help us understand both historical and modern changes of animal diets.

Key words: **trehalase, diet, pseudogenization, bats, mammals.**

Introduction

Diet has been thought to play a crucial role in shaping animal diversity since Darwin (1859). Living mammals are remarkably diverse in terms of diet, and lineages with distinct trophic strategies (i.e., herbivory, carnivory, or omnivory) exhibit striking differences in diversification rates (Price et al. 2012). The fossil record suggests that crown clade mammals originated in the Jurassic, with jaw and dental morphologies similar to living insectivorous species, and then radiated into a wide range of diverse dietary niches after the Cretaceous/Paleogene (K/Pg) boundary (Kemp 2005; Luo 2007; O'Leary et al. 2013). Among all mammalian orders, Chiroptera (bats) represents the largest dietary radiation, with independent origins of frugivory, nectarivory, carnivory, omnivory, and even sanguivory (Altringham 1996; Rojas et al. 2011; Dumont et al. 2012). Ancestral bats are thought to have been insectivorous based on fossil evidence and phylogenetic analyses (Simmons and Geisler 1998; Gunnell and Simmons 2005; Simmons et al. 2008).

The mammalian fossil record preserves phenotypic evidence of many dietary shifts. For example, dental microwear and morphology could be used to infer dietary preferences of fossil mammals (Walker et al. 1978; Ungar 1996, 1998; Wilson et al. 2012), because the shape and structure of teeth are generally suited to their natural diets. Moreover, numerous studies used the isotopic ratios of carbon and nitrogen of fossil bone and tooth collagen to predict ancient diets of mammals (Bocherens et al. 1994; Drucker and Bocherens 2004). In comparison, genetic changes followed by dietary diversification in mammals may have retained in their genomes. For example, taste receptor genes may act as a molecular signature of dietary shifts. Previous studies identified convergent pseudogenization of the umami taste receptor gene in the giant panda and red panda associated with shifts to a diet of bamboo, a genetic change that occurred presumably because these panda species do not need to use umami taste to perceive meat flavor as did their carnivorous ancestors (Zhao, Yang, et al. 2010; Hu et al. 2017). Likewise, vampire bats have lost many taste receptor genes accompanying their dietary shift from insectivory to sanguivory (Zhao, Zhou, et al. 2010; Hong and Zhao 2014). Additional examples were also observed, such as pseudogenization of the sweet

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taste receptor gene in some carnivorous mammals (Jiang et al. 2012), pseudogenization of the umami taste receptor gene in bats (Zhao et al. 2012) and pinnipeds (Sato and Wolsan 2012), and widespread losses of taste receptor genes in whales (Feng et al. 2014) and snakes (Emerling 2017), although some of these examples are more straightforward to link with dietary specialization than others (Zhao and Zhang 2012).

It appears that taste perception is sometimes evolutionarily reduced when mammals shift into a distinct, narrow dietary niche, most likely because some basic tastes are useless in selecting food items within the new niche. However, effective feeding requires more than just selecting and ingesting food items. After selecting appropriate food by taste, food must be digested into nutrients through a series of biochemical pathways. The evolution of digestive enzymes, which play key roles in liberating nutrients from food in the digestive system, may thus also be intimately associated with dietary shifts in mammals. For example, the chitinase genes (*CHIA*s), encoding enzymes capable of digesting insect exoskeletal chitin, were found to have three to five functional copies in species with a diet consisting almost entirely of invertebrates, in contrast to one or no *CHIA*s in species with a minimum amount of invertebrate consumption (Emerling et al. 2018). Regression analyses confirmed a positive correlation between the number of functional *CHIA*s in placental mammals and the abundance of invertebrates in their diets (Emerling et al. 2018), suggesting an important role of *CHIA*s in digesting insect exoskeletal chitin and promoting dietary diversification.

To test whether living mammals broadly exhibit molecular signatures of dietary shifts beyond those in taste perception genes and chitinase genes, we turn to examine the trehalase gene (*Treh*) across diverse lineages of mammals. Trehalase, a digestive enzyme (Karasov et al. 2011), is used by mammals for digestion of dietary trehalose (Dahlqvist and Thomson 1963). The substrate trehalose is the main blood sugar in insects, which use it as an energy source for their flight (Thompson 2003). Trehalose makes up approximately 7.2% of the average dry mass of an insect (Bell 1990), whereas only trace amounts are present in most plants (Grennan 2007) and none is detected in vertebrates (Arguelles 2014). Previous studies have shown that trehalase activity is largely reduced in bats that have few insects in their diets (Hernandez and Martinez del Rio 1992; Schondube et al. 2001), but little is known about genetic changes of *Treh* in bats and other mammals after dietary shifts from insectivorous to noninsectivorous dietary niches.

In this study, we separately examined *Treh* evolution in a data set of 26 bat species and a larger data set included 93 additional mammal species that also show remarkable dietary radiations from insectivorous to noninsectivorous niches. Using these data, we attempted to test two hypotheses. First, we hypothesized that *Treh* has undergone relaxed selection and become pseudogenes in unrelated lineages with noninsectivorous diets. The use of bats as the study group is particularly helpful to test this hypothesis because they represent the largest dietary radiation seen within a single mammalian order, and because several dietary habits (e.g., frugivory, nectarivory) evolved more than once in this clade (Altringham 1996; Rojas et al. 2011; Dumont et al. 2012). Second, we tested whether the evolutionary pattern of *Treh* in bats recapitulates patterns seen more broadly in mammals, which represent a deeper evolutionary timeframe.

Results

Collection of Bat *Treh* Sequences

We identified *Treh* genes through TBlastN searches against 16 publicly available bat genomes (supplementary table S1, Supplementary Material online). All identified sequences were divided into three categories: complete genes (referring to those with all 15 exons and a putative start and stop codon), partial genes (referring to those with at least 13 exons and an intact open reading frame [ORF]), and pseudogenes (referring to those with an interrupted ORF). We detected eight complete genes, four partial genes, and two pseudogenes through this process (supplementary table S1, Supplementary Material online). Due to either incomplete genome sequencing or poor genomic assembly, in one bat species we detected only one exon (*Megaderma lyra*), whereas in another we found abundant "N"s (i.e., unsequenced nucleotides) in the coding region (*Myotis lucifugus*) (supplementary table S1, Supplementary Material online); both of these were excluded from subsequent analysis. Although both complete and partial genes retain an intact ORF and are thus putatively functional, pseudogenes carry an interrupted ORF and are thus putatively nonfunctional. We further amplified and sequenced *Treh* genes from four bat species that have both genetic material and available genome sequences (supplementary tables S2 and S3, Supplementary Material online). Our newly acquired *Treh* sequences of *Eonycteris spelaea*, *Hipposideros armiger*, and *Desmodus rotundus* are consistent with those identified from their available genome sequences. We also sequenced exon 10 of *Treh* in *Myotis davidii* to replace the continuous "N"s in the identified sequence. We next generated *Treh* sequences of 12 bat species that do not have publicly available genome sequences, with different lengths that varied from four exons (438 nucleotides) to all 15 exons (1,746 nucleotides) (supplementary tables S2 and S3, Supplementary Material online). In sum, we assembled a total of 26 *Treh* sequences from representatives of most major lineages of bats with diverse diets, including ten species of insectivorous bats, nine species of frugivorous bats, two species of nectarivorous bats, two species of sanguivorous bats, one species of carnivorous bat, and two species of omnivorous bats (fig. 1).

Conservation of *Treh* in Insectivorous Bats

Consistent with previous observations in human, mouse, rat, and rabbit (Ruf et al. 1990; Ishihara et al. 1997; Oesterreicher et al. 1998; Oesterreicher et al. 2001), *Treh* is a single-copy gene in all bats examined in this study. We aligned *Treh* coding sequences from ten insectivorous bats and did not identify any inactivating mutations, indicative of strong functional constraint. Furthermore, we detected a conserved 14 amino acid domain (supplementary fig. S1, Supplementary

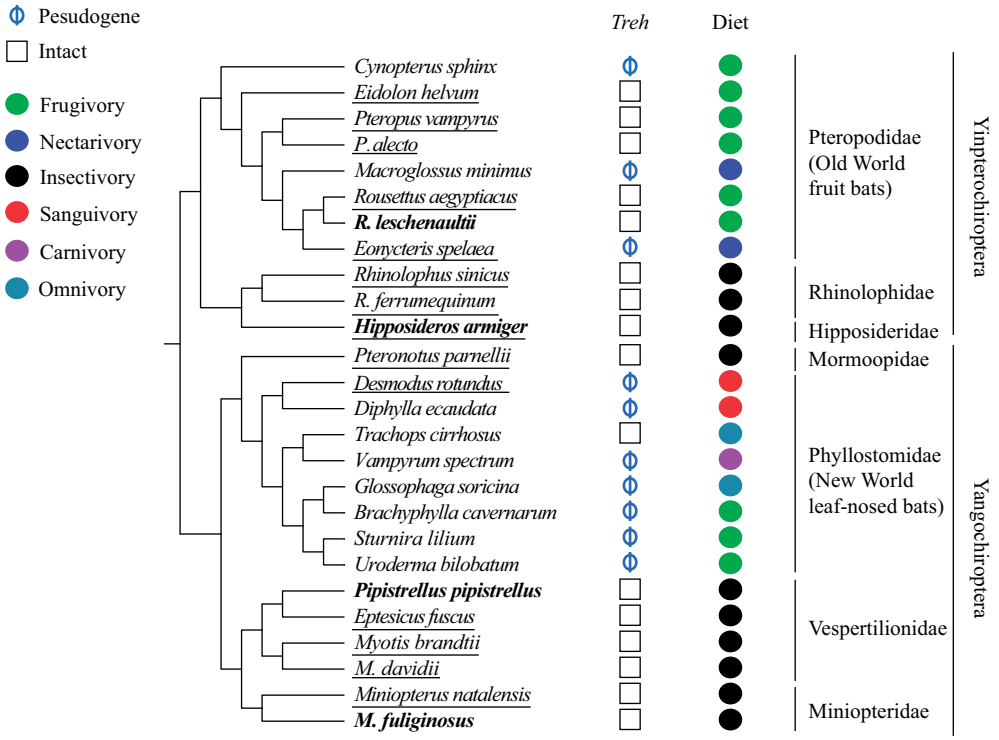

FIG. 1. Species tree depicting *Treh* evolution in 26 bat species. Topology of the tree is based on previous studies (Teeling et al. 2005; Miller-Butterworth et al. 2007; Roehrs et al. 2010; Almeida et al. 2011; Rojas et al. 2016). Fourteen species with available genome sequences are underlined and four species with RNA samples were shown in bold. Dietary preferences are indicated by colored circles.

Material online) that is believed to represent the signature sequence and catalytic site of trehalase (Henrissat and Bairoch 1993; Kopp et al. 1993; Oesterreicher et al. 2001). In addition, two critical amino acids of trehalase that are involved in the glycosyl hydrolysis (Henrissat and Bairoch 1993), 171E and 176D, were found to be identical among the insectivorous bats and two outgroup species in our data set (supplementary fig. S1, Supplementary Material online). To test whether *Treh* has undergone purifying selection in insectivorous bats, we conducted selection pressure analysis on the data set that included *Treh* sequences from all ten insectivorous bats (table 1). We estimated ω (i.e., nonsynonymous to synonymous substitution rate ratio) to infer the patterns of selection pressure: purifying selection ($\omega < 1$), neutral evolution ($\omega = 1$), and positive selection ($\omega > 1$) (Xu and Yang 2013). Under the assumption of a same ω for all lineages, ω was estimated to be 0.196 (model A in table 1), which is significantly less than 1 ($P = 4.98\text{E-}73$; see the comparison between models A and B in table 1). These findings unambiguously suggest that strong purifying selection and functional constraint have dominantly shaped *Treh* evolution in insectivorous bats.

Repeated Losses of *Treh* in Noninsectivorous Bats

In striking contrast to the conserved mode of evolution of *Treh* in insectivorous bats, the gene has undergone dramatic divergence and repeated pseudogenizations leading to loss of function in noninsectivorous bats (fig. 2). Here, we identified three types of inactivating mutations leading to loss of function: indels (referring to insertions or deletions that cause frameshifts and/or premature stop codons), nonsense mutations (referring to nucleotide changes that lead to premature stop codons and truncated proteins), and splice site mutations (referring to changes that lead to aberrant transcripts and production of nonfunctional proteins) (fig. 2). Of note, we were not able to recover all inactivating mutations for some bat species, because their *Treh* sequences identified from our genome searches were not complete. Our sample includes 16 noninsectivorous bats from 2 divergent families, Pteropodidae (Old World fruit bats) and Phyllostomidae (New World leaf-nosed bats) (fig. 1), which diverged by the early Eocene but contain the majority of noninsectivorous species among bats (Altringham 1996; Rojas et al. 2011; Dumont et al. 2012; Shi and Rabosky 2015; Amador et al. 2018). In the family Pteropodidae, where five frugivorous species retain a putatively functional *Treh* without inactivating mutations, two nectarivorous and one frugivorous species possess a pseudogenized *Treh* with one or multiple inactivating mutations (figs. 1 and 2). In the family Phyllostomidae, *Treh* is pseudogenized in all noninsectivorous bats in our samples except for one omnivorous species *Trachops cirrhosus* (figs. 1 and 2).

Despite the observation of many inactivating mutations in noninsectivorous bats, none of them are shared in any species pairs (fig. 2). Among the Old World fruit bats, *Cynopterus sphinx* has a 13-bp deletion in the end of exon 7. *Macroglossus minimus* has two 1-bp deletions and one 4-bp insertion in exon 7, and a splice site mutation in intron 7. *Eonycteris spelaea* has one 1-bp deletion in exon 8 (fig. 2). Among the New World leaf-nosed bats, the common vampire bat *D. rotundus* has two nonsense mutations, three 1-bp

Table 1. Selection Pressure Analysis on Bat *Treh* Genes.

Models	Number of Parameters	d_N/d_S (ω)			ln L[a]	Models Compared	2Δ(ln L)[b]	P-Value[c]
		ω_0	ω_1	ω_2				
Data set I: 11 sequences (all insectivorous bats)								
A. All branches have one uniform ω	**20**	**0.196**			**−4856.546**	**B vs. A**	**326.719**	**4.98E-73**
B. All branches have the same $\omega = 1$	**19**	**1.000**			**−5019.905**			
Data set II: 24 sequences (all but two fruit bats[d])								
C. All branches have the same ω	**48**	**0.271**			**−4246.387**	**C vs. D**	**29.523**	**5.53E-08**
D. Branches connecting noninsectivorous bats have ω_1 whereas other branches have ω_0	**49**	**0.187**	**0.492**		**−4231.626**			
E. Branches connecting noninsectivorous bats have $\omega_1 = 1$ whereas other branches have ω_0	**48**	**0.187**	**1.000**		**−4243.265**	**E vs. D**	**23.278**	**1.40E-06**
F. Branches connecting the six noninsectivorous bats with an intact *Treh* have ω_2, branches connecting other noninsectivorous bats with a pseudogenized *Treh* have ω_1, whereas the remaining branches have ω_0	**50**	**0.187**	**0.516**	**0.368**	**−4231.294**	**D vs. F**	**0.663**	**0.415**

[a]The natural logarithm of the likelihood value.
[b]Twice the difference in ln L between the two models compared.
[c]P-values were calculated by comparing the two models with a likelihood ratio test.
[d]*Treh* sequences generated from *Cynopterus sphinx* and *Macroglossus minimus* were excluded due to their short lengths.
P-values smaller than 0.05 are shown in bold.

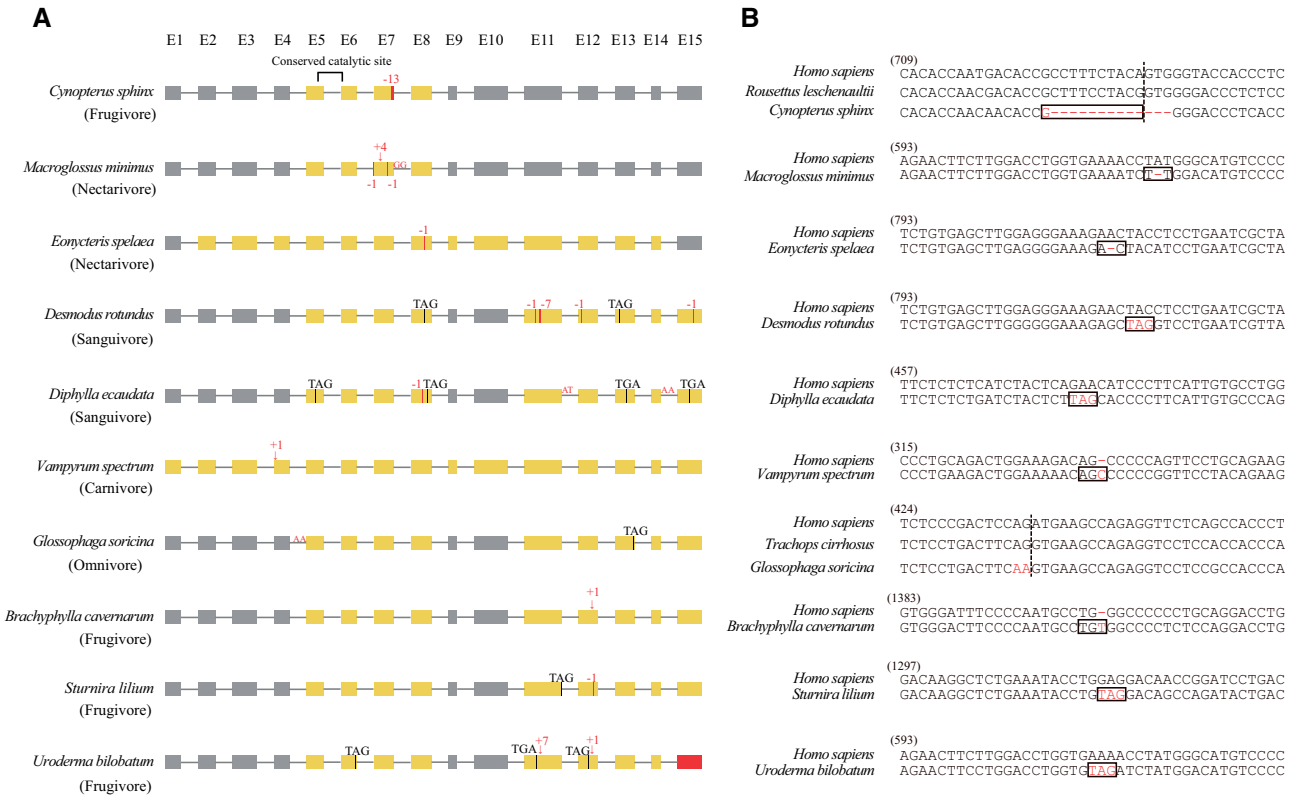

Fig. 2. Inactivating mutations of *Treh* in noninsectivorous bats. (*A*) Schematic showing locations of inactivating mutations. Exons (E1–E15) are shown as boxes (drawn to scale), and introns are shown as gray lines (not drawn to scale). Yellow boxes are exons that were amplified and sequenced in our study, whereas gray boxes are exons that were unamplified. The single red box indicates the exon with low sequence similarity due to numerous mutations. A red vertical line indicates a frameshifting deletion (the size of a deletion is given in base pairs with a minus sign), whereas a vertical arrowhead indicates a frameshifting insertion (the size of an insertion is given in base pairs with a plus sign). A black vertical line indicates a nonsense mutation, and splice site mutations are indicated by mutated dinucleotides with red color. The conserved catalytic site (also known as the trehalase signature) is indicated. (*B*) Nucleotide alignments with the first ORF-disrupting mutations boxed. Black dashed lines indicate exon–intron boundaries and splice site mutations. Dashes represent alignment gaps and numbers in parentheses indicate nucleotide positions following human *Treh*.

deletions and one 7-bp deletion. *Diphylla ecaudata* has four nonsense mutations, one 1-bp deletion and two splice site mutations. *Vampyrum spectrum* has one 1-bp insertion. *Glossophaga soricina* has one nonsense mutation and one splice site mutation. *Brachyphylla cavernarum* has one 1-bp insertion. *Sturnira lilium* has one nonsense mutation and one 1-bp deletion. *Uroderma bilobatum* has three nonsense mutations, one 1-bp insertion and one 7-bp insertion (fig. 2).

Molecular Evolutionary Analysis

To test whether *Treh* has undergone relaxation of functional constraint in noninsectivorous bats, most of which have a pseudogenized *Treh*, we estimated ω values along the bat phylogeny. We found that model D (table 1) allowing two different ω values for insectivorous and noninsectivorous lineages fits the data significantly better than the null model (model C in table 1) assuming a uniform ω value for all lineages ($P = 5.53E\text{-}08$, table 1). This test suggested that the ω value for noninsectivorous bats is significantly greater than that for insectivorous bats (ω_1=0.492, ω_0=0.187; table 1). Furthermore, model D yielded a significantly better fit than another model (model E in table 1) that assumes the ω value for noninsectivorous bats is equal to 1, suggesting that the ω value for noninsectivorous bats is significantly lower than 1 ($P = 1.40E\text{-}06$, table 1). These results indicated that *Treh* is generally under purifying selection but relaxation of functional constraint appears to have occurred in noninsectivorous bats. Given that six noninsectivorous bats (five Old World fruit bats and one New World omnivorous bat *T. cirrhosus*) in our sample have an intact *Treh* (fig. 1), we tested whether selective pressure on the six noninsectivorous bats is significantly different from that on other noninsectivorous bats (model F in table 1). This test showed that ω for the six noninsectivorous bats is not significantly different from that for other noninsectivorous bats with an intact *Treh* ($P = 0.415$, table 1), indicating similar levels of selection pressure on all noninsectivorous bats. Furthermore, we ran the RELAX program (Wertheim et al. 2015) by setting insectivorous lineages as reference branches and noninsectivorous lineages as test branches. The noninsectivorous lineages include six bat species with an intact *Treh* and eight bat species with a pseudogenized *Treh*. Our results showed that noninsectivorous lineages are under relaxed selection relative to insectivorous lineages after comparing the alternative model with the null model ($K = 0.427$, $P = 0.006$, supplementary table S4, Supplementary Material online). The partitioned exploratory model also supports this view ($P = 0.003$, supplementary table S4, Supplementary Material online).

Trehalase Activity Assays

To compare functional differences in trehalase among bats, we performed enzymatic assays of intestinal trehalase for the three Old World bat species (suborder Yinpterochiroptera), including two frugivorous bats (*Rousettus leschenaultii* and *C. sphinx*) and one insectivorous bat (*H. armiger*) (fig. 3*A* and *B*). We found that the three species show distinct patterns of intestinal trehalase activity, with the insectivorous bat showing significantly higher intestinal trehalase activity than the two frugivorous species ($P < 0.001$, one-way ANOVA, fig. 3*A* and *B*). In the two frugivorous species, their intestinal trehalase activities are significantly different ($P < 0.001$, one-way ANOVA, fig. 3*A*), although we did not estimate the difference in enzyme concentration. Specifically, *R. leschenaultii* showed a relatively weak activity of trehalose hydrolysis, whereas *C. sphinx* showed essentially no activity (fig. 3*A*), suggesting that the complete and intact *Treh* in the former species may have retained some level of residual function but the pseudogene in the latter has lost the function (figs. 1 and 3). Consistent with observations in the Old World bats, after examining 16 species of New World bats (suborder Yangochiroptera) with various diets such as insectivory, nectarivory, frugivory, omnivory, and sanguivory, a clear evolutionary pattern was also revealed that dietary shifts from insectivory to noninsectivory were accompanied by decreased trehalase activity (fig. 3*C*) (Schondube et al. 2001). Specifically, one sanguivore, three nectarivores, six frugivores, and one omnivore showed no or trace-level trehalase activity, whereas one omnivore and four insectivores showed a substantially high trehalase activity (Schondube et al. 2001). Thus, this and a previous study revealed a similar evolutionary pattern in both suborders of bats.

Widespread Losses of *Treh* in Noninsectivorous Mammals

To obtain a broad picture of *Treh* evolution in mammals, we identified *Treh* sequences from 107 mammal species with available genome assemblies, of which the 14 bat species mentioned above were included (fig. 4, supplementary fig. S2 and table S1, Supplementary Material online). We found that although most mammals retain an intact and putatively functional *Treh*, widespread losses of *Treh* were detected in noninsectivorous mammal lineages (fig. 4). We detected inactivating mutations leading to loss of function including frameshifting indels, nonsense mutations, and splice site mutations in 42 species from 9 mammalian orders: Primates, Rodentia, Perissodactyla, Eulipotyphla, Pilosa, Hyracoidea, Carnivora, Cetartiodactyla, and Chiroptera (mentioned above) (figs. 4 and 5). However, shared inactivating mutations are rare, occurring in only three lineages: all whales shared one such mutation, and two Old World monkeys and two artiodactyls each shared different inactivating mutations (figs. 4 and 5). With one exception, all mammals with inactivating mutations are all noninsectivorous, including taxa evolved to be carnivores or piscivores (cat, sea otter, pinnipeds, and whales) or herbivores (giant panda, sloth, rock hyrax, two rodents, two rhinoceroses, two Old World monkeys, and seven artiodactyls) (figs. 4 and 5). The only exception in our sample was the insectivorous species *Sorex araneus*, which has a pseudogenized allele that resulted from 1-bp deletion in exon 12 (fig. 5, supplementary fig. S3, Supplementary Material online), whereas its close relatives *Erinaceus europaeus* and *Condylura cristata* retain intact for both alleles (supplementary fig. S2, Supplementary Material online).

To examine whether the percentage of insects in the diet predicts loss or retention of *Treh*, we performed phylogenetic logistic regression analyses for binary dependent variables using two methods (fig. 6 and supplementary table S5,

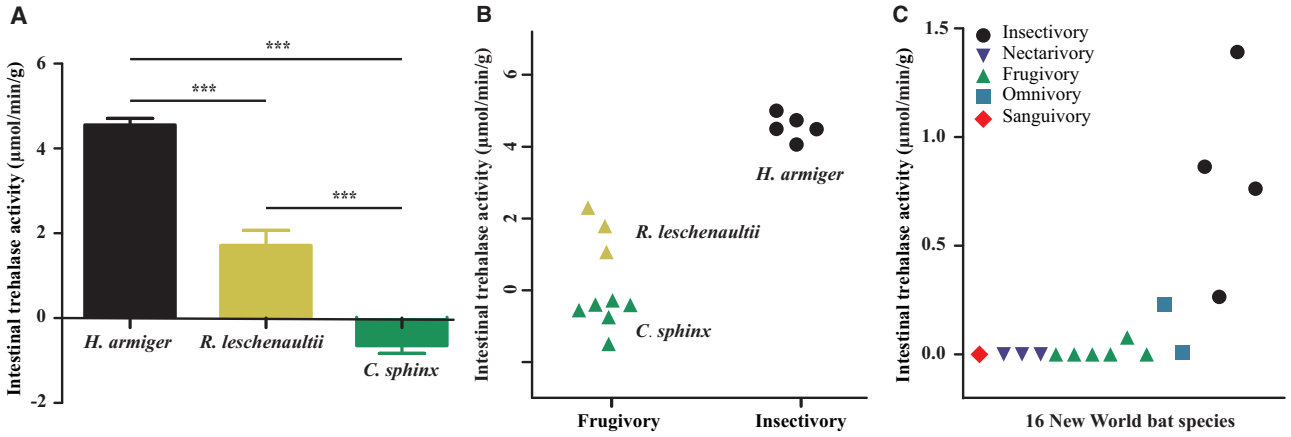

FIG. 3. Intestinal trehalase activity in 19 bat species. (*A*) Pairwise comparisons among three species of Old World bats examined in this study. *Rousettus leschenaultii* and *Cynopterus sphinx* are frugivorous, whereas *Hipposideros armiger* is insectivorous. All values are shown as means ± SE (***$P < 0.001$, one-way ANOVA). (*B*) Trehalase activity in 14 bat individuals examined in this study. A circle or a triangle represents one individual. (*C*) Trehalase activity in 16 species of New World bats examined in a previous study (Schondube et al. 2001). Dietary preferences are indicated by various colors as noted.

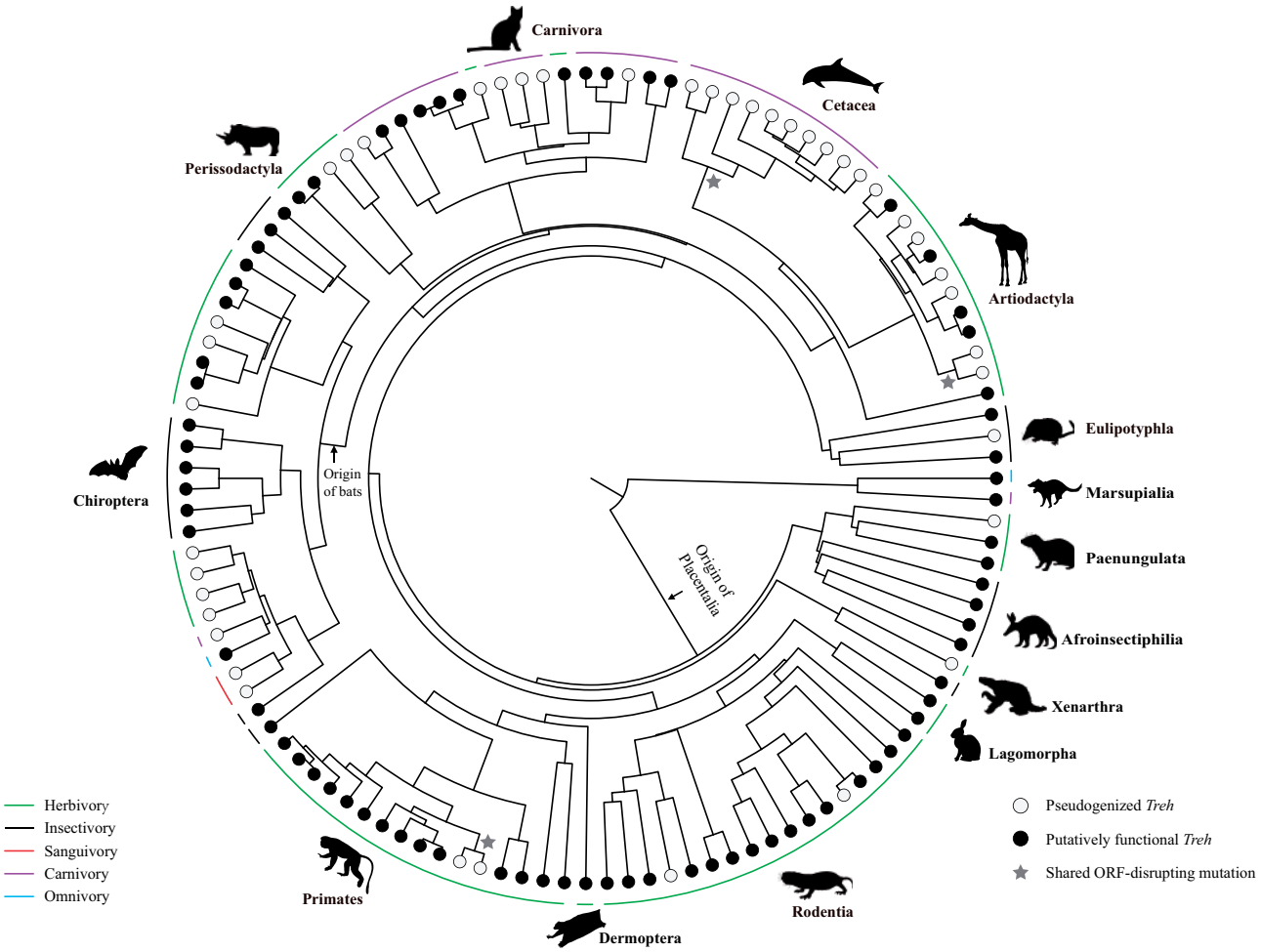

FIG. 4. Phylogenetic tree of mammals showing species with a pseudogenized *Treh*. Tree topology is based on multiple recent studies of mammalian phylogeny (see Materials and Methods). Dietary preferences were indicated with various colors as indicated. Silhouettes of mammals were taken from phylopic.org. This tree is simplified from supplementary figure S2 (Supplementary Material online).

Supplementary Material online) (Ives and Garland 2010; Ho and Ane 2014). Our results recovered a significant positive correlation between the percentage of insects in the diet and the retention of *Treh* (b1 = 0.044, $P = 6.19E-04$ for logistic_MPLE method; b1 = 0.048, $P = 2.06E-03$ for logistic_IG10 method, fig.6 and supplementary table S5,

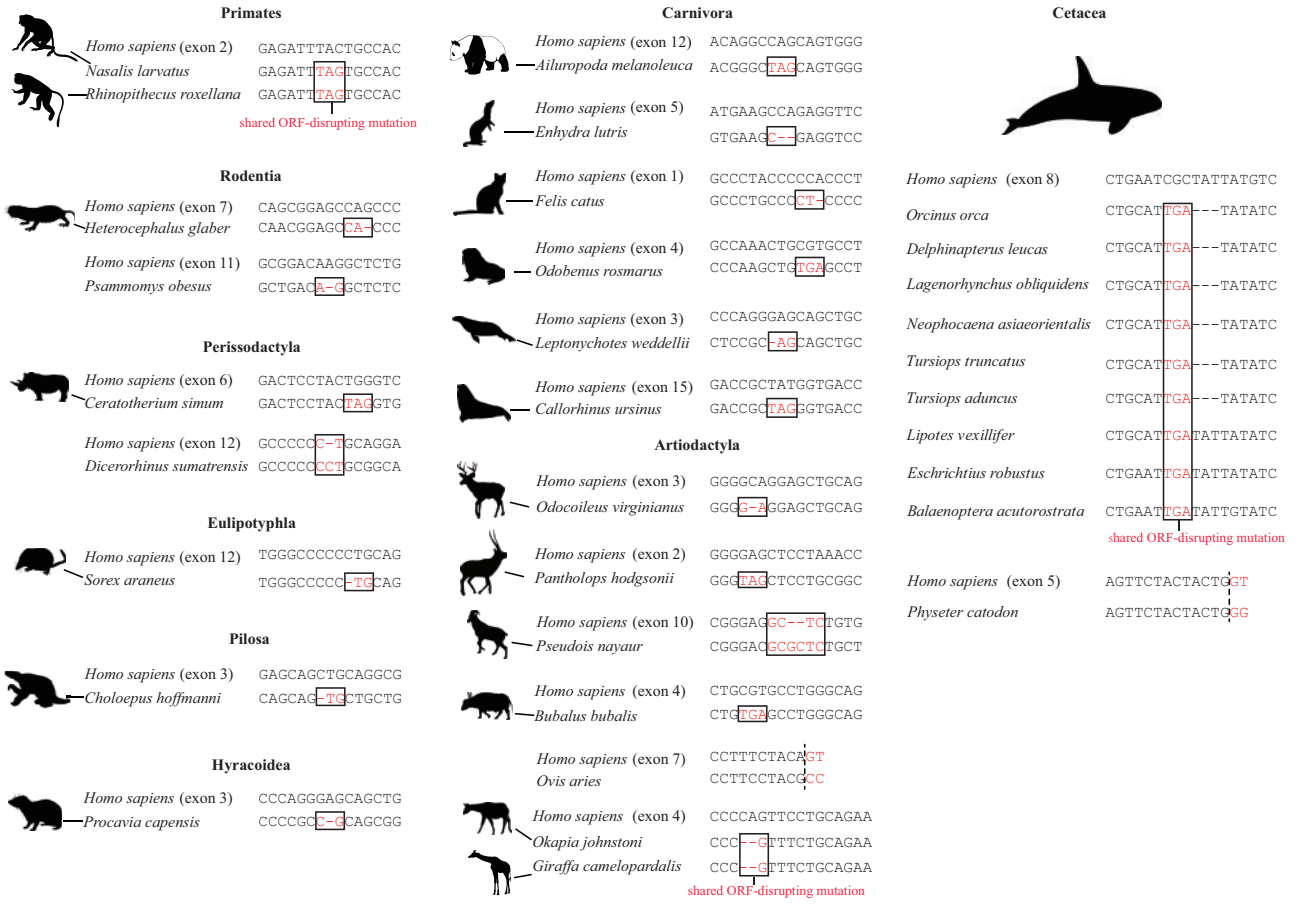

Fig. 5. Alignments of *Treh* pseudogenes in nonchiropteran mammals. Each pseudogene is aligned with human functional *Treh*. The first inactivating mutations are shown. Boxes indicate ORF-disrupting mutations, whereas black dashed lines indicate exon–intron boundaries and splice site mutations. Shared mutations were noted in the figure. All silhouettes of mammals were taken from phylopic.org (last accessed March 23, 2019) except the silhouette of giant panda that was obtained from http://www.supercoloring.com/silhouettes/giant-panda (last accessed March 23, 2019).

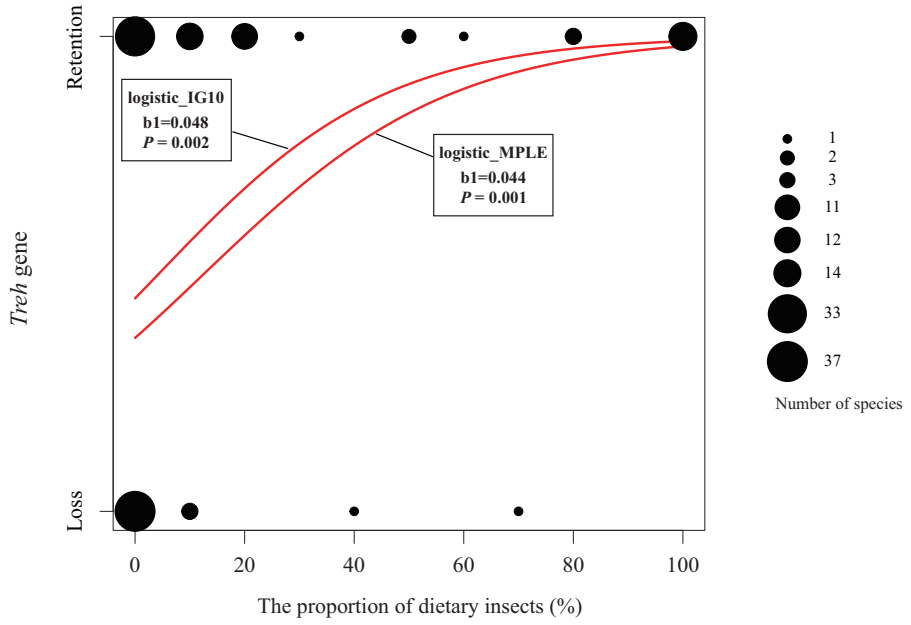

Fig. 6. Phylogenetic logistic regression analyses. Two methods (logistic_IG10 and logistic_MPLE) were used to evaluate the effect of the proportion of dietary insects on the retention/loss of *Treh* gene across mammals ($n = 119$).

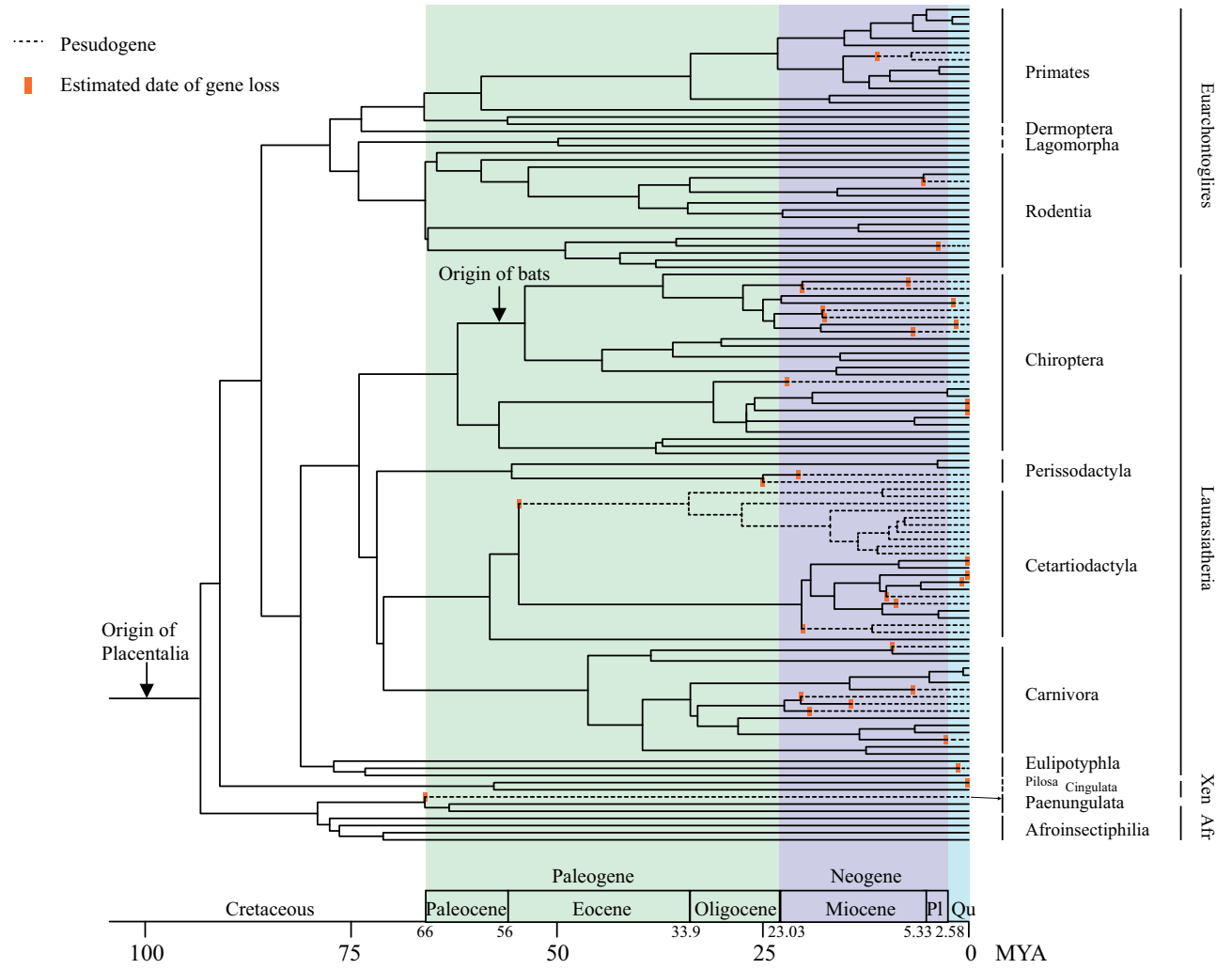

FIG. 7. Estimates of the timing of *Treh* losses along the mammalian phylogeny. An orange rectangle indicates the timing of each *Treh* loss (see Materials and Methods for details of analysis and dating methods). An arrow indicates that a species of Paenungulata (hyrax) has a pseudogenized *Treh*. Abbreviations: Xen, Xenarthra; Afr, Afrotheria; Pl, Pliocene; Qu, Quaternary.

Supplementary Material online), suggesting that low insect consumption may have resulted in the loss of *Treh*.

To date the loss of *Treh* along the mammalian phylogeny, we followed methods from a previous study (Meredith et al. 2009) (see Materials and Methods for details). We identified *Treh* losses in 42 of 119 mammalian species (107 species with available genomes plus 12 additional bat species with a newly sequenced *Treh*) (fig. 4). Due to the observation of apparently ancestral losses in three lineages (all ten cetaceans, two Old World monkeys, and two artiodactyls; figs. 4 and 5), we counted one *Treh* loss for each of these three lineages. Thus, a total of 31 independent losses were detected in our mammalian survey of *Treh* (figs. 4 and 7). Our estimates of the timing of 31 independent losses showed that the earliest *Treh* pseudogene appears to have occurred in the rock hyrax lineage (fig. 7). This must be an early loss, because the ω value for this branch is greater than 1 (supplementary table S6, Supplementary Material online). Of the remaining 30 *Treh* losses, 2 occurred in the Paleogene (23.03–66 Ma), 17 in the Miocene (5.33–23.03 Ma), 2 in the Pliocene (2.58–5.33 Ma), and 9 in the Quaternary (0–2.58 Ma) (fig. 7) (Cohen et al. 2013). As a result, the majority of *Treh* losses (~90%) apparently took place in the Neogene and the Quaternary (0–22.05 Ma), some quite recently, whereas only three losses can be dated back to more ancient time periods (25.00–66.04 Ma) (fig. 7). Of note, the dating method used in this study assumed that ω values for branches with a pseudogene should range from 0 to 1, our estimates of the timing would not be precise if ω values were inferred to be greater than 1 for those branches (supplementary table S6, Supplementary Material online).

Discussion

Animal diversification is often accompanied by dietary diversification. Diet typically imposes strong selective constraints on phenotypic traits, hence we hypothesized that dietary shifts and adaptive radiations involving dietary changes may also leave molecular records in the genome. In this work, we separately studied *Treh* evolution in 26 bats and a larger data set of 119 mammal species which show parallel dietary radiations from insectivorous to noninsectivorous niches, but which have contrasting depths of evolutionary timeframes.

Our study identified widespread convergent losses of *Treh* in bat and other mammal lineages as they independently radiated into noninsectivorous niches. Selection pressure analyses and enzymatic assays support our hypothesis that functional constraint of *Treh* has been relaxed during dietary shifts from insectivorous to noninsectivorous lifestyles in diverse mammalian clades.

Prior studies have revealed that dietary shifts in fish, birds, and mammals have been accompanied by adaptive shifts in digestive enzyme activities (Martínez del Rio 1990; Holbrook et al. 2000; Schondube et al. 2001; Birdsey et al. 2004; German et al. 2010; Liu et al. 2012), suggesting a close relationship between dietary changes and functional differences of digestive enzymes. By contrast, genetic changes of digestive enzyme genes underlying dietary changes in mammals remain understudied. Trehalase is one such enzyme, whose evolution had not previously been adequately addressed in molecular evolutionary analysis, possibly because trehalase is not the main component of dietary carbohydrates in most mammals. Our study provides the first molecular evidence for repeated losses of the trehalase gene (*Treh*) in bats and other mammals (fig. 4). Bats diversified in the early Eocene, whereas most placental mammals diverged in the late Cretaceous (Bininda-Emonds et al. 2007; Foley et al. 2016); the two groups accordingly represent somewhat different evolutionary timeframes. Despite widespread losses of *Treh* in noninsectivorous species, the most parsimonious inference is that *Treh* was intact and functional in ancestral bats and the most recent common ancestor of placental mammals, which is consistent with their insectivorous diets predicted by fossil evidence (Kemp 2005; Simmons et al. 2008). Following dietary radiations into noninsectivorous niches, mammals would not need trehalase to digest dietary trehalose, which is the principal sugar in the blood of most insects (Thompson 2003). As a result, the *Treh* gene encoding trehalase would have undergone relaxation of functional constraint, which has led to *Treh* pseudogenization in many noninsectivorous species (fig. 4). Note that there are three noninsectivorous species (*G. soricina*, *T. cirrhosis*, and *Rattus norvegicus*) consuming a considerable proportion of insects ($\geq$30%) (supplementary table S7, Supplementary Material online), of which two species (*T. cirrhosus* and *Ra. norvegicus*) have an intact *Treh* that may be used to digest dietary trehalose.

Chitinase genes (*CHIA*s), encoding another class of digestive enzymes capable of digesting insect exoskeletal chitin, were also found to have repeatedly lost in unrelated mammal lineages as they radiated into noninsectivorous niches. Chitinases and the trehalase may represent two complementary factors that have shaped the mammalian evolution from insectivorous to noninsectivorous niches. Indeed, species with a pseudogenized *Treh* generally have zero or one copy of *CHIA*, such as *Heterocephalus glaber*, *Nasalis larvatus*, *Ovis aries*, *Tursiops truncatus*, and *Odobenus rosmarus* (supplementary table S8, Supplementary Material online). The inactivation of both *Treh* and most (if not all) *CHIA*s suggests that genetic changes followed by dietary transitions from insectivory to noninsectivory may have been nearly completed in these species. We also found that some species with zero *CHIA* have a complete *Treh*, such as *Equus asinus*, *Ursus maritimus*, *Loxodonta africana*, and *Trichechus manatus* (supplementary table S8, Supplementary Material online), suggesting that functional relaxation on *CHIA*s occurred earlier than that on *Treh*. We did not find any species with three to five copies of *CHIA*s to have a pseudogenized *Treh* (supplementary table S8, Supplementary Material online), suggesting that both *Treh* and *CHIA*s are important in insectivorous species. In contrast to chitinase genes, which showed many inactivating mutations shared between multiples species from several mammalian clades (Emerling et al. 2018), we here observed only one large clade (Cetacea) with shared inactivating mutations (figs. 4 and 5). Thus, the majority of inactivating mutations in *Treh* evolved independently, suggesting that these mutations were relatively recent. Indeed, our dating of *Treh* losses indicates that most pseudogenization events (~90%) were younger than 22.05 Ma (Neogene and Quaternary) (fig.7), whereas most losses of chitinase genes have been dated back to 34–67 Ma (Paleocene and Eocene) (Emerling et al. 2018). This disparity between *Treh* and chitinase genes suggests that the losses of some chitinase genes could be partially compensated by other gene copies (Emerling et al. 2018), whereas *Treh* is a single-copy gene (Ishihara et al. 1997; Oesterreicher et al. 2001). Note that some nonoverlapping fragments may represent a distinct locus in our initial genome searches, but all the fragments are too short to make a good alignment and most of them contain inactivating mutations that have resulted in loss of function (supplementary table S9, Supplementary Material online), which suggested that these fragments are unlikely to form an additional copy of functional trehalase gene.

Although the patterns of *Treh* functionality (loss or retention) are largely correlated with dietary changes in mammals (fig.6 and supplementary table S5, Supplementary Material online), mismatches between *Treh* functionality and diets are still present. First, most mammalian orders have at least some noninsectivorous species carrying an intact and complete *Treh* (fig. 4 and supplementary fig. S2, Supplementary Material online). When a species does not typically consume insects, its *Treh* is expected to be under relaxed selection. However, the relaxation of functional constraint on *Treh* may take a long time to generate a pseudogene. Alternatively, *Treh* may have other functions and thus may not be readily disabled in some species. For instance, the flying lemur (*Galeopterus variegatus*) is folivorous and retains *Treh*, whereas two folivorous colobine monkeys (*N. larvatus* and *Rhinopithecus roxellana*) showed a disabled *Treh* (supplementary table S1, Supplementary Material online). Indeed, our gene expression analyses showed that *Treh* is predominately expressed in kidney and intestine tissues of three bat species (supplementary table S10, Supplementary Material online), suggesting that trehalase may have other functions, such as transport of glucose in mammalian kidney and intestine (Sacktor 1968). In some taxa, this function may be sufficiently important to prevent pseudogenization.

We also found that one species of a typical insectivore (European shrew, *S. araneus*) has a pseudogenized *Treh*. This species is the only exception as all other insectivorous

species in our sample have a complete *Treh* (fig. 4 and supplementary fig. S2, Supplementary Material online). Although traditionally considered insectivores, European shrews frequently consume a substantial amount of vertebrate prey or scavenge (~30%) (Wilman et al. 2014), and such a dietary preference may have resulted in the loss of *Treh*. Alternatively, if ancestral populations of this insectivore had lost the capacity to digest dietary trehalose, it is unlikely to have been regained in extant populations according to the Dollo's law of irreversibility (Dollo 1893). Such historical contingencies must be considered while understanding *Treh* evolution. Coincidentally, the same species only has two chitinase genes, which encode enzymes for digesting insect exoskeletal chitin, but most other insectivores have four or five (Emerling et al. 2018).

Together, this study suggests that the evolutionary patterns of the trehalase gene (*Treh*) are broadly consistent with the fossil record, providing a molecular record of dietary diversification in bats and other mammals. Consequently, identifying molecular signatures of dietary shifts followed by animal diversification would help us understand both historical and modern changes of animal diets.

Materials and Methods

Gene Identification

Using all published mammalian *Treh* gene sequences as queries, we performed TBlastN searches (Altschul et al. 1990) against 107 currently available mammalian genome sequences, including 14 bat genomes (supplementary table S1, Supplementary Material online), retrieved from National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, last accessed January 1, 2019). The coding sequences of *Treh* were determined manually after removing all introns by the canonical splice sites (GT-AG) (Oesterreicher et al. 2001). Here we classified *Treh* genes into three categories: complete genes, partial genes, and pseudogenes. Complete genes are full-length genes with an intact ORF and a putative start and stop codon; partial genes were defined as those with an intact ORF and at least 13 coding exons; pseudogenes are those with inactivating mutations. All sequences identified from available genome sequences were provided in the supplementary material S1 (Supplementary Material online).

Gene Sequencing

We additionally sequenced the *Treh* gene from 12 species of bats. Genomic DNAs were isolated from bat tissues (see sample resources in supplementary table S2, Supplementary Material online) that were stored at −20 °C, using the Qiagen DNeasy Kits. To amplify the nucleotide sequences of bat *Treh*, we designed a suite of primers (supplementary table S3, Supplementary Material online) based on the alignment of known *Treh* sequences. Polymerase chain reactions (PCRs) were performed as described previously (Lu et al. 2016; Wang et al. 2017). PCR products were purified by the QIAquick PCR Purification Kit (Qiagen) and then sequenced directly for both strands. For a few PCR products that did not work in direct sequencing, we cloned each of the PCR products into the pMD-19T vector (Takara) and sequenced three to five positive clones using the M13 universal primer pair.

We also performed reverse transcription polymerase chain reaction (RT-PCR) assays for three species with RNA samples (see sample resources in supplementary table S2, Supplementary Material online). Briefly, total RNAs were isolated from intestine or kidney tissue using Trizol (Invitrogen, CA). First-strand cDNAs were synthesized through the reverse transcription using PrimeScript RT reagent kit (Takara). RT-PCR assays were conducted following the manufacturer's instructions. Products were then purified and sequenced directly from both directions. All newly acquired bat *Treh* sequences were deposited into GenBank under accession numbers MH806876–MH806891.

The use and care of the bats in this study were reviewed and approved by the Ethics Committee of Guangdong Institute of Applied Biological Resource and Wuhan University. All samples were lawfully acquired and their use was conformed to the national and local laws and regulations.

Molecular Evolutionary Analysis

The bat *Treh* sequences generated in this study were inspected manually. To ensure the *Treh* gene identity, we confirmed the trehalase signature (Kopp et al. 1993), a 14 amino acid domain, in each bat *Treh* gene. We aligned translated amino acid sequences of all 26 bat *Treh* genes using the MUSCLE program (Edgar 2004), and found the inclusion of short sequences from *C. sphinx* and *M. minimus* would severely reduce the number of informative sites. We thus removed the sequences of those two species and generated a nucleotide sequence alignment according to the amino acid alignment. The nucleotide alignment was carefully checked by eye and then used for subsequent analyses. To investigate selective pressures on bat *Treh*, we estimated the ratio (ω) of the rate of nonsynonymous substitutions to the rate of synonymous substitutions using PAMLX (Xu and Yang 2013). Likelihood ratio tests were applied to compare nested models (Xu and Yang 2013). Furthermore, we examined whether noninsectivorous bat lineages have undergone relaxed selection using the program RELAX (Wertheim et al. 2015). Phylogenetic relationships of bats in our sample were obtained from previous studies (Teeling et al. 2005; Miller-Butterworth et al. 2007; Roehrs et al. 2010; Almeida et al. 2011; Rojas et al. 2016).

Enzymatic Activity Assays

We sampled intestinal tissues of three bat species, including two Old World fruit bats (*C. sphinx* and *R. leschenaultii*) and one insectivorous species (*H. armiger*). Following the methods of a previous study (Martínez del Rio et al. 1995), thawed tissues were homogenized in precooling phosphate buffered saline buffer using an OMNI tissue homogenizer. The substrate trehalose solution (56.0 mM) was prepared using 0.1 M maleate/NaOH buffer (pH = 6.5). Next, 100 µl tissue homogenates were incubated at 37 °C with 100 µl trehalose solutions. After 10 min incubation, 3 ml stop/develop reagents were added to arrest the reactions. The absorbance at

405 nm was evaluated using a spectrophotometer and standardized intestinal trehalase activity was measured as μmol/min/g (Martínez del Rio et al. 1995).

Phylogenetic Logistic Regression

We performed phylogenetic logistic regression analyses to test whether the percentage of insects in the diet can predict the retention or loss of *Treh* by two methods that implemented in the R package "phylolm" (Ives and Garland 2010; Ho and Ane 2014). The percentage of insects in the diet for each species was used as independent variable, and the retention (1) or loss (0) of *Treh* was treated as dependent binary trait.

Dating Gene Losses

To date *Treh* gene losses along the mammalian phylogeny, we followed methods of a recent study (Meredith et al. 2009) and examined changes in selective pressure (ω) along branches in the phylogeny to identify functional, mixed, and pseudogenic branches. Briefly, this method assumes that functional branches (ω_0) are under purifying selection ($0 < \omega_0 < 1$), mixed branches (ω_1) have both functional and pseudogenic components ($\omega_0 < \omega_1 < \omega_2$), pseudogenic branches (ω_2) are under neutral evolution ($\omega_2 = 1$). Our mammalian phylogeny with divergence times was obtained from multiple resources (Harrison et al. 2003; Chevret and Dobigny 2005; Gilbert et al. 2006; Eizirik et al. 2010; Perelman et al. 2011; Steiner and Ryder 2011; Zhou et al. 2011; Nyakatura and Bininda-Emonds 2012; Sato et al. 2012; Bibi 2013; Banguera-Hinestroza et al. 2014; Emerling et al. 2015; Tan et al. 2017; Arnason et al. 2018). We estimated when the functional constraint on *Treh* became relaxed in each pseudogene (supplementary table S6, Supplementary Material online) following the same approach (Meredith et al. 2009). In instances where the mixed branch ω was estimated to be greater than 1, including $Ks = 0$ but $Ka \neq 0$ in four cases, we considered the gene loss date to be same to the divergence time when the studied lineage separated from its sister lineage (supplementary table S6, Supplementary Material online). In instances where the mixed branch ω was estimated to be smaller than the functional branch ω, we considered the gene loss date to be zero (supplementary table S6, Supplementary Material online).

Diet Classification

Data on mammalian diets were obtained from a recent work (Wilman et al. 2014) and is provided in supplementary table S7 (Supplementary Material online). Following a standard method for assignment of avian diets (DeGolier et al. 1999; Wang and Zhao 2015), we defined the diet category for each mammal species using the 51% criterion: when a particular general food type makes up more than 50% of its diet, the species was assigned to that dietary category. As a result, we classified mammals into five categories according to their dietary components: herbivores (frugivores and/or nectarivores), insectivores, sanguivores, carnivores, and omnivores (fig. 4). Of note, species that utilize multiple food types with none constituting >50% of the diet were classified as omnivores.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

H.Z. conceived and designed the project. H.J. conducted the sequence analyses. L.Z. supervised the enzymatic activity assay. H.L., H.J., and H.W.X. performed the enzymatic activity assay. N.B.S. and L.Z. provided bat tissues. H.J. and H.Z. wrote the manuscript, N.B.S. and L.Z. edited the manuscript. All authors read and approved the manuscript.

References

Almeida FC, Giannini NP, DeSalle R, Simmons NB. 2011. Evolutionary relationships of the old world fruit bats (Chiroptera, Pteropodidae): another star phylogeny? *BMC Evol Biol*. 11:281.

Altringham JD. 1996. Bats: biology and behaviour. Oxford, New York, Tokyo (Japan): Oxford University Press.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol*. 215(3):403–410.

Amador LI, Arevalo RLM, Almeida FC, Catalano SA, Giannini NP. 2018. Bat systematics in the light of unconstrained analyses of a comprehensive molecular supermatrix. *J Mamm Evol*. 25(1):37–70.

Arguelles JC. 2014. Why can't vertebrates synthesize trehalose? *J Mol Evol*. 79:111–116.

Arnason U, Lammers F, Kumar V, Nilsson MA, Janke A. 2018. Whole-genome sequencing of the blue whale and other rorquals finds signatures for introgressive gene flow. *Sci Adv*. 4(4):eaap9873.

Banguera-Hinestroza E, Hayano A, Crespo E, Hoelzel AR. 2014. Delphinid systematics and biogeography with a focus on the current genus *Lagenorhynchus*: multiple pathways for antitropical and trans-oceanic radiation. *Mol Phylogenet Evol*. 80:217–230.

Bell GP. 1990. Birds and mammals on an insect diet: a primer on diet composition analysis in relation to ecological energetics. In: Michael L. Morrison, C. John Ralph, Jared Verner, Joseph R. Jehl Jr, editors. *Studies in avian biology*. San Diego, California: Cooper Ornithological Society. Vol. 13, p. 416–422.

Bibi F. 2013. A multi-calibrated mitochondrial phylogeny of extant Bovidae (Artiodactyla, Ruminantia) and the importance of the fossil record to systematics. *BMC Evol Biol*. 13:166.

Bininda-Emonds OR, Cardillo M, Jones KE, MacPhee RD, Beck RM, Grenyer R, Price SA, Vos RA, Gittleman JL, Purvis A. 2007. The delayed rise of present-day mammals. *Nature* 446(7135):507–512.

Birdsey GM, Lewin J, Cunningham AA, Bruford MW, Danpure CJ. 2004. Differential enzyme targeting as an evolutionary adaptation to herbivory in Carnivora. *Mol Biol Evol*. 21(4):632–646.

Bocherens H, Fizet M, Mariotti A. 1994. Diet, physiology and ecology of fossil mammals as inferred from stable carbon and nitrogen isotope biogeochemistry: implications for Pleistocene bears. *Palaeogeogr Palaeoclimatol Palaeoecol*. 107(3–4):213–225.

Chevret P, Dobigny G. 2005. Systematics and evolution of the subfamily Gerbillinae (Mammalia, Rodentia, Muridae). *Mol Phylogenet Evol*. 35(3):674–688.

Cohen KM, Finney SC, Gibbard PL, Fan JX. 2013. The ICS international chronostratigraphic chart. *Episodes* 36:199–204.

Dahlqvist A, Thomson DL. 1963. The digestion and absorption of maltose and trehalose by the intact rat. *Acta Physiol Scand*. 59:111–125.

Darwin C. 1859. On the origin of species by means of natural selection. London: John Murray.

DeGolier TF, Mahoney SA, Duke GE. 1999. Relationships of avian cecal lengths to food habits, taxonomic position, and intestinal lengths. *Condor* 101(3):622–634.

Dollo L. 1893. The laws of evolution. *Bulletin de la Société belge de géologie, de paléontologie et d'hydrologie*. 7: p.165–166.

Drucker D, Bocherens H. 2004. Carbon and nitrogen stable isotopes as tracers of change in diet breadth during middle and upper Palaeolithic in Europe. *Int J Osteoarchaeol*. 14(34):162–177.

Dumont ER, Davalos LM, Goldberg A, Santana SE, Rex K, Voigt CC. 2012. Morphological innovation, diversification and invasion of a new adaptive zone. *Proc R Soc B Biol Sci*. 279(1734):1797–1805.

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113.

Eizirik E, Murphy WJ, Koepfli K-P, Johnson WE, Dragoo JW, Wayne RK, O'Brien SJ. 2010. Pattern and timing of diversification of the mammalian order Carnivora inferred from multiple nuclear gene sequences. *Mol Phylogenet Evol*. 56(1):49–63.

Emerling CA. 2017. Genomic regression of claw keratin, taste receptor and light-associated genes provides insights into biology and evolutionary origins of snakes. *Mol Phylogenet Evol*. 115:40–49.

Emerling CA, Delsuc F, Nachman MW. 2018. Chitinase genes (*CHIA*s) provide genomic footprints of a post-Cretaceous dietary radiation in placental mammals. *Sci Adv*. 4(5):eaar6478.

Emerling CA, Huynh HT, Nguyen MA, Meredith RW, Springer MS. 2015. Spectral shifts of mammalian ultraviolet-sensitive pigments (short wavelength-sensitive opsin 1) are associated with eye length and photic niche evolution. *Proc R Soc B Biol Sci*. 282(1819):20151817.

Feng P, Zheng JS, Rossiter SJ, Wang D, Zhao H. 2014. Massive losses of taste receptor genes in toothed and baleen whales. *Genome Biol. Evol*. 6(6):1254–1265.

Foley NM, Springer MS, Teeling EC. 2016. Mammal madness: is the mammal tree of life not yet resolved? *Philos Trans R Soc Lond B Biol Sci*. 371(1699):20150140.

German DP, Nagle BC, Villeda JM, Ruiz AM, Thomson AW, Balderas SC, Evans DH. 2010. Evolution of herbivory in a carnivorous Clade of minnows (Teleostei: cyprinidae): effects on gut size and digestive physiology. *Physiol Biochem Zool*. 83(1):1–18.

Gilbert C, Ropiquet A, Hassanin A. 2006. Mitochondrial and nuclear phylogenies of Cervidae (Mammalia, Ruminantia): systematics, morphology, and biogeography. *Mol Phylogenet Evol*. 40(1):101–117.

Grennan AK. 2007. The role of trehalose biosynthesis in plants. *Plant Physiol*. 144(1):3–5.

Gunnell GF, Simmons NB. 2005. Fossil evidence and the origin of bats. *J Mamm Evol*. 12(1–2):209–246.

Harrison RG, Bogdanowicz SM, Hoffmann RS, Yensen E, Sherman PW. 2003. Phylogeny and evolutionary history of the ground squirrels (Rodentia: marmotinae). *J Mamm Evol*. 10(3):249–276.

Henrissat B, Bairoch A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J*. 293(3):781–788.

Hernandez A, Martinez del Rio C. 1992. Intestinal disaccharides in five species of phyllostomoid bats. *Comp Biochem Physiol B*. 103(1):105–111.

Ho, LSTAne C. 2014. A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. *Syst Biol*. 63:397–408.

Holbrook JD, Birdsey GM, Yang Z, Bruford MW, Danpure CJ. 2000. Molecular adaptation of alanine: glyoxylate aminotransferase targeting in primates. *Mol Biol Evol*. 17(3):387–400.

Hong W, Zhao H. 2014. Vampire bats exhibit evolutionary reduction of bitter taste receptor genes common to other bats. *Proc R Soc B Biol Sci*. 281(1788):20141079.

Hu Y, Wu Q, Ma S, Ma T, Shan L, Wang X, Nie Y, Ning Z, Yan L, Xiu Y, et al. 2017. Comparative genomics reveals convergent evolution between the bamboo-eating giant and red pandas. *Proc Natl Acad Sci U S A*. 114(5):1081–1086.

Ishihara R, Taketani S, Sasai-Takedatsu M, Kino M, Tokunaga R, Kobayashi Y. 1997. Molecular cloning, sequencing and expression of cDNA encoding human trehalase. *Gene* 202(1–2):69–74.

Ives AR, Garland T. 2010. Phylogenetic logistic regression for binary dependent variables. *Syst Biol*. 59(1):9–26.

Jiang P, Josue J, Li X, Glaser D, Li W, Brand JG, Margolskee RF, Reed DR, Beauchamp GK. 2012. Major taste loss in carnivorous mammals. *Proc Natl Acad Sci U S A*. 109(13):4956–4961.

Karasov WH, del Rio CM, Caviedes-Vidal E. 2011. Ecological physiology of diet and digestive systems. *Annu Rev Physiol*. 73:69–93.

Kemp TS. 2005. The origin and evolution of mammals. Oxford: Oxford University Press.

Kopp M, Muller H, Holzer H. 1993. Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*. *J Biol Chem*. 268(7):4766–4774.

Liu Y, Xu H, Yuan X, Rossiter SJ, Zhang S. 2012. Multiple adaptive losses of alanine-glyoxylate aminotransferase mitochondrial targeting in fruit-eating bats. *Mol Biol Evol*. 29(6):1507–1511.

Lu Q, Wang K, Lei F, Yu D, Zhao H. 2016. Penguins reduced olfactory receptor genes common to other waterbirds. *Sci Rep*. 6:31671.

Luo ZX. 2007. Transformation and diversification in early mammal evolution. *Nature* 450(7172):1011–1019.

Martínez del Rio C. 1990. Dietary, phylogenetic, and ecological correlates of intestinal sucrase and maltase activity in birds. *Physiol Zool*. 63(5):987–1011.

Martínez del Rio C, Brugger KE, Rios JL, Vergara ME, Witmer M. 1995. An experimental and comparative study of dietary modulation of intestinal enzymes in European starlings (*Sturnus vulgaris*). *Physiol Zool*. 68(3):490–511.

Meredith RW, Gatesy J, Murphy WJ, Ryder OA, Springer MS. 2009. Molecular decay of the tooth gene enamelin (*ENAM*) mirrors the loss of enamel in the fossil record of placental mammals. *PLoS Genet*. 5(9):e1000634.

Miller-Butterworth CM, Murphy WJ, O'Brien SJ, Jacobs DS, Springer MS, Teeling EC. 2007. A family matter: conclusive resolution of the taxonomic position of the long-fingered bats, Miniopterus. *Mol Biol Evol*. 24(7):1553–1561.

Nyakatura K, Bininda-Emonds ORP. 2012. Updating the evolutionary history of Carnivora (Mammalia): a new species-level supertree complete with divergence time estimates. *BMC Biol*. 10:12.

O'Leary MA, Bloch JI, Flynn JJ, Gaudin TJ, Giallombardo A, Giannini NP, Goldberg SL, Kraatz BP, Luo ZX, Meng J. 2013. The placental mammal ancestor and the post-K-Pg radiation of placentals. *Science* 339:662–667.

Oesterreicher TJ, Markesich DC, Henning SJ. 2001. Cloning, characterization and mapping of the mouse trehalase (*Treh*) gene. *Gene* 270(1–2):211–220.

Oesterreicher TJ, Nanthakumar NN, Winston JH, Henning SJ. 1998. Rat trehalase: cDNA cloning and mRNA expression in adult rat tissues and during intestinal ontogeny. *Am J Physiol Regul Integr Comp Physiol*. 274(5):R1220–R1227.

Perelman P, Johnson WE, Roos C, Seuánez HN, Horvath JE, Moreira MAM, Kessing B, Pontius J, Roelke M, Rumpler Y, et al. 2011. A molecular phylogeny of living primates. *PLoS Genet*. 7(3):e1001342.

Price SA, Hopkins SS, Smith KK, Roth VL. 2012. Tempo of trophic evolution and its impact on mammalian diversification. *Proc Natl Acad Sci U S A*. 109(18):7008–7012.

Roehrs ZP, Lack JB, Van den Bussche RA. 2010. Tribal phylogenetic relationships within Vespertilioninae (Chiroptera: Vespertilionidae) based on mitochondrial and nuclear sequence data. *J Mammal*. 91(5):1073–1092.

Rojas D, Vale A, Ferrero V, Navarro L. 2011. When did plants become important to leaf-nosed bats? Diversification of feeding habits in the family Phyllostomidae. *Mol Ecol*. 20(10):2217–2228.

Rojas D, Warsi OM, Davalos LM. 2016. Bats (Chiroptera: Noctilionoidea) challenge a recent origin of extant neotropical diversity. *Syst Biol*. 65(3):432–448.

Ruf J, Wacker H, James P, Maffia M, Seiler P, Galand G, Vonkieckebusch A, Semenza G, Mantei N. 1990. Rabbit small intestinal trehalase: purification, cDNA cloning, expression, and verification of glycosylphosphatidylinositol anchoring. *J Biol Chem*. 265:15034–15039.

Sacktor B. 1968. Trehalase and the transport of glucose in the mammalian kidney and intestine. *Proc Natl Acad Sci U S A*. 60(3):1007–1014.

Sato JJ, Wolsan M. 2012. Loss or major reduction of umami taste sensation in pinnipeds. *Naturwissenschaften* 99(8):655–659.

Sato JJ, Wolsan M, Prevosti FJ, D'Elía G, Begg C, Begg K, Hosoda T, Campbell KL, Suzuki H. 2012. Evolutionary and biogeographic history of weasel-like carnivorans (Musteloidea). *Mol Phylogenet Evol*. 63(3):745–757.

Schondube JE, Herrera-M LG, Martínez del Rio C. 2001. Diet and the evolution of digestion and renal function in phyllostomid bats. *Zoology (Jena)* 104(1):59–73.

Shi JJ, Rabosky DL. 2015. Speciation dynamics during the global radiation of extant bats. *Evolution* 69(6):1528–1545.

Simmons N, Geisler J. 1998. Phylogenetic relationships of Icaronycteris, Archaeonycteris, Hassianycteris, and Palaeochiropteryx to extant bat lineages, with comments on the evolution of echolocation and foraging strategies in Microchiroptera. *Bull Am Mus Nat Hist*. 235:4–182.

Simmons NB, Seymour KL, Habersetzer J, Gunnell GF. 2008. Primitive early Eocene bat from Wyoming and the evolution of flight and echolocation. *Nature* 451(7180):818–821.

Steiner CC, Ryder OA. 2011. Molecular phylogeny and evolution of the Perissodactyla. *Zool J Linn Soc*. 163(4):1289–1303.

Tan S, Wang ZH, Jiang LC, Peng R, Zhang T, Peng QK, Zou FD. 2017. Molecular phylogeny and phylogeography of genus Pseudois (Bovidae, Cetartiodactyla): new insights into the contrasting phylogeographic structure. *Ecol Evol*. 7(17):7047–7057.

Teeling EC, Springer MS, Madsen O, Bates P, O'Brien SJ, Murphy WJ. 2005. A molecular phylogeny for bats illuminates biogeography and the fossil record. *Science* 307(5709):580–584.

Thompson SN. 2003. Trehalose—the insect 'blood' sugar. *Adv In Insect Phys*. 31:205–285.

Ungar P. 1996. Dental microwear of European Miocene catarrhines: evidence for diets and tooth use. *J Hum Evol*. 31(4):335–366.

Ungar P. 1998. Dental allometry, morphology, and wear as evidence for diet in fossil primates. *Evol Anthropol*. 6(6):205–217.

Walker A, Hoeck HN, Perez L. 1978. Microwear of mammalian teeth as an indicator of diet. *Science* 201(4359):908–910.

Wang K, Hong W, Jiao H, Zhao H. 2017. Transcriptome sequencing and phylogenetic analysis of four species of luminescent beetles. *Sci Rep*. 7(1):1814.

Wang K, Zhao H. 2015. Birds generally carry a small repertoire of bitter taste receptor genes. *Genome Biol Evol*. 7(9):2705–2715.

Wertheim JO, Murrell B, Smith MD, Pond SLK, Scheffler K. 2015. RELAX: detecting relaxed selection in a phylogenetic framework. *Mol Biol Evol*. 32(3):820–832.

Wilman H, Belmaker J, Simpson J, de la Rosa C, Rivadeneira MM, Jetz W. 2014. EltonTraits 1.0: species-level foraging attributes of the world's birds and mammals. *Ecology* 95(7):2027.

Wilson GP, Evans AR, Corfe IJ, Smits PD, Fortelius M, Jernvall J. 2012. Adaptive radiation of multituberculate mammals before the extinction of dinosaurs. *Nature* 483(7390):457–460.

Xu B, Yang Z. 2013. PAMLX: a graphical user interface for PAML. *Mol Biol Evol*. 30(12):2723–2724.

Zhao H, Xu D, Zhang S, Zhang J. 2012. Genomic and genetic evidence for the loss of umami taste in bats. *Genome Biol Evol*. 4(1):73–79.

Zhao H, Yang J-R, Xu H, Zhang J. 2010. Pseudogenization of the umami taste receptor gene *Tas1r1* in the giant panda coincided with its dietary switch to bamboo. *Mol Biol Evol*. 27(12):2669–2673.

Zhao H, Zhang J. 2012. Mismatches between feeding ecology and taste receptor evolution: an inconvenient truth. *Proc Natl Acad Sci U S A*. 109(23):E1464.

Zhao H, Zhou Y, Pinto CM, Charles-Dominique P, Galindo GJ, Zhang S, Zhang J. 2010. Evolution of the sweet taste receptor gene *Tas1r2* in bats. *Mol Biol Evol*. 27(11):2642–2650.

Zhou X, Xu S, Yang Y, Zhou K, Yang G. 2011. Phylogenomic analyses and improved resolution of Cetartiodactyla. *Mol Phylogenet Evol*. 61(2):255–264.