ORIGINAL ARTICLE

# Molecular evolution and deorphanization of bitter taste receptors in the vampire bat

**Running title:** Bitter taste function in the vampire bat

Qin Lu[1], Hengwu Jiao[1], Yi Wang[1], Ngawang Norbu[2], and Huabin Zhao[1,2*]

[1]Department of Ecology, Tibetan Centre for Ecology and Conservation at WHU-TU, Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, Wuhan University, Wuhan 430072, China

[2]Research Center for Ecology, College of Science, Tibet University, Lhasa 850000, China

**Correspondence*: Huabin Zhao, Department of Ecology, College of Life Sciences, Wuhan University, 299 Bayi Road, Wuhan, Hubei 430072, China.

Tel: (86) 27 6875 3526; Fax: (86) 27 6875 2397

Email: huabinzhao@whu.edu.cn

ORCID: https://orcid.org/0000-0002-7848-6392

## ABSTRACT

Bats represent the largest dietary radiation in a single mammalian order, and have become an emerging model group for studying dietary evolution. Taste receptor genes have proven to be molecular signatures of dietary diversification in bats. For example, all three extant species of vampire bats have lost many bitter taste receptor genes (*Tas2r*s) in association with their dietary shift from insectivory to sanguivory. Indeed, only eight full-length *Tas2r*s were identified from the high-quality genome of the common vampire bat (*Desmodus rotundus*). However, it is presently unknown whether these bitter receptors are functional, since the sense of taste is less important in vampire bats, which have an extremely narrow diet and rely on other senses for acquiring food. Here we applied a molecular evolutionary analysis of *Tas2r*s in the common vampire bat compared with non-vampire bats. In addition, we provided the first attempt to deorphanize all bitter receptors of the vampire bat using a cell-based assay. We found that all *Tas2r* genes in the vampire bat have a level of selective pressure similar to that in non-vampire bats, suggesting that this species must have retained some bitter taste functions. We demonstrated that five of the eight bitter receptors in the vampire bat can be activated by some bitter compounds, and found that the vampire bat did not detect naturally occurring bitter compounds examined in this study. Our study demonstrates functional retention of bitter taste in vampire bats as suggested by cell-based functional assays, calling for an in-depth study of extra-oral functions of bitter taste receptors.

**Key words:** bitter taste, diet, evolution, selection, vampire bat

## INTRODUCTION

Bats (order Chiroptera) provide many critical ecosystem services such as pest control, seed dispersal, and pollination **(Kunz et al. 2011)**. They are the second most diverse order of mammals after Rodentia, consisting of >1400 species and representing ~20% of all mammals in the world – although more than half have unknown or decreasing population trends **(Frick et al. 2019)**. Bats are characterized by an enormous diversity and extraordinary adaptive radiations in both form and function, including the largest dietary radiation in a single mammalian order. They display independent origins of frugivory, nectarivory, carnivory and omnivory, as well as sanguivory that is unique in mammals **(Jiao et al. 2019)**. Approximately 70% of all bat species feed mainly on insects, and many also consume other animals such as non-insect arthropods, fish, amphibians, reptiles, birds, and mammals **(Altringham 1996; Zhao et al. 2010)**. Two divergent groups of bats have both independently obligate frugivory: the Old World fruit bats (family Pteropodidae within the suborder Yinpterochiroptera), and the New World fruit bats (subfamily Stenodermatinae of the family Phyllostomidae within the suborder Yangochiroptera) **(Wang et al. 2020b)**. Apparently unique in mammals, all three extant species of bats that are native to the Americas feed exclusively on blood: the common vampire bat, *Desmodus rotundus*; the white-winged vampire bat, *Diaemus youngi*; and the hairy-legged vampire bat, *Diphylla ecaudata* **(Turner 1975; Zhao et al. 2010; Hong and Zhao 2014; Chen and Zhao 2019)**.

Bats are an emerging model group of mammals for studying the evolution of dietary diversification; there is overwhelming evidence from bats demonstrating that molecular signatures of dietary diversification have been retained in their genome sequences **(Jiao et al. 2019)**. Previous studies identified clusters of olfactory receptor genes that are specifically linked to frugivorous bats **(Hayden et al. 2014; Han et al. 2016)**, suggesting that olfactory receptor genes could be molecular signatures of dietary shifts in bats. Two types of digestive enzyme genes (*CHIA*s and *Treh*) appeared to be reduced or lost in non-insectivorous bats, a molecular signature that occurred possibly because the non-insectivorous species do not need these enzymes to digest insects as their insectivorous relatives do **(Emerling et al. 2018; Jiao et al. 2019)**. Likewise, the sense of taste is intimately associated with dietary information, and thus taste receptor genes could also serve as molecular signatures of dietary diversification in bats **(Jiao et al. 2019)**. Indeed, the three extant species of vampire bats have lost sweet taste receptor genes (*Tas1r2* and *Tas1r3*) and many bitter taste receptor genes (*Tas2r*s) in association with their dietary shift from insectivory to sanguivory **(Zhao et al. 2010; Hong and Zhao 2014)**. After sequencing nine *Tas2r*s in all three species of vampire bats, we previously found that vampire bats have a significantly greater percentage of pseudogenes than other bats, along with some putatively functional genes **(Hong and Zhao 2014)**. Indeed, only eight full-length *Tas2r*s were identified from one of the three extant species of vampire bat (the common vampire bat; **(Jiao et al. 2018)** when the high-quality genome sequence became available **(Zepeda Mendoza et al. 2018)**. However, whether these bitter receptors are functional remains unknown; the sense of taste is less important in vampire bats, which have an extremely narrow diet and rely heavily on other senses for acquiring food **(Hong and Zhao 2014)**. In this study, we conducted a molecular evolutionary analysis of the eight full-length *Tas2r*s in the common vampire bat, and used a cell-based assay to deorphanize these bitter receptors.

## MATERIALS AND METHODS

### Gene sequences

All full-length *Tas2r*s in this study were taken from our earlier study **(Jiao et al. 2018)**, including the eight genes (*Tas2r1*, *Tas2r2*, *Tas2r3, Tas2r4*, *Tas2r7*, *Tas2r18*, *Tas2r40*, and *Tas2r408*) from the common vampire bat, as well as orthologs of these genes from other bat species **(figure 1)**. The nomenclature of these genes was based on an earlier phylogenetic analysis in mammals **(Jiao et al. 2018)**. In total, we collected 14 *Tas2r1*s, 10 *Tas2r2*s, 15 *Tas2r3*s, 13 *Tas2r4*s, 13 *Tas2r7*s, 19 *Tas2r18*s, 10 *Tas2r40*s, and 44 *Tas2r408*s from a variety of bat species across their phylogeny **(figure 1, table 1)**.

### Phylogenetic analysis

To conduct a phylogenetic analysis for each of these eight *Tas2r* genes, we additionally included outgroups for each gene. We selected two sequences as outgroups for each gene that is most closely related to the phylogenetic clustering of that gene based on the Bayesian phylogenetic tree of 255 bat *Tas2r* genes **(Jiao et al. 2018)**. Specifically, we selected *Rhinolophus_sinicus_Tas2r2* and *Pteronotus_parnellii_Tas2r2* as outgroups for the *Tas2r1* tree, *Rhinolophus_sinicus_Tas2r4* and *Pteronotus_parnellii_Tas2r4* for the *Tas2r2* tree, *Hipposideros_armiger_Tas2r10* and *Myotis_lucifugus_Tas2r10* for the *Tas2r3* tree, *Hipposideros_armiger_Tas2r2* and *Pteronotus_parnellii_Tas2r2* for the *Tas2r4* tree, *Hipposideros_armiger_Tas2r67 and Eptesicus_fuscus_Tas2r67* for the *Tas2r7* tree, *Rousettus_aegyptiacus_Tas2r42* and *Myotis_lucifugus_Tas2r42* for the *Tas2r18* tree, *Hipposideros_armiger_Tas2r39* and *Rousettus_aegyptiacus_Tas2r39* for the *Tas2r40* tree, and *Pteronotus_parnellii_Tas2r13* and *Megaderma_lyra_Tas2r13* for the *Tas2r408* tree **(figure 1) (Jiao et al. 2018)**.

Nucleotide sequences were aligned with MEGA X **(Kumar et al. 2018)** following protein sequence alignments. Phylogenetic trees for each gene were reconstructed with the Bayesian method, which is similar to that described in detail in our previous studies **(Feng et al. 2014; Hong and Zhao 2014; Wang and Zhao 2015; Wu et al. 2018)**. In brief, we selected the best-fit substitution models for each data set of the eight gene trees **(figure 1)** using the jModelTest2 program **(Darriba et al. 2012)**, and ran six Markov chains simultaneously with one million generations using the MrBayes 3.1.2 program **(Huelsenbeck and Ronquist 2001)**.

### Molecular evolution

To test whether selective constraints have been relaxed in the bitter taste receptor genes of the common vampire bat, we used the Codeml program in PAML **(Xu and Yang 2013)** to estimate the ratio ($\omega$) of nonsynonymous to synonymous substitutions ($d_N/d_S$). The $\omega$ value is an indicator of selective pressure; $\omega<1$ indicates purifying selection, $\omega=1$ indicates neutral evolution, and $\omega>1$ indicates positive selection **(Hong and Zhao 2014; Wang et al. 2020a)**. The input trees are the established species tree for each gene, except *Tas2r18* and *Tas2r404*, which used the newly reconstructed gene trees due to gene duplication events **(figure 1)**. We fitted each data set to two models to test whether differential selective pressures have acted on the vampire bat and other bats for each gene. The first model estimates one $\omega$ value for all branches, whereas the second model estimates two $\omega$ values, one of which is for the branch of the common vampire bat, and the other is for all other branches **(table 1)**. Likelihood-ratio tests **(Anisimova et al. 2001)** were applied to identify significant evidence of differential selective pressure by comparing the two models.

### Functional assays

To deorphanize the eight bitter receptors in the vampire bat, we collected 19 bitter compounds, including seven natural and 12 synthetic chemicals **(table 2)**. All bitter compounds were purchased from Sigma-Aldrich (see **table 2** for product catalog numbers). Our selection of chemicals was not based on the natural occurrence of bitter compounds in the vampire bat, because vertebrate blood intrinsically lacks bitter compounds that are common in nature. Deorphanization of bitter receptors was undertaken by cell-based functional assays, as previously described in detail elsewhere **(Jiao et al. 2018)**. Briefly, we synthesized complete coding sequences of bitter receptors and inserted each of them into the expression vector pEAK10. We cultured human embryonic kidney 293 (HEK293) cells (Peakrapid), which were subsequently transiently transfected by a Tas2r construct with a coupling chimeric G protein Gα16-gust44. Cells were assayed for their responses to bitter compounds using a FlexStation III spectrometer (Molecular Devices). Both relative fluorescence unit (RFU) and calcium mobilization trace were recorded. For each bitter compound, we only tested one concentration, which was shown to be the optimal in human bitter receptors **(Meyerhof et al. 2010)**. All of our experiments were run twice as technical replicates, but only one of the two replicates was plotted **(figure 2)**. Of note, the time course of fluorescence intensity for a functional taste receptor is typically a n-shaped curve, whereas that for a mock-transfected negative control or a non-functional taste receptor is typically a flat curve **(figure 2)**.

## RESULTS

### Phylogenetics of bat bitter taste receptors and selection tests for the vampire bat

Among the eight bitter taste receptor genes (*Tas2r*s), six (*Tas2r1*-*Tas2r4*, *Tas2r7*, and *Tas2r40*) are single-copy genes, with one copy from each species **(figure 1)**. By contrast, *Tas2r18* was found to have multiple copies in *Myotis* bats, which is comparable to the lineage-specific duplication of *Tas2r16* in this bat genus **(Jiao et al. 2018)**. Notably, *Tas2r408* was duplicated in all examined species except the vampire bat (*Desmodus rotundus*) and *Pteropus alecto* **(figure 1)**, suggesting that *Tas2r408* duplication most likely arose in the common ancestor of bats, much earlier than *Tas2r18* and *Tas2r16* duplications.

We aligned sequences of each gene and inferred phylogenetic trees. The topology of the phylogenetic trees for *Tas2r1*, *Tas2r2*, *Tas2r4*, and *Tas2r40* **(figure 1)** was similar to the established family-level phylogenetic tree of bats **(Teeling et al. 2005)**, suggesting that the evolutionary history of these genes may have generally followed that of these bat species. In contrast, the phylogenetic trees for *Tas2r3* and *Tas2r7* displayed a topology strikingly different from the species tree. Specifically, in the tree of *Tas2r3*, the Old World fruit bats (*Pteropus*, *Eidolon*, and *Rousettus*) were expected to group with *Rhinolophus* and *Hipposideros* following the species tree. However, they unexpectedly united with the clade consisting of *Myotis* and *Desmodus* with a low Bayesian posterior probability of 0.48 **(figure 1)**. Likewise, in the *Tas2r7* tree, *Rhinolophus* and *Hipposideros* were expected to cluster with the Old World fruit bats, yet they united with *Myotis* and *Desmodus* also with a low Bayesian posterior probability of 0.47 **(figure 1)**. These nodes showing a low posterior probability support have resulted from the alignments gaps of these rapidly evolved *Tas2r* genes **(Shi et al. 2003)**. Although multiple gene copies were found in *Tas2r18* and *Tas2r408*, the clustering of bat species is generally consistent with the established phylogenetic tree of bats **(figure 1) (Teeling et al. 2005)**.

We used the species tree (single-copy genes) or newly generated gene trees (multiple-copy genes) to test for differential selective pressures between the vampire bat and other bat species. First, we assumed the same ω across all branches of the trees for each species; ω was estimated to vary from 0.302 (*Tas2r40*) to 1.049 (*Tas2r408*), with an average of 0.656 **(table 1)**. While protein-coding genes in mammals typically have a ω ranging from 0.155 to 0.351 (average 0.219) **(Nikolaev et al. 2007)**, these bat *Tas2r* genes appear to have undergone an accelerated rate of sequence evolution compared with other genes, consistent with their dramatic variations found across vertebrates **(Shi and Zhang 2006)**. Second, we assumed two different ω values for each gene, one of which is for the branch of the vampire bat, and the other is for all other branches **(figure 1, table 1)**. After comparing the two models **(table 1)**, we found that the ω value of the vampire bat is not significantly different than that of the other lineages in all tested genes (*P*-value ranging from 0.161 to 0.945, **table 1)**. Given that ω of each gene is generally less than 1 **(table 1)**, these genes are most likely to have undergone overall purifying selection and functional constraint in all bats examined, although we cannot rule out the possibility that some lineages or sites have experienced relaxed selection or positive selection. In addition, *Tas2r408* has an overall ω close to 1 (ω=1.049, **table 1**), however selection on this gene is unlikely to have been relaxed from functional constraint, because we did not see any disruptive mutations in any species. This finding strongly suggests that the vampire bat and other bats have similar levels of selective pressure for each of the eight *Tas2r*s. We note that those nodes of gene trees showing a low posterior probability support should not impact the overall results of our selection pressure analysis, as we did not test those branches.

## Deorphanization of bitter taste receptors in the vampire bat

We undertook cell-based functional assays in a heterologous expression system to deorphanize the bitter taste receptors in the vampire bat. We collected 19 commercially available bitter compounds **(table 2)** that have shown to be able to activate human bitter receptors **(Lossow et al. 2016)**, including seven natural and 12 synthetic chemicals **(table 2)**. We tested the eight Tas2r receptors of the vampire bat (i.e. DrTas2r1-DrTas2r4, DrTas2r7, DrTas2r18, DrTas2r40, and DrTas2r408) for their responses toward the 19 bitter compounds **(figure 2, table 3)**. Activation of each receptor was recorded by measuring its fluorescence intensity with a FlexStation III spectrometer.

After screening all tested compounds, we found that five of the eight Tas2r receptors are able to recognize either two or seven compounds. Specifically, DrTas2r1 detects chloramphenicol and denatonium benzoate, DrTas2r2 detects acesulfame K and denatonium benzoate, DrTas2r4 detects chloramphenicol and denatonium benzoate, DrTas2r7 detects denatonium benzoate and yohimbine hydrochloride, and DrTas2r408 detects seven compounds: chloramphenicol, denatonium benzoate, D-salicin, phenylthiocarbamide, ranitidine hydrochloride, sodium thiocyanate, and 6-propyl-2-thiouracil **(figure 2, table 3)**. It appears that DrTas2r408 is more broadly tuned to respond to a wide range of compounds relative to the other four receptors (DrTas2r1, DrTas2r2, DrTas2r4, and DrTas2r7). However, three receptors (DrTas2r3, DrTas2r18, and DrTas2r40) were not activated by any compound tested **(table 3)**.

After counting compounds, we found that bitter compounds differed in the number of receptors that showed responsiveness. Specifically, seven compounds can be recognized by one receptor: acesulfame K (DrTas2r1), D-salicin (DrTas2r408), phenylthiocarbamide (DrTas2r408), ranitidine hydrochloride (DrTas2r408), sodium thiocyanate (DrTas2r408), yohimbine hydrochloride (DrTas2r7), and 6-propyl-2-thiouracil (DrTas2r408) **(table 3)**. One compound (chloramphenicol) can be detected by three receptors (DrTas2r1, DrTas2r4, and DrTas2r408), and one compound (denatonium benzoate) by five receptors (DrTas2r1, DrTas2r2, DrTas2r4, DrTas2r7, and DrTas2r408) **(table 3)**. However, 10 compounds (amygdalin, arbutin, camphor, chloroquine diphosphate salt, chlorpheniramine maleate salt, diphenidol hydrochloride, papaverine hydrochloride, phenanthroline, quinine, and saccharin) were not able to be recognized by any receptors **(table 3)**. Among the seven naturally occurring compounds, five (amygdalin, arbutin, camphor, quinine, and papaverine hydrochloride) cannot be detected by any receptors tested, and the other two (D-salicin, and yohimbine hydrochloride) can be detected by only one receptor **(table 3)**.

## DISCUSSION

In this study, we undertook a molecular evolutionary analysis of the bitter taste receptor gene repertoire (*Tas2r*s) in the common vampire bat (*Desmodus rotundus*) compared with non-vampire bats. In addition, we provided the first attempt to deorphanize all bitter receptors of this vampire bat using a cell-based assay. We found that all *Tas2r* genes in the vampire bat have a level of selective pressure similar to that in non-vampire bats. Moreover, we demonstrated that five of the eight bitter receptors in the vampire bat can be activated by some bitter compounds.

We assumed that non-vampire bats must have retained the functions of these *Tas2rs* for their survival, because the bitter taste plays a major role in detecting bitter and potentially toxic chemicals. However, our molecular evolutionary analysis of each of the eight full-length *Tas2r* genes in the vampire bat suggested that this species must have retained some bitter taste functions. Indeed, our previous genetic study also found that several *Tas2r*s are under strong functional constraint and evolutionarily conserved in all three extant species of vampire bats, although these genes do not have full-length sequences **(Hong and Zhao 2014)**. A previous behavioral study demonstrated that the common vampire bat can still detect bitter tastants at relatively higher concentrations, providing additional evidence of functional bitter taste **(Thompson et al. 1982)**. Anatomical and electrophysiological studies observed normal taste buds and functional taste receptors in vampire bats **(Park and Hall 1951; Suthers 1970)**, further supporting our molecular evolutionary analysis. In fact, we certainly can not rule out the possibility that the maintenance of some bitter taste may be useful in the vampire bat to decide whether certain blood is acceptable, as proposed in a blood-sucking insect **(Pontes et al. 2014)**. Regardless, we must realize that the bitter taste is markedly reduced in vampire bats, because we observed the smallest number of full-length *Tas2r*s in the vampire bat **(Jiao et al. 2018)** and a significantly greater percentage of *Tas2r* pseudogenes **(Hong and Zhao 2014)** compared to all non-vampire bats.

Although our collection of bitter compounds is modest **(table 2)**, we have successfully deorphanized five of the eight bitter receptors in the vampire bat for the first time **(figure 2)**. We predict that the remaining three receptors should also have normal functions as do the five others, however we simply did not have enough bitter compounds to identify these functions. Of the five deorphanized receptors, DrTas2r408 appears to be broadly tuned, as it was able to detect seven compounds **(figure 2)**. The gene encoding this receptor was duplicated in nearly all bat species, suggesting an early duplication in ancestral bats. This receptor may thus represent an ancient functional need of taste in early bats, which would be interesting to investigate in the future. Among all of the 19 compounds tested, only chloramphenicol and denatonium benzoate could be detected by multiple bitter taste receptors **(table 3)**. Interestingly, these two chemicals can also be detected by other bitter receptors in bat **(Jiao et al. 2018)**, human **(Meyerhof et al. 2010)**, mouse **(Lossow et al. 2016)**, and cat **(Lei et al. 2015)**, suggesting that they might represent broad ligands for mammalian bitter receptors. Among the seven naturally occurring bitter compounds tested **(table 3)**, five could not be detected by any receptors, and the other two could be detected by only one receptor, which prompted us to hypothesize that vampire bats may have substantially reduced their ability to detect bitter compounds in nature. However, our small collection of bitter compounds calls for further tests on this hypothesis using more bitter compounds. This finding is consistent with the diet that vampire bats rely on, as mammalian or bird blood is unlikely to be bitter or toxic to these animals **(Hong and Zhao 2014)**, and vertebrate blood intrinsically lacks bitter chemicals that are commonly found in nature. As a result, these functional Tas2r receptors are unlikely to have played an important role in taste perception for vampire bats. Indeed, *Tas2r* genes can have extra-oral functions in addition to taste, such as roles in glucose homeostasis and gastric emptying **(Dotson et al. 2008; Glendinning et al. 2008)**. Therefore, future studies should pay more attention to extra-oral functions of bitter taste receptors in vampire bats and other animals.

## Acknowledgments:

We thank Bing-Jun Wang and Kai Wang for technical assistance in data analysis, and Dr Jacquelin De Faveri for her valuable comments and for editing our English. This work was supported by the National Natural Science Foundation of China (31722051) and Natural Science Foundation of the Hubei Province (2019CFA075).

## REFERENCES

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

Anisimova M, Bielawski JP, Yang Z 2001. Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution. *Molecular Biology and Evolution* 18:1585-1592.

Chen Y, Zhao H 2019. A review of research on the dietary specialization of vampire bats. *Acta Theriologica Sinica* 39:202-208.

Darriba D, Taboada GL, Doallo R, Posada D 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9:772-772.

Dotson CD, Zhang L, Xu H, Shin Y-K, Vigues S, Ott SH, Elson AET, Choi HJ, Shaw H, Egan JM 2008. Bitter taste receptors influence glucose homeostasis. *PLoS ONE* 3:e3974.

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

Feng P, Zheng J, Rossiter SJ, Wang D, Zhao H 2014. Massive losses of taste receptor genes in toothed and baleen whales. *Genome Biology. and Evolution* 6:1254-1265.

Frick WF, Kingston T, Flanders J 2019. A review of the major threats and challenges to global bat conservation. *Annals of the New York Academy of Sciences*: https://doi.org/10.1111/nyas.14045.

Glendinning JI, Yiin YM, Ackroff K, Sclafani A 2008. Intragastric infusion of denatonium conditions flavor aversions and delays gastric emptying in rodents. *Physiology & Behavior* 93:757-765.

Han B, Wang K, Jiao H 2016. Molecular evolution of olfactory receptor genes in bats revealed by a genome-wide analysis. *Acta Theriologica Sinica* 36:422-428.

Hayden S, Bekaert M, Goodbla A, Murphy WJ, Davalos LM, Teeling EC 2014. A cluster of olfactory receptor genes linked to frugivory in bats. *Molecular Biology and Evolution* 31:917-927.

Hong W, Zhao H 2014. Vampire bats exhibit evolutionary reduction of bitter taste receptor genes common to other bats. *Proceedings of the Royal Society B: Biological Sciences* 281:20141079.

Huelsenbeck JP, Ronquist F 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754-755.

Jiao H, Wang Y, Zhang L, Jiang P, Zhao H 2018. Lineage-specific duplication and adaptive evolution of bitter taste receptor genes in bats. *Molecular Ecology* 27:4475-4488.

Jiao H, Zhang L, Xie H, Simmons N, Liu H, Zhao H 2019. Trehalase gene as a molecular signature of dietary diversification in mammals. *Molecular Biology and Evolution* 36:2171-2183.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.

Kunz TH, Braun de Torrez E, Bauer D, Lobova T, Fleming TH 2011. Ecosystem services provided by bats. *Annals of the New York Academy of Sciences* 1223:1-38.

Lei W, Ravoninjohary A, Li X, Margolskee RF, Reed DR, Beauchamp GK, Jiang P 2015. Functional analyses of bitter taste receptors in domestic cats (*Felis catus*). *PLoS ONE* 10:e0139670.

Lossow K, Hubner S, Roudnitzky N, Slack JP, Pollastro F, Behrens M, Meyerhof W 2016. Comprehensive analysis of mouse bitter taste receptors reveals different molecular receptive ranges for orthologous receptors in mice and humans. *Journal of Biological Chemistry* 291:15358-15377.

Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufe B, Appendino G, Behrens M 2010. The molecular receptive ranges of human TAS2R bitter taste receptors. *Chemical Senses* 35:157-170.

Nikolaev SI, Montoya-Burgos JI, Popadin K, Parand L, Margulies EH, National Institutes of Health Intramural Sequencing Center Comparative Sequencing P, Antonarakis SE 2007. Life-history traits drive the evolutionary rates of mammalian coding and noncoding genomic elements. *Proceedings of the National Academy of Sciences of the United States of America* 104:20443-20448.

Park H, Hall ER 1951. The gross anatomy of the tongues and stomachs of eight New World bats. *Transactions of the Kansas Academy of Science* 54:64-72.

Pontes G, Minoli S, Insaurralde IO, de Brito Sanchez MG, Barrozo RB 2014. Bitter stimuli modulate the feeding decision of a blood-sucking insect via two sensory inputs. *Journal of Experimental Biology* 217:3708-3717.

Shi P, Zhang J, Yang H, Zhang YP 2003. Adaptive diversification of bitter taste receptor genes in mammalian evolution. *Molecular Biology and Evolution* 20:805-814.

Shi P, Zhang J 2006. Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. *Molecular Biology and Evolution* 23:292-300.

Suthers RA. 1970. Vision, olfaction, taste. In: Wimsatt WA, editor. Biology of bats. New York: Academic Press.

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* 307:580-584.

Thompson RD, Elias DJ, Shumake SA, Gaddis SE 1982. Taste preferences of the common vampire bat (*Desmodus rotundus*). *Journal of Chemical Ecology* 8:715-721.

Turner DC. 1975. The vampire bat: A field study in behavior and ecology. Baltimore, Maryland: Johns Hopkins University Press.

Wang BJ, Xia JM, Wang Q, Yu JL, Song Z, Zhao H 2020a. Diet and adaptive evolution of alanine-glyoxylate aminotransferase mitochondrial targeting in birds. *Molecular Biology and Evolution* 37:786-798.

Wang K, Zhao H 2015. Birds generally carry a small repertoire of bitter taste receptor genes. *Genome Biology. and Evolution* 7:2705-2715.

Wang K, Tian S, Galindo-González J, Dávalos LM, Zhang Y, Zhao H 2020b. Molecular adaptations and convergent evolution of frugivory in Old World and New World fruit bats. *Molecular Ecology* 29:4366-4381.

Wu J, Jiao H, Simmons NB, Lu Q, Zhao H 2018. Testing the sensory trade-off hypothesis in New World bats. *Proceedings of the Royal Society B: Biological Sciences* 285:20181523.

Xu B, Yang Z 2013. PAMLX: a graphical user interface for PAML. *Molecular Biology and Evolution* 30:2723-2724.

Zepeda Mendoza ML, Xiong Z, Escalera-Zamudio M, Runge AK, Theze J, Streicker D, Frank HK, Loza-Rubio E, Liu S, Ryder OA, et al. 2018. Hologenomic adaptations underlying the evolution of sanguivory in the common vampire bat. *Nature Ecology and Evolution* 2:659-668.

Zhao H, Zhou Y, Pinto CM, Charles-Dominique P, Galindo-Gonzalez J, Zhang S, Zhang J 2010. Evolution of the sweet taste receptor gene *Tas1r2* in bats. *Molecular Biology and Evolution* 27:2642-2650.

**Acknowledgments:** We thank Bing-Jun Wang and Kai Wang for technical assistance in data analysis, and Dr Jacquelin De Faveri for her valuable comments and for editing our English. This work was supported by the National Natural Science Foundation of China (31722051) and Natural Science Foundation of the Hubei Province (2019CFA075).

---

**Author contributions:** H.Z. conceived the study, Q.L., H.J., Y.W., and N.N. performed the research, H.Z. and Q.L. wrote the manuscript.

**Competing interests:** The authors declare that they have no competing interests.

**Data availability:** All data needed to evaluate the conclusions are present in the paper.

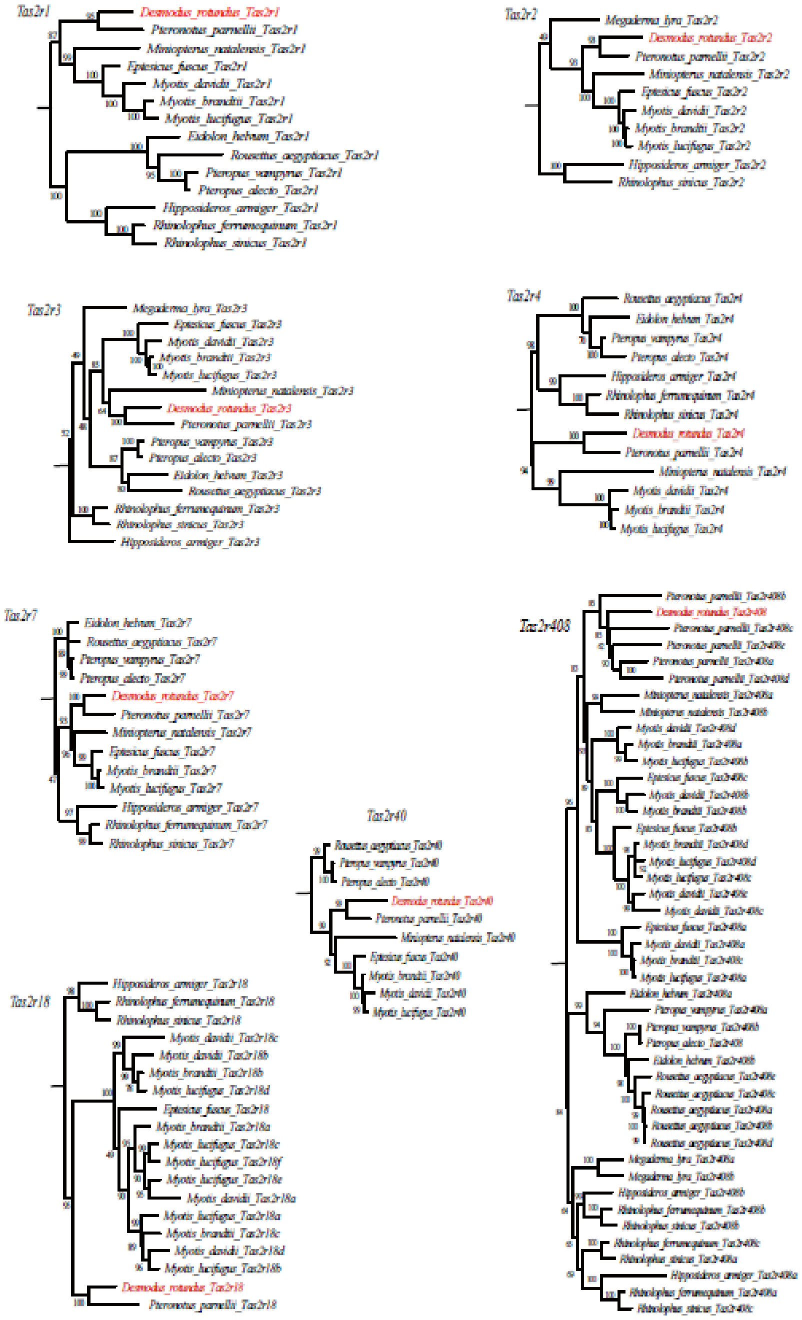

**Figure 1** Phylogenetic trees for each bitter taste receptor gene. Genes from the common vampire bat (*Desmodus rotundus*) are highlighted in red. The eight bitter taste receptor genes in this study are *Tas2r1*, *Tas2r2*, *Tas2r3*, *Tas2r4*, *Tas2r7*, *Tas2r18*, *Tas2r40*, and *Tas2r408*. Numbers at each node are the Bayesian posterior probabilities, shown as percentages. Phylogenetic trees were built with outgroups, which have subsequently been removed from this figure due to space limitations.

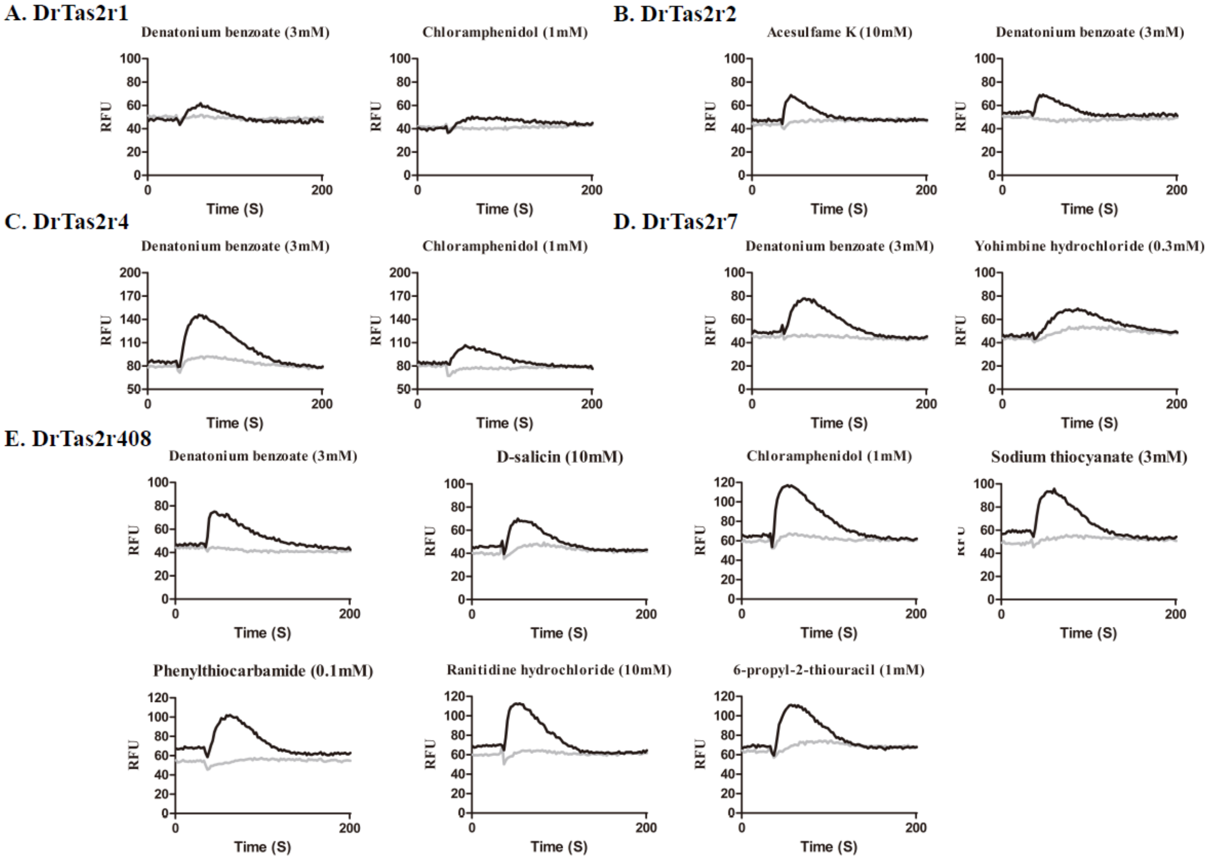

**Figure 2** Calcium mobilization of five bitter taste receptors to bitter compounds. Three of the eight bitter taste receptors (Tas2rs) from the common vampire bat (*Desmodus rotundus*) did not show responses to any of the compounds tested. Black traces, calcium mobilization of the five DrTas2rs (i.e. *Desmodus rotundus* Tas2rs); grey traces, calcium mobilization of mock-transfected cells used as a control.

**Table 1** Likelihood ratio tests of selective pressure on the eight *Tas2r* genes in bats

| **Models** | **ω ($d_N/d_S$)** | **ln*L*[a]** | **np[b]** | **Models compared** | **2Δ(ln*L*)[c]** | ***P*-values** |
|---|---|---|---|---|---|---|
| ***Tas2r1* (14 sequences):** (A) All branches have one ω | ω=0.782 | -5473.29 | 27 | | | |
| (B) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1$=0.780,<br>$\omega_2$=0.823 | -5473.28 | 28 | B vs. A | 0.022 | 0.883 |

| | | | | | | |
|---|---|---|---|---|---|---|
| (C) All branches have one $\omega=1$ | $\omega=1$ | -5478.33 | 26 | C vs. A | 10.08 | 0.001 |
| ***Tas2r2*** **(10 sequences):** (D) All branches have one $\omega$ | $\omega=0.646$ | -3860.36 | 17 | | | |
| (E) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=0.668$,<br>$\omega_2=0.493$ | -3859.86 | 18 | E vs. D | 0.997 | 0.318 |
| (F) All branches have one $\omega=1$ | $\omega=1$ | -3870.61 | 16 | F vs. D | 20.5 | $5.96\times10^{-6}$ |
| ***Tas2r3*** **(15 sequences):** (G) All branches have one $\omega$ | $\omega=0.530$ | -5941.03 | 29 | | | |
| (H) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=0.587$,<br>$\omega_2=0.830$ | -5940.51 | 30 | H vs. G | 1.042 | 0.307 |
| (I) All branches have one $\omega=1$ | $\omega=1$ | -5966.19 | 28 | I vs. G | 50.32 | $1.3\times10^{-12}$ |
| ***Tas2r4*** **(13 sequences):** (J) All branches have one $\omega$ | $\omega=0.580$ | -5028.26 | 25 | | | |
| (K) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=0.596$,<br>$\omega_2=0.410$ | -5027.55 | 26 | K vs. J | 1.418 | 0.234 |
| (L) All branches have one $\omega=1$ | $\omega=1$ | -5053.18 | 24 | L vs. J | 49.84 | $1.67\times10^{-12}$ |
| ***Tas2r7*** **(13 sequences):** (M) All branches have one $\omega$ | $\omega=0.596$ | -3793.14 | 25 | | | |
| (N) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=0.595$,<br>$\omega_2=0.611$ | -3793.13 | 26 | N vs. M | 0.005 | 0.945 |
| (O) All branches have one $\omega=1$ | $\omega=1$ | -3806.67 | 24 | O vs. M | 27.06 | $1.97\times10^{-7}$ |
| ***Tas2r18*** **(19 sequences):** (P) All branches have one $\omega$ | $\omega=0.766$ | -4986.92 | 37 | | | |
| (Q) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=0.763$,<br>$\omega_2=0.813$ | -4986.91 | 38 | Q vs. P | 0.027 | 0.869 |
| (R) All branches have one $\omega=1$ | $\omega=1$ | -4992.25 | 36 | R vs. P | 10.66 | 0.001 |
| ***Tas2r40*** **(10 sequences):** (S) All branches have one $\omega$ | $\omega=0.302$ | -3208.67 | 19 | | | |
| (T) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=0.313$,<br>$\omega_2=0.243$ | -3208.32 | 20 | T vs. S | 0.703 | 0.402 |
| (U) All branches have one $\omega=1$ | $\omega=1$ | -3273.67 | 18 | U vs. S | 130 | $4.1\times10^{-30}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Tas2r408* (44 sequences):** (V) All branches have one $\omega$ | $\omega=1.049$ | -11358.35 | 87 | | | |
| (W) The vampire bat branch has $\omega_1$, and other branches have $\omega_2$ | $\omega_1=1.063$,<br>$\omega_2=0.723$ | -11357.44 | 88 | W vs. V | 1.824 | 0.177 |
| (X) All branches have one $\omega=1$ | $\omega=1$ | -11358.79 | 86 | X vs. V | 0.88 | 0.348 |

Note – [a]Natural logarithm of the likelihood value. [b]Number of parameters. [c]Twice the difference in ln*L* between the two models compared.

**Table 2** Sources of the 19 bitter compounds and their concentrations used in our assays

| **Bitter Compound** | **Product Catalog** | **Source** | **Concentration (mM)** |
|---|---|---|---|
| Acesulfame K | 4054 | Synthetic | 10 |
| Amygdalin | A6005 | Natural | 30 |
| Arbutin | A4256 | Natural | 30 |
| Camphor | 148075 | Natural | 1 |
| Chloramphenicol | C0378 | Synthetic | 1 |
| Chloroquine diphosphate salt | C6628 | Synthetic | 10 |
| Chlorpheniramine maleate salt | C3025 | Synthetic | 0.1 |
| Denatonium benzoate | D5765 | Synthetic | 10 |
| Diphenidol hydrochloride | 5F4-06 | Synthetic | 0.1 |
| D-salicin | S0625 | Natural | 10 |
| Papaverine hydrochloride | P3510 | Natural | 0.01 |
| Phenanthroline | 131377 | Synthetic | 1 |
| Phenylthiocarbamide | P7629 | Synthetic | 1 |
| Quinine | Q1125 | Natural | 0.01 |
| Ranitidine hydrochloride | R101 | Synthetic | 10 |
| Saccharin | 240931 | Synthetic | 10 |

|  |  |  |  |
|---|---|---|---|
| Sodium thiocyanate | S7757 | Synthetic | 3 |
| Yohimbine hydrochloride | Y3125 | Natural | 0.15 |
| 6-propyl-2-thiouracil | P3755 | Synthetic | 1 |

**Table 3** Attempts to deorphanize the eight bitter taste receptors in the vampire bat. Receptors that have succeeded or failed to deorphanize are indicated with check marks (☑) or crosses (×). Three receptors that have failed to deorphanize in this study are shaded in grey. The seven naturally occurring compounds are indicated in boldface, whereas the 12 synthetic compounds are shown in plain text. Number of receptors that can be activated by each compound is shown in the rightmost column, and number of compounds that can activate each receptor is indicated in the bottom row.

| **Bitter compounds** | **DrTas2r1** | **DrTas2r2** | **DrTas2r3** | **DrTas2r4** | **DrTas2r7** | **DrTas2r18** | **DrTas2r40** | **DrTas2r408** | **Total** |
|---|---|---|---|---|---|---|---|---|---|
| Acesulfame K | × | ☑ | × | × | × | × | × | × | 1 |
| **Amygdalin** | × | × | × | × | × | × | × | × | 0 |
| **Arbutin** | × | × | × | × | × | × | × | × | 0 |
| **Camphor** | × | × | × | × | × | × | × | × | 0 |
| Chloramphenicol | ☑ | × | × | ☑ | × | × | × | ☑ | 3 |
| Chloroquine diphosphate salt | × | × | × | × | × | × | × | × | 0 |
| Chlorpheniramine maleate salt | × | × | × | × | × | × | × | × | 0 |
| Denatonium benzoate | ☑ | ☑ | × | ☑ | ☑ | × | × | ☑ | 5 |
| Diphenidol hydrochloride | × | × | × | × | × | × | × | × | 0 |
| **D-salicin** | × | × | × | × | × | × | × | ☑ | 1 |
| **Papaverine** | × | × | × | × | × | × | × | × | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **hydrochloride** | | | | | | | | | |
| Phenanthroline | × | × | × | × | × | × | × | × | 0 |
| Phenylthiocarbamide | × | × | × | × | × | × | × | ☑ | 1 |
| **Quinine** | × | × | × | × | × | × | × | × | 0 |
| Ranitidine hydrochloride | × | × | × | × | × | × | × | ☑ | 1 |
| Saccharin | × | × | × | × | × | × | × | × | 0 |
| Sodium thiocyanate | × | × | × | × | × | × | × | ☑ | 1 |
| **Yohimbine hydrochloride** | × | × | × | × | ☑ | × | × | × | 1 |
| 6-propyl-2-thiouracil | × | × | × | × | × | × | × | ☑ | 1 |
| Total | 2 | 2 | 0 | 2 | 2 | 0 | 0 | 7 | |

## Graphical Abstract

The vampire bat showed sequence conservation of a reduced number of bitter taste receptor genes, and retained some bitter taste function, as suggested by a cell-based assay, but these receptors generally cannot detect naturally occurring bitter compounds.