A New World Monkey Resembles Human in Bitter Taste Receptor Evolution and Function via a Single Parallel Amino Acid Substitution

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Letter

Abstract

Bitter taste receptors serve as a vital component in the defense system against toxin intake by animals, and the family of genes encoding these receptors has been demonstrated, usually by family size variance, to correlate with dietary preference. However, few systematic studies of specific Tas2R to unveil their functional evolution have been conducted. Here, we surveyed *Tas2R16* across all major clades of primates and reported a rare case of a convergent change to increase sensitivity to β-glucopyranosides in human and a New World monkey, the white-faced saki. Combining analyses at multiple levels, we demonstrate that a parallel amino acid substitution (K172N) shared by these two species is responsible for this functional convergence of Tas2R16. Considering the specialized feeding preference of the white-faced saki, the K172N change likely played an important adaptive role in its early evolution to avoid potentially toxic cyanogenic glycosides, as suggested for the human *TAS2R16* gene.

Key words: **convergent evolution, bitter taste receptors, Tas2R16, human, white-faced saki, *Pithecia pithecia*, adaptive evolution.**

Selection imposed by diet is a critical factor for adaptation of species to their ecological environments, with the perception of bitter taste being especially important as many natural bitter tasting substances are toxic or even lethal (Chandrashekar et al. 2006). Perception of a bitter taste usually results in aversion in animals, thus, avoiding the intake of toxins. In mammals, bitter taste perception is mediated by the bitter taste receptors (Tas2Rs), which belong to the superfamily of G protein-coupled receptors (GPCRs) and are encoded by a family of intronless genes (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000). In general, Tas2Rs bind bitter compounds in the extracellular environment and trigger a cascade of intracellular reactions that lead to bitter perception (Margolskee 2002). During the last two decades, we have seen an explosive increase in the number of *Tas2R* studies and research into the evolution of *Tas2R* gene family repertoires has clearly demonstrated associations with preferences in diet (Shi et al. 2003; Shi and Zhang 2006; Dong et al. 2009; Hayakawa et al. 2014; Li and Zhang 2014). These investigations provided a general view of the adaptive evolution of *Tas2R* genes at the large scale of vertebrate evolution; however, obtaining a comprehensive understanding of bitter taste perception and adaptation requires refined studies. Researches on one of the *Tas2R* gene family member, *Tas2R38*, have shown molecular and phenotypic variances and independent evolution among different species (e.g., Wooding et al. 2006; Suzuki et al. 2010; Wooding 2011; Purba et al. 2020). Whether other *Tas2R* members followed

the same evolutionary trajectory and how they contributed to animal adaptation remain largely unknown. A detailed survey of a specific *Tas2R* member in a group of closely related species, with diverse feeding preferences, should help dissecting the molecular mechanisms responsible for adaptation to diverse diets. To this end, primates appear to be appropriate candidates as their phylogenetic relationships are well established (Perelman et al. 2011) and, more importantly, their diet covers a dramatically diverse range of foods including leaves, fruits, insects, and even fishes and crabs (Rosenberger 1992; Liu et al. 2014). In this study, we scrutinized one of the best studied bitter taste receptors, Tas2R16, in a wide range of primate species, including experimentally testing their functional properties. Previous studies found that human Tas2R16 responds to a group of chemicals called β-glucopyranosides (Bufe et al. 2002), which contain some highly toxic cyanogenic glycosides (Drewnowski and Gomez-Carneros 2000). Tas2R16 sensitivity to β-glucopyranosides therefore is believed to play a vital role in avoiding such toxins. Interestingly, it was also suggested that decreased Tas2R16 sensitivity might be beneficial for protection against malaria in central Africa (Soranzo et al. 2005), although a subsequent investigation with larger African sample size did not find such association (Campbell et al. 2014). Here, by combining evolutionary, 3D structure modeling and functional assay analyses, we demonstrate the adaptive evolution of primate Tas2R16 and highlight a rare case of convergent evolution between human and a New World monkey.

Results

Primate *Tas2R16* Genes Underwent Positive Selection

In addition to sequences retrieved from available databases and previous studies, we amplified the *Tas2R16* gene from 15 additional primate species and also from the common tree shrew (*Tupaia glis*) as an outgroup. Altogether, we collected sequences from 41 primate species, with the samples covering the major clades of primate phylogeny; to our knowledge, this represents the most detailed study of the *Tas2R16* gene in primates to date (see Materials and Methods and fig. 1A; supplementary table S1, Supplementary Material online). To trace the evolutionary history of *Tas2R16*, we measured the selective pressures acting on this gene using the maximum likelihood (ML) method implemented in PAML4.7 (Yang 1997, 2007). Both sets of comparisons between neutral and selection models (M1a vs. M2a and M7 vs. M8) showed significant signs for positive selection ($P < 0.001$, table 1). In addition, four and seven sites having ω (ratio of d*N* to d*S*) >1 with posterior probabilities higher than 0.95 were identified with models M2a and M8, respectively. Notably, the identified sites are distributed in the transmembrane regions TM5 and TM7 of Tas2R16, which are involved in ligand–receptor interaction (Sakurai et al. 2010). Considering the diverse dietary preferences among primates, whose food sources include leaves, fruits, insects, and even fishes and crabs (Rosenberger 1992; Liu et al. 2014), our evolutionary analyses hinted that positive selection might have driven functional variations of Tas2R16s among these species, and the selective forces may have helped primate species adapt to specialized diets encountered in different habitats.

In Vitro Assay Reveals a Wide Range of Sensitivity of Primate Tas2R16s Responding to Bitter Compounds

To examine the above hypothesis, we surveyed the functional responses of 11 representative primate Tas2R16s to salicin and helicin, 2 bitter compounds known to activate human TAS2R16 (Bufe et al. 2002), in a functional heterologous cell expression assay (Bufe et al. 2002; Meyerhof et al. 2010). Previously reported functional data (Imai et al. 2012; Itoigawa et al. 2019) were also included into our analysis. Altogether, our functional assays included Tas2R16s from 17 representative primates which covered all major clades in the primate phylogeny (fig. 1B) and thus, should reflect the common response patterns of primate Tas2R16 to the corresponding bitter compounds in vitro. Dose−response curves revealed diverse activation of the primate Tas2R16s to salicin, ranging from nonresponsive to highly sensitive (fig. 1B; supplementary fig. S1, Supplementary Material online), which supports the above speculation and a previous study (Imai et al. 2012). Tests with helicin showed similar results. In general, when EC_{50} values were mapped to the primate phylogeny, we found that human and apes exhibited the highest sensitivity to salicin (0.49−1.0 mM), Old World monkeys showed intermediate sensitivities (2.3−4.2 mM), whereas strepsirrhines and New World monkeys generally presented a wider sensitivity range from non- to medium-responsive. Notably, the white-faced saki (*Pithecia pithecia*), a New World monkey, showed an extremely high sensitivity to salicin ($EC_{50} = 0.51 \pm 0.03$ mM). The sensitivity of the white-faced saki Tas2R16 resembles that seen with the human receptor ($EC_{50} = 0.49 \pm 0.08$ mM), suggesting a functionally convergent increase in sensitivity to salicin by Tas2R16s between these two species.

Parallel Amino Acid Evolution Identified between Human and the White-Faced Saki

We then carefully investigated the Tas2R16 sequences, trying to find an underlying characteristic for the observed change in salicin sensitivity. We noticed that both the human and the white-faced saki Tas2R16 protein sequences had an identical amino acid (asparagine, N) at position 172, which are encoded by different codons between these two species, that is, AAT in human and AAC in the saki monkey. This is a unique cooccurrence among all of the primate species samples tested, as the other sequences had either lysine (K) or methionine (M) at position 172 (fig. 1). Surprisingly, even when we extended the alignment to a wider range of nonprimate mammalian species, this cooccurrence remained exclusive to human and the white-faced saki (supplementary fig. S2, Supplementary Material online). Intriguingly, the K172N substitution appears to have experienced positive selection within human populations, with the 172N-type protein showing a higher sensitivity to a series of tested bitter compounds (Soranzo et al. 2005). This hinted that convergent/parallel evolution of *Tas2R16* might have occurred between these two species. As site 172 is polymorphic within

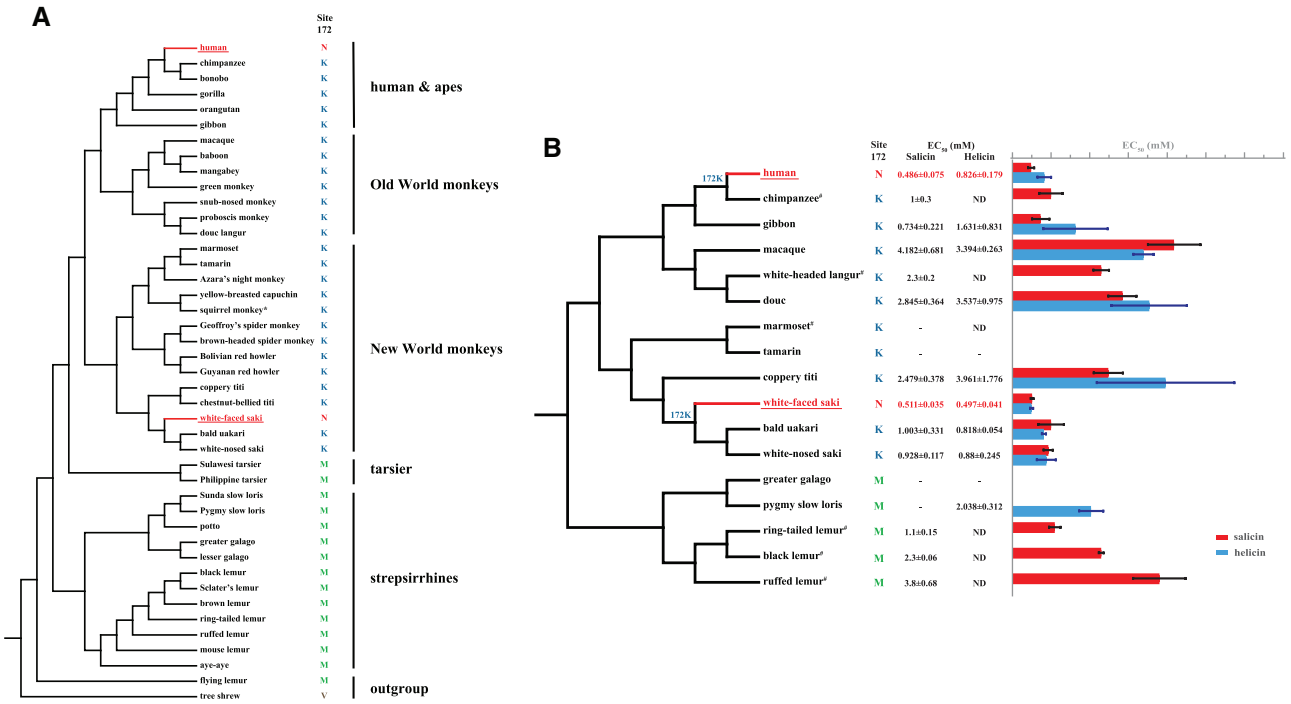

Fig. 1. Amino acid states at site 172 of Tas2R16 in primate species involved in this study and their in vitro function in representative species. (*A*) Phylogeny of the 41 primate species involved in this study. Amino acid states at site 172 of Tas2R16 in each species are mapped along the phylogenetic tree. The two branches that experienced parallel evolution are underlined. Asterisk indicates the sequence contains premature stop codons. (*B*) Half maximal effective concentrations (EC_{50}) for salicin and helicin in representative primate Tas2R16s. States for the amino acid at site 172 and the EC_{50} for salicin and helicin for each Tas2R16 assayed are mapped along with the accepted phylogeny. Residues on interior nodes indicate inferred ancestral states. The two branches that experienced parallel evolution are underlined. #, Data are from Imai *et al.* (2012) and Itoigawa *et al.* (2019); ND, not determined; -, nonresponsive.

Table 1. Likelihood Ratio Tests for Positive Selection in Primate *Tas2R16*s.

Models	ln *L*	ω(dN/dS)	Models' Comparison	*P* Value	Positively Selected Sites (BEB)[a]
M1a (NearlyNeutral)	−6,553.715264	0.7523			
M2a (PositiveSelection)	−6,533.193751	1.0219	M1a versus M2a	1.22E−09	179Q 182T 198F 255R
M7 (beta)	−6,555.454121	0.7404			
M8 (beta&ω)	−6,533.011973	0.9962	M7 versus M8	1.79E−10	82T 174H 179Q 180A 182T 198F 255R

[a] BEB= Bayes Empirical Bayes analysis. Amino acids with posterior probability >0.95 are listed.

human populations, we endeavored to collect additional eight unrelated white-faced saki individual samples to determine whether a similar polymorphism existed in this species. Surprisingly, all *Tas2R16* sequences obtained are identical, hinting that the 172N substitution likely predominate in the species. Although the possibility of polymorphism in the saki population cannot be completely excluded, the 172N allele most likely represents the major type of sequence in the white-faced saki. To better distinguish convergent/parallel substitutions, we inferred ancestral Tas2R16 protein sequences for each interior node of the primate species tree using ML and maximum parsimony methods (Yang 2007). Both inferences suggested a lysine (K) at site 172 as the ancestral state within the Simiiformes clade, which includes New World Monkeys, Old World Monkeys, human and apes, revealing that the asparagine (N) in human and white-faced saki descended from the ancestral lysine (K) in parallel (fig. 1), thus a parallel substitution has occurred at site 172 of Tas2R16 in both human and white-faced saki.

Although, in general, parallel evolution suggests adaptive evolution, the observation of parallel changes can sometimes arise by random chance as protein sequences evolve stochastically among at most 20 amino acid states at each site (Zhang and Kumar 1997). To examine the possibility that this observed change was just due to random chance, we applied a statistical test using the JTT-f_{gene} model and the most stringent JTT-f_{site} model (Zhang and Kumar 1997; Zou and Zhang 2015). Result of these tests showed that the null hypothesis of "parallel evolution due to random chance" is rejected by both models ($P = 0.0079$ in JTT-f_{gene} and $P = 0.0121$ in JTT-f_{site}), suggesting that a selective rather than stochastic process led to the parallel K172N substitutions, which then implicates a critical role of 172N in bitter taste perception.

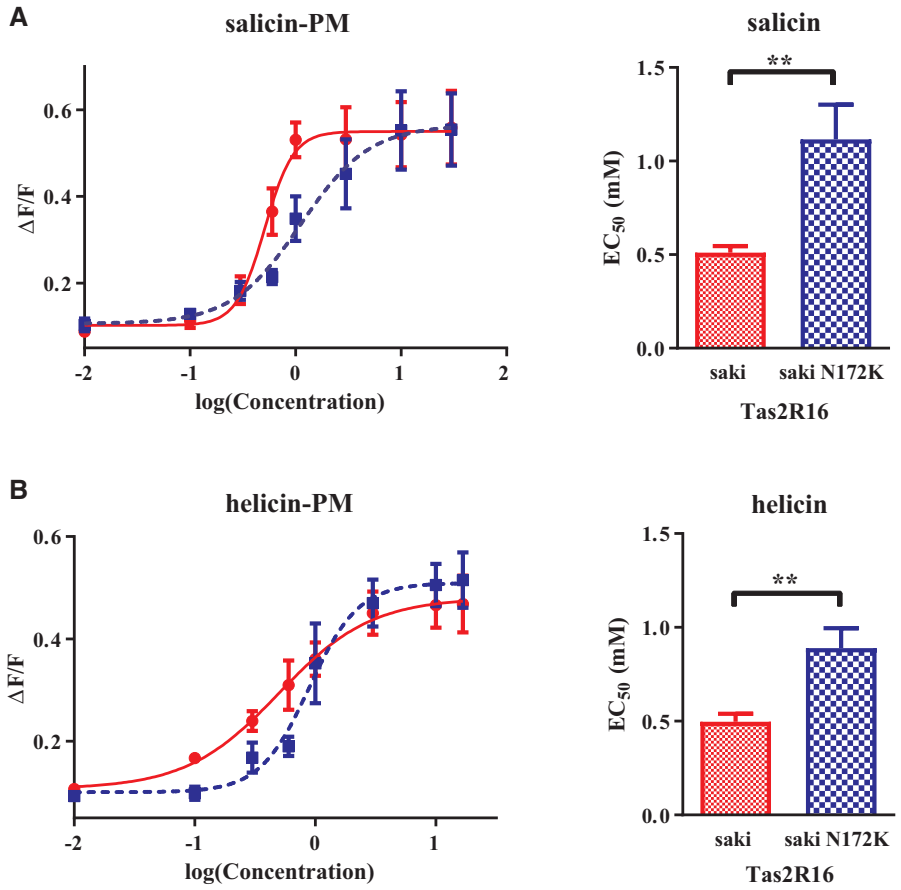

FIG. 2. Concentration−response curves and EC_{50} comparisons of wild-type and N172K mutated white-faced saki Tas2R16 for salicin (*A*) and helicin (*B*). Response curves of wild-type and N172K mutated Tas2R16 are represented by solid and dotted lines, respectively. **, Differences are significant at the 1% level ($P < 0.01$).

The Parallel Change K172N Accounts for the Convergent In Vitro Sensitivity Increase in Both Human and White-Faced Saki Tas2R16s

To verify the role of the 172N substitution in saki monkeys, we constructed a reverse 172K mutant of the white-faced saki Tas2R16 protein by mutagenesis, and determined its sensitivity using the functional assay described above. The EC_{50} of the 172K saki mutant protein was 1.12 ± 0.19 mM, making it about 50% as sensitive as the wild-type ($P < 0.001$) (fig. 2*A*). In addition, we tested the mutated protein with helicin and it also yielded a nearly 2-fold increased EC_{50} value of 0.89 ± 0.11 mM, indicating ~50% sensitivity, compared with the wild-type value of 0.50 ± 0.04 mM ($P < 0.05$) (fig. 2*B*). Similar patterns were previously reported in human Tas2R16 172K mutagenesis assays (Soranzo et al. 2005). These results together reveal that the single parallel amino acid change K172N observed in human and the white-faced saki Tas2R16 proteins is likely responsible for the convergent sensitivity increase in both species.

N172K Change Affects the Binding Affinity but not the Cell Surface Expression Level of White-Faced Saki Tas2R16

We next wanted to determine how the lysine/asparagine alteration at site 172 in Tas2R16 affects the sensitivity to bitter compounds. One possible explanation is that asparagine at site 172 may facilitate the increased trafficking of Tas2R16 to the cell surface to function as a GPCR, as previously reported in human Tas2R16 studies (Campbell et al. 2014; Thomas et al. 2017). To test this hypothesis, we measured and compared the cell surface expression of the white-faced saki Tas2R16 between the 172N and 172K variants. Unexpectedly, the cell surface expression levels of the two Tas2R16 forms were not statistically different (60.67 ± 1.16 vs. 59.73 ± 1.16, mean fluorescence intensity, $P = 0.8125$), suggesting that the lysine/asparagine alteration does not alter trafficking of the saki Tas2R16 to the cell surface, which is different from that observed for human Tas2R16.

Another potential reason for the difference in sensitivity is that the lysine/asparagine alteration may change the binding affinity between Tas2R16 and its ligands. To test this hypothesis, we modeled the white-faced saki Tas2R16 protein structure, into which its ligand salicin was docked using AutoDock Vina (Trott and Olson 2010). The ligand-binding energy was then predicted using the Cartesian_ddg application from Rosetta (Leman et al. 2020). The results show that salicin is bound by a ligand-binding pocket formed by portions of transmembrane regions 3, 5, 6, and 7 (TM3, TM5, TM6, and TM7). The aromatic ring and sugar moiety of salicin are directed toward the extracellular and the cytoplasmic

side of the cell membrane, respectively (fig. 3A). Ser-185 forms hydrogen bonds with the sugar moiety, whereas Gln-82 hydrogen bonds with the hydroxymethyl group on the aromatic ring. Residues, including Leu-186, His-181, Phe-178, His-174, and Leu-177, are involved in hydrophobic interactions with salicin (fig. 3B). Although Asn-172 is located in the TM5 and is far from the binding pocket, it can still possibly affect binding by changing the direction of side chains of Phe-178 and His-174 via a TM conformational transformation (fig. 3C). Calculation of binding energy reflects this possibility, where it is −20.4573 kcal/mol for the wild-type (172N) model and −19.7974 kcal/mol for the 172K mutant. An increase in the amount of binding energy is indicative of weaker binding affinity, and thus the N172K substitution significantly decreases the binding affinity of salicin ($P = 7.9e{-}10$) (fig. 3D). Our modeling prediction suggests that the sensitivity to salicin would be decreased if asparagine is replaced by lysine at site 172 in the saki Tas2R16 protein sequence, which is in accord with our in vitro functional assay results (fig. 2). Collectively, the above results suggest that the lysine/asparagine substitution affects the sensitivity of the Tas2R16 through an impact on binding affinity rather than due to change in the level of cell surface expression in the white-faced saki.

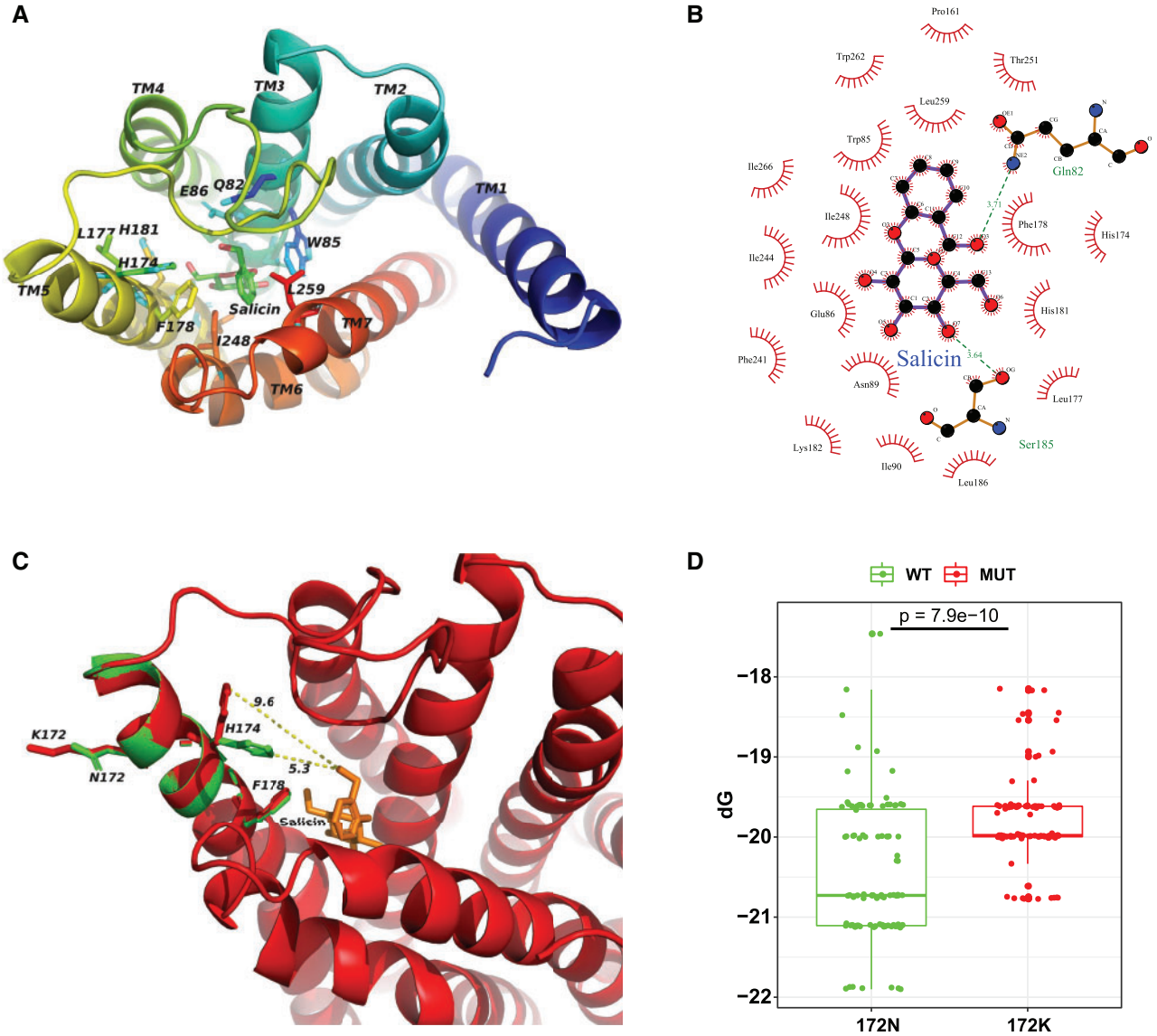

FIG. 3. Binding mode and energy change for salicin to the white-faced saki Tas2R16. (A) Overview of the binding mode for salicin to saki Tas2R16. Secondary structural elements are colored from blue (N-terminus) to red (C-terminus). Potential binding sites are highlighted. Transmembrane domains are labeled as TM1−TM7. (B) Corresponding interaction diagram for the Tas2R16−salicin complex. Residues with green labels are involved in hydrogen bonds. Red radial lines denote hydrophobic interactions, and green lines represent hydrogen bonds. All distances are in Å. (C) Close-up view of the binding sites and mutated region of saki Tas2R16. Wild-type and mutant structures are colored in green and red, respectively. Salicin is colored in orange. The side chains of the residues in the mutant region are shown as sticks. Yellow lines and the numbers represent the minimum distances between salicin and the residues. All distances are in Å. (D) Boxplot showing the binding energy change between the wildtype (WT) and N172K mutant (MUT).

Discussion

Since the identification of the bitter taste receptor (*Tas2R*) genes, their evolution has been of great interest to scientists because Tas2Rs may influence animals' diet choice and even their survival. Many of the researchers focused on *Tas2R* gene family size variation because the number of *Tas2R* genes were commonly regarded to reflect the importance of recognizing bitter toxins for each species, and thus may contribute to species-specific diet preferences. In general, plant tissues contain more potential bitter chemicals than animal tissues do, and consistently herbivores and omnivores tend to have larger *Tas2R* gene family size comparing to carnivores (Shi and Zhang 2006; Dong et al. 2009; Hayakawa et al. 2014; Li and Zhang 2014; Liu et al. 2016). Comparative genomics and evolutionary analyses revealed *Tas2R*s as a highly diverse family in vertebrates, experiencing diverse selective forces and showing high extent in sequence divergence (reviewed in Shi and Zhang 2009; Yang and Shi 2017). A few studies further aimed at a single receptor, mainly Tas2R38, to demonstrate its evolutionary patterns (Wooding et al. 2004, 2006; Wooding 2011; Behrens et al. 2013; Widayati et al. 2019; Purba et al. 2020). Notably, a study with 40 primate species discovered extensive variation in *Tas2R38* gene, showing high level of diversification (Wooding 2011). Interestingly, nontasters to the bitter compound phenylthiocarbamide (a ligand of Tas2R38) were reported in both humans and chimpanzees, however, the shared phenotype was evolved independently in the two species, that is, three nonsynonymous amino acid substitutions in human Tas2R38 result in the nonresponsiveness, whereas a mutation at the initiation codon changing ATG to AGG is responsible for the nontaster status in chimpanzee Tas2R38 (Wooding et al. 2006). In contrast, our findings exhibit a rare case of parallel evolution between human and the white-faced saki *Tas2R16*s, that convergent sensitivity increases were caused by the same amino acid substitution in these two species. To demonstrate this parallel change is adaptive, we integrated evidences from multiple aspects, as Zhang (2006) has suggested. First, by in vitro functional assay, we showed similar sensitivity increase of Tas2R16 in the two independent lineages of human and the saki. Second, parallel amino acid substitution of K172N was identified in these two lineages. Third, using the modified statistical test model, we demonstrated the parallel substitution did not occur by chance. Finally, via mutagenesis, we verified that the parallel substitution of K172N was responsible for the convergent functional change of Tas2R16 in these two lineages. Despite the predominant reports of diversified and divergent evolution of *Tas2R*s, our results revealed a special case of adaptive parallel evolution of primate *Tas2R16*s involving human, which would further extend our knowledge of bitter taste evolution.

In this study, we demonstrated convergent changes in functional sensitivity via parallel amino acid substitutions in human and the saki Tas2R16s. However, the reason why such a convergence occurred is still not clear. Considering the biological properties of Tas2Rs, the most likely selective reason involves dietary preferences in these two species. Previous research has revealed that human TAS2R16 mediates the recognition of β-glucopyranosides (Bufe et al. 2002), a class of ubiquitous plant compounds which includes some toxic cyanogenic glycosides (Drewnowski and Gomez-Carneros 2000). Human *TAS2R16* was shown to have experienced positive selection during an early stage of human evolution. The selected sensitive 172N variant predominated in human populations, and it was speculated to be associated with the increased protection against toxic cyanogenic glycosides, which can release lethal cyanide ions (CN^-) during degradation (Soranzo et al. 2005). Coincidently, the white-faced saki is well-known for its unique diet preference that it primarily feeds on unripe fruit and its seeds (Kinzey 1992; Rosenberger 1992; Kinzey and Norconk 1993; Norconk 2020), whereas most other frugivores eat ripened fruits. This specialized feeding behavior enabled the sakis to occupy a unique ecological niche that provides them with an ample food supply without competition from sympatric frugivores (Kinzey 1992; Palminteri et al. 2012). Meanwhile, as immature fruit and seed specialists, sakis are at higher risk of encountering greater amounts of lethal toxins while foraging, because newly formed plant tissues generally contain highest cyanogenic glycosides, for example, in young leaves, newly formed shoots and young reproductive organs, and the cyanogenic potential decreases as they mature (reviewed in Gleadow and Møller 2014). We therefore speculate that the parallelly evolved Tas2R16 in sakis may help with a sensitive perception of β-glucopyranosides to avoid the toxin-containing plants from their diets during their evolution, and thus resembles the pattern of Tas2R16 evolution seen in humans (Soranzo et al. 2005). Undoubtedly, the hypothesis requires further evidences, especially the precise cyanogenic toxicity measurement in diet. As more detailed studies are conducted and more data accumulate in the future, we expect to build better knowledge on this issue.

In conclusion, our study revealed an uncommon case of convergent evolution of bitter taste receptors between human and a New World monkey. Combining various types of analyses, at multiple levels, we identified a critical parallel amino acid substitution (K172N) that is responsible for this functional convergence. The results extended our knowledge of bitter taste receptor evolution and may shed light on the comprehensive understanding of molecular adaptation to specific ecological niches by animals.

Materials and Methods

Primate and other mammalian *Tas2R16* gene sequences were retrieved from previous studies (Fischer et al. 2005; Hayakawa et al. 2014; Meyer et al. 2015; Liu et al. 2016; Itoigawa et al. 2019) and available genome databases at Ensembl (http://www.ensembl.org, last accessed September 05, 2021) and HGSC (http://www.hgsc.bcm.edu/, last accessed September 05, 2021). Sequences from an additional 15 species were amplified from genomic DNA using a pair of degenerate primers (5′-TCCCTAYATTCVGATGTGATAGC-3′ and 5′-TCTTKTAYCCTGTGCCTCAGG-3′) (supplementary table S1, Supplementary Material online). Evolutionary analyses and ancestor reconstruction were performed using the ML

method implemented in PAML4.7 (Yang 2007). Extant sequences were then compared with the ancestors to identify convergent/parallel states. The probability that the observed convergent and parallel changes occurred by random chance was measured using the statistical test proposed by Zhang and Kumar (1997) and a recently updated statistical model (Zou and Zhang 2015).

HSV-tagged pcDNA5/FRT expression vectors containing primate *Tas2R16* genes were constructed and point mutation was introduced using the QuikChange Site-Directed Mutagenesis Kit. Constructs were transiently transfected into HEK293 cells that stably express the chimeric G protein subunit $G\alpha16\text{-gust}_{44}$ (Ueda et al. 2003). Bitter-stimulated calcium mobilization was recorded with FlexStation 3 (Molecular Devices). Concentration−response curves and EC_{50} values were calculated by the nonlinear regression equation for Sigmoidal dose−response (variable slope). Cell surface expression was measured by TissueFaxs Plus cytometer and analyzed using StrataQuest 6.0 (TissueGnostics, Austria). Data were obtained from 2,500 cells for each *Tas2R16* construct.

The structure of white-faced saki Tas2R16 was modeled using the I-TASSER server (Zhang 2008; Roy et al. 2010). Salicin structure (CID: 439503) was downloaded from PubChem (Li et al. 2010) and docked into the predicted structure of Tas2R16 using Autodock Vina v1.1.2 (Trott and Olson 2010), which is widely used and has shown very good accuracy for GPCR docking (Launay et al. 2012; Sandal et al. 2013; Labbe et al. 2015). The effect of the N172K mutation on ligand-binding energy was predicted using the Cartesian_ddg application (Park et al. 2016; Leman et al. 2020) from Rosetta version 2020.28.61328. The Cartesian_ddg protocol has high accuracy with a correlation coefficient of 0.88 between the predicted and experimental values according to a previous study (Strokach et al. 2019). A total of 100 independent runs were performed for the wild-type and mutant Tas2R16−salicin complex structures. The 3D structure of Tas2R16−salicin complex was visualized in Pymol (Lill and Danielson 2011). Diagrams of the protein–ligand interactions were generated by using LigPlot+ (Laskowski and Swindells 2011).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

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Data Availability

The newly reported *Tas2R16* sequences are available in the GenBank Nucleotide Database at https://www.ncbi.nlm.nih.gov/nucleotide/ (last accessed September 05, 2021), and can be accessed with accession numbers MZ945495−MZ945509.

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