Common ancestors of bats were omnivorous suggested by resurrection of ancestral sweet receptors

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Common ancestors of bats were omnivorous suggested by resurrection of ancestral sweet receptors

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The origins of powered flight and laryngeal echolocation in bats are widely cited as evidence that ancestral bats evolved as insectivores [1]. Indeed, the emergence of major bat lineages in the Eocene has also been linked to insectivory, with bat diversification arising due to the increase in insect abundance, in turn attributed to angiosperm radiation [2]. Further indication that early bats were insectivorous comes from the presence of tympanate moth families in Eocene deposits, which probably evolved simple hearing organs for avoiding echolocating bats [3]. Despite these observations, and the fact that insectivory is the dominant dietary specialisation among modern bat lineages, arguments linking the evolution of echolocation and flight to insectivory are not universally accepted. In particular, others have hypothesized that the first bats were diurnal frugivores, and that insectivory emerged secondarily for protein supplementation [1]. This scenario, if correct, suggests modern frugivorous and nectarivorous bats [4] might have retained ancestral adaptations, rather than undergone derived specializations. Unfortunately, direct evidence relating to the diets of ancestral bats is lacking, reflecting a depauperate fossil record [5]; however, insights may come from studying molecular adaptations in diet-related genes. By conducting the first resurrection and functional assays of sweet receptors in ancestral bat lineages, we found that the ancestral sweet receptor of all extant bats was functionally sensitive to natural sugars, with a lower level of sugar sensitivity than modern pteropodid bats, suggesting that they were omnivorous.

Of the main sensory modalities, taste is especially tied to diet [6]. Mammals possess five basic taste sensations (sweet, umami, bitter, salt, and sour), of which the perception of sweetness and umami are controlled by a family of type 1 taste receptors. The sweet taste receptor is formed by a dimer of Tas1r2 and Tas1r3, encoded by the genes *Tas1r2* and *Tas1r3*, respectively. Comparative studies of mammalian sweet receptors uncover a close relationship between sweet receptor presence and diet, with multiple gene losses across carnivorous lineages [7]. In bats, protein assays and behavioural studies both indicate that frugivorous species from both suborders can sense natural sugars, whereas insectivorous species cannot [8]. To obtain insights into the early evolution of diet in bats, we performed the first examination of taste receptors in ancestral bat lineages. By resurrecting and measuring the functional properties of ancient proteins from six ancestral taxa, we assess whether ancestral bats were able to sense natural sugars [8].

We used the Maximum Likelihood (ML) method with the amino acid model to reconstruct the protein sequences of sweet receptors for key ancestral nodes in the bat phylogeny **(Dataset S1, Supplementary Methods)**. Proteins were resurrected *in vitro*, and their phenotypic responses to natural sugars (sucrose and fructose) measured using calcium mobilization assays. We recorded clear responses to both natural sugars for the sweet receptors of the common ancestor of all extant bats (~10%), the ancestor of the suborder Yinpterochiroptera (~10%), and the ancestor of the family Pteropodidae (i.e., Old World fruit bats), with the latter showing the highest intensity (~30%) **(Fig. 1)**. In contrast, ancestral lineages leading to the suborder Yangochiroptera, and two clades within this suborder, showed no detectable response to the two sugars **(Fig. 1)**. All receptors without response to natural sugars showed clear responses to an artificial sweetener control (NHDC) **(Dataset S2)**, confirming that our heterologous expression system worked [8]. Co-expression levels of *Tas1r2* and *Tas1r3* in each species were similar **(Fig. S1, Supplementary Methods),** confirming equivalent expression levels of Tas1r2 and Tas1r3 for the ancestral protein studies. To assess the robustness of our findings, we repeated the ancestral sequence reconstructions under the codon model, and assessed functional responses of the corresponding synthesized sweet receptors **(Datasets S3-S5, Supplementary Methods)**. Consistent results were observed across both sets of proteins **(Dataset S6)**. We further used the Bayesian Inference (BI) and Maximum Parsimony (MP) methods to re-infer ancestral sequences, both sets of sequences showed a high consistency with those inferred by the ML method with the amino acid model (BI vs. ML: 98.5%; MP vs. ML: 99.3%) and the codon model (BI vs. ML: 98.7%; MP vs. ML:99.2%) **(Datasets S7-S8, Supplementary Methods)**.

Since all six resurrected ancestral bat sweet receptors comprised pairs of intact Tas1r2 and Tas1r3 subunits (hereafter T2 and T3), the observed losses of the sweet response could not be attributed to known loss-of-function mutations. Therefore, to determine the underlying causes of observed losses of sweet perception in bats, we generated chimeric dimers in which we paired mismatching T2 and T3 subunits. Briefly, we generated four pairs of mismatched sweet receptors:

ChiT2-YanT3, YanT2-ChiT3, GloT2-NfbT3, and NfbT2-GloT3 **(Fig. 2)**. Our results showed that ChiT2-YanT3 retained clear responses to both sugars, while YanT2-ChiT3 lost such function **(Fig. 2A-2B)**, indicating that Tas1r2 is responsible for the loss of sweet taste in the ancestor of Yangochiroptera. Both GloT2-NfbT3 and NfbT2-GloT3 pairs showed no detectable response to sucrose that can be detected by the nectar-feeding species *Glossophaga soricina* **(Fig. 2C)**, suggesting that mutations in both Tas1r2 and Tas1r3 have resulted in the regain of the sweet taste in this New World bat **(Fig. 1)**.

Our experiments provide the first evidence that the ability to sense natural sugars was present in the common ancestor of extant bats **(Fig. 1)**. Based on the correspondence between taste and diet in extant bats [8], we thus suggest that ancestral bats were likely omnivorous, feeding on a mixture of fruits and insects. We also recorded sugar-sensitive taste receptors for the ancestors of the subfamily Yinpterochiroptera and the family Pteropodidae **(Fig. 1)**, implying that the ability to perceive sweetness has been retained throughout the evolutionary history of Old World fruit bats. In contrast, the resurrected receptor of the ancestor of the suborder Yangochiroptera showed no such response to sugars, pointing to an earlier transition to an insectivorous diet in this suborder. Despite this, sensitivity to natural sugars was again present in some New World leaf-nosed bats **(Fig. 1),** consistent with an adaptive regain linked to their independent transition to a plant-based diet, as also found in hummingbirds [9]. We note that two New World frugivirous bats (*S. lilium*, and *A. jamaicensis*) have not gained sensitivity to natural sugars yet **(Fig. 1)**, as shown in our earlier work (9), possibly due to the short divergence times within this clade of neotropical bats with exceptional bursts of adaptive radiation [10]. Additionally, downstream genes of sweet taste signaling pathway may also have an impact on sweet taste function, which could be tested in the future.

Omnivory occurs in several extant bat lineages [11], and switches between animal- and plant-based diets have occurred multiple times in bats and other mammals, including the giant panda, which has evolved sweet taste perception relating to its bamboo-dominated diet **(Fig. 1)** [12]. If the ancestral bat was indeed omnivorous, then this calls into question the common view that bats evolved flight and echolocation for hunting insects. Previously, contrarian speculation that ancestral bats were diurnal frugivores was based on reasoning that, if flight evolved before echolocation, then the small eyes of bats would be ill-adapted to a nocturnal niche [1]. Although this theory predates major phylogenetic revisions of the bat clade, it is nevertheless arguably more credible in light of the discovery of the first transitional fossil bat *Onychonycteris*, an Eocene taxon that is suggested to possesses morphological characters consistent with an ability to fly but not echolocate [13, 14], but see [15] . While it is thus plausible that the first bats hunted for insects and fruit without echolocation, caveats remain. Notably, approximately ten millions of years separate the origin of bats and the earliest known fossils [5], raising the possibility that sugar sensing in the ancestor of modern bats is itself a derived state. Moreover, recent bat phylogenies place Eocene fossils outside of modern lineages [13], which, if correct, would imply that ancient adaptations inferred from protein reconstructions cannot be directly related to extinct taxa.

Appendix A. Supplementary materials

Supplementary materials to this article can be found online.

Data Availability

All data in this study are included in the article and/or supporting information.

Acknowledgments

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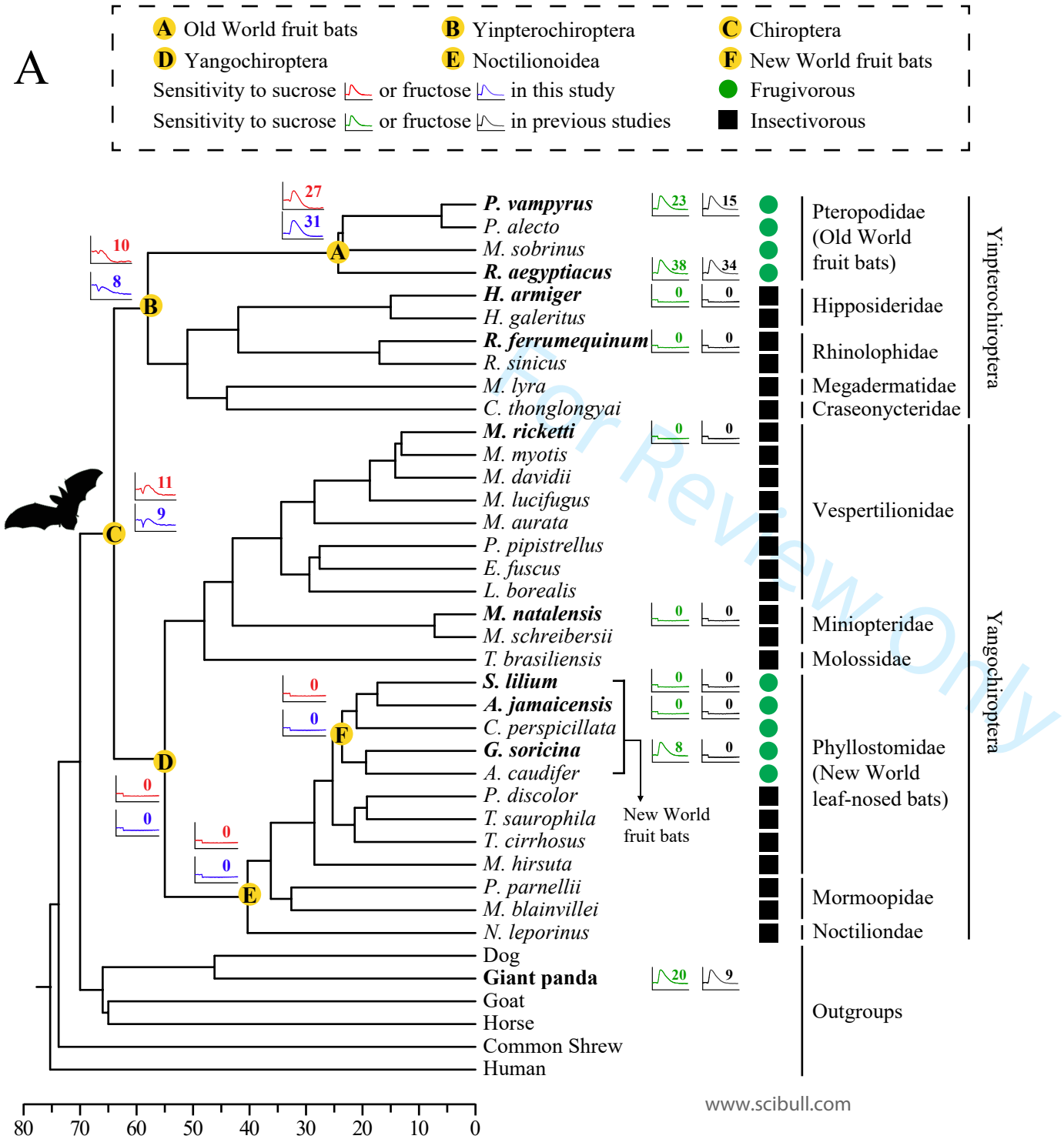
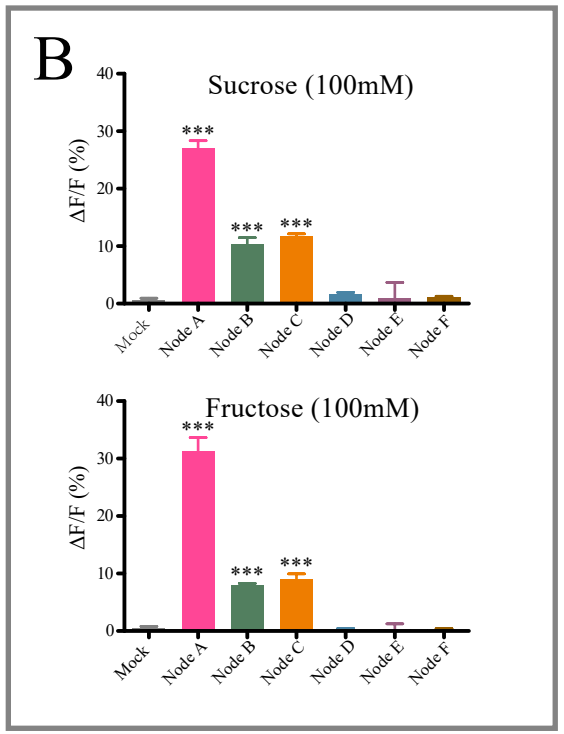
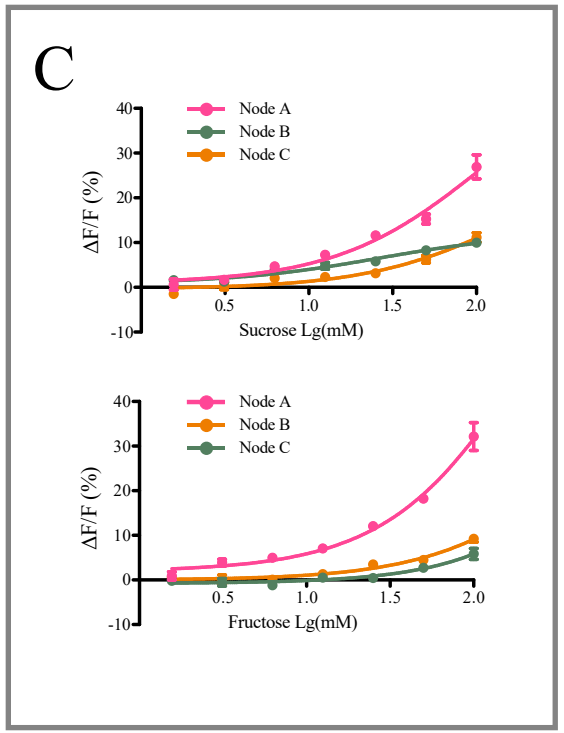
Figure Legends:

Figure 1. Functional evolution of sweet receptors in bats. (A) Receptor responses to sugars. Response lines with a number indicate the intensity of response to sucrose or fructose. Responses of extant species to sugars were obtained from previous studies [8, 12]. (B) Quantitative analysis of responses of ancestral sweet receptors of the six early lineages (mean ± SEM; ***p<0.001, one-way ANOVA). (C) Dose-dependent responses of ancestral sweet receptors to sugars. The bat silhouette was taken from PhyloPic.

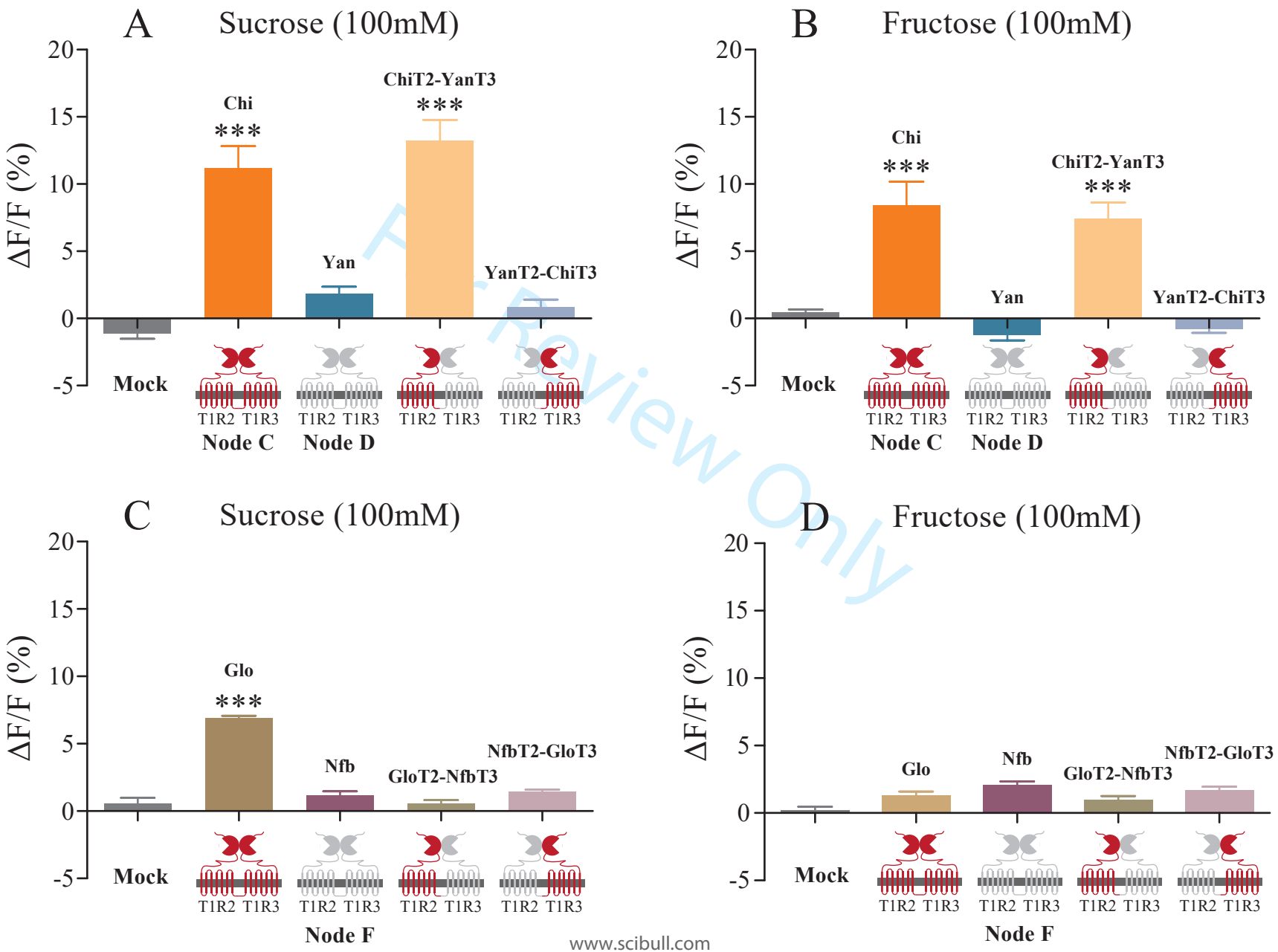
Figure 2. Responses of mismatched sweet receptors to natural sugars. (A, B) Quantitative analysis of responses of sweet receptors to sucrose and fructose (mean ± SEM; ***p<0.001, one-way ANOVA). ChiT2-YanT3 denotes a mismatched receptor of Chiroptera Tas1r2 and Yangochiroptera Tas1r3, and other mismatched receptors are indicated in a similar fashion. (C, D) No responses of mismatched sweet receptors to sucrose or fructose.







Node C = Chiroptera (Chi) Node D = Yangochiroptera (Yan)
Node F = New World fruit bats (Nfb) Glo = *Glossophaga soricina*



Supplementary Material for

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Datasets S1 to S8
Supplementary References

Supplementary Text

Supplementary methods

Ancestral sequence reconstruction

Our dataset included the full-length coding sequences of *Tas1r2* and *Tas1r3* from 32 bat species [1] and six non-bat mammals as outgroups (human, common shrew, horse, goat, dog, and giant panda). We used a Maximum Likelihood (ML) method with the amino acid model to infer the ancestral protein sequences of bat sweet receptors with the JTT model in PAML version 4 [2]. All sequences were aligned with the Muscle program [3]. The input tree was taken from previous studies [1, 4]. We selected ancestral amino acid sequences with the highest posterior probabilities for subsequent analyses. For both receptor genes (*Tas1r2* and *Tas1r3*), more than 95% amino acids of the inferred ancestral sequences had high posterior probabilities (>0.95). Ancestral sequences of six early lineages of bats were synthesized commercially to perform the subsequent cell-based functional assays. To validate the robustness of our functional results, we also used a codon method in PAML to perform the ancestral sequence reconstruction, and performed the same assays on these receptors. The Bayesian Inference (BI) method in Mrbayes version 3.2.7a [5] and Maximum Parsimony (MP) method in Mesquite version 3.81 [6] were also used to confirm ancestral sequence reconstruction based on the ML method in PAML.

Sweet compounds

Two natural sugars (sucrose and fructose) and one artificial sweetener (NHDC, neohesperidin dihydrochalcone) were purchased from Sigma-Aldrich for functional assays. All compounds were dissolved in DPBS buffer (Thermo Fisher, pH 7.4). The highest concentrations of sucrose, fructose, and NHDC used in functional assay were 100mM, 100mM, and 4mM, respectively.

Construction of expression vectors

The reconstructed ancestral coding sequences of six bat early lineages were chemically synthesized and inserted into the pcDNA3.1(+) vector incorporating 5'-EcoRI and 3'-NotI restriction sites. Codon optimization was employed to increase protein expression in Peakrapid cells. The Kozak sequence was inserted before the start codon for efficient translation. To validate equivalent protein expression levels of Tas1r2 and Tas1r3, the C-terminus HSV glycoprotein D epitope and 3×Flag tags were fused to Tas1r2 and Tas1r3, respectively. All plasmids were verified by Sanger sequencing.

Functional assays of bat sweet receptors

Responses of sweet receptors to compounds were measured by calcium mobilization assays as previously described [7]. Briefly, HEK293-derived peak rapid cells were cultured in Opti-MEM supplemented with 6% fetal bovine serum. Healthy HEK cells were plated at a density of 50,000 per well in 96-well microplates. After 24 hours, the cells were transiently transfected with Gα16-gust44, Tas1r2, and Tas1r3 using Lipofectamine 2000. After being washed once with DPBS at 48 hours after transfection, cells were dyed with Fluo-4 AM and Pluronic F-127 in the dark for one hour. Fluorescence changes were measured by the flexstation 3 system (Molecular Devices) after washing three times with DPBS. The flexstation 3 system was set as follows: fluorescence was recorded every two seconds for a total of 200 seconds, and sweet compounds at desired concentrations were added at 30 seconds. Calcium mobilization ($\Delta F/F$) was quantified as the percentage of the difference (ΔF) between the peak fluorescence and the baseline fluorescence relative to the baseline fluorescence (F). All results were replicated independently at least three times, and the average of independent replicates was used to quantify the response to sweet compounds.

Immunocytochemistry Assay

After 2 days of transfection in 12-well plates, HEK293 cells were subsequently fixed 4% paraformaldehyde (PFA) for 20 min, washed three times with phosphate-buffered saline (PBS) buffer, incubated with 0.1% Triton X-100 for 20 min, washed with PBS three times, and blocked

with 10% fetal bovine serum (FBS) for 1 h. Then, cells were incubated with primary antibodies (Rb pAb to HSV tag, 1:400, ab19355, Abcam; DDDDK tag Mouse McAb, 1:400, 66008-3-1g, Proteintech) in PBS plus 10% FBS for 1 h. Cells were washed three times with PBS. Secondary antibodies (Cy3-conjugated Affinipure Goat anti-Rabbit IgG(H+L), 1:800, SA00009-2, Proteintech; CoraLite488-conjugated Goat Anti-Mouse IgG(H+L), 1:800, SA00013-1, Proteintech) were used for the detection of HSV and 3×Flag tags. After three washes with PBS, the nucleus was stained with DAPI for 5min. Slides were mounted with antifading fluorescent mounting medium (YEASEN, Cat#36307ES08), and analyzed by confocal laser scanning microscopy (Leica TCS SP8). Seven independent images for each groups were counted for calculation of coexpression levels.

Figure S1. Co-expression level of ancestral Tas1r2 and Tas1r3 sequences of six early lineages of bats reconstructed based on the amino acid model in PAML. The average co-expression levels and their standard errors of the mean (SEM) were showed in the left, with seven independent images for each group counted. Blue, DAPI (nuclei); green, Tas1r2-HSV; red, Tas1r3-3×Flag. Scale bar=50μm.