Bitter taste facilitates the detection of potentially harmful substances and is perceived via bitter taste receptors (TAS2Rs) expressed on the tongue and oral cavity in vertebrates. In primates, TAS2R16 specifically recognizes β-glucosides, which are important in cyanogenic plants' use of cyanide as a feeding deterrent. In this study, we performed cell-based functional assays for investigating the sensitivity of TAS2R16 to β-glucosides in three species of bamboo lemurs (Prolemur simus, Hapalemur aureus and H. griseus), which primarily consume high-cyanide bamboo. TAS2R16 receptors from bamboo lemurs had lower sensitivity to β-glucosides, including cyanogenic glucosides, than that of the closely related ring-tailed lemur (Lemur catta). Ancestral reconstructions of TAS2R16 for the bamboo-lemur last common ancestor (LCA) and that of the Hapalemur LCA showed an intermediate sensitivity to β-glucosides between that of the ring-tailed lemurs and bamboo lemurs. Mutagenetic analyses revealed that P. simus and H. griseus had separate species-specific substitutions that led to reduced sensitivity. These results indicate that low sensitivity to β-glucosides at the cellular level—a potentially adaptive trait for feeding on cyanogenic bamboo—evolved independently after the Prolemur–Hapalemur split in each species.

1. Introduction

Taste is essential to evaluate food quality for mammals. Basic tastes are classified as sweet, umami, bitter, salty and sour. Bitterness is often associated with the detection of potentially harmful substances in food, while not all toxic substances are bitter and vice versa [1]. Bitterness is perceived via bitter taste receptors (TAS2Rs) expressed in lingual taste buds and on the palate epithelium [2,3]. TAS2Rs are G protein-coupled receptors (GPCRs) and recognize various bitter substances as ligands. TAS2R16 is one of the best-studied TAS2Rs and mainly recognizes β-glucosides including plant-derived toxins in millimolar concentrations [4–7]. While there are interspecies differences in β-glucoside sensitivity of TAS2R16, several studies suggest that β-glucosides specifically agonize TAS2R16 in primates, including humans [5,8,9]. Furthermore, a
and phylogenetic analyses. We found that the sensitivity of \( \beta \)-glucosides in three species of bamboo lemurs (\textit{Hapalemur}) to cyanogenic compounds in plants is correlated with its bitterness [10]. These studies suggest that primates can perceive the bitterness of \( \beta \)-glucosides in plants via TAS2R16.

Bamboo lemurs (\textit{Prolemur} and \textit{Hapalemur}) are endemic to Madagascar and are unusual among primates because they fill an ecological niche similar to giant pandas (\textit{Ailuropoda melanoleuca}) and red pandas (\textit{Ailurus fulgens}) in primarily feeding on bamboo or other grassy plants. Furthermore, bamboo lemurs may share morphological and gut microbial adaptations with giant and red pandas as well [11–15]. Three sympatric species of bamboo lemurs (\textit{Prolemur simus}, \textit{Hapalemur aureus} and \textit{Hapalemur griseus}) feed on a variety of bamboo species, some of which contain cyanogenic compounds in the form of \( \beta \)-glucosides [16]. They heavily rely on Madagascar giant bamboo (\textit{Cathariostachys madagascariensis}), which is one of the most cyanogenic plants in the world [14,16,17]. In particular, \textit{P. simus} and \textit{H. aureus} are obligate bamboo specialists [15].

Various species of plants use cyanide as a deterrent to herbivory [18,19]. Cyanogenic plants enzymatically release hydrogen cyanide (HCN) from preformed cyanogenic compounds in response to cell damage, which can lead to acute poisoning in herbivores [19]. Most such plants store cyanide in the form of \( \beta \)-glucosides (e.g. linamarin, linustatin and lotaustralin in cassava; taxiphyllin, linamarin and lotaustralin in bamboo; amygdalin in almonds; prunasin in \textit{Eucalyptus} plants) [20–22]. Some \( \beta \)-glucosides are known ligands of TAS2R16 [4,7], allowing mammals to detect cyanogenic glucosides through bitter taste. This is thought to cause an aversive response to avoid the poison. However, since bamboo lemurs mainly feed on cyanogenic bamboo, we hypothesized that bamboo lemurs would sense less bitterness of \( \beta \)-glucosides including cyanogenic glucosides.

To address this hypothesis, we characterized the functional features of TAS2R16 sensitivity to \( \beta \)-glucosides in three species of bamboo lemurs (\textit{P. simus}, \textit{H. aureus} and \textit{H. griseus}) and the closely related ring-tailed lemurs (\textit{Lemur catta}) using cell-based functional assays, molecular modelling and phylogenetic analyses. We found that the sensitivity of TAS2R16 to \( \beta \)-glucosides in bamboo lemurs was lower than in \textit{L. catta}; in particular, \textit{P. simus} showed far lower sensitivity to all tested \( \beta \)-glucosides due to species-specific amino acid substitutions. These findings contribute to uncovering the gustatory adaptations underlying the dietary specialization in bamboo lemurs.

2. Results

(a) Sensitivity of TAS2R16 to \( \beta \)-glucosides in bamboo lemurs

We first identified that TAS2R16 orthologues are present as single copies in bamboo-lemur genomes (electronic supplementary material, figure S1). The receptors showed over 93% amino acid sequence identity among bamboo lemurs (electronic supplementary material, table S2) and were properly expressed at the cellular membrane (electronic supplementary material, figure S2). We then evaluated their \( \beta \)-glucoside sensitivity by cell-based functional assays using three plant-derived \( \beta \)-glucosides selected for their ability to agonize our receptor of interest: linamarin, a cyanogenic glucoside, and salicin and arbutin, well-known ligands of TAS2R16 [4,7]. TAS2R16 of three bamboo lemurs showed lower responses to linamarin and salicin than that of \textit{L. catta} and did not show any responses to arbutin (figure 1). Interestingly, \textit{P. simus} showed almost no responses to the ligands at the concentrations used in the assays. These results imply lowered bitter perception of \( \beta \)-glucosides, including cyanogenic glucosides, in bamboo lemurs compared to their closest relative, \textit{L. catta}.

(b) Sensitivity of ancestral TAS2R16 to \( \beta \)-glucosides

To investigate the evolutionary history of TAS2R16 functions, we inferred TAS2R16 sequences of the last common ancestor (LCA) in bamboo lemurs (anc-Bamboo lemur TAS2R16) and that of the genus \textit{Hapalemur} (anc-Hapalemur TAS2R16) (figure 2a), and we then evaluated the responses of these reconstructed receptors to linamarin, salicin and arbutin. Only two positions (249 and 282) had multiple states (249: isoleucine or valine, 282: serine or leucine) in anc-Bamboo

Figure 1. Responses of TAS2R16 to \( \beta \)-glucosides in bamboo lemurs. HEK293T cells expressing TAS2R16 from each lemur with \textit{G\alpha 16/gust44} were stimulated with increasing concentrations of (a) linamarin, (b) salicin and (c) arbutin (see top of panel for chemical structures). Changes in fluorescence (\( \Delta F/F \)) upon ligand application were monitored (mean ± s.e.m.). Detailed values of parameters and statistics are shown in electronic supplementary material, tables S3 and S5. * Indicates the significant differences compared to those of \textit{L. catta} (\( p < 0.05 \), two-sided Welch’s t-test with BH correction). (Online version in colour.)
Compared to the ancestral receptors, bamboos showed lower signal amplitudes in response to arbutin (figure 2d). Anc-Hapalemur TAS2R16 showed the same degree of response to linamarin as in L. catta and lower response to salicin (figure 2c). EC_{50} values of anc-Bamboo lemur TAS2R16 were similar to those of L. catta for arbutin; however, the reconstructed receptor showed maximal signal amplitudes that tended to be lower than those of L. catta (figure 2d and electronic supplementary material, table S4). Anc-Hapalemur TAS2R16 showed the same degree of response to tested compounds as in anc-Bamboo lemur TAS2R16 (figure 2b–d). Anc-Hapalemur TAS2R16 showed lower responses to linamarin and salicin than did L. catta TAS2R16 (figure 2c). While the arbutin response of Anc-Hapalemur TAS2R16 was similar to that of L. catta, the maximal signal amplitude was lower than that of L. catta but similar to anc-Bamboo lemur TAS2R16 (figure 2d). These low maximal signal amplitudes of responses to arbutin were probably caused by the amino acid difference at position 249 (84.5% ML probability) and serine at position 282 (85.4%) in anc-Bamboo lemur TAS2R16. Anc-Bamboo lemur and anc-Hapalemur TAS2R16 receptors showed higher responses to arbutin but similar responses to linamarin and salicin (electronic supplementary material, tables S3 and S5). Although there is some variation among ligands, these results indicate that the LCA of bamboo lemurs exhibited a bitterness intermediate between those of modern-day L. catta and bamboo lemurs.

(c) Identification of the amino acid residues responsible for low sensitivity in TAS2R16

While the ancestral TAS2R16 receptors showed such intermediate β-glucoside sensitivities, TAS2R16 receptors of extant bamboo lemurs did show strikingly lower β-glucoside sensitivity than the ancestral TAS2R16 receptors. To identify the causal substitutions responsible for low sensitivity to β-glucosides, we first generated single-point mutants of the three bamboo lemurs and screened the causal mutations by the responses to salicin, which is one of the best-studied ligands in TAS2R16. We then evaluated the effects of identified substitutions in the ancestral receptors using all three ligands to trace the receptor’s evolution. We found 13 such substitutions in bamboo lemur species by comparing amino acid sequences of TAS2R16 among extant lemurs and ancestral bamboo lemurs (figure 3e). Of these 13 mutations, point mutations of P. simus at positions 144 and of H. griseus at position 147 showed higher responses to salicin compared with each wild-type (figure 3f; electronic supplementary material, figure S4 and table S6). Of those, the point mutations of P. simus at position 144 and of H. griseus at position 251 with the largest recovery of responses to salicin were considered the candidates mainly responsible for low sensitivity. Next, we generated anc-Bamboo lemur TAS2R16 with the mutation at position 144 (anc-Bamboo lemur
between human and bamboo-lemur orthologues. The
assume that the binding site has the required features to accom-
moiety of ligands are accommodated [23] (electronic supple-
human TAS2R16 [23] are conserved in bamboo lemurs. The
binding of the glucose moiety of arbutin and salicin in
previous study [23]. The receptor positions involved in the
representative conformations of human TAS2R16 model from a
substitution at position 1444.62 did not
tors and similar responses to the wild-types of extant bamboo
species-specific substitution at position 2516.59.
lower responses to
sensitivity to
P. simus
H. griseus
H. aureus
Y71H
W79L
N220D
L256F
S147N
L251S
P. simus
H. griseus
V178L
K257R

Figure 3. Key substitutions responsible for low sensitivity in bamboo-lemur
TAS2R16. Amino acids specifically substituted in bamboo lemurs are listed in
(a). Responses of single-point mutants of TAS2R16 to salicin in (b) P. simus
(L144S) and (c) H. griseus (S251L). HEK293T cells expressing each mutant of
TAS2R16 with Gz16/gust44 were stimulated with increasing concentrations of
salicin. Changes in fluorescence (ΔF/F) upon ligand application were moni-
tored (mean ± s.e.m.). Detailed values of parameters and statistics are
shown in electronic supplementary material, tables S6 and S7. (Online version
in colour.)

S144 L) and anc-Hapalemur TAS2R16 with the mutation at
position 2516.59 (anc-Hapalemur L251S) and evaluated their
sensitivity to the three β-glucosides. The mutants showed
lower responses to β-glucosides than ancestral TAS2R16 recep-
tors and similar responses to the wild-types of extant bamboo
lemurs (figure 4). The substitution at position 1444.62 did not
completely mimic the responses of wild-type P. simus to salicin,
which may be caused by the multiple effect from the other
P. simus-specific substitutions (L129F and T140A) and S282 L,
which is a shared substitution between P. simus and L. catta.
In summary, these results indicate that low sensitivity to β-glu-
cosides in P. simus was acquired by the substitution at position
1444.62 after diverging from the genus Hapalemur and that low
sensitivity to β-glucosides in H. griseus was caused by a separate,
species-specific substitution at position 2516.59.

(d) Structural basis of low sensitivity to β-glucosides
in bamboo lemurs
To identify the localization of the key residues in the recep-
tor, we generated three-dimensional models of P. simus
TAS2R16/ligand complexes. Models were based either on
X-ray structures (RHO or β2AR) or on the optimized repre-
sentative conformations of human TAS2R16 model from a
previous study [23]. The receptor positions involved in the
binding of the glucose moiety of arbutin and salicin in
human TAS2R16 [23] are conserved in bamboo lemurs.
Similarly, hydrophobicity of the bottom part of the binding
cavity is conserved in them, and this is where the aglycon
moiety of ligands are accommodated [23] (electronic supple-
mentary material, Analysis S2). Therefore, it is reasonable to
assume that the binding site has the required features to accom-
modate the ligands and that binding could occur similarly
between human and bamboo-lemur orthologues. The
reliability of the models was assessed using available experi-
mental mutagenesis data for human TAS2R16 in complex
with arbutin and salicin [24,25]. Seven residues previously
suggested to play a role in binding within the orthosteric bind-
ing site [24,25] are located within 5 Å from the ligands in the
models based on human TAS2R16 (figure 5a), directly interact-
ing with the ligand or having a role in shaping the binding
cavity (figure 5b–d). By contrast, the models based on the
X-ray templates did not agree with the mutagenesis
data (electronic supplementary material, Analysis S1). There-
fore, the models based on human receptor complexes are
discussed in the rest of the analysis. The localization of
positions with bamboo-lemur specific substitutions are
summarized in electronic supplementary material, figure S5.
In our final models, position 1444.62 was located at the
border between TM4 and extracellular loop (ECL) 2, while
position 2516.59 is located at the border between TM6 and
ECL3. Both key residues (position 1444.62 and 2516.59) were
not predicted to belong to the orthosteric binding site and
are not expected to be in direct contact with the bound
ligand (figure 5). This modelling indicated that the key resi-
dues have indirect effects to ligand binding and their effects
are probably related to ligand gating, rather than to direct
ligand recognition in the binding site.

3. Discussion
In this study, we characterized the functional and evolutionary
features of TAS2R16 in response to β-glucosides in bamboo
lemurs using cell-based functional assays, homology model-
ing and phylogenetic analyses (figure 6). β-glucosides are
specific agonists of TAS2R16 in humans [9] and in non-
human primates such as macaques and lemurs as well [5,8].
We found that bamboo-lemur TAS2R16 orthologues had low
sensitivity to β-glucosides including cyanogenic glucosides
(figure 1). Furthermore, this loss of sensitivity is not related
to any TAS2R16 gene-duplication event in any bamboo-
lemur lineage, as was demonstrated in a M y o t i s bat, in which
one paralog lost β-glucose sensitivity [6]. Because of the
lack of evidence for TAS2R16 duplication in bamboo lemurs,
and due to the lower sensitivity of their TAS2R16 orthologues
to β-glucosides, our results suggest that bamboo lemurs,
especially P. simus, are relatively insensitive to the bitterness
of β-glucosides. Interestingly, β-glucoside sensitivity is higher
in H. aureus than H. griseus and P. simus (figure 1), despite
the fact that cyanide intake is similarly high in P. simus and
H. aureus but comparatively low in H. griseus [26]. Hence,
there is no clear correlation between β-glucose sensitivity of
TAS2R16 and cyanide consumption within the bamboo-
lemur clade. To clarify whether this difference in sensitivity
influences food choice strategy among bamboo lemurs, further
studies on the relationship between receptor sensitivity and
ligand concentration contained in bamboo are required.

Our findings show some gustatory convergence between
bamboo lemurs and giant pandas—two distantly related mam-
malian taxa that have independently adapted to exploit similar
ecological niches [11]. A recent study showed that the giant
pandas of the Qinling Mountains in China, which consume
more bamboo leaves than pandas in other regions, have a
variant of TAS2R20 with low sensitivity to the bitter rhamno-
side quercitrin contained in Chinese bamboo, suggesting
that low bitter perception enables intake of high-quercitrin
Figure 4. Responses of mutants of ancestral TAS2R16 to β-glucosides. Responses of TAS2R16 of the LCA of bamboo lemurs (anc-Bamboo lemur TAS2R16) with the substitution at position 1444.62 to (a) linamarin, (b) salicin and (c) arbutin. Responses of TAS2R16 of the LCA of the genus Hapalemur (anc-Hapalemur TAS2R16) with the substitution at position 2516.59 to (d) linamarin, (e) salicin and (f) arbutin. HEK293T cells expressing each mutant of TAS2R16 with Gα16/gust44 were stimulated with increasing concentrations of ligands. Changes in fluorescence (ΔF/F) upon ligand application were monitored (mean ± s.e.m.). Detailed values of parameters and statistics are shown in electronic supplementary material, tables S8 and S9. (Online version in colour.)

Figure 5. Homology model of bamboo-lemur TAS2R16. (a) Residues (in ribbon representation) within 5 Å from the docked ligand (arbutin, in green licorice) are coloured in orange. Residues within 5 Å and experimentally suggested as potentially involved in the binding site definition are coloured and labelled in red. The position of the residues altering the activation level when mutated (blue ribbon) does not overlap with the binding site residues. Receptor’s TM1 and a portion of the ECL2 are not shown for sake of clarity. (b–d) Schrödinger Maestro interaction diagram for arbutin, salicin, and linamarin, respectively. For the sake of clarity, only residues within 3 Å from the ligand are shown (and not 5 Å as in (a)). Arrows indicate hydrogen bonds. (Online version in colour.)
bamboo [27]. Likewise, insensitivity to bitterness of β-glucosides in bamboo lemurs would be a similarly adaptive trait for feeding on high-cyanide bamboo such as C. madagascariensis [14,16,17]. Some bamboo species, including Bambusa vulgaris consumed by P. simus, contain taxiphyllin, a different cyanogenic glucoside [12,28–30]. However, the chemical structures of cyanogenic compounds in C. madagascariensis are still unknown. To determine the precise relationship between feeding on cyanogenic bamboo (i.e. C. madagascariensis) and TAS2R functions, identification of the cyanogenic compounds in C. madagascariensis and cell-based studies using bamboo extracts or purified cyanogenic compounds of Malagasy bamboo are required.

Our analysis revealed that ancestral receptors of bamboo-lemur TAS2R16 had an intermediate sensitivity to β-glucosides between extant L. catta and bamboo lemurs (figure 2). These ancestral receptors showed similar or low salicin-sensitivity to TAS2R16 orthologues from two confamilial lemurs whose TAS2R16 sensitivity has already been characterized (Eulemur macaco: EC\textsubscript{50} = 2.3 mM, Varecia variegata: EC\textsubscript{50} = 3.8 mM; see Itoigawa et al., [8]). Such functional features of the bamboo-lemur LCA suggest that it had a similar, or intermediated lowered, bitter taste response to β-glucosides compared to other extant lemurids including the frugivorous L. catta and the frugivorous E. macaco and V. variegata. Thus, bamboo lemurs’ reduction of β-glucoside sensitivity may have started in the LCA of bamboo lemurs. Mutagenetic analyses revealed that the species-specific substitutions at position 144\textsuperscript{K} to S in P. simus and position 251\textsuperscript{L} to S in H. griseus cause extremely low sensitivity to β-glucosides in each species (Figures 3a,c and 4). The drastic effect of the P. simus-specific substitution at position 144\textsuperscript{K} indicates that this species independently acquired extremely low sensitivity after diverging from the genus Hapalemur (figure 4a–c). Furthermore, the difference of β-glucoside sensitivity within the genus Hapalemur was caused by the amino acid difference at position 251\textsuperscript{L} and only in the species H. griseus (figure 1 and figure 4d–f). These results suggest that β-glucoside sensitivity was independently decreased after the two genera diverged.

Neither of the amino acid residues responsible for low sensitivity (L144\textsuperscript{K} and S251\textsuperscript{L}) is in direct contact with the bound ligand in TAS2R16 (figure 5), indicating that their substitutions are more likely to be involved in receptor activation or in ligand entry to the binding site. Position 144\textsuperscript{K} is located at the border between ECL2 and TM4 (figure 5a, electronic supplementary material, figure S6). In human TAS2R16, the substitution from serine to leucine or alanine at position 144\textsuperscript{K} decreases sensitivity to several β-glycosidic ligands [25]. Molecular dynamics simulations using human TAS2R16 indicate that S144\textsuperscript{K} interacts with extracellular water molecules and is able to form hydrogen bonds with other hydrophilic residues of ECL2 including N148\textsuperscript{ECL2}, Q151\textsuperscript{ECL2} and E158\textsuperscript{ECL2} [23]. N148\textsuperscript{ECL2} in humans corresponds to K148\textsuperscript{ECL2} in lemur, while Q151\textsuperscript{ECL2} and E158\textsuperscript{ECL2} were conserved among humans and lemurs. K148\textsuperscript{ECL2} can potentially form hydrogen bonds with S144\textsuperscript{K}. The lack of these interactions in P. simus, due to the hydrophobic K148 L substitution, could affect the loop conformation of ECL2 and, hence, the ligand gating to the binding pocket. On the other hand, position 251\textsuperscript{L} is located at the border between TM6 and ECL3 in lemurs (figure 3a and figure 5a) and corresponds to position 249\textsuperscript{K} to catarrhine primates. One such lineage, the macaques shows lower sensitivity to β-glucosides than other catarrhines including humans, chimpanzees and langurs due to a L247 M substitution [31]. Thus, mutations at this TM6/ECL3-bordering position appear to have occurred multiple times during primate evolution, leading to similar effects on ligand sensitivity. In our homology models, residue 251\textsuperscript{L} is in close contact with the long ECL2 TM5 (electronic supplementary material, figure S6). Hence, if this residue is changed from hydrophobic (L) to polar (S), it may modify the interaction with ECL2. As suggested above for residue 144\textsuperscript{K}, such alterations may affect ligand gating and lead to substantive consequences for ligand–receptor interactions.

Parallel loss of taste receptors associated with parallel ecological and/or dietary adaptation is reported in several mammals. Giant pandas and red pandas (Ailurus fulgens), distantly related carnivorous bamboo specialists, have convergently lost umami taste receptor TAS1R1 by different indel mutations [32]. A previous study suggests that otarioids and phocids, the two main lineages of seals, have also independently lost TAS1R genes with their independent aquatic adaptation from terrestrial ancestors [33]. In the case of bamboo lemurs, they have maintained intact TAS2R16 receptors, but those sensitivities to (cyanogenic) β-glucosides were independently decreased after the two genera of bamboo lemurs diverged, similar to what was proposed for strychnine-sensitive TAS2Rs in several mammals [34]. So, did adaptation to high-cyanide bamboo occur separately in each lineage? Both lineages of bamboo lemurs share dentitions specialized to process fibre-rich bamboo [14,35,36]. By contrast, there is considerable variation in gut passage rates among bamboo lemurs. P. simus has passage rates of 9 h, three times as fast as Hapalemur species, suggesting that slow digestion may not be a shared strategy for detoxification in bamboo lemurs [26,37,38]. At present, there is no fossil record of the common ancestor of bamboo lemurs and ancestral reconstructions of dietary specialization in the bamboo-lemur LCA are equivocal [17]. Therefore, it is still uncertain whether the adaptation to high-cyanide bamboo occurred once or twice among bamboo-lemur species. However, β-glucoside sensitivity of TAS2R16 in the LCA of bamboo lemurs is lower than, but
not largely different from, that of other lemurids, and β-glucoside sensitivity decreased independently in two of three species of bamboo lemurs. Such results would justify future studies to more precisely pinpoint when the remarkable dietary specialization in bamboo lemurs occurred in the evolution of this taxon.

Low bitter sensitivity would be advantageous to feeding on bitter plants such as *C. madagascariensis*. However, because of the high cyanogenic potential of this plant species, it is also critical to improve detoxification mechanisms to feed on such toxic plants. A previous study found that the cyanide derivatives releasing cyanide upon acidification such as cyanide and thiocyanate are present in the urine of bamboo lemurs [26], implying that dietary cyanide is absorbed in the gastrointestinal tract and converted to non-toxic derivatives via enzymes such as rhodanese (E.C. 2.8.1.1). While the functional characteristics of rhodanese in bamboo lemurs are unknown, its expression and activity in the liver and kidney is higher in giant pandas, an obligate bamboo specialist, than in cats, but not higher than in rabbits [39]. Although the evolutionary histories of dietary specialization in bamboo lemurs are not completely revealed in the present study, further studies on the evolution of rhodanese and other enzymes related to cyanide detoxification and bitter taste receptors at the genome level and protein (functional) level would shed light on the evolutionary history of bamboo lemurs.

In conclusion, our results clarified the functions of TAS2R16 in bamboo lemurs, which is an important bitter taste receptor recognizing toxic compounds contained in cyanogenic bamboo. Lowered sensitivity of TAS2R16, which was independently acquired in each species of bamboo lemur, may be advantageous to feeding on cyanogenic bamboo, which is very rich in bitter cyanogenic β-glucosides. The combination of cell-based functional assays with phylogenetic analyses can discover functional convergence not only in putatively pseudogenized taste receptors [32,33] but also in intact taste receptors. Such work contributes to a deeper understanding of gustatory adaptation in various feeding habits in mammals.

4. Material and methods

(a) Sequence determination of TAS2R16

TAS2R16 sequences of 11 lemur species including three bamboo lemurs (P. simus, H. aureus and H. griseus) were identified in whole-genome assemblies summarized in electronic supplementary material, table S1 as previously described [40]. Briefly, tblastn searches were performed into each genome assembly. Reciprocal tblastn searches into the human genome assembly GKh38.p13 and the reconstruction of a neighbour-joining tree were performed to confirm the orthology of identified sequences. The open reading frames with minimal flanking sequences were evaluated for the presence of seven transmembrane domains using TOPCONS [41].

(b) Construction of expression vectors for TAS2R16 and point mutants

TAS2R16 sequences of three bamboo lemurs were synthesized using the gBlocks Gene Fragment services (Integrated DNA Technologies Inc., Coralville, IA). The synthesized sequences were tagged at the N terminus with the first 45 amino acids of rat somatostatin receptor type 3 to improve cell-surface trafficking and at the C terminus with the last eight amino acids of bovine rhodopsin as an epitope tag. The tagged sequences were inserted into the mammalian expression vector pEAK10 (Edge Biosystems Inc., Gaithersburg, MD) using the In-Fusion HD Cloning Kit (Clontech, Fremont, CA). Point mutant vectors were constructed using the QuikChange Lightning Multi Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and the overlap extension PCR method. All mutations were checked by direct sequencing. The expression vector of ring-tailed lemurs (*L. catta*) was prepared in a previous study [8].

(c) Calcium assay

Cell culture, transfection and calcium assays were performed as previously described [8]. Salicin, arbutin (Sigma-Aldrich, St Louis, MO) and linamarin (Santa Cruz Biotechnology, Dallas, TX) were used as ligands. As calcium indicators, Calcium 4 (Molecular Devices, Sunnyvale, CA) was used for the assays with salicin and arbutin, and Calcium 5 (Molecular Devices) were used for those with linamarin. Data were collected from 3–5 independent experiments. The calcium response is expressed as the normalized peak response (F) relative to background fluorescence (F0): ΔF/ F0 = (F–F0)/F0. The response of cells transfected with the empty pEAK10 vector (no insert) and Gal16/gus44 was defined as the TAS2R-independent response and was subtracted from all responses. To calculate dose–response relationships, ΔF/F values were fitted to the nonlinear regression model (y = a + b(x – c))/d, where x is the test compound concentration and b is the Hill coefficient, using the drc package in R [42]. Threshold concentrations (TH) were defined as the lowest substance concentration where the normalized fluorescence (ΔF/F) was higher than that in 0 mM (Dunnett’s test, p < 0.05). A lack of detectable TH indicates that the receptors have no response to the substances. Maximal signal amplitudes (Amax) were defined as the maximum normalized fluorescence (ΔF/F) within the tested substance concentrations. Statistical comparisons of the results were performed by two-sided Welch’s t-test with Benjamini–Hochberg (BH) correction or Dunnett’s test. To screen the substitutions responsible for ligand sensitivity, we compared EC50 and/or Amax of point mutations with those of each wild-type using Dunnett’s test. We then considered the mutations with the largest response changes the candidates mainly responsible for the ligand sensitivity.

(d) Phylogenetic analysis and ancestral reconstruction

Sequences of intact TAS2R16 orthologues in other lemurids were obtained from a previous study [8]. To compare sequences of TAS2R16 orthologues, a multiple sequence alignment was generated based on the amino acid sequences using MAFFT version 7 [43]. A maximum-likelihood (ML) tree with 1000 bootstrap replicates was reconstructed based on this alignment using MEGA X [44]. The JTT + F + G model, which was determined as the best substitution model by AICc values using the model selection test in MEGA X, was used to correct for multiple substitutions [45]. The ancestral amino acid sequences of bamboo-lemur TAS2R16 were inferred using ML-based ancestral reconstruction in MEGA X.

(e) Molecular modelling

Three-dimensional models of *P. simus* TAS2R16 were generated using MODELLER [46]. The structures employed as templates were human TAS2R16 from a previous study [23]. Specifically, Fierro and co-workers built the human model using the GOMoDo webserver [47] for template selection (β(2) adrenergic receptor: β2AR, PDB ID: 4LDE) and MODELLER for model building. In that paper, several ligands including arbutin and salicin were docked into the human TAS2R16 model and the molecular dynamics (MD) simulations [48] were performed for 800 ns each.
Here, we selected the MD frame with the lowest RMSD to the average structure calculated along with the MD simulations for human TAS2R16 in complex with arbutin in the so-called ‘TM binding mode’ as a template. The alignment between the target and template sequences was performed using BLAST [49]. Another model was similarly built based on salicin MD trajectory in the same binding mode. Through superimposition of the human models with the lemur ones performed with Chimera [50], salicin and arbutin were placed into the respective lemur model in the orthosteric binding site, followed by minimization of the complex performed with Schrödinger Maestro [51]. The lemur TAS2R16/linamarin complex was obtained by alchemical substitution of the arbutin phenol aglycon from lemur TAS2R16/arbutin complex with the corresponding linamarin substituent, acetone cyano-hydrin. The analysis of three-dimensional structures was achieved with Chimera, Chimera X [52], Schrödinger Maestro and VMD [53]. Superscript numbers in amino acid residues located in transmembrane domains (TM) were represented following the Ballesteros–Weinstein (BW) numbering method [54] based on the human TAS2R16 numbering reported in the GPCRdb [55]. For the residues of lemur TAS2R16, located in TM regions and corresponding to gaps in the alignment with human TAS2R16, the numbers obtained by adding ‘xN’ (N = 2, 3, 4, …) to the previous BW numbering in humans were represented (e.g. position 183 in lemur: 5.44x2). Additional models were obtained using MODELLER based on rhodopsin (RHO, PDB ID: 1U19) or β2AR X-ray template, but these models were less compatible with experimental mutagenesis data [24,25] and were discarded (electronic supplementary material, Analysis S1).

Ethics. Acquisition of *H. griseus* blood for sequencing was approved by IACUC of Duke University (A272-17-12). Acquisition of *H. aureus* tissue for sequencing was approved by Madagascar National Parks, CAFF/CORE, and the Parc Botanique et Zoologique de Tsimbazaza.

The use of genetic samples of *L. catta* was approved by the Japan Monkey Centre (no. 2017-018). This is Duke Lemur Center publication no. 1477.

Data accessibility. DNA sequences: DDBJ accessions LC579432 and LC579433. Original data for the calcium assays are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.fttdz08x7 [56].

Authors’ contributions. A.J. designed and conducted experiments, analysed, interpreted data and wrote the original draft. F.F., M.E.L. and T.H. conducted experiments, analysed and interpreted data, and wrote and revised the draft. A.J.T., M.Y.N. and H.I. designed experiments and wrote and finalized the draft. All authors approved the final version of the manuscript.

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