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Discoveries

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Functional Decline of a Bitter Receptor Gene in New World Vultures

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Abstract

The bitter taste perception, crucial for avoiding harmful foods, is mediated by Tas2r taste receptors in vertebrates. Vultures are obligate scavengers of considerable conservation concern, consisting of Old World and New World vultures. While vultures primarily subsist on carrion, which contains various bitter secondary metabolites produced by microbes, their ability to sense bitterness remains unclear. In this work, we identified all *Tas2r* genes from the genomes of 6 vultures and 22 other Accipitriformes birds. Our analysis revealed that every species, except the osprey, possessed intact *Tas2r1* and *Tas2r2* genes. We observed the lack of genetic divergence in *Tas2r1* among all species and relaxation of functional constraint in *Tas2r2* in New World vultures. Molecular docking simulations revealed reduced binding affinity of Tas2r2 in New World vultures after testing 843 bitter compounds. Additionally, we conducted cell-based functional assays for *Tas2r2* to assess its responsiveness to 24 natural bitter compounds with diverse chemical structures, and confirmed lower responsiveness in New World vultures compared to other birds. These findings suggest a functional decline of bitter taste perception in New World vultures, not observed in Old World vultures, aligning with functional relaxation and reduced binding affinity of Tas2r2 predicted in New World vultures. The functional decline of bitter taste may compromise their natural defense against synthetic bitter pesticides or veterinary drugs, highlighting the potential risks faced by New World vultures in contemporary environments.

Keywords: vultures, diet, conservation, bitter, taste

Introduction

The sense of taste plays a crucial role in providing vital information about food constituents prior to consumption. In general, tastes are classified into five primary categories: sweet, umami, bitter, sour, and salty. Among these, bitter taste holds particular importance for animal survival as it aids in identifying and avoiding the ingestion of most natural toxic substances, which commonly have a bitter taste (Yarmolinsky et al. 2009). In vertebrates, the perception of bitter taste is primarily mediated by bitter taste receptors, a group of G protein-coupled receptors encoded by the type 2 taste receptor (*Tas2r*) genes (Yarmolinsky et al. 2009). Despite birds having a significantly smaller *Tas2r* gene repertoire compared to other vertebrates, bitter taste generally remains indispensable for their dietary selection (Wang and Zhao 2015). Moreover, there was a positive correlation observed between the number of avian *Tas2r* genes and the presence of potential toxins in diets, indicating the significant role of diet during the evolution of birds (Wang and Zhao 2015).

Vultures, classified within the Accipitriformes order, are unique as the sole obligate scavengers among extant vertebrates, providing invaluable ecological services in decomposition and nutrient recycling (Campbell 2015). The term "vulture" itself denotes three distinct groups, each converging in lifestyles, morphological traits, and feeding habits (Campbell 2015). Two subfamilies Gypaetinae and Aegypiinae within the Accipitridae form the Old World vultures, while the Cathartidae family forms the New World vultures (Campbell 2015). Currently, among the 22 existing vultures worldwide, 16 (73%) are at risk of extinction, with 14 (64%) experiencing declining population trends (IUCN 2024), and some showing low genomic heterozygosity, rendering them particularly vulnerable (Zou et al. 2021). A significant factor contributing to the endangerment of most vultures is their specialization in carrion feeding (Campbell 2015). It is widely known that carrion often contains various bitter substances, primarily secondary metabolites produced by microbes, and occasionally residual chemical drugs (Oaks et al. 2004; Reddy et al. 2021). For instance, the widespread veterinary use of diclofenac resulted in drug residues in deceased livestock, ultimately leading to catastrophic vulture declines in South Asia two decades ago (Oaks et al. 2004). However, the extent to which vultures perceive bitterness during food selection has yet to be elucidated.

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In this study, we focused on the evolution of *Tas2r* genes in vultures and assumed that the bitter taste perception may reflect the adaptation to scavenging diets. Indeed, functional diversification of *Tas2r* genes has been detected in multiple mammalian groups with diverse diets. For example, *Tas2r1*, *Tas2r4,* and *Tas2r16* in primates with different dietary items were found to respond to different amounts of bitter compounds, indicating adaptations to different feeding habits (Imai et al. 2012; Tsutsui et al. 2016). Moreover, functional differentiations of *Tas2r118*, *Tas2r119,* and *Tas2r143* between two blind mole rat populations living in two contrasting soil environments have been associated with their distinct food resources (Jiao et al. 2021a).

To test potential differences in bitter taste perception between vultures and their non-scavenging relatives, as well as between Old World and New World vultures, we initially identified all *Tas2r* genes from published genome sequences of 28 Accipitriformes species, including 6 representative species of vultures (supplementary table S1, Supplementary Material online). Next, we estimated ω (i.e. nonsynonymous to synonymous substitution rate ratio) to evaluate differences in selective pressure between vultures and other Accipitriformes species for each *Tas2r* gene (Yang 2007). Moreover, we estimated whether *Tas2r* genes in different vulture clades were subjected to similar selective pressure, since vultures consist of three independent clades. Meanwhile, we separately performed molecular docking of Tas2r1 and Tas2r2 against 843 bitter compounds, to predict functional differences in bitter taste perception between vultures and other Accipitriformes species. Finally, we chose *Tas2r* genes showing significant genetic divergence between vultures and other birds for conducting cell-based functional assays in a heterologous expression system (Jiao et al. 2018, 2021b).

Results

Tas2r Identification of 28 Accipitriformes Species

Through the identification of *Tas2r* genes from the genome sequences of 28 Accipitriformes species, we discovered that every species, except the osprey *Pandion haliaetus*, possessed 2 intact *Tas2r* genes (*Tas2r1* and *Tas2r2*), while *Tas2r3* was either absent or pseudogenized in all examined species (Fig. 1, supplementary fig. S1, Supplementary Material online). All identified intact sequences of *Tas2r1* and *Tas2r2* genes were provided in supplementary dataset S1, Supplementary Material online. Synteny analysis further corroborated this finding by revealing that the seven species lacking *Tas2r3* still retained the two adjacent genes (*mep1a*, *ankrd66*) in the genome, affirming the completeness of genome sequencing and the true absence of *Tas2r3* in the genome (supplementary table S2, Supplementary Material online).

Evolution of *Tas2r* Genes in Vultures

To investigate selective pressures on *Tas2r* genes in the vulture-specific lineages, we estimated the nonsynonymous (d_N) to synonymous (d_S) substitution rate ratio (ω, or d_N/d_S) using a likelihood approach (Yang 2007). First, we examined all Accipitriformes species in this study and estimated the same ω for all branches (model A in supplementary table S3, Supplementary Material online). The ω is significantly lower than 1 in both *Tas2r* genes (Fig. 2, see also the comparison with model B in supplementary table S3, Supplementary Material online), indicating that *Tas2r1* (ω<1, $P = 1.07 \times 10^{-16}$, Likelihood ratio test) and *Tas2r2* (ω<1, $P = 1.29 \times 10^{-9}$) have been under purifying selection and strong functional constraint in Accipitriformes. Second, we compared the ω values between the six vultures and other Accipitriformes species for each *Tas2r*. We found that for *Tas2r1* genes, a model (model C in supplementary table S3, Supplementary Material online), allowing a variation in ω between six vultures and other birds, was not significantly better than the simpler model A ($P = 0.159$). By contrast, *Tas2r2* had a significantly larger ω in six vultures (ω=1.217, $P = 0.004$) than in other birds, although the ω in vultures did not differ significantly from 1 (Fig. 2, and model D in supplementary table S3, Supplementary Material online, $P = 0.576$), suggesting a relaxation of functional constraint acting on *Tas2r2*.

Given that vultures are comprised of three independent clades (Aegypiinae, Gypaetinae, and Cathartidae), we next estimated the variation in ω between each independent clade and other birds (models E, F, and G in supplementary table S3, Supplementary Material online). We found that *Tas2r1* genes were at a similar level of purifying selection between each independent vulture clade and other relatives (Fig. 2), after comparing with a corresponding simpler model assuming the same ω for all branches (model A in supplementary table S3, Supplementary Material online). Therefore, we inferred that the *Tas2r1* genes lack genetic divergence between vultures and other birds. For *Tas2r2* genes, the ω was significantly higher in Cathartidae (ω=1.116, $P = 0.002$) than in other birds, whereas the Aegypiinae or Gypaetinae clade did not show a significantly different ω from that in other birds (Fig. 2). Subsequent analyses indicated that the ω of *Tas2r2* in Cathartidae did not differ significantly from 1 (Fig. 2, model H in supplementary table S3, Supplementary Material online, $P = 0.719$), suggestive of a relaxation of functional constraint. Consistently, when using sliding-window analysis to visualize changes in selective pressure on *Tas2r2*, we also observed overall higher d_N and lower d_S in New World vulture compared to other Accipitriformes species (Fig. 3).

To test whether the relaxation of *Tas2r2* detected in New World vultures is part of genome-wide relaxation, we also evaluated the selective pressures on one mitochondrial gene (*Cytb*) and one nuclear gene (*KCTD21*), which are publicly available for vultures and other Accipitriformes birds. *KCTD21* is located on the same scaffold of *Tas2r2*. We separately designated all 6 vultures and each independent clade of vultures (i.e. Aegypiinae, Gypaetinae, and Cathartidae) as foreground branches, and other Accipitriformes relatives as background branches (supplementary table S4, Supplementary Material online). The two genes showed no significant difference in ω between foreground and background branches ($P > 0.05$) and were under purifying selection in Accipitriformes species ($P < 0.05$), indicative of strong functional constraints (supplementary table S4, Supplementary Material online). Combined with the observation that *Tas2r1* was also under purifying selection in all examined species, we suggested that the relaxation of *Tas2r2* detected in New World vultures is not part of genome-wide relaxation but rather a lineage-specific event.

Calculated Binding Affinities of Tas2r to Bitter Compounds Between New World Vultures and Other Accipitriformes

Using a protein structure modeling approach, we inferred three-dimensional (3D) structures of Tas2r1 and Tas2r2 receptors in 17 Accipitriformes species, including three New World vultures, three Old World vultures, and 11

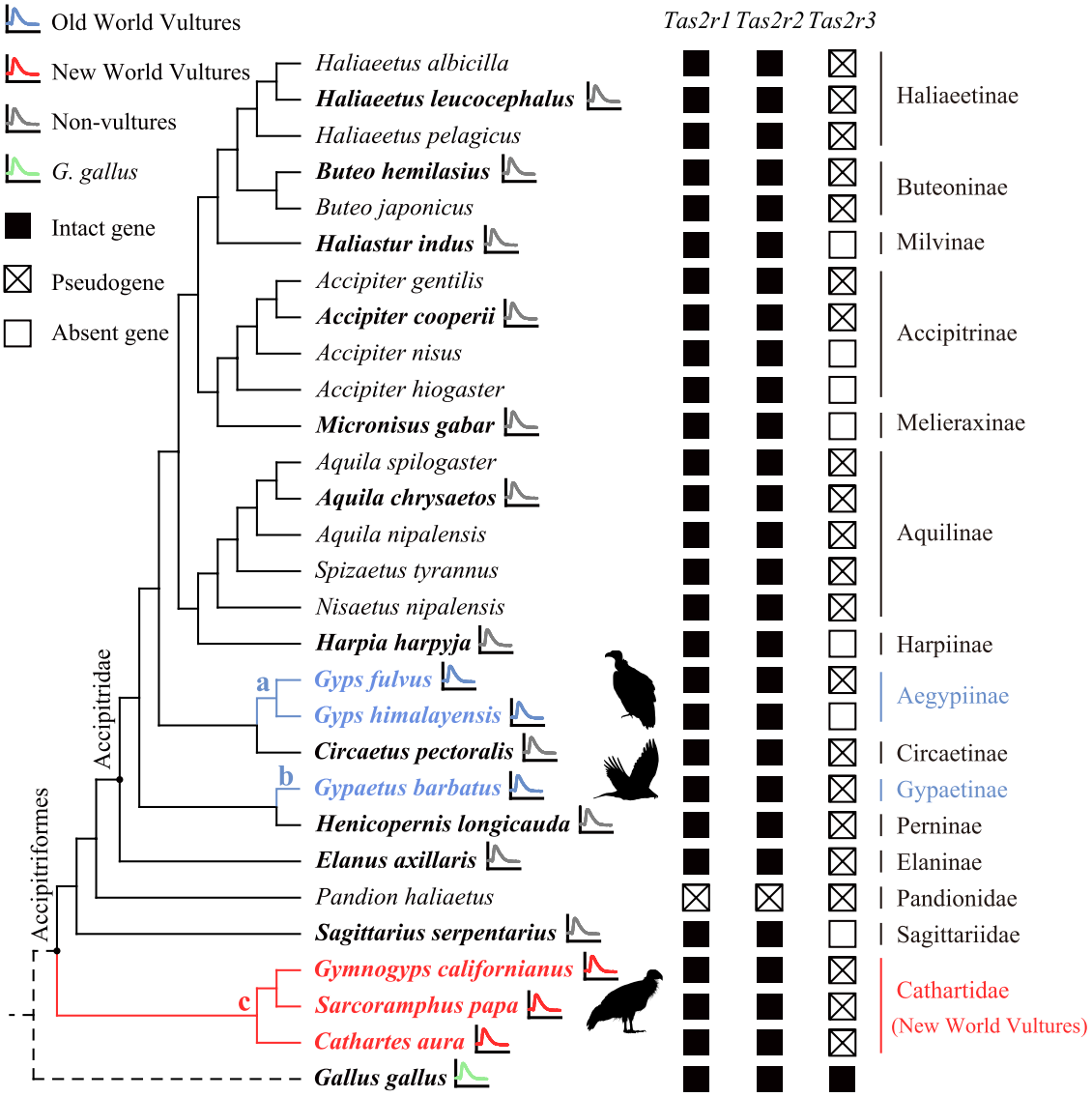

Fig. 1. Summary of bitter receptor genes in 28 accipitriformes species. Phylogenetic relationships of Accipitriformes were referred to in previous studies (Prum et al. 2015; Mindell et al. 2018; Stiller et al. 2024). A total of 17 Accipitriformes species selected for molecular docking analyses and cell-based functional assays are indicated in bold. *Gallus gallus* (Chicken), used as a positive control in the functional assays, is also shown in bold. Old World Vultures (nodes a and b) and New World Vultures (node c) are highlighted. Silhouettes of vultures were taken from phylopic.org.

representatives of other Accipitriformes species (Fig. 1). We then selected bitter compounds in BitterDB (Wiener et al. 2012) and downloaded their 3D structures. To conduct virtual molecular docking, each Tas2r with its 3D structure was tested against 843 bitter compounds with their 3D structures, and affinity scores were calculated and provided in supplementary datasets S2 to S3, Supplementary Material online. Our findings showed no significant differences in affinity scores for Tas2r1 receptors among New World vultures, Old World vultures, and other Accipitriformes species (Fig. 4 and supplementary fig. s2, Supplementary Material online). In contrast, Tas2r2 receptors in New World vultures exhibited significantly lower affinity scores compared to those in Old World vultures and other Accipitriformes species ($P < 0.01$, *t*-test, Fig. 4 and supplementary fig. S2, Supplementary Material online), implying a potential reduction of bitter taste perception mediated by Tas2r2 in New World vultures. Conversely, the affinity of Tas2r2 receptors to bitter compounds in Old World vultures did not significantly differ from that in other Accipitriformes species (Fig. 4, $P > 0.05$). Subsequently, we estimated the affinity scores of the Tas2r1 and Tas2r2 receptors for 33 bitter compounds, which are more likely to originate from animals—including amino acids, peptides, esters, nucleosides, and bile acids—and are potentially present in carrion. This analysis aimed to further evaluate the receptor affinity levels in New World vultures, Old World vultures, and other Accipitriformes species (supplementary datasets S4 to S5, Supplementary Material online). The results for these 33 compounds revealed patterns similar to those observed for the larger dataset of 843 bitter compounds (supplementary fig. S3, Supplementary Material online). Specifically, the Tas2r2 receptor in New World vultures showed significantly lower affinity scores compared to those in Old World vultures and other Accipitriformes species. In contrast, no significant differences were observed in the affinity scores of the Tas2r1 receptor among these groups (supplementary fig. S3, Supplementary Material online). Additionally, based on protein structure modeling, we

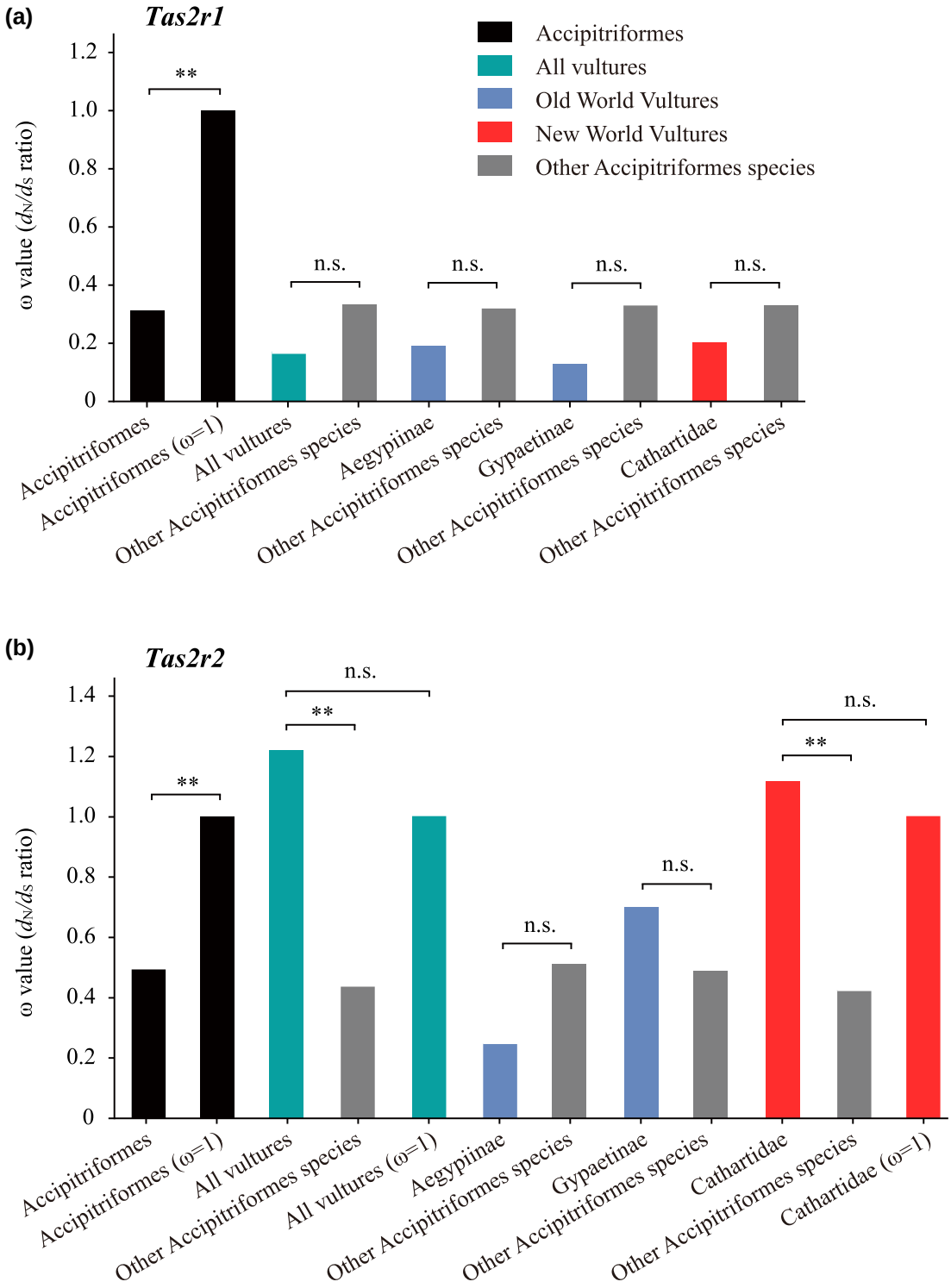

Fig. 2. Histogram plot illustrating the differences in ω between various groups. a) Differences in ω for *Tas2r1* genes across different groups. b) Differences in ω for *Tas2r2* genes across different groups. ω was estimated by the branch model implemented in PAML (Yang 2007). Statistical significance was assessed using a Chi-square test. "n.s." indicates no significance ($P > 0.05$), while $*P < 0.05$ and $**P < 0.01$ indicate significant differences.

compared lineage-specific amino acids in Tas2r2 receptors of New World vultures with those of other Accipitriformes species, using the turkey vulture (*Cathartes aura*) and Cooper's hawk (*Accipiter cooperii*) as representative examples (supplementary fig. S4, Supplementary Material online). Among the 15 sites unique to New World vultures, 5 were located within the binding pocket region, as predicted by molecular docking analyses, suggesting potential structural changes that could impact bitter taste perception (supplementary fig. S4, Supplementary Material online).

Functional Differences of *Tas2r2* Genes Between New World Vultures and Other Accipitriformes

Given the absence of genetic divergence in *Tas2r1* and the predicted lower binding affinity to 843 bitter compounds

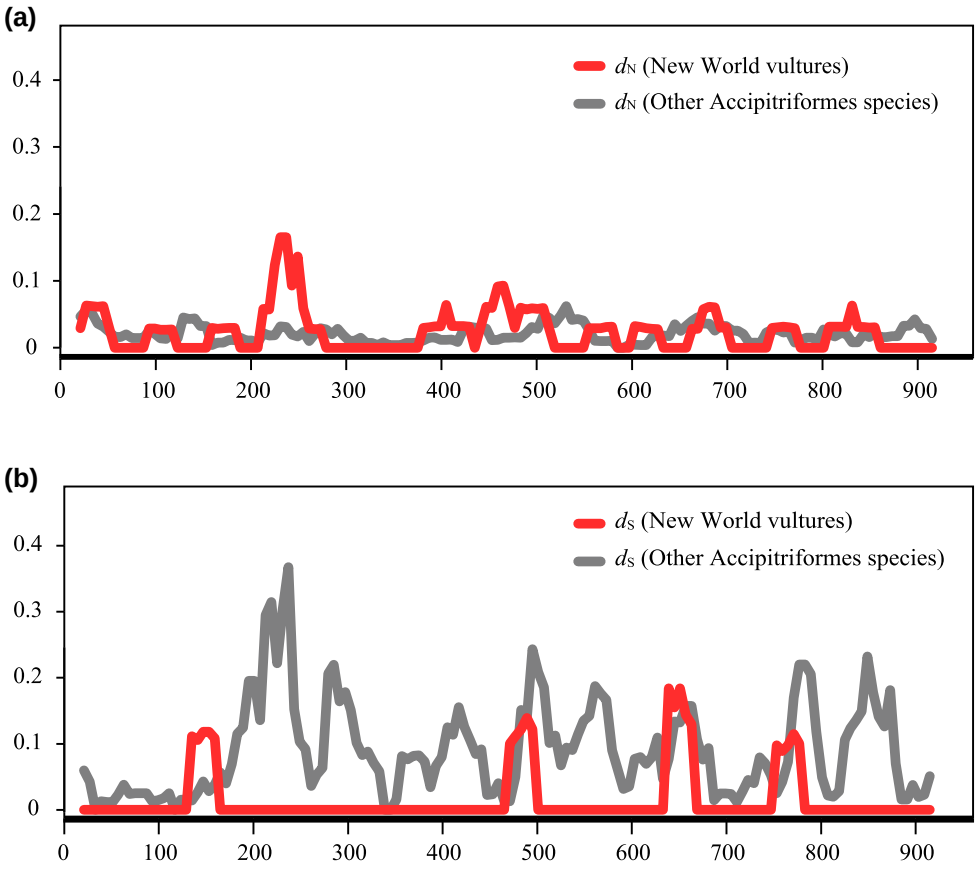

Fig. 3. Sliding-window analysis of evolutionary changes along the *Tas2r2* gene between New World vultures and other Accipitriformes species. a) Distribution of the nonsynonymous substitution rate (d_N) between the two groups. b) Distribution of the synonymous substitution rate (d_S) between the two groups.

observed in *Tas2r2* in vultures compared to other birds (Figs. 2 and 4), we proceeded with cell-based functional assays for *Tas2r2*, with the aim to assess potential functional disparities between vultures and other Accipitriformes birds, as well as between Old World and New World vultures. A total of 6 vultures and 11 other Accipitriformes relatives, representing the main taxa of the Accipitriformes, were selected to evaluate the responsiveness of their Tas2r2 receptors to 24 naturally occurring bitter compounds (supplementary table S5, Supplementary Material online). We also assessed the expression levels of 17 Tas2r2 receptors in HEK293 cells using an immunofluorescence assay and found similar expression levels across various receptors (ranging from 10.28% to 16.61%), ruling out the possibility that functional differences were due to variations in expression levels (supplementary fig. S5, Supplementary Material online). Among the six species of vultures tested, only *Gyps himalayensis* and *G. fulvus* responded to two and three bitter compounds, respectively, whereas nine of 11 other Accipitriformes species exhibited responses to a variety of bitter compounds (Figs. 5 and supplementary fig. S6, Supplementary Material online). The number of responses activated by bitter compounds in other Accipitriformes birds, ranged from 0 (*Melierax gabar* and *Sagittarius serpentarius*) to eight (*Circaetus pectoralis*), with an average of 2.91 (Fig. 5). The success rate of bitter compound activation by the Tas2r2 receptor in Aegypiinae (5 out of 48 tests, or 10.42%) or Gypaetinae (0 of 24) was comparable to their Accipitriformes relatives (32 of 264, $P > 0.05$, Fisher's exact test) (Fig. 5). By contrast, 3 New World vultures (0 of 72) showed a significantly lower success rate of bitter compound activation than other Accipitriformes relatives ($P < 0.001$, Fisher's exact test) (Fig. 5). Additionally, we tested 21 compounds potentially present in carrion using the same assay for the same 17 Accipitriformes species (supplementary table S6, Supplementary Material online), further confirming that Tas2r2 receptors in New World vultures did not respond to these bitter compounds (supplementary fig. S7, Supplementary Material online). Together, these results indicate a functional decline of bitter taste perception in New World vultures rather than Old World vultures, aligning with the observations of functional relaxation and reduced binding affinity of Tas2r2 predicted in New World vultures (Figs. 2 and 4).

Discussion

In this study, we performed the first in-depth study of bitter taste receptor genes in vultures compared to other Accipitriformes relatives, combining sequence analyses, molecular docking simulation, and cell-based functional assays. We found that *Tas2r1* has been under purifying selection and strong functional constraint in Accipitriformes, and *Tas2r2* has undergone a lineage-specific relaxation of functional constraint in Cathartidae, the New World vultures. Molecular docking simulation revealed reduced binding affinity of Tas2r2 in New World vultures compared to other birds after testing 843 bitter compounds. Indeed, our cell-based functional assays on *Tas2r2* also suggest a functional decline

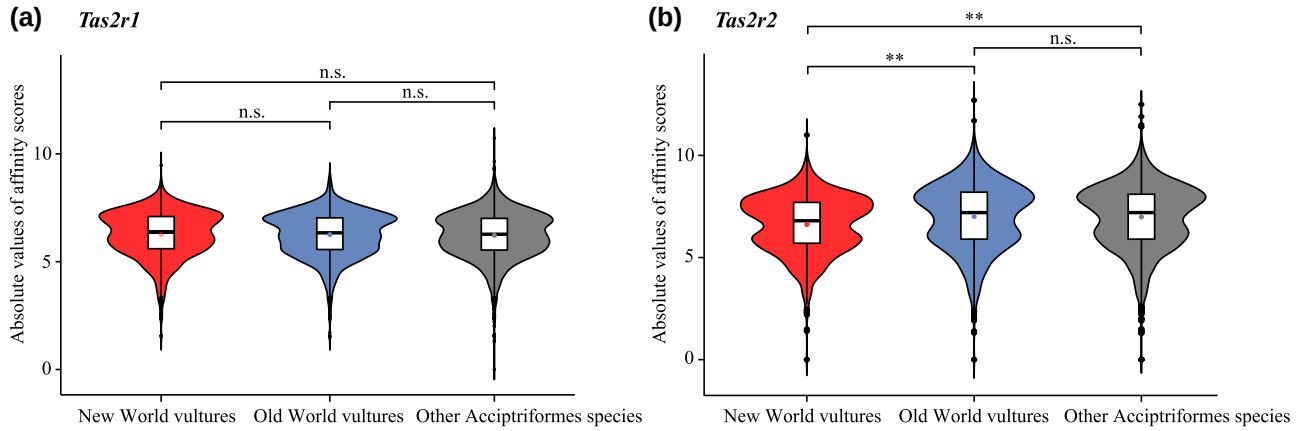

Fig. 4. Differences in affinity scores of bitter taste receptors Tas2r1 a) and Tas2r2 b) between vultures and other Accipitriformes species based on 843 tested bitter compounds. In the box plots, the edge of the box nearest to zero represents the 25th percentile, while the edge farthest from zero represents the 75th percentile. A bold line within the box indicates the median, and a dot represents the mean. Violin plots illustrate the distribution shapes of affinity scores for different groups. The significance level was assessed using a *t*-test. "n.s." indicates no significance ($P > 0.05$), while $^{**}P < 0.01$ denotes significant differences.

of bitter taste perception in New World vultures relative to other birds.

After identifying bitter taste receptor genes, we confirmed that except for the osprey *P. haliaetus*, every Accipitriformes species examined in this study has two intact *Tas2r* genes (*Tas2r1* and *Tas2r2*). *Tas2r3* was absent or pseudogenized and was further confirmed to be nonfunctional in Accipitriformes by the high conservation of two adjacent genes in synteny analysis. Unlike *Tas2r1* and *Tas2r2*, *Tas2r3* shows the greatest variability in copy number among birds with different feeding habits, suggesting its important role in bitter taste perception (Wang and Zhao 2015). In contrast to the intact *Tas2r3* found in the closely related Strigiformes, the pseudogenization of *Tas2r3* in Accipitriformes is a lineage-specific event (Wang and Zhao 2015). This pseudogenization may be partially linked to their carnivorous diets, similar to the loss of Tas2r3 function observed in Sphenisciformes and the red-throated loon (Wang and Zhao 2015; Zhao et al. 2015). Additionally, the pseudogenization of all 3 *Tas2r* genes in ospreys may link to their specialized and narrow diet of fish, which differs from the feeding habits of other Accipitriformes birds (Wilman et al. 2014). Similarly, the Sphenisciformes and the red-throated loon were also reported to undergo pseudogenization in all *Tas2r* genes (Wang and Zhao 2015; Zhao et al. 2015; Cole et al. 2022). As a whole, compared to the relatively large and variable number of *Tas2r* genes in Passeriformes and Apodiformes (Wang and Zhao 2015), the Accipitriformes mainly feeding on meat present a small and relatively constant number of *Tas2r* genes.

Signals of purifying selection were detected in *Tas2r1* of all Accipitriformes species examined in this study, suggesting that genetic diversification of *Tas2r1* in vultures did not significantly differ from that in other Accipitriformes birds. Although the evolution of vertebrate *Tas2r* genes was generally shaped by diets or ecological niches (Shi and Zhang 2006; Davis et al. 2010), *Tas2r1* in scavenging or carnivorous Accipitriformes was highly conserved in sequence, indicative of a strong functional constraint. By contrast, *Tas2r2* in branches connecting to 6 vultures showed a significantly higher ω than other birds. Further analyses suggested that this phenomenon was due to the bias of the relaxation of *Tas2r2* in New World vultures. However, *Tas2r2* genes of the Old World vultures did not show a significantly different ω from that of other Accipitriformes relatives. These results suggested that New World vultures have undergone a lineage-specific relaxation of functional constraint, which might lead to a functional decline of bitter taste perception.

Subsequently, using computational simulation, we carried out a molecular docking analysis of 843 bitter compounds to Tas2r1 and Tas2r2 receptors from 17 Accipitriformes species. All tested bitter compounds covered the majority of bitter compounds in BitterDB (Wiener et al. 2012). Consistent with the results of selective pressure analyses, molecular docking of bitter receptors with 843 bitter compounds indicated that Tas2r2 receptors in three New World vultures showed a significantly lower affinity to bitter substances than those in Old World vultures and other Accipitriformes species. Further analyses of 33 bitter compounds potentially present in carrion showed patterns similar to those observed for the full dataset of 843 compounds. Notably, the binding pockets of Tas2r2 predicted based on eight experimentally verified active sites of chicken Tas2r1 (Di Pizio et al. 2017) might cause some limitations to our molecular docking results. As a result, in this study, we performed cell-based functional assays to examine the bitter taste perception of Tas2r2 receptors in New World vultures.

Indeed, our cell-based assays demonstrated that Tas2r2 bitter receptors of three New World vultures cannot be activated by 24 bitter compounds, indicating a significantly lower success rate of bitter compound activation (Fig. 5). Moreover, functional assays on the same 17 Accipitriformes species showed that New World vultures did not respond to 21 additional bitter compounds potentially present in carrion (supplementary fig. S7, Supplementary Material online). This is consistent with the relaxation of functional constraints detected in selective pressure analyses. Meanwhile, Tas2r2 bitter receptors of two Aegypiinae vultures can sense several bitter compounds like most Accipitridae birds. However, Tas2r2

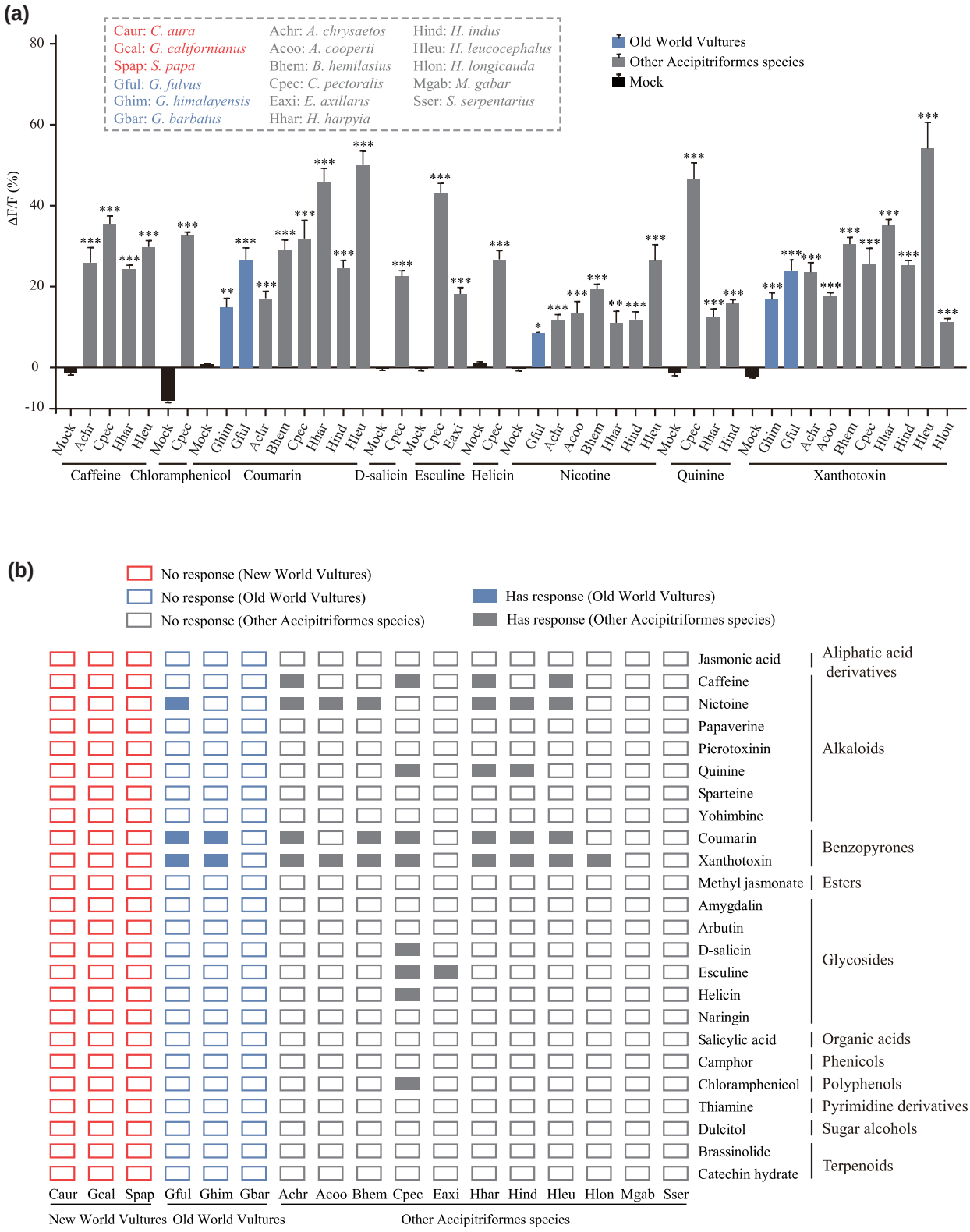

Fig. 5. Functional differences of *Tas2r2* between New World vultures and other Accipitriformes birds. a) Calcium mobilization of bitter receptors in response to bitter compounds. The abbreviations of species names were indicated within the dashed box. Only reactions that can be activated by bitter compounds are shown. Cells transfected only with Gα16-gust44 served as negative controls (mock transfection). The response intensity of Tas2r2 to each bitter compound is represented as the percentage change in fluorescence value (ΔF/F). Student's *t*-tests were conducted to assess the significance between the mock and studied species. $*P<0.05$, $**P<0.01$, $***P<0.001$. b) Responses of 17 Tas2r2 receptors to 24 bitter compounds. Solid rectangles indicate a response, while empty rectangles indicate no response.

of the bearded vulture *Gypaetus barbatus* was found irresponsive to each of the 24 bitter compounds. Both Aegypiinae and Gypaetinae vultures showed no significant difference in bitter taste perception of *Tas2r2* compared to other Accipitriformes relatives. Due to the specialization of the bearded vulture feeding on bone marrow, more sampling of Gypaetinae vultures (such as the Egyptian vulture *Neophron percnopterus*) for cell-based functional assays or even behavioral experiments would better elucidate the bitter taste perception of Gypaetinae vultures. Nonetheless, we concluded that compared to other Accipitriformes species, New World vultures exhibit a significant functional decline in bitter perception that differs from Old World vultures.

Since the Old World and New World vultures have evolved independently as scavengers, diverging approximately 62 million years ago (Stiller et al. 2024), we speculate that the two lineages may have developed distinct mechanisms for obligating scavenging, potentially representing a classical example of convergent or parallel evolution. Different animal species often develop distinct adaptations in response to similar environmental pressures or ecological niches (Muegge et al. 2011; Wang et al. 2023). Indeed, vultures exhibit convergent evolution in immunoregulatory and anti-pathogenic genes as an adaptation to scavenging diets (Roggenbuck et al. 2014; Zou et al. 2021), a pattern also observed in carrion crows (Hu et al. 2024). Of note, the decline of bitter taste perception in New World vultures does not imply that Old World vultures have developed a broad sensitivity to various bitter substances (Fig. 5) or the ability to avoid all drug-laden carrion. Moreover, compared to many non-Accipitriformes species, all Accipitriformes species—except for the osprey—possess only 2 intact *Tas2r* genes (*Tas2r1* and *Tas2r2*), indicating a generally limited capacity for bitter perception (Wang and Zhao 2015). In numerous animal groups adapted to highly restricted diets or exceptionally harsh environments, such as vampire bats and penguins, *Tas2r* pseudogenization has led to the reduction or loss of bitter taste (Hong and Zhao 2014; Zhao et al. 2015). Conversely, in the case of vultures, while the *Tas2r* gene remains intact, functional decline still occurs, possibly attributable to critical mutations within the gene, similar to those observed in other taste receptor genes (Jiao et al. 2021b; Li et al. 2023).

Our study suggests that New World vultures present a relaxation of the functional constraint of *Tas2r2* and a functional decline in bitter perception different from Old World vultures, despite their convergent evolution of obligate scavenging. Hence, we speculate that the reduced perception of New World vultures may potentially avoid aversive reactions to bitter substances in carrion. Historically, prior to the 18th century, bitter compounds primarily stemmed from secondary metabolites present in bacteria, fungi, or plants (Reddy et al. 2021). However, the onset of industrialization ushered in a modern era dominated by human activities, broadening the range of bitter substances to encompass a plethora of harmful compounds, including synthetic drugs, toxins, and inorganic ions (Meyerhof et al. 2005). Consequently, the functional decline in bitter taste perception in New World vultures may have rendered them equally unresponsive to artificial bitter substances. In fact, mortality events caused by poisoning from pesticides or veterinary drugs occurred in both New World and Old World vultures. For example, in the Americas, exposure to carbofuran (a carbamate pesticide) and dichlorodiphenyldichloroethylene (DDE, an organochlorine pesticide) has been identified as a potential threat to the survival of Andean condors and California condors (Alarcon and Lambertucci 2018; Plaza et al. 2019). In South Asia, the vulture crisis has been primarily attributed to poisoning from the veterinary drug diclofenac (Oaks et al. 2004; Ogada et al. 2012). Meanwhile, the decline of vultures in Africa and Europe is driven by various factors, including poisoning by carbamate pesticides (e.g. methomyl, carbofuran, and furadan), organophosphorus compounds, and strychnine (Ogada et al. 2012; Plaza et al. 2019). However, due to the distinct geographical distributions of New World and Old World vultures, data on pesticide or veterinary drug use in these groups are limited and often regionally biased, with little emphasis on comparing the effects of the same substances across regions. Future research should comprehensively assess the potential threats posed by these drugs to vulture species globally. This approach will help uncover the differential impacts of drug poisoning on various species and provide critical scientific evidence to inform drug regulation and support vulture conservation efforts.

Taken together, the functional decline in bitter taste perception in New World vultures may have rendered them a reduced perception to detect artificial bitter substances. This implies that, owing to the impact of anthropogenic activities on Earth's ecosystems, the originally advantageous low sensitivity to bitterness in New World vultures for scavenging purposes may now yield detrimental fitness effects in contemporary environments. As such, the functional decline of bitter taste perception in New World vultures could compromise their natural defense against synthetic bitter pesticides or veterinary drugs, highlighting the potential risks faced by New World vultures in modern environments. In addition, this study implies that the sense of taste may play a more significant role than previously appreciated in the field of conservation biology.

Materials and Methods

Selection of 28 Accipitriformes Species

Considering the availability of 87 Accipitriformes genomes from the NCBI database (including 6 vultures; last accessed September 15, 2022), we ultimately selected 28 representative species representing all major lineages of the order Accipitriformes to identify all putative *Tas2r* genes. Specifically, all 6 vultures, referring to three Cathartidae vultures and three Accipitridae vultures (two Aegypiinae vultures and one Gypaetinae vulture) were chosen as representatives of three independent clades of vultures. Within the Accipitridae, 20 additional species were selected to encompass all subfamilies except Aegypiinae and Gypaetinae. Since both the Pandionidae and Sagittariidae only have one species, the *Pandion haliaetus* and *Sagittarius serpentarius* were also selected. Genome assemblies of 28 Accipitriformes species were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). Detailed information for each assembly is given in supplementary table S1, Supplementary Material online.

Identification of *Tas2r* Genes of 28 Accipitriformes Species

The tblastn program was conducted to search against each genome with an *E*-value cutoff of 1e-10, using known intact *Tas2r* protein sequences of human, mouse, chicken, African clawed frog, and Chinese alligator as queries (Li and Zhang 2014; Jiao et al. 2018). Intact, partial and pseudogenized *Tas2r* genes

were determined according to previous studies (Wang and Zhao 2015; Jiao et al. 2018). Moreover, seven transmembrane domains were predicted for each identified intact *Tas2r* by the TMHMM method (Krogh et al. 2001), to ensure the normal bitter taste function. Finally, we used the blastp program to check whether the best hit generated by tblastn was a real bitter receptor gene, and obtained the final set of bitter receptor genes. All newly identified *Tas2rs* were provided in supplementary dataset S1, Supplementary Material online.

Phylogenetic Analysis

A total of 76 *Tas2rs* (54 intact genes and 22 pseudogenes) were analyzed, with an alligator *V1r1* gene (GenBank: XM_006031313) as the outgroup, since vertebrate *V1r* genes were closely related to *Tas2rs* (Shi and Zhang 2006). To determine the category of each *Tas2r* gene, we used three *Tas2r* genes of chicken as references. After removing insertions or deletions (indels), and premature termination codons, all pseudogenes were subsequently aligned with other intact *Tas2r* genes using the MUSCLE program (Edgar 2004). The resulting alignment was obtained by removing gaps and highly variable regions through Gblocks version 0.91 (Castresana 2000). The best-fitting substitution model for reconstructing a phylogenetic tree was determined by the MrModeltest version 2.4 program (Nylander 2004), following the Bayesian information criterion (Posada and Buckley 2004). Next, a Bayesian Inference phylogenetic tree was reconstructed using MrBayes version 3.1.2 program (Ronquist and Huelsenbeck 2003) with 7 million generations. Results showed that *Tas2r* genes of the Accipitriformes were classified into three major types: *Tas2r1*, *Tas2r2* and *Tas2r3* (supplementary fig. S1, Supplementary Material online). Meanwhile, synteny analyses were conducted to determine whether these *Tas2r3* genes were truly absent from the genomes.

Selective Pressure Tests for *Tas2r* Genes

To assess the patterns of selective pressure on each *Tas2r* in vultures, we estimated the nonsynonymous to synonymous substitution rate ratio (ω) using the codeml program implemented in PAML version 4.9j (Yang 2007). $\omega>1$, $\omega=1$, and $\omega<1$ indicate positive selection, neutral evolution, and purifying selection, respectively. Given that *Tas2r3* was pseudogenized or absent in 28 Accipitriformes species, only intact *Tas2r1* and *Tas2r2* sequences were chosen for analyses. The nucleotide sequence alignment of each *Tas2r* gene was generated according to the protein sequence alignment and was carefully checked by eye. The resulting alignment, along with the species tree topology (Fig. 1) obtained from previous studies (Prum et al. 2015; Mindell et al. 2018; Stiller et al. 2024), was used as input for selective pressure analyses.

For *Tas2r1* and *Tas2r2*, we separately conducted five and seven tests (supplementary table S3, Supplementary Material online). First, we tested whether the overall ω is significantly smaller than 1 in all examined species. Second, we tested whether there is a significant difference in ω between vultures (foreground branches: all branches connecting to 6 vultures) and other Accipitriformes relatives. Third, we test whether ω was divergent between each of the three independent vulture clades (i.e. the Aegypiinae, Gypaetinae, and Cathartidae) and other Accipitriformes relatives. For *Tas2r2*, tests with ω significantly larger in foreground branches (i.e. all branches connecting to 6 vultures, and branches connecting to the Cathartidae vultures) than background branches, additional two corresponding null models were conducted as the same as the alternative models, except the ω of foreground branches fixed at 1. Since Cathartidae vultures showed a significantly different ω value in *Tas2r2* compared to other Accipitriformes species, we performed sliding-window analyses to visualize the distribution of nonsynonymous (d_N) and synonymous (d_S) substitutions per site along the *Tas2r2* gene between the two groups. These analyses were performed using SWAAP version 1.0.2 (Pride 2000), applying the Nei-Gojobori method (Nei and Gojobori 1986), with a sliding window of 30 codons and a step size of six codons. Moreover, we conducted selective pressure tests of two other genes (*Cytb* and *KCDT21*) as controls to differentiate the selective pressure pattern of *Tas2r2* from a general genome-wide relaxation. All sequences were obtained from genomic data of 28 Accipitriformes species used in this study.

Molecular Docking of Tas2r With Bitter Compounds

To evaluate the potential binding ability of each Tas2r with bitter compounds, we performed virtual molecular docking analyses to estimate the affinity of Tas2r1 and Tas2r2 receptors from 17 Accipitriformes species (3 New World vultures, 3 Old World vultures, and 11 representatives of other Accipitriformes species) to a variety of bitter compounds. Briefly, we first employed AlphaFold2 (Mirdita et al. 2022) to conduct the 3D structure modeling of each Tas2r. Considering the close relationship between avian Tas2r1 and Tas2r2, and the absence of known active sites for Tas2r2, we used eight experimentally verified active sites of chicken Tas2r1 to predict the binding pocket of each Tas2r receptor (Di Pizio et al. 2017). The binding site for bitter compounds on each Tas2r was defined by forming a cube with the dimensions $25\times25\times28$ around the protein with a grid point spacing of 0.375 Å. Next, we selected all 1,041 known bitter compounds provided by BitterDB (Wiener et al. 2012), and downloaded three-dimensional (3D) structures from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) via the PubChem compound identifier (CID) of each bitter compound. Due to the lack of 3D structures of some bitter compounds, we finally obtained 843 bitter compounds with 3D structures for subsequent analyses. Subsequently, Tas2r1 and Tas2r2 receptors with 3D structures from 17 Accipitriformes species were docked with 843 bitter compounds with 3D structures by AutoDock Vina (Trott and Olson 2010). Furthermore, since carrion may contain bitter compounds derived from animals, we identified 33 such compounds—comprising amino acids, peptides, esters, nucleosides, and bile acids—from a total of 843 bitter compounds, focusing on those more likely to originate from animals rather than plants. Next, we estimated the affinity scores of the Tas2r1 and Tas2r2 receptors in 17 Accipitriformes species for these 33 bitter compounds. The binding affinity scores were negatively correlated to the estimated binding ability of bitter receptors. Affinity scores greater than zero were ignored, which were considered impossible for bitter receptors to capture the tested bitter compounds.

Bitter Compounds Selected for Functional Assays

Since organic compounds in carrion are greatly influenced by the presence of primary microbial colonizers (Kotze et al. 2021), the bitter compounds from various carrion, often a mixture of various compounds produced during the

decomposition process, are comparably difficult to distinguish to date. Bitter compounds in nature are mostly derived from glycosides or alkaloids in plants (Meyerhof 2005). However, due to commercial availability, we were only able to obtain a selection of these compounds from suppliers. Since nearly no literature reported that bitter receptors can only sense bitter compounds derived from animals or microbes, we chose bitter compounds mostly from plants to explore the difference in bitterness perception between vultures and other Accipitriformes relatives. Ultimately, 24 naturally occurring bitter compounds that were accessible to our lab were selected, which spanned diverse classes of chemical structures, including most of the common classes like alkaloids, glycosides, terpenoids, and polyphenols, alongside some uncommon classes such as organic acids and esters (supplementary table S5, Supplementary Material online). All bitter compounds used in this study were recorded in BitterDB (Wiener et al. 2012), and detailed information was provided in supplementary table S5, Supplementary Material online. Additionally, of the 33 bitter compounds potentially present in carrion, we obtained all commercially available ones (21 in total), which represent all five chemical categories (supplementary table S6, Supplementary Material online). Functional assays were conducted on these compounds to assess differences in bitter taste perception for the same 17 Accipitriformes species. The highest concentration of each bitter compound used in this study followed previous studies with some modifications (Maehashi et al. 2008; Maehashi and Huang 2009; Meyerhof et al. 2010; Upadhyaya et al. 2010; Kohl et al. 2013; Behrens et al. 2014; Yan and Tong 2023; Ziegler et al. 2023).

Functional Assays of *Tas2r2*

Tas2r2 of 6 vultures and 11 other Accipitriformes species were chosen for cell-based functional assays. All examined species represented the main taxa of Accipitriformes and covered the three independent vulture clades (Fig. 1). The assay on chicken *Tas2r2* responding to Caffeine (0.3 mM) was selected as a positive control, since its sensitivity to Caffeine was previously confirmed (Behrens et al. 2014). All coding sequences were codon-optimized and synthesized (GENEWIZ, China), after which they were inserted into the expression vector pcDNA3.1(+), introduced 5′-EcoRI and 3′-NotI restriction sites. The Kozak sequence was incorporated at the 5′end before the start codon to promote efficient translation, and a signal peptide derived from the first 45 amino acid residues of rat somatostatin receptor 3 was incorporated at the 5′ end of the *Tas2r2* genes. All constructs were verified by Sanger sequencing.

Our functional assays were conducted as previously described (Jiao et al. 2018; Hao et al. 2023). In brief, human embryonic kidney 293 (HEK293)-derived peak rapid cells were cultured in Opti-MEM with 6% fetal bovine serum. Healthy cells were seeded in 96-well plates at a density of 40,000 to 50,000 per well. When cell density reaches 80% to 90% confluence, cells were transiently transfected with Gα16-gust44 (0.10 μg per well) and Tas2r2 (0.10 μg per well) using Lipofectamine 2000 (0.50 μl per well). Cells transfected only with Gα16-gust44 were used as negative controls (mock transfection). After 24 h, the cells were washed once with Dulbecco's phosphate-buffered saline (DPBS), then loaded with Fluo-4 AM (2.50 μM; Invitrogen) for 1 h in the dark at room temperature. After washing the cells three times with DPBS to remove excess dye, responses to bitter compounds were detected using FlexStation III spectrometer (Molecular Devices). Calcium mobilization was quantified as the percentage of fluorescence changes (ΔF, i.e. the peak of fluorescence minus baseline) relative to the baseline (F). The response intensity of the bitter taste receptor to the bitter compounds is represented as the percentage change in fluorescence value ($\Delta F/F$). All experiments were conducted in triplicate. Student's *t*-tests were used for statistical analysis ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Immunocytochemical Assays

Immunocytochemical assays were carried out as described previously (Jiao et al. 2021a; Li et al. 2023). After transfection for 24 h in 12-well plates, HEK293 cells were washed three times with phosphate-buffered saline (PBS) and then were placed at 4 °C for 1 h. Cell surface was stained with concanavalin A, Alexa Fluor 633 Conjugate (C21402, Thermo Fisher, 1 mg/ml) for 1 h. After three rinses with PBS, cells were fixed for 15 min with 4% paraformaldehyde (PFA). Then, they were incubated for 10 min with 0.1% Triton X-100 in PBS and blocked for 1 h with 10% fetal bovine serum (FBS) in PBS to avoid unspecific binding. Next, cells were incubated for 2 h with primary antibodies (HSV-Tag mouse monoclonal antibody, 1:200, T607, Signalway Antibody) in PBS with 10% FBS. Secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse antibody, 1:800, 115-545-003, Jackson ImmunoResearch) were used to detect the HSV-Tag. Finally, 4′,6-diamidino-2-phenylindole (DAPI) was employed to stain the nucleus for 5 min. The cells were washed three times with PBS after each treatment. Confocal laser scanning microscopy (Leica TCS SP8) was used to capture images. Three independent areas were counted to evaluate the expression level of Tas2r2 in HEK293 cells.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

Author Contributions

H.Z. designed research. Y.C., H.J., and H.Z. performed sequence analysis, L.X., Y.C., Y.L., C.H., and Q.L. performed functional assays. L.X., Y.C., and H.Z. wrote the paper.

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Conflict of Interests

The authors declare no competing interests.

Data Availability

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

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