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Evolutionary biology

Functional characterization of the TAS2R38 bitter taste receptor for phenylthiocarbamide in colobine monkeys

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Bitterness perception in mammals is mostly directed at natural toxins that induce innate avoidance behaviours. Bitter taste is mediated by the G protein-coupled receptor TAS2R, which is located in taste cell membranes. One of the best-studied bitter taste receptors is TAS2R38, which recognizes phenylthiocarbamide (PTC). Here we investigate the sensitivities of TAS2R38 receptors to PTC in four species of leaf-eating monkeys (subfamily Colobinae). Compared with macaque monkeys (subfamily Cercopithecinae), colobines have lower sensitivities to PTC in behavioural and *in vitro* functional analyses. We identified four non-synonymous mutations in colobine *TAS2R38* that are responsible for the decreased sensitivity of the TAS2R38 receptor to PTC observed in colobines compared with macaques. These results suggest that tolerance to bitterness in colobines evolved from an ancestor that was sensitive to bitterness as an adaptation to eating leaves.

1. Introduction

Mammals discriminate five basic flavour qualities: bitterness, sweetness, umami, saltiness and sourness. Bitterness is mostly associated with natural toxins that induce innate avoidance behaviours in animals [1]. The ability to taste bitterness is mediated by the G protein-coupled receptor TAS2R in taste cells [2]. One of the best-studied TAS2Rs, TAS2R38 mediates the perception of the bitterness of synthetic phenylthiocarbamide (PTC), propylthiouracil (PROP) and phytotoxins, such as goitrin, limonin and sinigrin [3,4]. PTC is a prototypical agonist for TAS2R38 in humans and some primates, and allelic variants in *TAS2R38* result in different intraspecific sensitivities to PTC [5–7].

Plants have evolved protective mechanisms against herbivory, for example, by synthesizing toxic secondary compounds [8]. Colobine monkeys (subfamily Colobinae) are folivorous primates with digestive systems that are specialized for digesting leaves [9]. Analysing the sensitivity of colobines to bitterness could improve our understanding of their dietary choices. Here, we report the behavioural responses of colobines to PTC and the functional characteristics of colobine TAS2R38 receptors.

2. Material and methods

(a) Animals

PTC sensitivity was analysed using 13 captive colobine monkeys of four species (*Presbytis femoralis*, *Nasalis larvatus*, *Trachypithecus cristatus* and *T. auratus*) and individual Heck's macaque (*Macaca hecki*) at the Ragunan Zoo, Jakarta, Indonesia (table 1).

Table 1. Monkey performance in behavioural tests. A trial is defined as the act of a monkey putting an apple slice into its mouth followed by acceptance or rejection of the slice.

			PTC-treated			control				
object (code)	sex	age	acceptance	trial	%	acceptance	trial	%	total	*p*-value[a]
P. femoralis (F)	female	adult	28	28	100	54	54	100	82	0.2871
T. auratus 1 (A1)	female	adult	17	22	77.27	38	39	97.43	61	0.003751
T. auratus 2 (A2)	female	adult	2	4	50	6	6	100	10	0.01092
T. auratus 3 (A3)	male	adult	21	21	100	36	36	100	57	0.5243
T. auratus 4 (A4)	male	adult	33	33	100	53	53	100	86	0.2619
T. auratus 5 (A5)	male	adult	2	2	100	3	3	100	5	0.2803
T. cristatus 1 (C1)	female	adult	12	12	100	22	22	100	34	0.8344
T. cristatus 2 (C2)	female	adult	16	16	100	27	28	100	44	0.939
T. cristatus 3 (C3)	female	adult	41	41	100	80	80	100	121	0.1077
T. cristatus 4 (C4)	female	adult	44	44	100	77	77	100	121	0.108
T. cristatus 5 (C5)	male	adult	14	15	93.33	26	27	96.30	42	0.9487
T. cristatus 6 (C6)	male	adult	4	4	100	8	8	100	12	0.7788
N. larvatus (N)	male	adult	7	7	100	17	17	100	24	0.8961
M. hecki	male	adult	0	10	0	12	12	100	22	2.20×10^{-16}

[a]Probability that the proportion of acceptance in the PTC-treated session is the same as that in the control session [10].

(b) Behavioural experiments

Animals were given apple slices that had been soaked overnight in control (water) or test (2 mM PTC in water) solution, as previously reported [11]. Details are provided in the electronic supplementary material, Methods.

(c) TAS2R38 genotyping

Genomic DNA was extracted from faecal samples collected from the cages (see electronic supplementary material, Methods for details). The *TAS2R38* sequences from colobines were aligned to previously determined *TAS2R38* orthologues from colobine (*Colobus guereza*) and taster individuals of Japanese and rhesus macaques, humans and chimpanzees (accession numbers: JQ272224.1, AB623012.1, AB623025.1, AY258597.1 and JQ272199.1, respectively) using ATGC software (Genetyx Corporation, Tokyo, Japan). A phylogenetic tree was constructed based on the *TAS2R38* alignment using maximum-likelihood (ML) implemented in MEGA5.2 [12]. The Jones–Taylor–Thornton model was used to calculate pairwise distances between amino acid sequences [13]. Validation of trees was performed using bootstrap resampling (500 replicates).

(d) Functional assays of TAS2R38

Functional properties of the TAS2R38 receptor in colobines were evaluated using calcium imaging methods, as described previously [6,14,15]. To calculate responses to PTC, the amplitude of fluorescence was plotted against PTC concentration using IGOR Pro (WaveMetrics) and curves were fitted using nonlinear regression $f(x) = I_{min} + (I_{max} - I_{min})/(1 + (x/EC_{50})^h)$, where x is the ligand concentration and h is the Hill coefficient used to calculate EC_{50} (half maximal effective concentration) for the ligand–receptor interaction between PTC and TAS2R38.

(e) Statistical analysis

Differences in the frequency of acceptance between PTC and the control were assessed by the proportion test [10] using the stats package in R v. 3.3.1 (R Core Team (https://www.R-project.org/)). EC_{50} was compared between *M. fuscata* receptors and mutant receptors mimicking those of colobines by nonlinear model fitting to dose–response data [16] using the drc package in R.

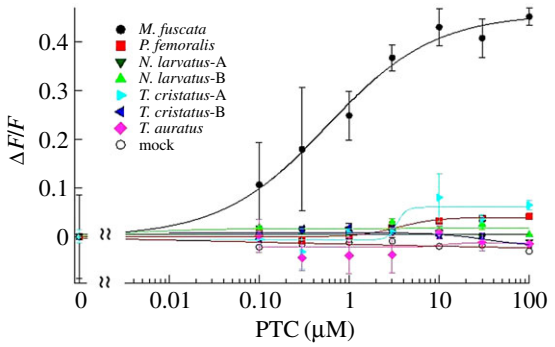

Figure 1. *In vitro* responses of TAS2R38 of *M. fuscata* and colobines transfected into HEK 293T cells were assayed, using an intracellular calcium indicator (Calcium4). The responses to various concentrations of PTC were calculated as the normalized peak response (F) relative to background fluorescence ($F0$): $\Delta F/F$ [$=(F - F0)/F0$]. Average responses ($n = 3$) for each time course are plotted against the concentration of PTC. (Online version in colour.)

3. Results

Most individual colobines ate both PTC-treated (50–100%) and control (90–100%) apple slices. In contrast, the one *M. hecki* individual strictly rejected PTC-treated apples (0%), although it readily ate control apple slices (100%; table 1); this is similar to past studies of *M. fuscata* [11]. This suggests that most colobines could not discriminate between PTC-treated and control apple slices. Thus, behavioural sensitivity to PTC was lower in colobines than in the macaque. The genetic basis for this behaviour was investigated by determining the nucleotide sequences

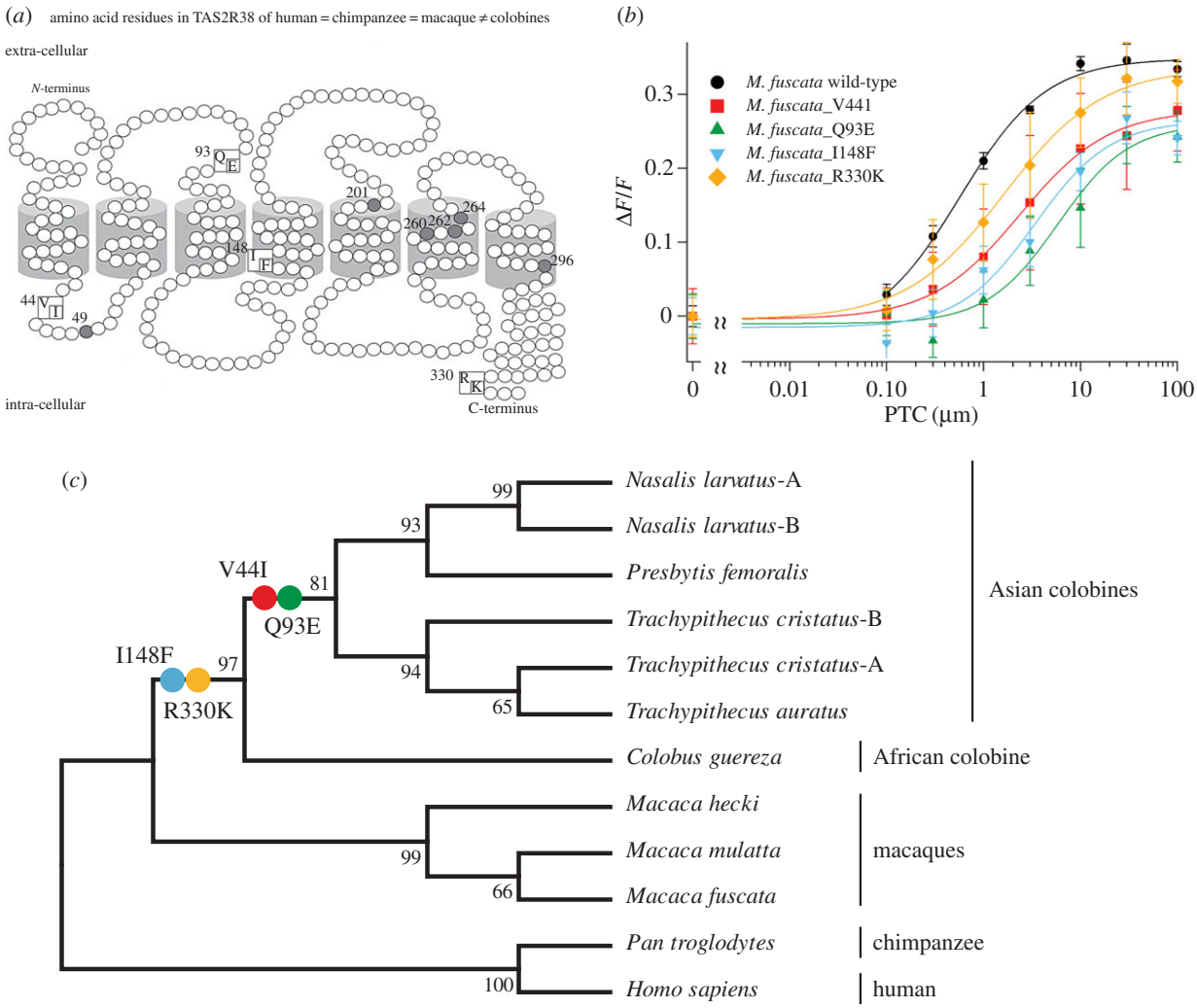

Figure 2. Possible amino acid substitutions associated with the decreased response to PTC. (*a*) Schematic transmembrane topology of the TAS2R38 receptor of colobines. The structure shown is based on the structure of bovine rhodopsin. Grey circles represent known functional sites for PTC [18]. The positions of the four amino acid substitutions observed in colobines are shown in squares: human = chimpanzee = macaque ≠ colobines. (*b*) The *in vitro* responses of *M. fuscata TAS2R38* mutated to match the colobine-type receptor. Multiple *M. fuscata TAS2R38* mutants were created to evaluate the effects of amino acid changes on receptor sensitivity. Average responses ($n = 4$) for each time course are plotted against the concentration of PTC. The results for mutants, except R330K ($p = 0.070$), were significantly different from those for *M. fuscata* ($p < 0.05$). (*c*) Phylogenetic relationships among amino acid sequences of TAS2R38 in primates. The numbers at the nodes on the phylogeny represent bootstrap values. The relationships are consistent with the species phylogeny obtained using other molecular data, *Alu* mobile elements, 11 nuclear loci (SRY, DBY5, SMCY7, SMCY11, UTY18, vWF11, ZFYLI, ALB3, *IRBP3*, *TNP2* and *TTR1*) and mitochondrial genome [18].

of *TAS2R38*, which encodes the TAS2R38 receptor in colobines. We found the sequences of *TAS2R38* orthologues from each species were intact, except for that of *T. cristatus*-B, which encoded a premature stop codon at nucleotide position 552. The *TAS2R38* sequences from colobines encoded amino acids different from those encoded by functional *TAS2R38* in macaques, chimpanzees and humans (electronic supplementary material, figure S1). Cells transfected with TAS2R38 expressing a vector from *P. femoralis* and *T. cristatus*-A responded very slightly to PTC and the EC_{50} could not be measured (figure 1). We also found that translated TAS2R38 from *N. larvatus*, *T. auratus* and *T. cristatus*-B did not respond to PTC, even at the highest PTC concentration (100 μM). Taken together, lower responses were observed for colobine TAS2R38 receptors than for the *M. fuscata* TAS2R38 receptor, which responded to PTC in a concentration-dependent manner with EC_{50} 0.58 ± 0.17 μM. This result was consistent with the PTC-tolerant behaviour of colobines.

The critical amino acid positions for human TAS2R38 functionality [17] are not altered in the colobine TAS2R38 receptor. We found that all colobines differed at four amino acid positions that are conserved in humans, chimpanzees and macaques (figure 2*a*). Valine was mutated to isoleucine at amino acid 44 (V44I), glutamine to glutamic acid at amino acid 93 (Q93E), isoleucine to phenylalanine at amino acid 148 (I148F) and arginine to lysine at 330 (R330K). To check whether these four amino acids are responsible for the low response of TAS2R38 to PTC, we mimicked those receptors by performing site-directed mutagenesis of the vector containing *TAS2R38* of *M. fuscata*. Single-site mutants of macaque TAS2R38 that mimic colobine TAS2R38 show remarkably decreased responses to PTC, as indicated by EC_{50} values. The mutations V44I, Q93E, I148F and R330K increased the EC_{50} values of the receptor (2.22 ± 0.44, 5.59 ± 2.34, 2.80 ± 1.31 and 1.35 ± 0.54 μM, respectively) compared with the wild-type (figure 2*b*). Mutations in *N. larvatus*-B *TAS2R38* mimicking those of the wild-type macaque failed to restore the sensitivity of the receptor to PTC (electronic supplementary material, figure S2), suggesting an additional mutation is responsible for the low sensitivity in colobines.

4. Discussion

We found that colobines are less responsive to PTC than are macaques. The decreased sensitivity to bitterness in colobines might be an advantageous adaptation to eating leaves. The common ancestor of primates is assumed to have had high sensitivity to PTC based on TAS2R38 [18]. In non-colobine primates, amino acid positions 44, 93, 148 and 330 are conserved. Substitutions of these amino acids might be a colobine-specific mechanism to reduce their sensitivity to PTC (as evidenced by the mutated macaque TAS2R38, which mimics the colobine receptor). Based on the phylogenetic relationships among the primate predicted TAS2R38 proteins, the amino acid substitutions arose in colobines after their divergence from the lineage that led to macaques. Two substitutions (V44I and Q93E) occurred after the separation of the Asian and African colobine lineages (figure 2*c*). Although the TAS2R38 sequences and sensitivities of other colobine TAS2R loci to PTC should be investigated, we hypothesize that sensitivities to bitter flavours became weak specifically in the colobine lineage as an adaptation to their leaf-eating behaviour.

Ethics. Behavioural experiments were performed in accordance with the guidelines of Bogor Agricultural University. The protocol was approved by the Animal Ethic Commission of Research and Community Service Institute, Ministry of Research, Technology and Higher Education, Bogor Agricultural University to B.S. (no. 35-2016 IPB). *In vitro* experiments were performed in accordance with the guidelines of Kyoto University. The protocol was approved by the Genetic Research Committee of Agency for Health, Safety and Environment, Kyoto University to H.I. (no. 120197).

Data accessibility. DNA sequences: DDBJ accessions LC167091–LC167096. Original functional data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.36d35 [19].

Authors' contributions. L.H.P.S.P. conducted experiments, wrote the original draft, and analysed and interpreted data. K.A.W. conducted experiments and analysed and interpreted data. K.T. and N.S.-H. designed the experiments, conducted experiments and analysed the data. T.H. and S.N. conducted experiments. B.S. designed the experiments, wrote the paper and revised the draft. H.I. designed the experiments, wrote the paper and finalized the draft. All authors agree to be held accountable for the content in the manuscript and approve the final version.

Competing interests. We have no competing interests to report.

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