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ORIGINAL ARTICLE

Lineage-specific evolution of bitter taste receptor genes in the giant and red pandas implies dietary adaptation

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Abstract

Taste 2 receptors (TAS2R) mediate bitterness perception in mammals, thus are called bitter taste receptors. It is believed that these genes evolved in response to species-specific diets. The giant panda (*Ailuropoda melanoleuca*) and red panda (*Ailurus fulgens styani*) in the order Carnivora are specialized herbivores with an almost exclusive bamboo diet (>90% bamboo). Because bamboo is full of bitter tasting compounds, we hypothesized that adaptive evolution has occurred at TAS2R genes in giant and red pandas throughout the course of their dietary shift. Here, we characterized 195 TAS2R genes in 9 Carnivora species and examined selective pressures on these genes. We found that both pandas harbor more putative functional TAS2R genes than other carnivores, and pseudogenized TAS2R genes in the giant panda are different from the red panda. The purifying selection on *TAS2R1*, *TAS2R9* and *TAS2R38* in the giant panda, and *TAS2R62* in the red panda, has been strengthened throughout the course of adaptation to bamboo diet, while selective constraint on *TAS2R4* and *TAS2R38* in the red panda is relaxed. Remarkably, a few positively selected sites on *TAS2R42* have been specifically detected in the giant panda. These results suggest an adaptive response in both pandas to a dietary shift from carnivory to herbivory, and TAS2R genes evolved independently in the 2 pandas. Our findings provide new insight into the molecular basis of mammalian sensory evolution and the process of adaptation to new ecological niches.

Key words: bitter taste receptor gene, dietary adaptation, giant panda, red panda

INTRODUCTION

The mammalian sensory system of taste, including bitter, umami, sweet, sour and salty, dedicated to the evaluation of the physical and chemical features of their diets, is vital for their survival and reproduction. Among these, bitter taste perception is particularly vital to mammalian survival, as it is a critical defense mechanism enabling animals to avoid ingesting toxic substances, because poisons usually taste bitter and cause aversion (Garcia & Hankins 1975; Li & Zhang 2014). It has been shown that the perception of bitterness is mediated by a large group of seven-transmembrane G protein-coupled receptors (GPCRs), which are encoded by the taste 2 receptor gene family in the membrane of taste receptor cells (Bachmanov & Beauchamp 2007). Studies on

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taste sensory evolution have revealed that loss of taste receptor function in mammals is widespread and directly related to feeding specialization, such as pseudogenized sweet and/or umami taste receptor genes in cats (feliformia, obligate carnivores), giant and red pandas (caniformia, obligate bamboo feeders), vampire bats (desmodontines, obligate blood feeders), dolphins and pinnipeds (cetaceans, swallowing food whole without chewing) (Li *et al.* 2005; Zhao *et al.* 2010; Jiang *et al.* 2012; Sato & Wolsan 2012; Zhu *et al.* 2014; Hu *et al.* 2017). Moreover, the TAS2R repertoire vary greatly in size among species from a few to approximately 60, and losses and gains in the repertoire are frequent in mammalian evolution (Li & Zhang 2014; Hayakawa *et al.* 2014; Liu *et al.* 2016). Furthermore, Li and Zhang (2014) found that more functional TAS2R genes in herbivores than in carnivores as plant tissues contain more toxic compounds than animal tissues do, and, thus, concluded that the number of TAS2R genes in a species correlates with the fraction of plants in its diet. Given that, dietary toxins are a major selective force driving the evolution of the TAS2R genes in mammals (Davis *et al.* 2010; Li & Zhang 2014), and, therefore, the degeneration and adaptation of TAS2R genes is an evolutionary response to dietary changes.

The giant panda (*Ailuropoda melanoleuca*) and the red panda (*Ailurus fulgens*) are 2 sympatric species distributed at the edge of the Qinghai-Tibetan Plateau that have captured much interest from the community of wildlife conservationists because of their conservation status, phylogenetic distinctiveness and specialized feeding ecology (Glatston 1994; Wei *et al.* 1999, 2012; Yu *et al.* 2011). They evolved divergently from a meat-eating ancestor approximately 43 Ma (Eizirik *et al.* 2010), with distinct phylogenetic positions in the order Carnivora: the giant panda belongs to the family Ursidae and the red panda belongs to the family Ailuridae. Although they belong to the order Carnivora, both pandas are well known for their dietary shifts from carnivory to herbivory, whereby >90% of their modern diet comprises bamboo (Jin *et al.* 2007; Zhao *et al.* 2010; Hu *et al.* 2017). Bamboo, like most vascular plants, contains abundant bitter secondary compounds such as phenolics, terpenoids, alkaloids, flavonoids and cyanogenic glycosides (Keski-Saari *et al.* 2008; Huang *et al.* 2016), which appear to protect them against mammalian herbivores. Therefore, we hypothesized that adaptive evolution should have occurred at TAS2R genes in the giant and red pandas during their transition to a bamboo diet. To test this hypothesis, we characterized TAS2R genes from 9 carnivoran species (Fig. 1) for which genome assemblies were available, and conducted evolutionary analyses on TAS2R genes to identify molecular mechanisms in response to dietary shifts in pandas.

MATERIAL AND METHODS

Data mining

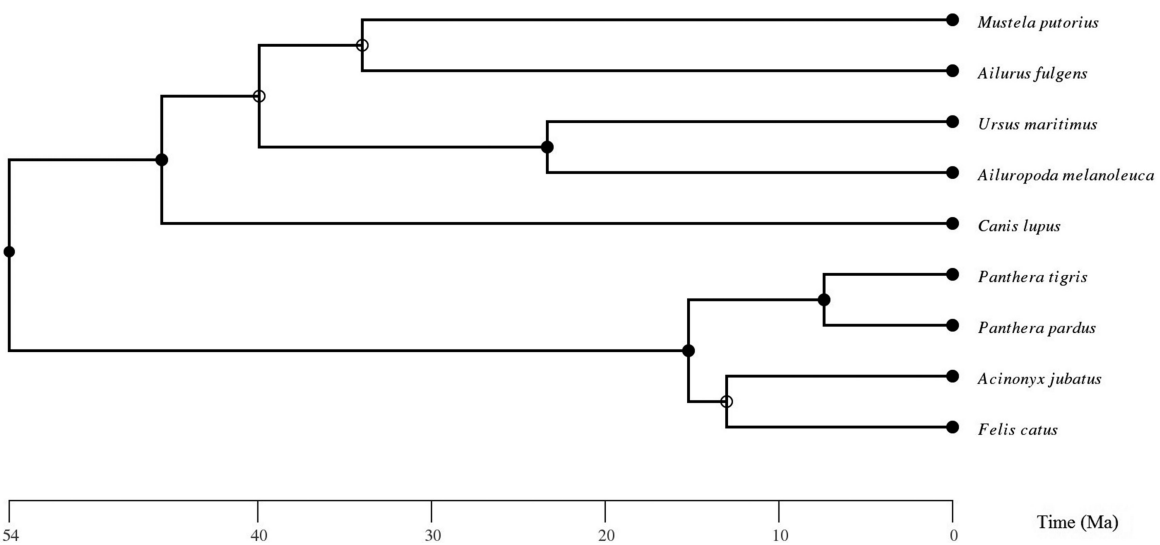

Figure 1 A species tree of 9 carnivores studied in this work, assessed using TimeTree v3.0 (http://www.timetree.org/, last accessed 30 June 2016) (Hedges *et al*. 2006)

The 9 carnivore genome assemblies were obtained from Ensembl (http://www.ensembl.org/index.html) and NCBI (https://www.ncbi.nlm.nih.gov/), which are summarized in the supplementary materials (Suppl. Table S1). We characterized TAS2R genes following a previous study (Shi & Zhang 2006) with a little modification. Initially, we retrieved previously annotated TAS2R gene sequences of these species from the NCBI, and then used these sequences as queries for TBLASTN searches in each whole-genome assembly. Because TAS2R genes are single-exon genes, we looked for intact TAS2R genes with continuous sequences from the start to stop codons, and pseudogenized TAS2R genes with ≥750 bp sequences disrupted by start loss, stop gain and/or indel, on the basis of high BLAST E-values of 1e-5. All the overlapping sequences of hits with the same orientations at the contig level were merged. The candidate TAS2R genes were verified by the TransMembrane helices prediction using hidden Markov models (TMHMM) method (online version 2.0) for the presence of 7 transmembrane domains (Krogh *et al.* 2001), and the intact ones were examined by BLASTP searches against the entire GenBank to ensure that the best hit with an annotation is a known TAS2R gene. All characterized TAS2R gene sequences and genomic locations are listed in the supplementary material. Here, we follow the nomenclature of the TAS2R genes established in Laurasiatheria by Hayakawa *et al.* (2014).

Phylogenetic and selection analyses

To determine the orthologous and paralogous relationships between the TAS2R genes, a neighbor-joining (NJ) tree was constructed based all alignable TAS2R genes used MEGA7 (Kumar *et al.* 2016). Then, selection analyses were conducted on all intact TAS2R genes with indels and stop codons removed, and a series of evolutionary models implemented in PAML (Yang 2007) were conducted and compared in a likelihood framework, using the species tree shown in Fig. 1. Positive selection analyses were restricted to the giant panda and red panda branch, respectively. Pseudogenization of genes is a sign of relaxed selective constraints (Conte *et al.* 2003), thus pseudogenized TAS2R genes were excluded from the further natural selection analyses.

A maximum-likelihood approach was used to calculate the rate ratio (ω) of nonsynonymous (d_N) to synonymous (d_S) substitutions ($\omega = d_N/d_S$), where $d_N/d_S > 1$ indicates positive selection, $d_N/d_S < 1$ negative selection and $d_N/d_S = 1$ neutrality. First, we tested one-ratio model, a more general model assuming a single ω ratio for all branches. Subsequently, we used two-ratio model that allow 2 ω ratios that differ between the background branches and the branch of interest (the giant or red panda branch). For null hypotheses, we used the one-ratio model with a fixed ω = 1 for all branches and a two-ratio model with a fixed ω = 1 on the branch of interest. Then, the likelihood ratio tests (LRTs) were used to evaluate the level of significance of differences between the 2 nested models, where twice the difference of log-likelihood between the models ($2\Delta \ln L$) would follow a χ^2-distribution, and the degree of freedom was the difference in number of parameters between the nested models. Furthermore, the recommended Test 2 was used to detect positively selected sites on the branch of interest, which compares the modified branch-site model A (model = 2 NSsites = 2) with the corresponding null model with a fixed ω = 1 (fix_omega = 1 and omega =1) on the branch of interest (Yang *et al.* 2005; Zhang *et al.* 2005). The Test has been shown to have power to differentiate positive selection from relaxation of selection (Zhang *et al.* 2005). Finally, the posterior probabilities of positively selected sites were calculated using the Bayes empirical Bayes (BEB) method as implemented for the modified model A in the CODEML program of PAML 4 package (Yang 2007). We considered those sites with a posterior probability >0.8 as candidates undergoing positive selection.

RESULTS

Taste 2 receptor gene characterization and phylogenetic analysis

A total of 195 TAS2R genes (121 complete and 74 disrupted) were characterized from the 9 carnivoran species (Fig. 2), including 26 that were characterized for the first time in this study (16 in the red panda, the *TAS2R62* in the leopard, *TAS2R408B* in the cheetah, and *TAS2R18* in all species except the polar bear). However, 3 putative TAS2R genes (*TAS2R5*, *TAS2R16* and *TAS2R408B*) were not found in the cheetah, dog and red panda genome assembly, respectively. Collectively, only 10–16 intact TAS2R genes were identified for individual species. The intact TAS2R genes are those annotated without premature stop codons or frame shift mutations, thus suggesting the presence of a functional TAS2R protein. The giant and red pandas have the same number of intact TAS2R genes (both with 16), which is the largest number in comparison to the number of intact TAS2R genes from the other 7 carnivoran species. Generally,

Figure 2 Gene tree of taste 2 receptor (TAS2R) genes in 9 Carnivora inferred using the neighbor-joining method in MEGA7 (Kumar *et al*. 2016). A bootstrap test with 1000 replicates was taken to represent the evolutionary relationships of TAS2R genes. Node with bootstrap values of >50% are shown in the tree. Evolutionary distances were computed using the Jukes–Cantor method and are in the units of the number of base substitutions per site pair, and gap sites were removed pairwise. Rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

the 3 species from the Caniformia have a relatively larger number of intact TAS2R genes than the number in the 4 species from the Feliformia. The cheetah has the smallest intact TAS2R repertoire (only 10 intact TAS2R genes). In addition, the pseudogenized TAS2R genes are different between the 2 pandas. The NJ tree suggest-

ed 20 one-to-one orthologs with the exception of *TAS2R408A* and *TAS2R408B* (Fig. 2), including 3 pseudogenized across all species, and the other 17 comprising 107 intact and 45 pseudogenized TAS2R genes among the 9 species.

Signatures of adaptive evolution

To look for evidence of adaptive evolution of TAS2R genes in the giant and red panda branches, the 17 orthologs (with pseudogenized TAS2R genes excluded) were examined in a series of selection analyses. The one-ratio model revealed that the average ω for 12 TAS2R genes (*TAS2R1*, *TAS2R2*, *TAS2R3*, *TAS2R4*, *TAS2R5*, *TAS2R7*, *TAS2R9*, *TAS2R10*, *TAS2R38*, *TAS2R40*, *TAS2R41* and *TAS2R42*) are significantly less than 1 (Suppl, Table S2), indicative of a generally strong purifying selection acting on these TAS2R genes across the branches. In contrast, the average ω for the other 5 TAS2R genes (*TAS2R8*, *TAS2R12*, *TAS2R39*, *TAS2R62*, *TAS2R67*) are not significantly different from 1 (Suppl. Table S2), suggesting that purifying selection pressure on these receptor genes is generally relaxed. Further, a two-ratio model showed that ω for *TAS2R1*, *TAS2R9* and *TAS2R38* on the giant panda branch (ω_g), and *TAS2R62* on the red panda branch (ω_r) are significantly less than the ratio for all other branches (ω_0), indicating a divergence in the selective pressure between the giant or red panda branch and the other branches, and, thus, suggesting that the purifying selection pressure on these genes is lineage-specifically strengthened. Although the two-ratio model showed that ω_g for *TAS2R42*, and ω_r for *TAS2R4* and 38 are also significantly different from the average ω, they are not significantly different from a two-ratio model where ω_g or $\omega_r = 1$, respectively (Suppl. Table S2), suggesting the purifying selection pressure on these genes is lineage-specifically relaxed. The ω_r for *TAS2R4* is less than 1, indicative of a relaxation of selective constraint on this gene in the red panda. The ω_g for *TAS2R42* and ω_r for *TAS2R*38 are greater than 1, which indicates that the *TAS2R42* on the giant panda branch and *TAS2R38* on the red panda branch have accumulated more nonsynonymous than synonymous mutations. Despite this, the two-ratio tests did not evidently support positive selection on the *TAS2R42* and *TAS2R38*, as we cannot exclude the possibility of relaxation of selection. Naturally, positive selection will act on only a few sites and for a short period of evolutionary time (Shen *et al.* 2010); thus, the signal for positive selection usually is swamped by the continuous negative selection that occurs on most sites in a gene se-

Table 1 Candidate amino acid sites under positive selection identified on *TAS2R42* in the giant panda using the PAML method

	Site class	0	1	2a	2b	Ln $L^{\dagger}$	np‡	$2\Delta(\ln L)^{\S}$	*P*-value	Positive sites¶
Model A	Proportion	0.27042	0.66566	0.01846	0.04545	−2790.89	21			49 T 0.980, 62 S 0.939
	Background ω_0	0.00864	1	0.00846	1					113 Q 0.912, 121 W 0.944
	Foreground ω_g	0.00864	1	159.7084	159.7084					173 S 0.905, 212 N 0.933
Model A0	Proportion	0	0	0.28586	0.71414	−2794.32	20	6.86	0.0088	242 F 0.928, 300 H 0.939
	Background ω_0	0.00051	1	0.00051	1					308 E 0.940
	Foreground ω_g	0.00051	1	1	1					

†ln(likelihood) value. ‡Number of parameters. §Twice the difference of ln(likelihood) between the 2 models compared. ¶Posterior probabilities of a Bayes empirical Bayes (BEB) analysis (Yang *et al.* 2005) with $P > 0.8$ considered as candidates for selection.

quence (Zhang *et al.* 2005). Even after a short period of positive selection, this is commonly followed by a long period of purifying selection, which would obscure the selective processes (Shen *et al.* 2010). Therefore, positive selection on genes is difficult to detect. Branch-site models, in contrast to branch models in which ω varies only among branches, allows variation in the selective pressure to occur at both amino acid sites and on lineages; thus, these models are considered to be powerful in distinguishing positive selection from the relaxation of purifying selection (Zhang *et al.* 2005). We thus used branch-site models to further examine for possible positive selective pressure on the branch leading to giant panda and red panda. Remarkably, a significant positive selection on *TAS2R42* was detected in the giant panda ($P = 0.0088$; Table 1). Nine amino acid sites were identified as candidate sites that had undergone positive selection with posterior probabilities of ≥90%. However, no evidence for significant positive selection on *TAS2R38* was detected in the red panda, suggesting a relaxation of selection on this gene.

DISCUSSION

We included 195 TAS2R genes for 9 carnivoran mammals according to the latest assemblies. We speculate that the most recent common ancestor (MRCA) of modern carnivoran mammals had at least 19 intact TAS2R genes, but only 10–16 intact TAS2R genes were retained in modern Carnivora, which is fewer than that in herbivores (such as cows and horses) and omnivores (such as humans and mice) (Hu & Shi 2013; Li & Zhang 2014; Hayakawa *et al.* 2014). This result supports earlier speculation that the number of TAS2R genes in a species correlates with the fraction of plants in its diet (Li & Zhang 2014). Accordingly, the largest intact TAS2R repertoire size as found in the giant and red pandas is likely the result of adaptation to dietary shift, as more functional TAS2R genes are required to detect bitter substances in bamboo. In contrast, more pseudogenized TAS2R genes were identified in other meat-eating carnivores, which may be due to a lack of specific bitterness in their diets and greater dispensability of TAS2R genes (Conte *et al.* 2003; Shi *et al.* 2003).

Generally, purifying selection was detected for the 12 TAS2R genes, indicating the functional importance of these receptor genes in the carnivoran evolution. However, the ω for the 12 genes excepting *TAS2R41* are still relatively high in comparison to an average ω of 0.23 for mammalian genes (Zhang 2000), which shows that TAS2R genes in carnivoran mammals are generally less conserved, probably because of their diets containing less toxins. In contrast, the other 5 TAS2R genes were tested under the relaxation of selection, suggesting that these genes became functionally less important for detecting bitter tastants, where we speculate that the volume of bitter compounds in responding to these genes decreased in the mammalian evolution. However, bamboo contains a large volume of bitter compounds (Keski-Saari *et al.* 2008; Huang *et al.* 2016); thus, pandas should experience stronger selective pressure on TAS2R genes than that in other meat-eating mammals for detecting toxins. Indeed, our study showed that purifying selection pressure is lineage-specifically strengthened on *TAS2R1*, *TAS2R9* and *TAS2R38* in the giant panda, and *TAS2R62* in the red panda, which suggests that these 4 genes are responsive to the bitter compounds from bamboo. Remarkably, the positive selection, powerful evidence suggesting an adaptive evolution, on *TAS2T42* in the giant panda indicates that new sites were evolved for detecting the newly encountered bitter compounds in bamboo diet, suggesting an evolutionary response to the dietary shift. However, selective constraint is markedly relaxed on *TAS2R4* and *TAS2R38* in the red panda, which suggests that some functional sites on the 2 genes have become less important, probably due to the specific bitter compounds changed during the course of the red panda's diet shifting from carnivory to herbivory. No evidence for significant positive selection was detected on any of the TAS2R genes in the red panda; in combination with the different TAS2R genes evolved in response to the dietary shift between the 2 pandas, our results suggests that the TAS2R genes evolved independently in the 2 pandas. This may seem counterintuitive, because convergent evolution should be expected on at least some of TAS2R genes between the 2 pandas. To us, when the 2 pandas' diet shifted to herbivory (Hu *et al.* 2017), bitter compounds from bamboos became the major force driving the TAS2R evolution in the 2 species. During the evolutionary process, new variations should have arisen for detecting newly encountered tastants in bamboos. Because each TAS2R receptor is commonly responsive to several bitter substances, and the same bitter tastants can activate different bitter taste receptors (Meyerhof *et al.* 2010), the new variations for detecting the same bitter bamboo compounds could arise independently in different TAS2R genes in the giant and red pandas. New TAS2R genes arise from the duplication of old TAS2R genes (Shi *et al.* 2003), which provided different possible evolutionary routes

for achieving the same functional solution. Consequently, although the giant and red pandas face the same bitter bamboo compounds, the same compounds can be detected by different bitter taste receptors. Similar results have been found in chimpanzees and humans, where although the 2 species shared variable taste sensitivity to bitter compounds mediated by phenylthiocarbamide receptor variants, the molecular basis of the variation has arisen twice, independently, in the 2 species (Wooding *et al.* 2006). This phenomenon has been suggested as convergence occurred at a functional level of genes (Nery *et al.* 2016). The findings presented in this study provide us with new insight into the molecular basis of evolutionary adaptation in giant and red pandas to dietary shift, and support previous discoveries that bitter taste receptor genes have evolved independently in mammals in response to dietary specialization (Wooding *et al.* 2006; Jiang *et al.* 2012; Li & Zhang 2014; Liu *et al.* 2016; Risso *et al.* 2017).

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SUPPLEMENTARY MATERIALS

Table S1 The whole-genome assemblies used in this study

Table S2 Analyses of selective pressure on the branch of the giant panda and/or red panda for 17 TAS2R genes

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