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**Lineage-specific duplication and adaptive evolution of bitter taste receptor genes in bats**

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ABSTRACT

By generating raw genetic material and diverse biological functions, gene duplication represents a major evolutionary mechanism that is of fundamental importance in ecological adaptation. The lineage-specific duplication events of bitter taste receptor genes (Tas2rs) have been identified in a number of vertebrates, but functional evolution of new Tas2r copies after duplication remains largely unknown. Here we present the largest data set of bat Tas2rs to date, identified from existing genome sequences of 15 bat species and newly sequenced from 17 bat species, and demonstrate lineage-specific duplications of Tas2r16, Tas2r18 and Tas2r41 that only occurred in Myotis bats. Myotis bats are highly speciose and represent the only mammalian genus that is naturally distributed on every continent except Antarctica. The occupation of such diverse habitats might have driven the Tas2r gene expansion. New copies
of Tas2rs in Myotis bats have shown molecular adaptation and functional divergence. For example, three copies of Tas2r16 in Myotis davidii showed differential sensitivities to arbutin and salicin that may occur in their insect prey, as suggested by cell-based functional assays. We hypothesize that functional differences among Tas2r copies in Myotis bats would increase their survival rate through preventing the ingestion of an elevated number of bitter-tasting dietary toxins from their insect prey, which may have facilitated their adaptation to diverse habitats. Our study demonstrates functional changes of new Tas2r copies after lineage-specific duplications in Myotis bats and highlights the potential role of taste perception in exploiting new environments.

**KEYWORDS:** Adaptation, Tas2r, Molecular evolution, Taste, Diet

1. **INTRODUCTION**

Gene duplication is prevalent across all three domains of life (Zhang 2003) and represents a major evolutionary mechanism that is of fundamental importance in ecological adaptation by generating raw genetic material and diverse biological functions (Ohno 1970; Zhang 2003). After duplication, newly derived gene copies may undergo functional loss, conservation, modification, or innovation (Zhang 2003). The vertebrate bitter taste receptor genes (Tas2rs) represent a classic case study of gene duplication (Zhang 2003), with massive numbers of duplicates identified in a number of vertebrates (Li & Zhang 2014), which are linked to adaptive evolution of lineage-specific characteristics, such as dietary changes, sensory trade-offs, and ecological adaptations (Davis et al., 2010; Hayakawa et al., 2014; Li & Zhang 2014; Liu et al., 2016; Wang & Zhao 2015).
Vertebrate *Tas2r* encode bitter taste receptors, which confer the ability to taste bitter compounds. Because toxic substances such as plant alkaloids and insect defensive secretions typically taste bitter, bitter taste is considered an important natural defense in animals (Hong & Zhao 2014). In general, herbivorous mammals possess more *Tas2r* duplicates than carnivorous mammals (Li & Zhang 2014), and insectivorous birds tend to have more *Tas2r* duplicates than carnivorous birds feeding on noninsect animals (Wang & Zhao 2015). Moreover, the massive duplication of *Tas2rs* may also have been related to a sensory trade-off (Hayakawa et al., 2014). For example, the increase in the number of *Tas2rs* in anthropoids (simians, monkeys and apes) relative to other euarchontoglires species suggests an increased reliance on bitter taste (Hayakawa et al., 2014). Because anthropoids have poor olfactory abilities relative to strepsirrhine primates (Barton 2006; Matsui et al., 2010), olfactory degeneration may have been partly compensated for by an increased reliance on vision, and their enhanced taste abilities may also have partially replaced roles of olfaction to evaluate dietary information (Hayakawa et al., 2014). In addition, ecological adaptation may underlie the duplication of *Tas2rs*. For example, despite the fact that birds generally carry a small repertoire of *Tas2rs* (n=4 on average) (Wang & Zhao 2015), a massive duplication of 15 *Tas2rs* was detected specifically in a New World sparrow (Davis et al., 2010); this expansion occurred at a time when the Emberizinae began their initial migration and radiation into the New World, suggesting a role of *Tas2rs* in ecological adaptation (Davis et al., 2010). Such discovery is an echo of an earlier conjecture that mammalian *Tas2rs* may undergo adaptive diversification following gene duplication during exploration of new habitats and diets (Shi et al., 2003). Of note, *Tas2r* gene numbers do not necessarily represent...
the average tuning width of bitter taste receptor repertoires, because the small gene number could be partially compensated by broadly tuned receptors, while the large gene number may be correlated with more specialized receptors (Behrens et al., 2014; Lossow et al., 2016).

Lineage-specific Tas2r duplication events were also observed in members of the mammalian order Chiroptera – the bats, the nocturnal flying mammals – but these observations were identified from two insect-eating species with available genome sequences: Myotis lucifugus and Myotis davidii (Hong & Zhao 2014; Li & Zhang 2014; Zhou et al., 2009). Despite the limited sampling, the authors argued that insect-eating bats demand more Tas2r duplicates to detect insect defensive secretions than their fruit-eating relatives (Hong & Zhao 2014; Zhou et al., 2009). This argument remains true in the Laurasiatherian mammals (Liu et al., 2016) and birds (Wang & Zhao 2015) but has not yet been tested among more species of insect-eating bats. Bats, numbering over 1,100 species, are the second-most speciose group of mammals after rodents, accounting for approximately 20% of all living mammal species (Simmons 2005). Although bats exhibit a huge diversity in diets, including insects, fish, amphibians, mammals, birds, fruits, flowers, nectar, pollen, foliage, and blood (Altringham 1996; Zhao et al., 2010), most species (>900) exclusively feed on insects (Simmons 2005). In addition, bats are widely distributed, being absent only in polar regions (Simmons 2005). This occupation of diverse habitats may also drive the evolution of Tas2rs as bats inhabit new environments.

Here we present the first in-depth analysis of molecular evolution of Tas2rs in bats, combining sequence analysis with cell-based functional assays. We present the largest data set of bat Tas2rs to date that were identified from 15 existing bat genomes and newly
sequenced from 17 bat species, and we characterize functional changes of \( \text{Tas2r} \) copies following lineage-specific duplication. We aimed to test (1) whether lineage-specific duplications of \( \text{Tas2r} \)s have occurred just in \( \text{Myotis} \) or in all insect-eating bats, (2) whether new copies of \( \text{Tas2r} \)s have undergone positive selection to facilitate molecular adaptation of functional changes, and (3) whether these new copies show functional differentiation.

2. MATERIALS AND METHODS

2.1 Genomic data

The genome assemblies of 15 bat species were retrieved from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, last accessed April 25, 2018). Detailed information for each assembly is given in Supplementary Table S1.

2.2 Annotation and nomenclature of \( \text{Tas2r} \) genes

\( \text{Tas2rs} \) are intronless genes that encode bitter taste receptors with seven-transmembrane domains (Matsunami et al., 2000). Using the previously published intact \( \text{Tas2r} \) protein sequences in human, mouse, chicken, and zebra fish as queries, we conducted tBLASTn searches (Altschul et al., 1990) against each of 15 genome sequences to identify \( \text{Tas2r} \) genes with an e-value of 1e-10, following a previous study (Wang & Zhao 2015). The BLAST hits with a length less than 300 bp were discarded. An intact \( \text{Tas2r} \) gene was considered to have a coding sequence with proper initiation codon and stop codon and length greater than 270

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amino acids. All full-length candidate genes were checked to predict whether the seven-transmembrane domains were present using the TMHMM method (Krogh et al., 2001). Those hits with disrupted ORFs (open reading frames) were regarded as pseudogenes. The remaining hits with truncated ORFs resulting from either incomplete genome sequencing or poor genome assembly were considered to be partial Tas2r genes. To ensure Tas2r gene identity, BLASTn searches against the entire GenBank were performed. All intact Tas2r sequences newly identified from this study are provided in Data Set S1.

We here used the nomenclature of Tas2rs previously proposed in Euarchontoglires and Laurasiatheria (Hayakawa et al., 2014; Liu et al., 2016). To do this, we built a gene tree (Figure S1) with the Bayesian approach (Ronquist & Huelsenbeck 2003) based on the multiple alignment of nucleotide sequences of bat Tas2rs and representative genes from the 28 Tas2r clades in Boreoeutheria (Hayakawa et al., 2014).

2.3 Reconstruction of Tas2r gene repertoire evolution

The deduced Tas2r sequences were aligned using the MUSCLE program (Edgar 2004). The Tas2r gene trees were constructed by the maximum likelihood (ML) and the Bayesian methods. The ML trees were built by PhyML, version 3.0 (Guindon et al., 2010), with 1,000 bootstrap replications, and the Bayesian trees were recovered by MrBayes, version 3.1.2 (Ronquist & Huelsenbeck 2003), with 1×10^6 Markov chain Monte Carlo generations. The best-fitting nucleotide substitution models were selected by the program jModelTest 2 (Darriba et al., 2012).
To infer the evolutionary changes of *Tas2r* gene numbers in bats, we used the reconciliation method to predict the gene gains and losses in NOTUNG, version 2.6 (Chen et al., 2000), following a recent study (Wang & Zhao 2015). This method compares the species tree and the gene tree (Chen et al., 2000). The species tree is shown in **Figure 1**; the gene tree was inferred by the Bayesian method (**Figure S2**).

To examine whether diet has driven the evolution of *Tas2r* gene repertoire in bats, we performed a regression analysis of *Tas2r* gene numbers against diet, following previous studies (Li & Zhang 2014; Wang & Zhao 2015). We coded dietary preference of each bat as 0 (frugivorous) and 1 (insectivorous) and then conducted a phylogenetically independent contrast (PIC) analysis using the package Analyses of Phylogenetics and Evolution (Paradis et al., 2004). We also performed a PIC analysis by coding the non-*Myotis* species as 0 and *Myotis* species as 1, to test whether *Myotis* species possess more *Tas2r* genes than do non-*Myotis* species. The input tree was the established species tree (**Figure 1**). The branch lengths were estimated by divergence times obtained from the TimeTree (Hedges et al., 2006).

### 2.4 Sequencing of *Tas2r16* and *Tas2r41* genes

Genomic DNAs of the 17 bat species (**Table S2**) were isolated from wing membranes frozen at -80°C using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. All wing membranes of bats were obtained from the mammal tissue archive held at the Guangdong Institute of Applied Biological Resources (China). A suite of
primers (Table S3) were used to conduct polymerase chain reaction (PCR) amplifications for *Tas2r16* and *Tas2r41* genes. To isolate the different copies of *Tas2r16* and *Tas2r41* genes, we cloned the PCR products into the pMD19-T vector and then sequenced them directly using the M13 universal primers. For one PCR product, at least three positive clones were picked for sequencing. The resulting sequences were assembled at 99% similarity level to allow for 1% mutations by considering both Taq polymerase-derived errors and allelic variations, aiming to avoid inclusion of the same *Tas2r* multiple times. Each assembled sequence was counted as one copy. The sequences newly acquired in the present study were deposited to the GeneBank database under accession numbers MG200308- MG200350.

### 2.5 Selection tests for *Tas2r16*, *Tas2r18*, and *Tas2r41* genes

To determine whether natural selection acts on the *Myotis*-specific lineages, we estimated the ratio ($\omega$) of the rate of nonsynonymous substitutions ($d_N$) to the rate of synonymous substitutions ($d_S$) using the codeml program implemented in PAML, version 4 (Yang 2007), with $\omega$>1, $\omega=1$, $\omega$<1 indicating positive selection, neutral evolution, and purifying selection. We conducted two tests for each of the three genes (*Tas2r16*, *Tas2r18*, and *Tas2r41*). First, we tested whether positive selection affects a few sites along the *Myotis* lineages (foreground branches: all branches connecting *Myotis* genes) using the improved branch-site test of positive selection (Zhang et al., 2005); the corresponding null model is same as the alternative model, except the $\omega$ of foreground branches fixed at 1 (Yang & Nielsen 2002; Zhang et al., 2005). Second, we tested whether $\omega$ was divergent between the
*Myotis* lineages (all branches connecting *Myotis* genes) and the non-*Myotis* background by comparing Clade model C (CmC) with its null model (M2a_ref) (Bielawski & Yang 2004; Weadick & Chang 2012). The gene trees reconstructed by the Bayesian approach were used to undertake these selection tests (Figures S3-S5). Likelihood ratio tests were applied to compare alternative models with null models.

### 2.6 Bitter compounds

Our collection of bitter compounds contained 8 synthetic and 8 natural compounds (Table S4). Among the 8 naturally occurring compounds, arbutin and salicin are likely to occur in the prey of *Myotis* bats, because they could be synthesized or sequestered by some insects, such as bees (Duffey 1980), beetles (Kuhn et al., 2004) and butterflies (Prudic et al., 2007). The remaining 6 compounds (Table S4) have the possibilities to be encountered by *Myotis* bats in their surrounding environments, as many *Myotis* bats are typical gleaners, catching their insect prey from foliage (Arlettaz 1996; Ruedi & Mayer 2001). Specifically, camphor, papaverine hydrochloride, picrotoxinin and quinine are natural products from plants (Calixto et al., 1984; Croteau et al., 2000; Porter 1967); Thiamine is a vitamin that was also found in plants (Asensi-Fabado & Munne-Bosch 2010); Chloramphenicol was first isolated from the soil bacterium *Streptomyces venezuelae* (Fernandez-Martinez et al., 2014).

With the exception of diphenidol hydrochloride (Reagent World), all tested compounds were purchased from Sigma-Aldrich (Table S4). These compounds were dissolved in Dulbecco’s phosphate-buffered saline (DPBS) or in a mixture of dimethyl sulfoxide (DMSO).
and DPBS, with a final DMSO concentration <0.1% to protect the transfected cells against toxicity. The highest concentrations of bitter compounds used in our study were taken from Meyerhof et al. (Meyerhof et al., 2010).

2.7 Construction of bat Tas2r16 expression plasmids

The complete coding sequences of bat Tas2r16 genes were amplified from the genomic DNAs using high-fidelity KOD-Plus-Neo DNA polymerase (Toyobo). PCR products were purified and then inserted into the expression vector pEAK10, with the first 45 amino acid residues of rat somatostatin receptor 3 as the signal peptide at the 5'-end of Tas2r16 genes. The sequences of all constructs were verified by the traditional Sanger sequencing.

2.8 Functional assays of Tas2r16

Functional assays were carried out as described previously (Lei et al., 2015). Briefly, the human embryonic kidney 293 (HEK293) cells (Peakrapid) were cultured in Opti-MEM supplemented with 5% fetal bovine serum and seeded in 96-well plates at a density of 50,000 per well. After 24-26 hours, the HEK293 cells were transiently transfected by a Tas2r16 construct (0.1 μg/well) with a coupling chimeric G protein Gα16-gust44 (0.1 μg/well) using Lipofectamine 2000 (0.5 μl/well). After 24 hours of transfection, cells were washed once with DPBS and loaded with the calcium-sensitive dye Fluo-4-AM (2.5 μM; Invitrogen) for 1 hour at room temperature. After three washes with DPBS to remove excess dye, cells were
assayed for their responses to bitter compounds using a FlexStation III spectrometer (Molecular Devices). Relative fluorescence units (excitation at 488 nm, emission at 525 nm, cutoff at 515 nm) were recorded every 2 seconds after addition of DPBS supplemented with 2× ligands. Calcium mobilization traces were recorded. All measurements were independently repeated at least three times.

Calcium mobilization was quantified as the percentage of the changes in fluorescence (ΔF) relative to F (the peak of fluorescence minus baseline fluorescence) from triplicate experiments. Calcium signal traces and bar graphs were generated with the GraphPad Prism 5 package (GraphPad Software, Inc.). Dunnett’s tests were used to perform the statistical analysis. Dose-dependent curves were plotted by a nonlinear regression using GraphPad Prism 5. Functional assay data generated from our study was provided in Data Set S2.

3. RESULTS

3.1 Evolution of Tas2r gene repertoires in bats

A total of 416 Tas2rs (mean, 28 per species; median, 26; range, 21-38) were identified in this study: 263 intact genes (mean, 18; median, 17; range, 8-29), 26 partial genes (mean, 2; median, 2; range, 0-5), and 127 pseudogenes (mean, 8; median, 9; range, 2-15) (Figure 1, Data Set S1). It appears that the numbers of partial genes are comparable across all species, including four with low-coverage genomes sequenced by the Illumina HiSeq platform (17-18×) (Figure 1, Table S1). Indeed, we found that the fractions of Tas2r partial genes are not correlated with the contig N50 lengths of the 15 genome assemblies (Spearman’s...
\( \rho = -0.526, P = 0.065 \) (Figure S6), suggesting that varying genome quality did not play a major role in determining \( \text{Tas2r} \) gene number and is thus acceptable for our analysis.

Through a phylogenetically independent contrast analysis, we found no significant relationship between \( \text{Tas2r} \) gene number in frugivorous and insectivorous bats, including number of intact genes \( (P = 0.799) \), number of putatively functional genes \( (P = 0.127) \), number of pseudogenes \( (P = 0.173) \), number of all identified genes \( (P = 0.777) \), and proportion of pseudogenes \( (P = 0.071) \) (Table S5). Thus, insectivorous bats apparently do not carry more \( \text{Tas2rs} \) than their frugivorous relatives in general. The number of putatively functional \( \text{Tas2rs} \) in three \( \text{Myotis} \) species (range, 28-31) is larger than that in other species (range, 8-21) (Figure 1), and the difference is significant after correcting for phylogenetic inertia (Spearman’s \( \rho = 0.763, P = 0.002 \) for intact genes; \( \rho = 0.793, P = 0.001 \) for putatively functional genes; \( \rho = 0.578, P = 0.030 \) for all identified \( \text{Tas2r} \) genes; Figure S7).

Using a reconciliation approach (Chen et al., 2000), we inferred evolutionary changes in the number of intact \( \text{Tas2rs} \) in ancestral and extant lineages by comparing the species tree with the gene tree (Wang & Zhao 2015). The species tree (Figure 1) followed previous studies (Almeida et al., 2011; Miller-Butterworth et al., 2007; Ruedi et al., 2013; Teeling et al., 2005), while the gene tree (Figure S2) was reconstructed using a Bayesian approach. We identified the greatest reduction of \( \text{Tas2r} \) gene number \( (n = 11) \) in the common ancestor of four species of Old World fruit bats (Pteropodidae) (Figure 1), suggesting that the ancestral lineage leading to the Old World fruit bats underwent a massive loss of taste receptor genes, although their extant lineages did not (Figure 1). By contrast, the greatest expansion of \( \text{Tas2r} \) gene number \( (n = 11) \) occurred in the common ancestor of three \( \text{Myotis} \) species (Figure 1).
3.2 Tas2r gene duplications in bats

To identify gene duplication events, we aligned the 263 intact Tas2rs from the 15 bat species (Figure 1), and reconstructed phylogenetic trees with both maximum likelihood (ML) and Bayesian approaches, using the mouse V1R1 gene as an outgroup. In both approaches, the partial Tas2r genes and pseudogenes were not included because they are either too short or too divergent to be aligned. We found that the Bayesian tree showed a topology similar to the ML tree, though some nodes of the ML tree were weakly supported (Figure S8). From the phylogenetic tree, all bat Tas2rs formed 22 clades, each of which consisted of 2-43 genes (Figure S2). Notably, we identified Myotis-specific duplication events in two clades (Tas2r16 and Tas2r18): three Myotis species have 3-4 copies of Tas2r16 and 3-6 copies of Tas2r18, whereas other bats possess only one copy of each of the two genes (Figure S2).

Another duplication (Tas2r41) was not specific to Myotis species; a non-Myotis species (Eptesicus fuscus) has two duplicates as well. For Myotis bats, this duplication occurred in the common ancestor of the three Myotis species (Figure S2). Additionally, we detected a large gene expansion of Tas2r408 (43 copies), in which duplication events occurred in nearly all bats examined; putative gene losses in the four Old World fruit bats were also found in five clades (Tas2r2, Tas2r10, Tas2r11, Tas2r18, and Tas2r67) (Figure S2), consistent with the massive reduction in the number of Tas2r genes in the common ancestor of Old World fruit bats (Figure 1). We also undertook tests of gene conversion in all Tas2rs using the Sawyer’s method (Sawyer 1989) and identified only four possible gene conversion events (Table S6), suggesting that such events play a minor role in bat Tas2r evolution.
To test whether *Myotis* species are unique in *Tas2r* gene duplication compared to other species, we examined *Tas2r16* and *Tas2r41* in 30 bat species (4 *Myotis* and 26 non-*Myotis* species; **Figure 2**). We did not examine *Tas2r18* in the same 30 bats in the present study, because there is no information about ligands of human TAS2R18, which may represent a challenge for detecting ligands for bat *Tas2r18* since our collection of bitter compounds is small. Our samples contained 15 species with available genome sequences and 17 species with available genetic material ready for sequencing. One species (*Myotis davidii*) has both available genome sequence and genetic material (**Figure 2**). Combining our newly generated 43 sequences (**Table S2**) with the identified genes from genomic data, our phylogenetic analyses (**Figures S3, S4**) showed that the vast majority of bats have only one copy of *Tas2r16* (or *Tas2r41*), whereas the four *Myotis* species have at least two copies (**Figure 2**). There are a few exceptions: *Cynopterus sphinx*, *Plecotus auritus*, and *Vespertilio superans* have two copies of *Tas2r16*, and *Eptesicus fuscus* has two copies of *Tas2r41* (**Figure 2**). Together, these findings show that lineage-specific duplications of *Tas2r16* and *Tas2r41* occurred in *Myotis* bats.

**3.3 Molecular adaptation of bat *Tas2r* genes after duplication**

To test whether the *Myotis*-specific duplicates were associated with molecular adaptation, we estimated \( \omega \) using a likelihood approach (Yang & Rannala 2012). In the data set for *Tas2r16*, we detected a small proportion of sites (7.7%) with a signature of positive selection \( (\omega_2=4.606, P=4.45E-06; \text{**Table 1**}) \), suggesting that the *Myotis*-specific duplicates occurred in *Myotis* bats.
underwent molecular adaptation. We also detected divergent selection between *Myotis* and non-*Myotis* lineages after comparing CmC with M2a_ref \((\omega_2/\omega_f=3.019/4.618, P=0.042,\text{ Table 1})\), suggesting that functional divergence occurred among *Myotis* duplicates. Similarly, the data sets for *Tas2r18* and *Tas2r41* also showed signatures of positive selection and divergent selection (see \(\omega\) and \(P\) values in Table 1). Therefore, *Myotis*-specific duplicates of *Tas2rs* were inferred to have undergone adaptive evolution and functional divergence.

### 3.4 Functional differentiation of new copies of *Tas2r16*

To determine whether gene duplications of bat *Tas2rs* were accompanied by functional changes or innovations, we performed cell-based functional assays of bat *Tas2r16* because the human ortholog *TAS2R16* is one of the best-characterized *Tas2r* genes (Buske et al., 2002; Imai et al., 2012). By contrast, the human ortholog *TAS2R41* has only two known ligands (Lossow et al., 2016; Thalmann et al., 2013). We examined the responsiveness of these receptors toward 16 available bitter compounds that are known to activate human TAS2Rs (Table S4) (Meyerhof et al., 2010), including D-salicin and arbutin, plant-derived compounds activating the human TAS2R16 (Parejo et al., 2001; Vane 2000). After measuring relative fluorescence changes (ΔF/F) with respect to the mock-transfected negative control, we found that four of the five bat Tas2r16 receptors recognized arbutin and D-salicin (Figure 4), while one copy (Mda_Tas2r16a) of *Myotis davidii* showed no response to any of the 16 compounds we tested. To test the sensitivities of these receptors toward arbutin and D-salicin, we obtained the dose-dependent curves for all examined receptors (Figure 4c, f). While Mda_Tas2r16a was not activated at any concentration of any ligands that we tested,
the other four receptors showed distinct sensitivities to arbutin and D-salicin (Figure 4c, f). Mda_Tas2r16b appears to be more sensitive than Mda_Tas2r16c to arbutin, while both Mda_Tas2r16b and Mda_Tas2r16c are more sensitive than the two outgroups, which showed similar sensitivity levels (Figure 4c). By contrast, Mda_Tas2r16b is less sensitive than Mda_Tas2r16c to D-salicin, while the two outgroups showed distinct sensitivity levels (Figure 4f). Based on their different degrees of sensitivity to the tested compounds, functional differentiations were apparent among the three copies of Mda_Tas2r16 and between Mda_Tas2r16 copies and the two outgroups (Figure 4).

Notably, the Mda_Tas2r16c receptor showed responsiveness to chloramphenicol (not a ligand for human TAS2R16), while other receptors did not recognize this compound at any concentration tested (Figure 4g-i), suggesting a new function of Mda_Tas2r16c. Likewise, denatonium benzoate activated Mda_Tas2r16b and Mda_Tas2r16c but did not activate Mda_Tas2r16a or the two outgroups (Figure 4j-l), indicating another new function. In addition, the dose-dependent curves showed that Mda_Tas2r16b appears to be more sensitive than Mda_Tas2r16c to denatonium benzoate (Figure 4l), suggesting differential functional characteristics for the two duplicate genes. Clearly, novel functions occurred in newly derived duplicates after Myotis-specific duplication. As expected, arbutin and D-salicin activated human TAS2R16, whereas denatonium benzoate and chloramphenicol did not (Figure S9), consistent with previous reports (Meyerhof et al., 2010). Therefore, our functional expression analyses demonstrated that Tas2r duplicates acquired distinct functionality and expanded bitter compound-detecting capacity.
DISCUSSION

In this work, we undertook the first in-depth study of bitter taste receptor genes (Tas2rs) in bats, combining sequence analysis with cell-based functional assays. We identified lineage-specific duplications of Tas2rs that occurred in Myotis bats, and new copies of Tas2rs were inferred to have undergone positive selection and functional divergence. Cell-based functional assays suggest that three copies of Tas2r16 in Myotis davidii developed functional differentiations and functional innovations following duplication.

In search of bat Tas2r gene repertoires, the common vampire bat Desmodus rotundus was found to have the smallest Tas2r repertoire (n=21), of which 8 Tas2r genes are complete and intact and thus putatively functional, whereas the remaining 13 genes are pseudogenized. This finding strongly suggests that bitter taste reception is reduced in Desmodus rotundus, which was also supported by previous genetic (Hong & Zhao 2014) and behavioral (Thompson et al., 1982) studies. In a recent study, however, Tas2r3 in Desmodus rotundus was found to have undergone positive selection and thus may be linked to blood consumption (Zepeda Mendoza et al., 2018). By contrast, Tas2r3 is a pseudogene in another vampire bat species Diphylla ecaudata (Hong & Zhao 2014), suggesting that the proposed link between Tas2r3 function and sanguivorous diet appears to be more complicated than anticipated.

In phylogenetic reconstructions inferred from intact Tas2r sequences of bats, gene duplication events were observed in only a few cases (Tas2r16, Tas2r18, Tas2r41, and Tas2r408) (Figures S2, S8). While Tas2r408 was duplicated in each species, other duplication events (Tas2r16, Tas2r18 and Tas2r41) occurred specifically in Myotis bats.

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(Figures S2, S8). Since the divergence between the New World species (Myotis lucigugus and M. brandti) and the Old World species (M. davidii) was at the origin of the common ancestor of all Myotis bats (Figure 1) (Kawai et al., 2003; Ruedi et al., 2013; Stadelmann et al., 2007), the lineage-specific duplications of Tas2rs observed in Myotis bats were likely to have taken place in the common ancestor of all Myotis species. Such gene duplications were not found in other insectivorous bats, suggesting that Tas2r gene duplications do not necessarily occur in all insect eaters, although many nocturnal insects release poisonous defensive secretions (likely bitter) that may function to deter insect-eating bats (Blum 1981; Hristov & Conner 2005; Ratcliffe & Fullard 2005). This finding does not support an earlier hypothesis that insectivorous bats have more Tas2r genes than their frugivorous relatives, possibly due to the scanty sampling of taxa in earlier studies (Hong & Zhao 2014; Zhou et al., 2009). Our study taxa included 4 species of frugivorous bats, 1 species of sanguivorous bat and 10 species of insectivorous species sampled across bat phylogeny (Figure 1), and our whole genome data of the 15 bat species enabled us to obtain all potential Tas2r genes from each species. Moreover, we expanded our study taxa to 6 frugivorous and 24 insectivorous bats in the surveys of two receptors, Tas2r16 and Tas2r41 (Figure 2), providing compelling evidence that Tas2r duplications occurred in Myotis but not in other insectivorous bats.

However, we acknowledge that we cannot completely rule out the potential bias in gene amplification and molecular cloning. An expansion of Tas2r genes only in Myotis bats did not support a sensory tradeoff between an increase of bitter taste and a loss of color vision, because all Myotis bats examined so far possess dichromatic color vision (Zhao et al., 2009a; Zhao et al., 2009b). In addition, no pseudogenes were detected in the Tas2r16 duplicates.
(Figure S10), suggesting that Tas2r16 duplicates in Myotis species are functionally important and purifying or positive selection has prevented them from pseudogenization.

Signals of positive selection were detected in branches connecting Myotis-specific duplicates (Table 1), suggesting that the observed Tas2r duplicates in Myotis bats are adaptive. Although the evolutionary history of vertebrate Tas2rs is generally shaped by adaptive evolution (Shi & Zhang 2006; Shi et al., 2003), lineage-specific adaptation of Tas2rs has rarely been reported. Such adaptive evolution points to an evolutionary mechanism associated with Myotis-specific biological characteristics. Signals of diversifying selection were also identified between Myotis duplicates and other Tas2r genes (Table 1), suggesting that functional divergence and specialization may have occurred in newly derived duplicates in Myotis. Indeed, in the case of Tas2r16 from Myotis davidii, the three copies of Tas2r16 have undergone functional differentiation and have evolved novel functions (Figure 4). Such functional changes in newly derived Tas2r duplicates are likely driven by adaptive evolution.

Our cell-based assays demonstrated functional differences of the three copies of Mdav_Tas2r16 (Figure 4). Specifically, Mdav_Tas2r16b is more sensitive than Mdav_Tas2r16c to arbutin, while the opposite is true to D-salicin. Chloramphenicol recognition was found in Mdav_Tas2r16c, but not in other receptors tested. Similarly, denatonium benzoate can activate Mdav_Tas2r16b and Mdav_Tas2r16c but can’t activate other receptors tested. A molecular mechanism of functional divergence of the three copies of Mdav_Tas2r16 was tentatively inferred. First, we ran GENECONV to detect gene conversion events that may contribute to the acquisition of new ligand recognition of Mdav_Tas2r16b and Mdav_Tas2r16c by adding the sequences of the three copies of Mdav_Tas1r16. We
detected the same four possible gene conversion events that were listed in Table S6. This finding suggests that no gene conversion event occurred between Mdav_Tas2r16b, Mdav_Tas2r16c and other intact Tas2r genes in Myotis davidii. Second, we aligned the amino acid sequences of five Tas2r receptors tested in our functional assays to identify the key residues differing among these receptors which may lead to the functional innovation. The alignment led to the identification of 12 residues, including 10 unique ones of Mdav_Tas2r16c that may underlie chloramphenicol recognition and two residues shared by Mdav_Tas2r16b and Mdav_Tas2r16c that may underlie denatonium benzoate recognition (Figure S11A). Such residues are found in both transmembrane helix and extracellular and intracellular loops of these receptors (Figure S11B). Future work using mutagenesis and cell-based functional assays will determine whether these residues are indeed critical for ligand-receptor interactions. Third, we conducted site models in PAML (Yang 2007) to identify positively selected sites that may lead to molecular adaptation (Table S7).

Intriguingly, two of the three positively selected sites are located in the same positions as the unique sites for Mdav_Tas2r16c (Figure S11). We predicted four Tas2rs (Mdav_Tas2r1, Mda_Tas2r4, Mdav_Tas2r10, Mdav_Tas2r39) to have the potential ability for denatonium benzoate or chlorphenicol recognition, because their one-to-one orthologous genes in humans can respond to the two compounds, although sequence-orthologous Tas2r receptors may have distinct ligand profiles (Lossow et al., 2016). We mapped the 12 key sites to the four Tas2rs based on an alignment of amino acid sequences of 25 intact Mdav_Tas2rs, and identified one site (164K) that was only shared by Mda_Tas2r16c and Mdav_Tas2r39. We thus propose that the shared site (164K) may account for functional convergence in the two receptors, although
this proposal needs to be substantiated by an in-depth functional assay in the future. Of note, although novel functions were found in new copies of Tas2r16 in Myotis davidii, we can not rule out a possibility that this would not increase the receptive field of the entire Tas2r repertoire due to functional redundancy of other Tas2rs. Therefore, testing additional naturally occurring bitter compounds that are relevant to the bat’s ecology and other bitter receptors may address this issue in the future.

Functional diversification of duplicate genes generally involves subfunctionalization and neofunctionalization (Zhang 2003). Based on our functional assays and genetic evidence for positive selection, we infer that adaptive functional diversification of Tas2r duplicates may have occurred in Myotis bats. Why did this occur specifically in Myotis bats? Earlier studies proposed that diet generally shapes Tas2r gene numbers in vertebrates (Hayakawa et al., 2014; Li & Zhang 2014; Liu et al., 2016; Wang & Zhao 2015). However, our data from bats are not consistent with this claim, because only Myotis bats have more Tas2r genes than frugivorous and other insectivorous bats (Figure 1). Despite the difference in dietary components, both Myotis and non-Myotis insectivorous bats appear to most commonly feed on insects from the orders Coleoptera (beetles), Lepidoptera (moths and butterflies), and Diptera (flies) (Hill & Smith 1984; Liu et al., 2014). Thus, there is no clear-cut correlation between diet and Tas2r gene number in bats. On the other hand, we identified an apparent association between ecological adaptation and Tas2r gene expansion in Myotis bats. The genus Myotis is cosmopolitan, representing the only mammalian genus that is naturally distributed on every continent except Antarctica, from the boreal to sub-Antarctic zones, tropical rain forests, or semidesertic habitats (Ruedi & Mayer 2001; Ruedi et al., 2013).
Myotis bats are highly speciose, with over 100 species living in most terrestrial habitats (except polar regions) (Simmons 2005). Indeed, these bats are the second most diverse genus (after Crocidura shrews) among mammals (Ruedi et al., 2013). They are ecologically diversified, with some taxa being water surface foragers, others aerial insect feeders, and others terrestrial gleaners from foliage and other surfaces (Findley 1972; Ruedi & Mayer 2001). We thus hypothesize that occupation of such ecological diversity in Myotis bats may have driven Tas2r gene expansion, permitting them to colonize novel habitats. According to our experimental data from cell-based functional assays, we found that 4 of the 16 bitter compounds are able to activate one or more bat Tas2r16 receptors (Figure 4). To the best of our knowledge, chloramphenicol and denatonium benzoate are unlikely to be present in bat diets. By contrast, arbutin and salicin are naturally occurring glycosides, both of which are secondary metabolites in the leaves of plants (Panusa et al., 2015). Previous studies showed that herbivorous insects have the ability to synthesize or sequester glucosides for defending against their predators, such as insect-eating bats (Kuhn et al., 2004; Nishida 2002; Opitz & Muller 2009; Zagrobelny et al., 2004). Due to differential sensitivities of Tas2r16 copies to bitter compounds, including arbutin and salicin that may occur in their insect prey (Kuhn et al. 2004, Oba et al. 2013), Myotis bats may tend to feed on safe foods in varying environments. As a result, we hypothesize that Myotis bats may have greater abilities to explore and adapt to new environments, which may be one of the reasons why they are widely distributed. Consistent with this hypothesis, passerines (order Passeriformes), the most diverse order among birds, with a global distribution, carry a larger Tas2r gene repertoire than most other bird species (Wang & Zhao 2015). Similarly, in the New World...
sparrow *Zonotrichia albicollis*, a species-specific expansion of *Tas2rs* occurred at the time when the Emberizinae began their initial migration and radiation into the New World (Davis et al., 2010). This is consistent with the hypothesis that the expansion of *Tas2rs* might be an adaptive mechanism as animals move into new environments.

**ACKNOWLEDGMENTS**

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DATA ACCESSIBILITY

The sequences newly acquired in the present study were deposited to the GeneBank database under accession numbers MG200308-MG200350.

AUTHOR CONTRIBUTIONS

H.Z. conceived and designed the project; P.J. supervised the cell-based functional assays; H.Z. and P.J. contributed reagents or analytical tools; H.J. analyzed data and conducted the functional assays; H.J. and Y.W. performed the sequencing work; L.Z. provided wing punches of bats; H.Z. and H.J. wrote the paper, and P.J. edited the paper.

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**FIGURE CAPTIONS**

**Figure 1.** The bitter taste receptor repertoires of 15 species of bats with available genome sequences. Four of the 15 species with low-quality genome assemblies are underlined. Dietary preferences are indicated by colored squares. Gene gain and loss events are mapped to the species tree, indicated by red and green numbers, respectively. Massive gene gains and losses (>10) are marked with red and green circles, respectively.

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Figure 2. Validation for *Myotis*-specific duplications by polymerase chain reaction amplifications. The copy numbers for *Tas2r16* and *Tas2r41* are listed for each species. The species with newly amplified sequences are indicated in boldface, whereas the species with available genome sequences are shown in plain type. The only species (*Myotis davidii*) with both newly amplified and available genome sequences is underlined, and the number of genes identified from its genome sequence is shown in parentheses. Four *Myotis* species are indicated by shading; n.a. (not available) indicates failed amplifications or absence from the genome sequence.

Figure 3. The Bayesian gene tree of bat *Tas2r16*. Node support values represent Bayesian posterior probabilities. The *Tas2r16* genes generated by amplification and sequencing are shown in boldface. The pink star indicates the node where *Myotis*-specific duplication occurred. Three *Myotis* clusters are marked by colored circles. Genes belonging to the *Myotis* genus are highlighted within a dashed box. Genes selected for cell-based functional assay are indicated with a response line.

Figure 4. Functional differentiations of *Tas2r16* receptors in *Myotis davidii*. HEK293 cells transiently expressing bat *Tas2r16* and *Ga16-gust44* were assayed for responses to 4 compounds. (a, d, g, j) Time course of fluorescence intensity: calcium mobilization of *Mdav_Tas2r16b* (red) or *Mdav_Tas2r16c* (green) and mock-transfected cells (transfected This article is protected by copyright. All rights reserved.
only with Gα16-gust44; gray) to arbutin (30 mM), D-salicin (10 mM), chloramphenicol (1mM) and denatonium benzoate (3mM). (b, e, h, k) Quantitative analysis of responses of bat Tas2r16 to arbutin, D-salicin, chloramphenicol and denatonium benzoate (mean ± SE, ***P<0.001, one-way ANOVA). (c, f, i, l) Dose-dependent responses of bat Tas2r16 to arbutin, D-salicin, chloramphenicol and denatonium benzoate. All data were fitted using GraphPad Prism 5.
Table 1. Adaptive functional diversification of Tas2r genes in Myotis bats.

<table>
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<th>Models</th>
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<th>$\ln L^b$</th>
<th>Models compared</th>
<th>$2\Delta (\ln L)^c$</th>
<th>$P$ value$^d$</th>
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*The $\omega$ values of each site class ($\omega_0$, $\omega_1$, and $\omega_2$) are shown with the percentage of each site class in parentheses. The $\omega_f$ value is the divergent site class of the foreground lineages, which has a separate value shown in boldface.

*The natural logarithm of the likelihood value.

*Twice the difference in $\ln L$ between the two models compared.

*P values were generated by comparing the two models with a chi-square test.

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### Table 1: Number of Tlx2r genes

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### Table 2: Tlx2r16 and Tlx2r41 Abbreviations

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