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The Bitter Taste Receptor Genes of the Raccoon Dog (*Nyctereutes procyonoides*)

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ABSTRACT

Mammals have five basic tastes, including sweet, bitter, umami, sour and salty. Among these tastes, the bitter sense is believed to help animals identify poisonous compounds. Bitter taste receptors coded by bitter taste receptor (*Tas2r*) genes has been identified in many species, but the evolution and repertoire of *Tas2r* genes in raccoon dog (*Nyctereutes procyonoides*) was still unknown. As a true omnivorous mammal, the *Tas2r* genes may play a critical role in food selection of raccoon dogs. Thus, we explored the evolution of *Tas2r* genes in raccoon dogs. In this study, *Tas2r* genes including eleven intact genes and four pseudogenes from raccoon dogs were identified for the first time. We also obtained *Tas2r* genes from fourteen additional species based on their genome sequences and our previous study, including thirteen carnivorous species and one omnivorous species. Then, we constructed a phylogenetic tree using *Tas2r* genes sequences from these fifteen species. Phylogenetic analyses showed that most *Tas2r* genes of raccoon dogs were closed to other canid-species, indicating that the *Tas2r* genes were very conservative. Besides, we also detected positive selection in 11 intact genes in raccoon dogs. The results of the positive selection analyses indicated that *Tas2r10* and *Tas2r67* had positively selected sites and other intact genes in raccoon dogs were under purifying selection. Overall, the phylogenetic and evolutionary relationship of *Tas2r* genes in raccoon dogs was first studied in this study. Most of the raccoon dog' *Tas2r* genes were under purifying selection, indicating that its true omnivorous diet was an important role in the *Tas2r* evolution. Our study provided valuable data of *Tas2r* genes in raccoon dogs, and provided novel insights into conservation concern of natural populations.

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Authors' Contribution
HHZ and XXT designed the experiments. SS, XYW and JC performed the experiments. SS, HMZ and QGW analyzed the data. HXZ, HHZ and XXT contributed material. SS wrote the paper.

Key words
Raccoon dog, *Tas2r* genes, Phylogenetic analysis, Positive selection, Diet.

INTRODUCTION

Mammals have five basic tastes, including sweet, bitter, umami, sour and salty (Li and Zhang, 2014). Bitter taste receptors which were coded by bitter taste receptor (*Tas2r*) genes play an important role in identifying the toxins and choosing food in mammals (Chandrashekar *et al*., 2000). The *Tas2r* gene repertoires of vertebrates diverge tremendously in size, from 10 pseudogenes in the dolphin (*Tursiops truncatus*) (Lei *et al*., 2015) to 80 in the *Latimeria chalumnae* (Syed and Korsching, 2014). The relationship between the number of *Tas2r* genes and diets in 54 vertebrate species suggested that dietary toxins were major selective force for shaping the diversity of *Tas2r* repertoire (Li and Zhang, 2014). By analyzing the evolutionary trajectories of *Tas2r* genes in the 41 Laurasiatherian species, Liu found that insectivores in Laurasiatherian tended to have more functional *Tas2r* genes in comparison to carnivoresand omnivores (Liu *et al*., 2016). However, no positive correlation were detected between diet composition and *Tas2r* genes in carnivores (Shang *et al*., 2017).

Although *Tas2r* genes have been identified in many species (He *et al*., 2017), including seven canids, the evolution and repertoire of *Tas2r* genes in raccoon dog (*Nyctereutes procyonoides)* were still unknown. As a nocturnal canid, the raccoon dog originally came from East Asia (Ward and Wurster-Hill, 1990). Raccoon dogs have spread over eastern, central and north Europe since its introduction from Asia (Kowalczyk and Bunevich, 2009). Previous studies mainly focused on the diet, habitat use, seasonal physiology, hair and reproduction of the raccoon dog (Kauhala *et al*., 1993; Hirasawa *et al*., 2006; Matsuyama *et al*., 2006; Kowalczyk and Bunevich, 2009). As a true omnivore (Hirasawa *et al*., 2006), the raccoon dog may face poisonous compounds in food selection, but the research of *Tas2r* genes in the raccoon dog was obscure. In the present study, we first identified and explored the evolution of *Tas2r* genes in raccoon dogs.

MATERIALS AND METHODS

Samples, primers and DNA extraction

The sample was obtained from Dalai Lake. Genomic DNA of the raccoon dog was isolated from the muscle

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tissue using QIAGEN DNeasy kits. The primers were designed by Primer premier 5.0 (Lalitha, 2000), which can be found in Supplementary Material S1. PCR condition was as follow: 95°C 30s, 52-58°C 40s, 72°C 45s, and 72°C elongation for 10 min. Then polymerase chain reaction (PCR) productions were sequenced in both directions. The sequences of the newly generated were submitted in GenBank under the accession number (KT426765-KT426779), and additional *Tas2r* gene sequences used in this study was present in Supplementary Material S2.

Ethical approvals

This research was approved by the Qufu Normal University Institutional Animal Care and Use Committee with the permit number of QFNU2015-004. This study has no harm for the canid species. Meanwhile, it may help us protect these animals better.

Sequencing data and phylogenetic analysis

The DNA sequences of intact genes were translated into protein sequences by the MEGA 6 (Tamura *et al.*, 2013). Then the protein sequences (Supplementary Material S2) were used for multiple sequence alignment by Multiple Sequence Comparison by Log-Expectation (MUSCLE), free available online at http://www.ebi.ac.uk/Tools/msa/muscle (version 3.8.31) (Edgar, 2004). The software was also used for analyzing the percent identity matrix. The aligned protein data were used for phylogenetic analysis using the software MEGA 6. The neighbor-joining statistical method of analysis and the bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985; Saitou and Nei, 1987). And the zebra fish *V1R* gene was used as outgroup. The best fitting model was calculated by ProtTest 3.4 (Abascal, 2005).

Selective pressure on Tas2r *genes*

In order to detect the selective pressure on the intact bitter taste receptor genes in raccoon dogs, we used the site model in the codeml program in PAML (Yang, 2007). The ω ratios of nonsynonymous to synonymous substitution (dN/dS) being less than 1, equal to 1 and more than 1 indicate purifying selection, neutral evolution and positive selection, respectively. Twice the log likelihood differences (2Δl) were detected in the Chi-square test. The LRT suggest positive selection if there is a significant difference between the two models. Then we use three distinct different models, including random effect likelihood (REL), fixed-effect likelihood (FEL) and single likelihood ancestor counting (SLAC) to analyze the potential selective pressure of 11 intact genes (Kosakovsky-Pond and Frost, 2005).

RESULTS

Tas2r *gene amplification and sequencing*

The dog *Tas2r* genes were used to design the primers of the raccoon dogs. The PCR products of *Tas2r* genes were obtained from the raccoon dogs, including 11 genes (at least 270 codons, start codon, stop codon and seven transmembrane domains) and 4 pseudogenes (Fig. 1). The similarity of the *Tas2r* protein sequence between dog and raccoon dog ranged from 97.6% to 99.25%, thus the newly *Tas2r* genes of the raccoon dog were reliable (Supplementary Material S3). Meanwhile, we also used *Tas2r* genes from 14 additional species (Fig. 1). The results showed that the intact and pseudogenes were different among 15 species. For example, several *Tas2r* intact genes in raccoon dog were pseudogenized in other species.

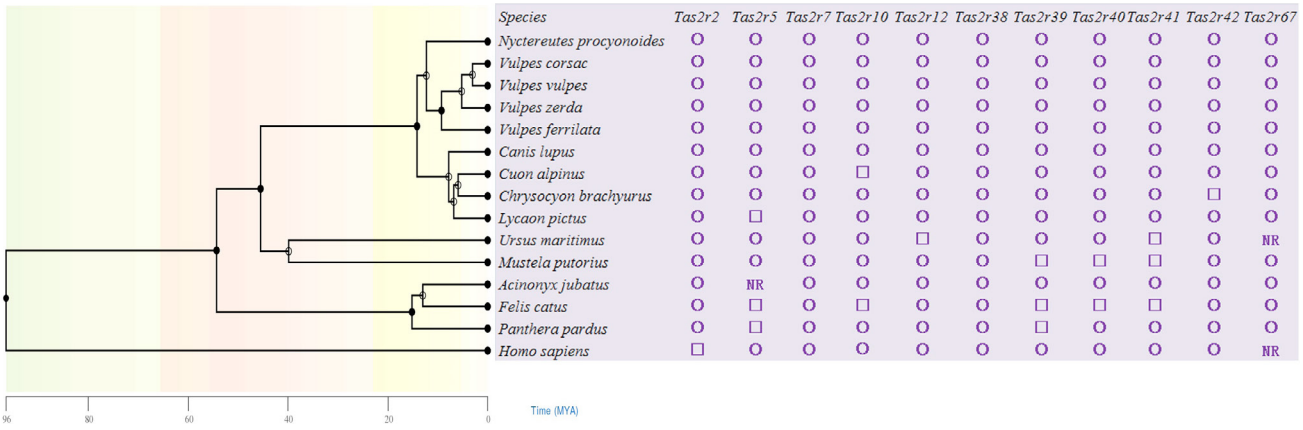

Fig. 1. The divergence time and difference in 11 *Tas2r* genes of 15 mammals. The phylogenetic relationships of these 15 mammals were assessed using TimeTree v3.0 (http://www.timetree.org/, last accessed September 8, 2017). Circles mean intact genes; square mean pseudogenes; NR means no record.

Phylogenetic analysis

In the present study, we carried out a phylogenetic analysis based on the intact *Tas2r* genes from 15 mammals. Intact *Tas2r* genes which were homologous to 11 intact *Tas2r* genes from the raccoon dog were used in the analysis. The pseudogenes and other intact genes were not used in the phylogenetic analysis. Total 145 *Tas2r* genes and one zebra fish *V1r* gene were used in the phylogenetic analysis. The result showed that most *Tas2r* genes of raccoon dog were closed to other canid species (except for *Tas2r40* and *Tas2r39*) (Fig. 2). And *Tas2r* genes from the Feliodea species tended to cluster together. *Tas2r* genes of canid species were near to the carnivorous species and far from the omnivorous species.

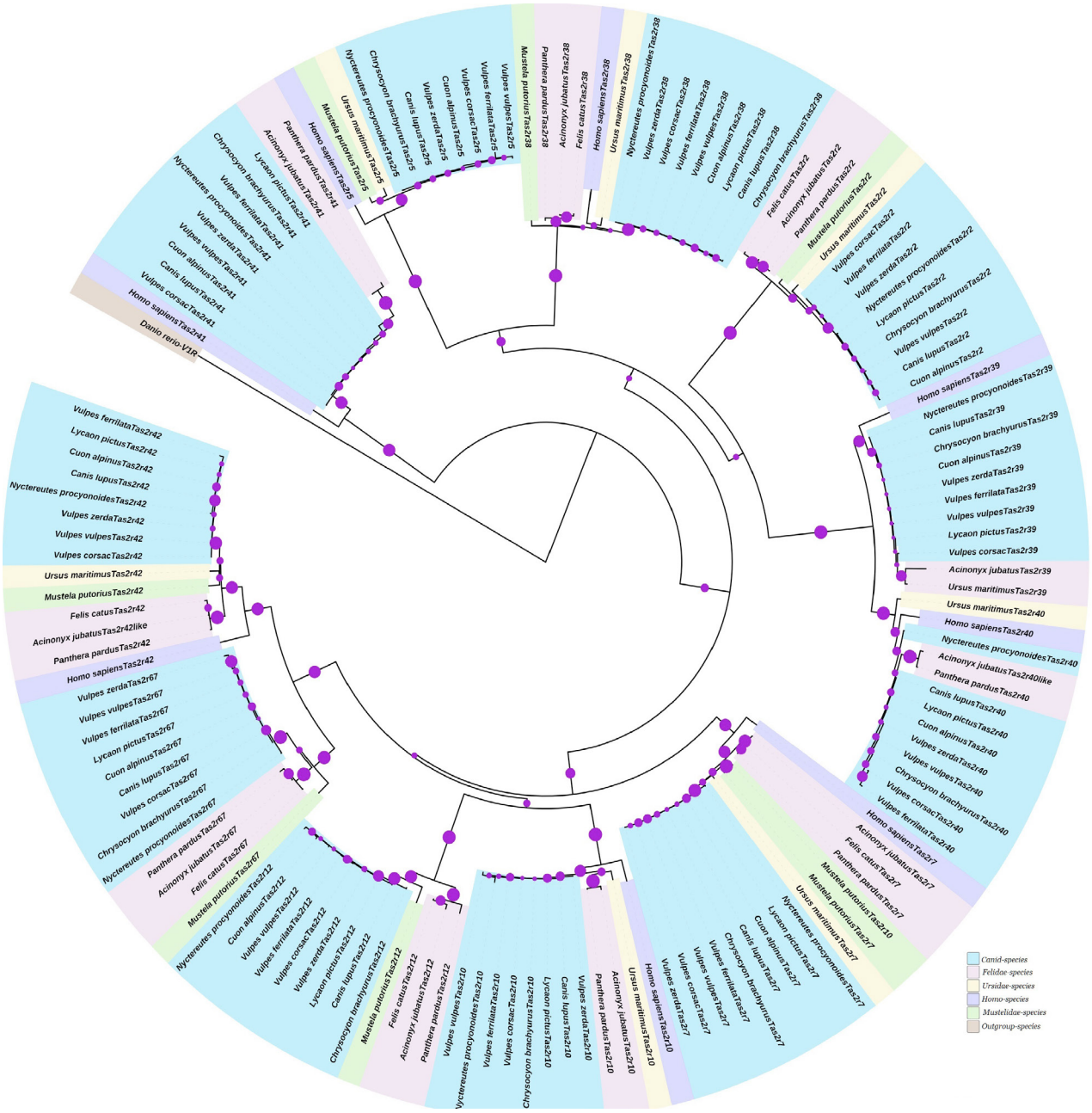

Fig. 2. Phylogenetic Analysis of *Tas2r* genes from 15 mammals. 146 amino acid sequences were involved in the phylogenetic analysis. The evolutionary history was inferred using the Neighbor-Joining method with the model Jones-Taylor-Thornton (JTT) + Gamma Distributed (G). Different species were marked in different colors.

Table I.- Positive selection of 11 intact *Tas2r* genes.

Gene	Species	Lnl M7	Lnl M8	P-values	M8	SLAC	FEL	REL
Tas2r2	8	-1402.88	-1401.38	0.22	31	0	0	31
Tas2r5	8	-1459.87	-1457.99	0.15	26 73 76	0	0	0
Tas2r7	9	-1425.02	-1423.17	0.15	15 26 30 31 180 213 308	0	0	0
Tas2r10	8	-1494.8	-1490.98	**0.02**	6 **7 9** 40 164 200 318			**7 9**
Tas2r12	9	-1523.52	-1523.19	0.72	171 262	0	0	0
Tas2r38	9	-1486.22	-1483.36	0.057	46 65 105 299 300 312	0	0	0
Tas2r39	9	-1535.46	-1534.66	0.45	149 205	0	0	0
Tas2r40	9	-1524.8	-1523.44	0.26	84 112 229	0	0	0
Tas2r41	9	-1508.97	-1508.97	0.99	0	0	0	0
Tas2r42	8	-1548.74	-1546.37	0.093	39 63 82 216 279 291 310 315 319 320 321 322	0	0	315 321
Tas2r67	9	-1573.53	-1565.84	**0.00045**	**86 308** 310 **312**	0	0	**86 308 312**

Positively selected sites with posterior probability >0.95 are in bold and < 0.95 in plain text.

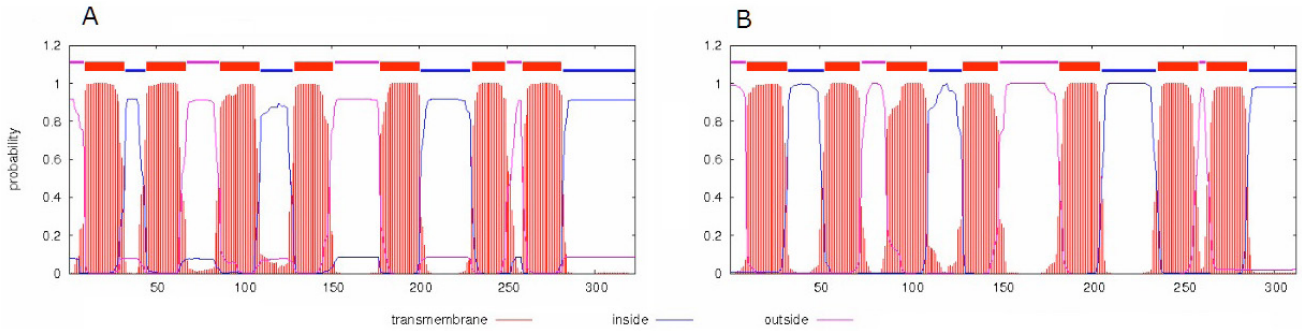

Fig. 3. Predicted transmembrane domains of *Tas2r10* and *Tas2r67*. The predicted transmembrane domains of *Tas2r10* (A) and *Tas2r67* (B). The red lines mean the transmembrane region; the pink lines mean the outside region; the blue lines mean the inside region.

Selective pressure of Tas2r *genes*

To explore the evolutionary forces on the *Tas2r* genes, we conducted an analysis of site models by using the *Tas2r2*, *Tas2r5*, *Tas2r7*, *Tas2r10*, *Tas2r12*, *Tas2r38*, *Tas2r39*, *Tas2r40*, *Tas2r41*, *Tas2r42* and *Tas2r67* sequences as input data. The likelihood values, estimated parameters, and predicted positively selected sites were listed in Table I. The LRT was significantly different in *Tas2r10* and *Tas2r67*, indicating that the positive selection was reliable in these genes. The results showed that two and three common positively selected sites were identified in *Tas2r10* and *Tas2r67,* respectively (Table I).

The two common positively selected sites (codon 7and 9) in *Tas2r10* were located on the transmembrane region (Fig. 3). And the common positively selected sites in *Tas2r67* were located on the transmembrane region (codon 86) and an intracellular domain (codon 308 and 312) (Fig. 3).

DISCUSSION

The *Tas2r* genes sequences were identified for the first time in the raccoon dog. The protein sequence identity between dog and raccoon dog ranged from 97.6% to 99.25% (Supplementary Material S3), suggesting that the dog gene-based primer sets can be used for the raccoon dog. Previous researches showed that the carnivorous species obtained 22 or more *Tas2r* genes (Hu and Shi, 2013; Li and Zhang, 2014; Liu *et al*., 2016; Shang *et al*., 2017), while in our research, 11 intact genes and 4 pseudogenes were obtained in the raccoon dog. The incomplete repertoire of *Tas2r* genes in the raccoon dog may be due to the high similarity but containing gaps in the template sequence at the annealing point of the primer (Monteiro-Ferreira *et al*., 2015). Several *Tas2r* intact genes in raccoon dog were pseudogenized in other species, for example, the *Tas2r67* and *Tas2r2* were not existed in human, but intact in raccoon dogs and other canid species; the *Tas2r39*,

Tas2r40 and *Tas2r41* was pseudogenized in ferret, but was intact in canid species. The pseudogenes and intact genes were different species, indicating that the *Tas2r* genes were species-specific.

The phylogenetic tree showed that most *Tas2r* genes of the raccoon dog were clustered together with canid species. We speculated that *Tas2r* genes were very conservative among species. Thus they could be used as a molecular marker to study the evolutionary relationships among animals (Shi and Zhang, 2006; Hu and Shi, 2013). The phylogenetic results also showed that 11 intact genes from raccoon dogs were intermingled across the tree which indicated that gene duplication events were not existent, which was similar to other carnivorous species (Hu and Shi, 2013). Due to lack of genome sequences in raccoon dog, the incomplete repertoire of *Tas2r* genes may not reflect the real events. More comparative data are needed to critically test it.

Previous study showed that the repertoire and evolution of *Tas2r* genes were shaped by many factors, including diet and feeding behaviors (Hong and Zhao, 2014; Li and Zhang, 2014; Liu *et al*., 2016; Shang *et al*., 2017; Zhong *et al*., 2017). Our results showed that the raccoon dog had a small number of *Tas2r* genes, which might suggest *Tas2r* genes playing a less important role. But minimal number of functional *Tas2r* genes did not imply a reduced importance of bitter taste, as it could be compensated by large tuning width (Behrens *et al*., 2014).

In human, the *Tas2r10* was reported to identify 20 natural bitter compounds (Meyerhof *et al*., 2010). The results of positive selection of *Tas2r* genes in the raccoon dogs showed that two intact genes had positively selected sites, the *Tas2r10* and *Tas2r67*. Meanwhile, except the *Tas2r10* and *Tas2r67*, the remaining intact genes in raccoon dogs had no positively selected sites, suggesting that most genes were under purifying selection. Raccoon dogs are omnivores that feed on meat (insects, rodents birds, fish, carrion and reptiles) and plants (fruits, nuts and berries) (Sasaki and Kawabata, 1994; Sutor *et al*., 2010). They faced the potential poisonous compounds, including the bile acids, venom, and skin secretion from carrion, reptiles, insects and toxic plants. We speculated that *Tas2r* genes might be functionally important during their evolution due to its true omnivorous diet (Ward and Wurster-Hill, 1990). These taste capabilities could protect raccoon dogs from harmful substances and thus be helpful for their survival.

CONCLUSIONS

Overall, the phylogenetic and evolutionary relationship of *Tas2r* genes in raccoon dogs was first studied in this study. Most of the raccoon dog's *Tas2r* genes were under purifying selection, indicating that its true omnivorous diet was an important role in the *Tas2r* evolution. Our study provided valuable data of *Tas2r* genes in raccoon dogs, and provided novel insights into conservation concern of natural populations.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2018.50.4.1361.1366

Statement of conflict of interest

Authors have declared no conflict of interest.

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