Human Molecular Genetics, 2004, Vol. 13, No. 21 **2671–2678**
doi:10.1093/hmg/ddh289
Advance Access published on September 14, 2004

Relaxation of selective constraint and loss of function in the evolution of human bitter taste receptor genes

Xiaoxia Wang, Stephanie D. Thomas and Jianzhi Zhang*

Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, USA

Received July 30, 2004; Revised and Accepted September 2, 2004 DDBJ/EMBL/GenBank accession nos AY736042–AY736066

Bitter taste perception prevents mammals from ingesting poisonous substances because many toxins taste bitter and cause aversion. We hypothesize that human bitter taste receptor (*TAS2R*) genes might be relaxed from selective constraints because of the change in diet, use of fire and reliance on other means of toxin avoidance that emerged in human evolution. Here, we examine the intra-specific variations of all 25 genes of the human *TAS2R* repertoire. Our data show hallmarks of neutral evolution, including similar rates of synonymous (d_S) and non-synonymous (d_N) nucleotide changes among rare polymorphisms, common polymorphisms and substitutions; no variation in d_N/d_S among functional domains; segregation of pseudogene alleles within species and fixation of loss-of-function mutations. These results, together with previous findings of large numbers of loss-of-function mutations in olfactory, pheromonal and visual sensory genes in humans, suggest surprisingly reduced sensory capabilities of humans in comparison with many other mammals.

INTRODUCTION

Humans and most mammals can perceive and discriminate among five major taste modalities: sweet, sour, bitter, salty and umami (the taste of sodium glutamate) (1,2). Among these, bitter perception has a special role of preventing animals from ingesting poisonous substances, because many toxins taste bitter and cause aversion (3,4). The sense of the bitter taste begins by binding of bitter compounds to bitter taste receptors that are found on the surface of the taste receptor cells of the tongue and palate epithelium (2,5). The receptors are encoded by a large family of seven-transmembrane G protein-coupled receptor genes named *TAS2R*s (or *T2R*s), and the roles of *TAS2R*s in bitter taste have been demonstrated by both *in vitro* and *in vivo* functional assays (5–9). Unlike visual pigment genes or olfactory receptor genes for which only one gene is expressed in each visual or olfactory receptor cell, multiple *TAS2R* genes are found to be expressed in each bitter taste receptor cell in rodents, providing a plausible explanation for the uniform taste of many structurally distinct toxins (5). *TAS2R* genes do not contain introns in their protein-coding regions, which facilitated their detection in sequenced genomes. Twenty-five putatively functional *TAS2R* genes [with open reading frames (ORFs)] and eight pseudogenes (with disrupted ORFs) have been identified from the human genome sequence and they are mapped to chromosomes 5, 7 and 12 (10). In comparison, the mouse genome contains 33 functional *Tas2r* genes and three pseudogenes (10). A phylogenetic analysis of these genes suggested considerable variation in the *TAS2R* repertoire among different mammalian lineages (10). Although only a minority of these *TAS2R* genes has been functionally characterized, their chromosomal location, sequence similarity and phylogenetic relationships strongly suggest that they are involved in bitter perception.

Because TAS2Rs are directly involved in the interaction between mammals and their dietary sources, it is likely that the evolution of these proteins responds to and reflects dietary changes in organismal evolution. For example, there were significant changes in diet during human evolution (11–13). In particular, the amount of meat in hominid diet began to increase about 2 million years (MY) ago, whereas the amount of plant materials decreased (11–13). This dietary shift may have caused a reduction in the importance of bitter taste and *TAS2R* genes because animal tissues contain fewer bitter and toxic compounds than plant tissues (4). Detoxification of poisonous foods, starting ~0.8 MY

*To whom correspondence should be addressed at: Department of Ecology and Evolutionary Biology, University of Michigan, 3003 Natural Science Building, 830 North University Avenue, Ann Arbor, MI 48109, USA. Tel: +1 7347630527; Fax: +1 7347630544; Email: jianzhi@umich.edu

Table 1. Intra- and inter-specific variations of human *TAS2R* genes

Gene names	Chr.[a]	L[b]	Synonymous changes					Non-synonymous changes					Total					
			S[c]	π (%)[d]	θ (%)[e]	D[f]	d[g]	S	π (%)	θ (%)	D	d	S	π (%)	θ (%)	D	d	d_N/d_S[h]
TAS2R1	5	825	1	0.04	0.10	−0.85	2	2	0.05	0.08	−0.56	4	3	0.05	0.08	−0.87	6	0.82
TAS2R3	7	864	2	0.22	0.18	0.37	2	0	0.00	0.00	0.00	1	2	0.06	0.05	0.37	3	0.21
TAS2R4	7	810	0	0.00	0.00	0.00	1	2	0.11	0.08	0.59	1	2	0.07	0.06	0.59	2	0.43
TAS2R5	7	801	1	0.04	0.10	−0.85	3	3	0.11	0.12	−0.15	7	4	0.09	0.11	−0.48	10	0.96
TAS2R7	12	876	0	0.00	0.00	0.00	2	0	0.00	0.00	0.00	4	0	0.00	0.00	0.00	6	0.82
TAS2R8	12	831	2	0.22	0.20	0.24	0	1	0.01	0.04	−1.12	2	3	0.07	0.08	−0.39	2	NA
TAS2R9	12	867	0	0.00	0.00	0.00	3	1	0.08	0.04	1.54	4	1	0.06	0.03	1.54	7	0.54
TAS2R10	12	849	2	0.12	0.20	−0.69	0	1	0.04	0.04	0.27	5	3	0.07	0.08	−0.40	5	NA
TAS2R13	12	837	0	0.00	0.00	0.00	2	2	0.09	0.08	0.36	11	2	0.07	0.05	0.36	13	2.14
TAS2R14	12	879	2	0.33	0.18	1.47	3	1	0.03	0.04	−0.53	4	3	0.11	0.08	0.57	7	0.53
TAS2R16	7	807	2	0.11	0.20	−0.83	2	3	0.12	0.12	−0.04	1	5	0.12	0.14	0.48	3	0.20
TAS2R38	7	945	0	0.00	0.00	0.00	3	4	0.21	0.14	1.16	2	4	1.15	0.10	1.16	5	0.28
TAS2R39	7	957	1	0.02	0.08	−1.12	1	3	0.03	0.10	−1.57	2	4	0.02	0.10	−1.76	3	0.79
TAS2R40	7	918	0	0.00	0.00	0.0	1	1	0.03	0.04	−0.14	2	1	0.02	0.03	−0.14	3	0.80
TAS2R41	7	870	3	0.14	0.26	−1.00	1	1	0.04	0.04	0.27	5	4	0.07	0.11	−0.71	6	2.14
TAS2R43	12	870	2	0.00	0.19	−1.30	1	7	0.21	0.26	−0.47	2	9	0.17	0.24	−0.84	3	0.78
TAS2R44	12	888	3	0.23	0.27	−0.28	5	7	0.27	0.26	0.18	8	10	0.26	0.26	0.02	13	0.66
TAS2R45	12	828	1	0.02	0.10	−1.12	2	6	0.45	0.23	2.39*	5	7	0.32	0.19	1.82	7	1.01
TAS2R46	12	804	1	0.04	0.10	−0.85	3	2	0.12	0.80	0.05	6	3	0.10	0.11	−0.40	9	0.78
TAS2R47	12	867	2	0.18	0.18	−0.03	0	2	0.08	0.07	0.12	0	4	0.11	0.11	0.05	0	NA
TAS2R48	12	753	2	0.45	0.20	2.19*	0	6	0.10	0.26	−1.62	6	8	0.20	0.24	−0.47	6	NA
TAS2R49	12	855	3	0.38	0.28	0.75	0	9	0.50	0.34	1.37	2	12	0.47	0.32	1.35	2	NA
TAS2R50	12	831	2	0.29	0.19	0.97	1	3	0.09	0.12	−0.53	5	5	0.15	0.14	0.15	6	2.01
hT2R55	12	885	1	0.10	0.09	1.60	3	4	0.24	0.15	1.57	7	5	0.20	0.13	1.91	10	0.95
TAS2R60	7	891	0	0.00	0.00	0.00	1	1	0.01	0.04	−0.85	8	1	0.01	0.03	−0.85	9	3.34
Sum		21 408	33	0.12	0.12	−0.05	42	72	0.12	0.11	0.09	104	105	0.12	0.11	−0.05	146	1.00

NA, not applicable because of zero synonymous differences.
[a]Chromosomal location.
[b]Number of sequenced nucleotides.
[c]Number of polymorphic sites.
[d]Nucleotide diversity per site.
[e]Watterson's θ per site.
[f]Tajima's D.
[g]Number of fixed nucleotide differences between human and chimpanzee.
[h]Number of fixed non-synonymous differences per non-synonymous site between human and chimpanzee, divided by the number of fixed synonymous differences per synonymous site.
*$P < 0.05$.

ago (14), by controlled use of fire may trigger a further functional relaxation in *TAS2R*s. We thus hypothesize that patterns of intra-specific polymorphism and recent evolution of human *TAS2R* genes should exhibit low selective constraints. Here, we characterize the intra- and inter-specific variations of all 25 members of the human *TAS2R* repertoire and provide strong evidence supporting this hypothesis.

RESULTS

Equal levels of synonymous and non-synonymous polymorphisms in human *TAS2R* genes

To examine the genetic variation of *TAS2R*s within humans, we sequenced all 25 functional genes in 22 humans of diverse geographic origins and one chimpanzee. Because synonymous nucleotide changes do not alter protein sequences and are more or less neutral whereas non-synonymous changes could be subject to natural selection, a comparison between them can reveal signals of selection (15,16). In most genes, the majority of non-synonymous sites are under selective constraints, resulting in lower rates of polymorphisms and substitutions at non-synonymous sites than at synonymous sites (15,16). In human *TAS2R*s, we identified 72 non-synonymous and 33 synonymous polymorphic sites from 21 408 nucleotide sites, including 15 242 non-synonymous and 6166 synonymous sites, respectively (Tables 1 and 2; Supplementary Material, Fig. S1). The number of polymorphisms per non-synonymous site (4.72×10^{-3}, Watterson's $\theta = 1.09 \times 10^{-3}$) is 88% of that per synonymous site (5.35×10^{-3}, $\theta = 1.23 \times 10^{-3}$), indicating that the overall

Table 2. Rates of synonymous and non-synonymous nucleotide changes in human *TAS2R*s

	Non-synonymous	Synonymous	*N*/*S* ratio
Number of sites sequenced	15 242	6166	2.47
Rare polymorphisms	33	15	2.20
Common polymorphisms	39	18	2.17
Fixed changes	104	42	2.48

None of the N/S ratio are significantly different from each other (Fisher's test).

Table 3. Comparison between *TAS2R* genes and neutrally evolving human sequences in intra- and inter-specific variations

Genomic regions (references)	Sequence length (nt)	π (%)[a]	θ (%)[b]	d (%)[c]	θ/d	HKA prob.[d]
25 *TAS2R* genes (this study)	21 408	0.121	0.115	0.831	0.138	
Non-coding region at 1q24 (24)	8991	0.058	0.095	0.623	0.152	0.669
β-Globin inititation region at 11p 15 (25)	6076	0.129	0.107	1.284	0.083	0.508
Non-coding region at 22q 11 (26)	9091	0.088	0.139	1.353	0.103	0.312
Dystrophin intron-dys44 at Xp21 (27)	7475	0.135	0.102	0.604	0.169	0.779
*PDHA*1 introns at Xp22 (28)	3530	0.225	0.211	0.992	0.213	0.648
Non-coding region at Xq13.3 (29)	10 200	0.045	0.083	0.922	0.090	0.369

[a]Nucleotide diversity per site; for X chromosome data, it is corrected by multiplication by 4/3.
[b]Watterson's estimate of polymorphism per site; for X chromosome, it is corrected by multiplication by 4/3.
[c]Number of nucleotide differences per site between human and chimpanzee sequences.
[d]Probability from the HKA test (30), with comparison to the *TAS2R* data.

selective constraints on the two types of sites are similar ($P = 0.31$; Fisher's exact test) in *TAS2R* genes. Consistent with this observation, the nucleotide diversity per site (15,16) is virtually identical between non-synonymous ($\pi_N = 1.22 \times 10^{-3}$) and synonymous ($\pi_S = 1.19 \times 10^{-3}$) sites (Table 1). Furthermore, although the *TAS2R* π_S is close to the average synonymous nucleotide diversity observed from a large number of human genes (1.1×10^{-3}) (17), π_N is about four times the corresponding average (0.28×10^{-3}) (17). These comparisons suggest that the similar magnitudes of π_N and π_S at *TAS2R*s are likely due to reduced selective constraints on non-synonymous sites.

Alternatively, high π_N may result from balancing selection, which retains beneficial alleles in a population for a long time (18). Balancing selection has been suggested to account for high π_N of the *TAS2R38* (also called *PTC*) gene (19), which is largely responsible for the well-known human polymorphism in tasting the synthetic compound phenylthiocarbamide (PTC) (20). A characteristic of balancing selection is the positiveness of $\pi - \theta$, which can be measured by Tajima's D statistic (21). For the present data, D is positive in 12 genes, 0 in one gene and negative in 12 genes, with an overall value of -0.048 for all the genes analyzed together (Table 1). D is not significantly different from 0 for the 25 genes, individually or collectively. When the synonymous and non-synonymous sites were separated, one gene (*TAS2R48*) had a significantly positive D at synonymous sites ($P < 0.05$) and another (*TAS2R45*) had a significantly positive D at non-synonymous sites ($P < 0.05$). But the two significant cases may simply be due to multiple testing as 50 tests were conducted and 2.5 significant cases (at 5%) were expected by chance. These observations do not support the balancing selection hypothesis for the human *TAS2R* repertoire, although failure to detect balancing selection here does not preclude its operation at a few *TAS2R* genes. Similar results were obtained by Fu and Li's tests (22) (data not shown). Wooding *et al.* (19) suggested the action of balancing selection on *TAS2R38* largely because of the observation of a positive Tajima's D (1.55). This D value was not significantly higher than 0 under the assumption that the population size is constant, but it became significantly higher than 0 under specific models of population expansion (19). They suggested that an instant population expansion from the effective size of 10 000 to 1 000 000 that occurred 100 000 years ago is most appropriate for humans (19,23, see also the manual of the DFSC program of S. Wooding at http://www.xmission.com/~wooding/DFSC/README). We evaluated this model by computing D for six apparently neutral loci (Table 3) of the human genome under this model. Surprisingly, five of the six loci (except the locus at Xq13.3) show significantly positive D values. Thus, we believe that use of the above population expansion model leads to false positive detection of balancing selection. Although this does not mean that use of the constant-population model is correct, it is certainly a more conservative and safer test for balancing selection, particularly when the details of human population expansion is still unclear.

Possible natural selection on human *TAS2R*s can be further scrutinized by comparing rare and common polymorphisms (31). We used the chimpanzee as the outgroup of humans to determine which human alleles are derived and which are ancestral, and then classified each polymorphism to either rare or common using 10% frequency for the derived allele as the cutoff (Table 2). We found that the ratio of the number of non-synonymous polymorphisms to that of synonymous polymorphisms was similar between rare ($33/15 = 2.20$) and common ($39/18 = 2.17$) categories ($P = 0.57$). This is consistent with the lack of purifying selection on *TAS2R*s, because purifying selection would prevent deleterious non-synonymous mutations from becoming common, and thus generate a lower non-synonymous/synonymous ratio for common polymorphisms than for rare ones (31).

Pseudogenization of human *TAS2R*s

In addition to the many non-synonymous polymorphisms, two nonsense polymorphisms were observed in human *TAS2R*s. The first was a C → T mutation at position 640 of *TAS2R7*, which changed an Arg residue to a premature stop codon and resulted in a receptor that contains only five transmembrane (TM) domains (Supplementary Material, Fig. S1). This apparently non-functional allele was observed once (in Andes Indian) in the 22 individuals surveyed. The relatively low frequency (2.3%) of the allele makes it difficult to distinguish whether it is neutral (but newly emergent) or deleterious. The second nonsense polymorphism (G → A at position 749) changed a Trp to a premature stop codon in *TAS2R46*, resulting in a truncated receptor with six TM domains (Supplementary Material, Fig. S1). This non-functional

allele was observed 11 times in our samples. The moderate frequency (0.25) of this allele and its presence in various human populations (African Americans, Caucasians, Southeast Asians, Chinese and Pacific Islanders) demonstrate that it is not under purifying selection. A comparison between the human and chimpanzee genome sequences indicates that chimpanzees have six *TAS2R* pseudogenes, whereas humans have two additional ones (*hps1* and *hps2*) (10), both having been fixed, as revealed by our sequencing of 22 humans (Supplementary Material, Figs S2 and S3). We also confirmed that the chimpanzee orthologs of *hps1* and *hps2* have intact ORFs by sequencing one individual. Therefore, in the 6–7 MY since the human–chimpanzee split (32), two functional *TAS2R* genes have turned into pseudogenes in the hominid lineage and another one or two are becoming pseudogenes in present-day humans. In contrast, no new *TAS2R* pseudogenes have been fixed in the chimpanzee lineage.

There are three residues that are absolutely conserved among all 63 mouse and rat Tas2rs (see later), suggesting that these residues are required for proper functioning of bitter taste receptors. However, two of them have changed in human TAS2Rs. Position 103 of human TAS2R13 is fixed with Phe, whereas this position is Leu in all mouse and rat Tas2rs. Similarly, human TAS2R14 is fixed with Met at position 205, whereas it is Leu in rodents. An *in vitro* study showed that human TAS2R14 responds to multiple bitter compounds (9). It will be interesting to examine if human TAS2R13 is functional.

Equal rates of synonymous and non-synonymous substitutions

When did the functional relaxation in human *TAS2R*s occur? This question may be addressed by comparing polymorphism and divergence data. A comparison between orthologous *TAS2R* genes from the 22 humans and one chimpanzee identified 104 non-synonymous and 42 synonymous substitutions that have been fixed between species (Tables 1 and 2). These numbers translate into identical rates of non-synonymous (6.82×10^{-3}) and synonymous (6.82×10^{-3}) substitutions per site ($P = 0.52$). When we measured the human–chimpanzee distance by comparing a randomly picked human allele with a randomly picked chimpanzee allele, as normally conducted, the synonymous and non-synonymous distances both became 8.3×10^{-3} per site. These numbers are significantly ($P < 0.05$) lower than the average human–chimpanzee distance (0.012 per site) observed from large genomic data (33,34) and may reflect mutation rate variation within genome (35). The McDonald–Kreitman test (36) did not detect any difference between the intra- and inter-specific patterns of non-synonymous and synonymous variations for human *TAS2R*s ($P = 0.38$; Table 2). From the single chimpanzee sequenced, three synonymous and eight non-synonymous polymorphisms were found. The non-synonymous/synonymous ratio ($8/3 = 2.67$) is comparable with the ratio for human polymorphisms ($72/33 = 2.18$) and the ratio for fixed substitutions between species ($104/42 = 2.48$). We also conducted an HKA test (30) by comparing the polymorphism and divergence data from human *TAS2R*s and those from six non-coding regions of the human genome that are at least 3000 nt long and are not known to be under natural selection, but no difference was found (Table 3). These results suggest that the functional relaxation already started in the common ancestor of humans and chimpanzees and that there is no detectable difference in selection between the human polymorphism and divergence data.

An alternative explanation for the above between-species data is a more complex scenario involving purifying selection at some sites and positive selection at some other sites of *TAS2R*s. Previous studies showed that transmembrane (TM) domains of TAS2Rs are more conserved than extracellular (EC) and intracellular (IC) domains and that EC domains may be subject to positive selection between rapidly diversifying paralogous genes, consistent with the presumable tastant-TAS2R binding sites being located in the EC domains (10). To test the hypothesis of positive and purifying selection on human and chimpanzee *TAS2R*s, we estimated the d_N/d_S ratio in the EC, TM and IC domains, respectively. For comparison, we used *Tas2r* genes from mouse and rat. To obtain these genes, we searched the rat genome sequence using all 33 mouse *Tas2r* genes as queries and identified 30 rat *Tas2r* genes. A phylogenetic tree of all putatively functional *TAS2R* genes from human, chimpanzee, mouse and rat was reconstructed (Fig. 1; Supplementary Material, Fig. S4). From this tree, 28 pairs of mouse and rat genes were apparently orthologous. We concatenated these 28 genes for mouse and rat, respectively, and estimated the average d_N and d_S between them. Figure 2 shows that the d_N/d_S ratio is significantly lower than 1 for each of the three functional regions (EC, TM and IC) in rodent *Tas2r* genes, demonstrating the operation of purifying selection between the orthologs. Among-domain variation in d_N/d_S, another characteristic of functional genes, is also evident, and TM domains show the lowest d_N/d_S. For both intra- and inter-specific data of human *TAS2R*s, d_N/d_S is not significantly different from 1 for any of the three functional regions or the entire genes. Furthermore, no variation in d_N/d_S among functional regions is seen. Compared with rodent *Tas2r*s, human *TAS2R*s have significantly elevated d_N/d_S in the TM domains. These observations are consistent with the hypothesis of functional relaxation in humans (and chimpanzees), but do not support the alternative hypothesis of a combination of positive and purifying selection, because the latter predicts an elevation in d_N/d_S in the EC domains whereas maintaining low d_N/d_S in functionally conserved TM domains.

Similarly, one may hypothesize that some *TAS2R*s of humans are under positive selection whereas some others are under purifying selection, giving an average d_N/d_S of ~ 1 for the repertoire. Table 1 lists the d_N/d_S ratio for each of the 25 human *TAS2R* genes and some ratios appear much higher than others, although none of them are significantly different from 1. We used computer simulation to test whether this among-gene variation was simply due to chance. In the simulation, we assumed that every gene has the expected d_N and d_S identical to the corresponding averages of the 25 human *TAS2R* genes and then generated an expected distribution of d_N/d_S from 5000 simulations (Fig. 3). When this distribution was compared with the observed distribution, no significant difference was found ($P = 0.65$, χ^2 test; Fig. 3), suggesting that the apparent among-gene variation in d_N/d_S shown in Table 1 is explainable by chance alone.

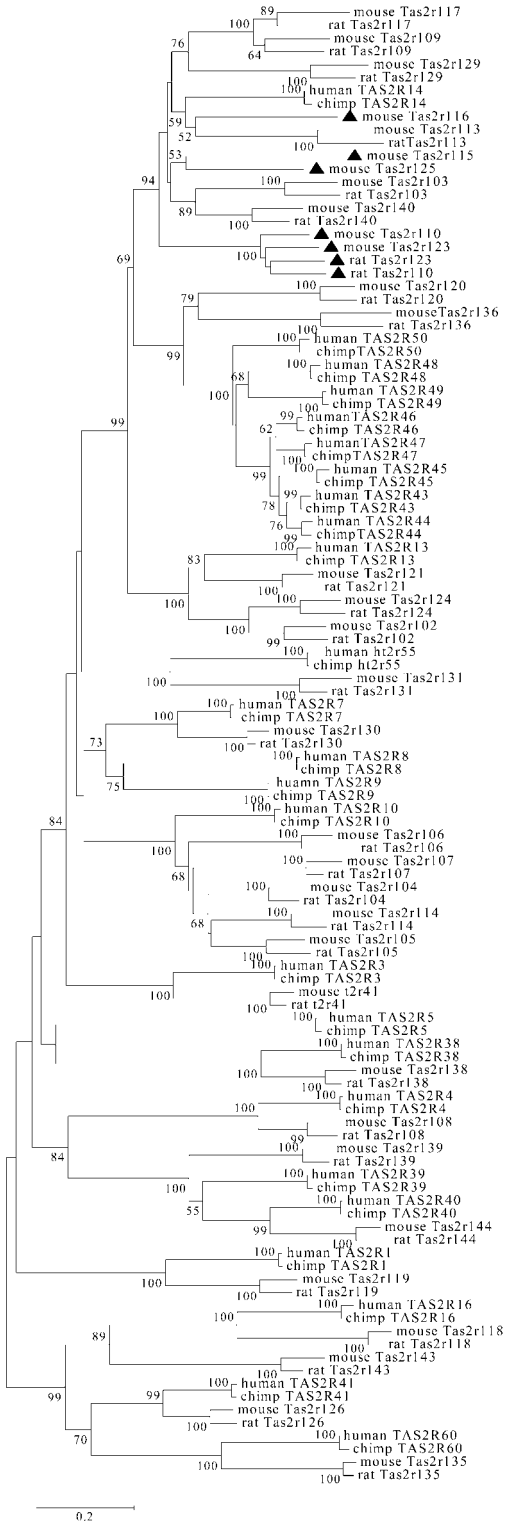

Figure 1. Evolutionary relationships of 113 putatively functional *TAS2R* genes from human, chimpanzee, mouse and rat. The tree is reconstructed by the neighbor-joining method with protein Poisson distances. Bootstrap percentages ($\geq$50) from 2000 replications are shown at interior nodes. The mouse and rat genes with uncertain orthology are marked with triangles and are not used in computing the d_N/d_S ratios presented in Figure 2. The protein sequence alignment used for the tree reconstruction is provided in Supplementary Material, Figure S4.

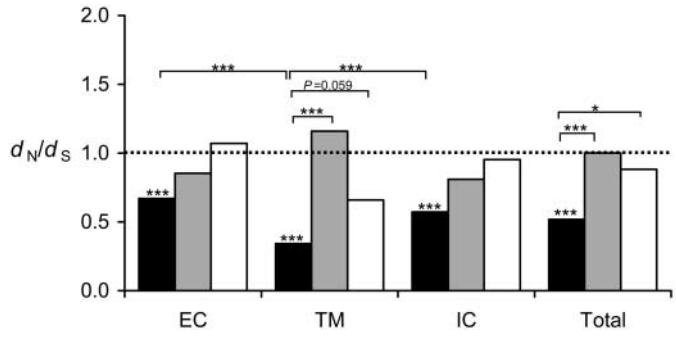

Figure 2. Comparison of d_N/d_S among different functional domains of TAS2Rs. Black bars show the d_N/d_S ratios computed from the concatenated sequences of 28 pairs of orthologous *Tas2r* genes of mouse and rat. Shaded bars show the d_N/d_S ratios of the fixed differences between human and chimpanzee for the concatenated sequences of 25 pairs of orthologous *TAS2R* genes. White bars show the d_N/d_S ratios computed from the human polymorphisms at the 25 *TAS2R* genes. All comparisons are conducted using Fisher's exact test following Zhang *et al.* (37). Significant levels: *, 5%; **, 0.5%; ***, 0.05%. The test result of the null hypothesis of $d_N/d_S = 1$ for each bar is indicated above the bar. Comparisons between two bars are indicated with brackets. Non-significant results are not indicated. EC, four extracellular domains; TM, seven transmembrane domains; IC, four intracellular domains; Total, entire proteins.

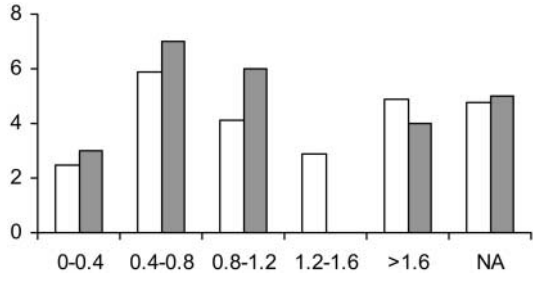

Figure 3. Expected and observed distributions of d_N/d_S among 25 human *TAS2R* genes. The expected distribution (white bars) under equal d_N/d_S ratios among genes was generated by computer simulation (see Materials and Methods), whereas the observed distribution (black bars) was from the d_N/d_S column in Table 1. NA, not applicable due to zero synonymous substitution. There is no significant difference between the two distributions ($P > 0.5$; χ^2 test).

DISCUSSION

In this work, we characterized the intra- and inter-specific variations of the entire human bitter taste receptor gene repertoire. Our intra-specific data show equal levels of synonymous and non-synonymous polymorphisms, equal non-synonymous/synonymous ratios for rare and common polymorphisms, equal non-synonymous/synonymous ratios among functional domains, segregation of non-functional alleles in populations and fixation of pseudogenes in the species. All these observations support the lack of selective constraint on human *TAS2R* genes. Our inter-specific comparison between the human and chimpanzee also suggests that *TAS2R* genes have been under neutral evolution without much constraint. If a complete functional relaxation occurred in the common ancestor of humans and chimpanzees, it is perplexing why fixations of mutations that disrupt *TAS2R* ORFs occurred only twice in humans and did not occur at all in chimpanzees. Using computer simulation (see Materials and Methods), we estimated

that the average half-life of human *TAS2R* genes is 6.77 MY in the absence of natural selection, meaning that after 6.77 MY of neutral evolution, a *TAS2R* gene has a 50% chance of becoming a fixed pseudogene. From the observation that only two out of 27 *TAS2R* genes have become fixed pseudogenes during hominid evolution, we obtained the maximum likelihood estimate of the starting time (T) of the complete functional relaxation to be 0.75 MY ago, though any T within 0.1–2.6 MY ago cannot be rejected at 5% significance level. It is likely that an incomplete functional relaxation started early in the ancestry of humans and chimpanzees but a second wave of more complete relaxation occurred recently in the hominid lineage alone. This would explain the lack of pseudogene formation in chimpanzees and the paucity of human pseudogenizations. Because each *TAS2R* recognizes multiple related toxic compounds (8,9) and each taste receptor cell expresses multiple *TAS2R* genes (5), it is possible that an incomplete functional relaxation occurs when the number or amount of toxins that a species encounter reduces.

As mentioned, a dietary change that started 2 MY ago (11–13) resulted in an increase of animal tissues and decrease of plant tissues in hominid diet, which reduced the number of toxic foods that hominids came across. Controlled use of fire was evident about 0.8 MY ago (14); the reliance on TAS2Rs to detect toxins further diminished because cooking significantly detoxifies poisonous food (11). It is interesting that the likelihood estimate of the starting time (0.75 MY ago) of the complete functional relaxation in human *TAS2R* genes coincides with the beginning of the fire use in hominid evolution, although our estimate has a large variance. Our data suggest that chimpanzee *TAS2R*s are likely under relaxed selective constraint as well, although to a lesser degree, as evident from the lack of pseudogenization. It has been noted that chimpanzees occasionally eat meat (2–13% of diet), whereas other great apes (gorillas and orangutans) almost never do so (13). Furthermore, among the plant foods, chimpanzees eat mostly ripe fruits whereas gorillas and orangutans eat more leaves, unripe fruits, bark and pith (13). Ripe fruits contain considerably fewer toxins than do leaves and unripe fruits (4). These factors, when combined, may have reduced the selective pressures on chimpanzee *TAS2R*s. A broader survey of *TAS2R* genes in primates may provide a better understanding of the ecology and selective agents behind the evolution of *TAS2R* genes and the bitter taste.

Our finding that the TAS2R bitter taste receptor gene repertoire of humans is under relaxed selective constraint has several implications. First, humans have lost and continue to lose *TAS2R* genes, which would result in a decrease in the number of bitter compounds that we can taste. Second, the segregation of non-functional *TAS2R* alleles in current human populations indicates among-individual variation in bitter sensitivity. Third, functional relaxation may also allow the appearance of new *TAS2R* alleles that can bind previously unrecognizable tastants. Although the emergence of these new alleles may increase the diversity in bitter recognition among humans, it likely has little effect on fitness because of the neutral nature of the new alleles. It should be noted that loss of selective constraint does not result in loss of function instantly. Probably because of the relatively short time since the loss of selective constraints on human *TAS2R* genes, many human *TAS2R* genes may still be functional and we can still taste many bitter compounds. But loss of function will result after sufficient time of evolution under no selective constraint.

Our finding is intriguing when compared with the evolutionary patterns of other human sensory genes. Olfactory receptor genes have been inactivated in the human lineage at a higher rate than in chimpanzees and other primates. In humans, >50% of olfactory receptor genes are pseudogenes, in contrast to 30–35% in other apes and <20% in rodents (38,39). In fact, humans have fewer than 400 functional olfactory receptor genes (40) and they appear to be under weak or no selective constraints (41), whereas mice have over 1000 functional genes (42). Humans, apes and Old World monkeys have lost important components of the vomeronasal pheromone signal transduction pathway and are insensitive to vomeronasal pheromones (43,44). Humans also have an unusually high frequency of red/green color blindness (e.g. 8% in male Caucasians) that is not found in wild apes or Old World monkeys (45). Deafness also occurs at a relatively high frequency (0.08%) in humans; non-functional GJB2 alleles, which are responsible for genetic deafness in many populations, have a total frequency of ~1.8% in the United States (46). Although genes involved in these sensory systems may have deteriorated at different times in the evolutionary lineage of humans, it is compelling that sensitivities to a number of sensory signals are weaker in humans than in many of our mammalian relatives. It is possible that in the evolution of apes, and particularly humans, sensory capabilities became a less important component of an individual's fitness.

MATERIALS AND METHODS

Sequencing human and chimpanzee *TAS2R* genes

Genomic DNAs of one chimpanzee (*Pan troglodytes*) and 22 unrelated humans (*Homo sapiens*) of diverse geographic origins (three Pygmy Africans, six African Americans, five Caucasians, three Southeast Asians, two Chinese, two Pacific Islanders and one Andes Indian) were purchased from Coriell Cell Repositories. Gene-specific primers for amplifying the 25 *TAS2R* genes were designed according to the human genome sequence. The protein-coding region of each *TAS2R* gene has 900–1000 nt, most of which were amplified in our experiments. After removing the primer-encoded regions, the total number of nucleotides examined here was 21 408, or on an average 856 per gene. Polymerase chain reactions (PCRs) were performed with high fidelity DNA polymerase under conditions recommended by the manufacturer (Invitrogen). PCR products were separated on 1.5% agarose gel and purified using the Gel Extraction Kit (Qiagen), before being sequenced from both directions using the dideoxy chain termination method with an automated DNA sequencer. Sequencher (GeneCodes) was used to assemble the sequences and to identify DNA polymorphisms. Two human pseudogenes (*hps1* and *hps2*) were also sequenced in the 22 humans and one chimpanzee. The primer sequences are available upon request.

Evolutionary analyses

Numbers of synonymous and non-synonymous sites and numbers of synonymous and non-synonymous nucleotide changes were counted following Zhang *et al.* (47). The number of synonymous changes per synonymous site (d_S) and the number of non-synonymous changes per non-synonymous sites (d_N) were then computed (16). Watterson's θ, nucleotide diversity π, Tajima's *D* test (21) and HKA test (30) were computed or conducted by DnaSP (48). Rare and common polymorphisms were defined using the cutoff of 10% frequency for the derived allele. Use of different cutoffs (5 and 15%) did not change our result. The sequence data of six non-coding regions used in the HKA test were from the literature (24–29). Mouse *Tas2r* gene sequences were obtained from the literature (10). Rat *Tas2r* genes were identified by BLAST searches of the rat genome sequence using the mouse genes as queries. A neighbor-joining tree (49) of the putatively functional *TAS2R* genes of the human, chimpanzee, mouse and rat was reconstructed using the protein Poisson distance (16). The bootstrap test (with 2000 replications) was used to examine the reliability of the observed branching patterns (50). MEGA2 (51) was used for the phylogenetic analysis. Twenty-eight pairs of orthologous *Tas2r* genes from mouse and rat were identified and used for computing d_S and d_N. Functional domains in TAS2Rs were defined following Adler *et al.* (7). Computer simulation was used to evaluate the expected variation in d_N/d_S among *TAS2R* genes. Because the total numbers of fixed synonymous and non-synonymous differences in the 25 orthologous genes of humans and chimpanzees were 42 and 104, respectively (Table 1), the average numbers per gene were 1.68 and 4.16, respectively. As nucleotide substitution is a Poisson process, we generated a Poisson random number with the mean of 1.68 as the number of synonymous substitution for a gene and an independent Poisson random number with the mean of 4.16 as the number of non-synonymous substitution for the gene. The d_N/d_S ratio for the gene was then computed following Zhang *et al.* (47), using the average numbers of synonymous (246.64) and non-synonymous (609.68) sites per gene for the *TAS2R* genes. When the number of synonymous substitution was 0, d_N/d_S was designated as NA. Such simulation was repeated 5000 times and a distribution of d_N/d_S was generated. This distribution was compared with the observed distribution from the 25 *TAS2R*s by a χ^2 test. We used the program PSEUDOGENE (43) to estimate the half-life ($t_{1/2}$) of each of the human *TAS2R* genes and obtained an average of 6.77 ± 0.05 MY. In the computation, we used a point nucleotide substitution rate of 6.4×10^{-10} per site per year, which was estimated from the synonymous substitution rates in *TAS2R* genes under the assumption of 6.5 MY as the time since the human–chimpanzee split (32). We also used a substitution rate of 0.81×10^{-10} per site per year for ORF-disruptive indels, which was estimated from human–chimpanzee genomic comparisons (43,52,53). The starting time of the complete functional relaxation (T), as well as its confidence interval, was estimated using a likelihood approach (43).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Soochin Cho, David Webb and two anonymous reviewers for valuable comments. This work was supported by a startup fund of the University of Michigan and a research grant from the National Institutes of Health (GM67030) to J.Z.

REFERENCES

1. Kinnamon, S.C. and Cummings, T.A. (1992) Chemosensory transduction mechanisms in taste. *Annu. Rev. Physiol.*, **54**, 715–731.
2. Lindemann, B. (1996) Taste reception. *Physiol. Rev.,* **76**, 718–766.
3. Garcia, J. and Hankins, W.G. (1975) The evolution of bitter and the acquisition of toxiphobia. In Denton, D.A. and Coghlan, J.P. (eds), *Olfaction and Taste V, Proceedings of the 5th International Symposium in Melbourne, Australia*. Academic Press, New York, pp. 39–45.
4. Glendinning, J.I. (1994) Is the bitter rejection response always adaptive? *Physiol. Behav.*, **56**, 1217–1227.
5. Chandrashekar, J., Mueller, K.L., Hoon, M.A., Adler, E., Feng, L., Guo, W., Zuker, C.S. and Ryba, N.J. (2000) T2Rs function as bitter taste receptors. *Cell*, **100**, 703–711.
6. Matsunami, H., Montmayeur, J.P. and Buck, L.B. (2000) A family of candidate taste receptors in human and mouse. *Nature*, **404**, 601–604.
7. Adler, E., Hoon, M.A., Mueller, K.L., Chandrashekar, J., Ryba, N.J. and Zuker, C.S. (2000) A novel family of mammalian taste receptors. *Cell*, **100**, 693–702.
8. Bufe, B., Hofmann, T., Krautwurst, D., Raguse, J.D. and Meyerhof, W. (2002) The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides. *Nat. Genet.*, **32**, 397–401.
9. Behrens, M., Brockhoff, A., Kuhn, C., Bufe, B., Winnig, M. and Meyerhof, W. (2004) The human taste receptor hTAS2R14 responds to a variety of different bitter compounds. *Biochem. Biophys. Res. Commun.*, **319**, 479–485.
10. Shi, P., Zhang, J., Yang, H. and Zhang, Y.P. (2003) Adaptive diversification of bitter taste receptor genes in mammalian evolution. *Mol. Biol. Evol.*, **20**, 805–814.
11. Harris, D.R. (1992) Human diet and subsistence. In Jones, S., Martin, R. and Pilbeam, D. (eds), *The Cambridge Encyclopedia of Human Evolution*. Cambridge University Press, Cambridge, pp. 69–74.
12. Leonard, W.R. (2002) Food for thought. Dietary change was a driving force in human evolution. *Sci. Am.*, **287**, 106–115.
13. Milton, K. (2003) The critical role played by animal source foods in human (Homo) evolution. *J. Nutr.*, **133**, 3886S–3892S.
14. Goren-Inbar, N., Alperson, N., Kislev, M.E., Simchoni, O., Melamed, Y., Ben-Nun, A. and Werker, E. (2004) Evidence of hominin control of fire at Gesher Benot Yaaqov, Israel. *Science*, **304**, 725–727.
15. Li, W.H. (1997) *Molecular Evolution*. Sinauer, Sunderland, MA.
16. Nei, M. and Kumar, S. (2000) *Molecular Evolution and Phylogenetics*. Oxford University Press, New York, NY.
17. Cargill, M., Altshuler, D., Ireland, J., Sklar, P., Ardlie, K., Patil, N., Shaw, N., Lane, C.R., Lim, E.P., Kalyanaraman, N. *et al.* (1999) Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat. Genet.*, **22**, 231–238.
18. Hughes, A.L. and Nei, M. (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature*, **335**, 167–170.
19. Wooding, S., Kim, U.K., Bamshad, M.J., Larsen, J., Jorde, L.B. and Drayna, D. (2004) Natural selection and molecular evolution in PTC, a bitter-taste receptor gene. *Am. J. Hum. Genet.*, **74**, 637–646.
20. Kim, U.K., Jorgenson, E., Coon, H., Leppert, M., Risch, N. and Drayna, D.(2003) Positional cloning of the human quantitative trait locus underlying taste sensitivity to phenylthiocarbamide. *Science*, **299**, 1221–1225.
21. Tajima, F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.
22. Fu, Y.X. and Li, W.H. (1993) Statistical tests of neutrality of mutations. *Genetics*, **133**, 693–709.
23. Rogers, A.R. (1995) Genetic evidence for a Pleistocene population expansion. *Evolution*, **49**, 608–615.

24. Yu, N., Zhao, Z., Fu, Y.X., Sambuughin, N., Ramsay, M., Jenkins, T., Leskinen, E., Patthy, L., Jorde, L.B., Kuromori, T. and Li, W.H. (2001) Global patterns of human DNA sequence variation in a 10-kb region on chromosome 1. *Mol. Biol. Evol.*, **18**, 214–222.
25. Fullerton, S.M., Bond, J., Schneider, J.A., Hamilton, B., Harding, R.M., Boyce, A.J. and Clegg, J.B. (2000) Polymorphism and divergence in the beta-globin replication origin initiation region. *Mol. Biol. Evol.*, **17**, 179–188.
26. Zhao, Z., Jin, L., Fu, Y.X., Ramsay, M., Jenkins, T., Leskinen, E., Pamilo, P., Trexler, M., Patthy, L., Jorde, L.B. *et al.* (2000) Worldwide DNA sequence variation in a 10-kilobase noncoding region on human chromosome 22. *Proc. Natl Acad. Sci. USA*, **97**, 11354–11358.
27. Zietkiewicz, E., Yotova, V., Jarnik, M., Korab-Laskowska, M., Kidd, K.K., Modiano, D., Scozzari, R., Stoneking, M., Tishkoff, S., Batzer, M. and Labuda, D. (1998) Genetic structure of the ancestral population of modern humans. *J. Mol. Evol.*, **47**, 146–155.
28. Harris, E.E. and Hey, J. (1999) X chromosome evidence for ancient human histories. *Proc. Natl Acad. Sci. USA*, **96**, 3320–3324.
29. Kaessmann, H., Heissig, F., von Haeseler, A. and Paabo, S. (1999) DNA sequence variation in a non-coding region of low recombination on the human X chromosome. *Nat. Genet.*, **22**, 78–81.
30. Hudson, R.R., Kreitman, M. and Aguade, M. (1987) A test of neutral molecular evolution based on nucleotide data. *Genetics*, **116**, 153–159.
31. Fay, J.C., Wyckoff, G.J. and Wu, C.I (2001) Positive and negative selection on the human genome. *Genetics*, **158**, 1227–1234.
32. Brunet, M., Guy, F., Pilbeam, D., Mackaye, H.T., Likius, A., Ahounta, D., Beauvilain, A., Blondel, C., Bocherens, H., Boisserie, J.R. *et al.* (2002) A new hominid from the Upper Miocene of Chad, Central Africa. *Nature*, **418**, 145–151.
33. Chen, F.C., Vallender, E.J., Wang, H., Tzeng, C.S. and Li, W.H. (2001) Genomic divergence between human and chimpanzee estimated from large-scale alignments of genomic sequences. *J. Hered.*, **92**, 481–489.
34. Ebersberger, I., Metzler, D., Schwarz, C. and Paabo, S. (2002) Genomewide comparison of DNA sequences between humans and chimpanzees. *Am. J. Hum. Genet.*, **70**, 1490–1497.
35. Ellegren, H., Smith, N.G. and Webster, M.T. (2003) Mutation rate variation in the mammalian genome. *Curr. Opin. Genet. Dev.*, **13**, 562–568.
36. McDonald, J.H. and Kreitman, M. (1991) Adaptive protein evolution at the Adh locus in *Drosophila. Nature*, **351**, 652–654.
37. Zhang, J., Kumar, S. and Nei, M. (1997) Small-sample tests of episodic adaptive evolution: a case study of primate lysozymes. *Mol. Biol. Evol.*, **14**, 1335–1338.
38. Gilad, Y., Man, O., Paabo, S. and Lancet, D. (2003) Human specific loss of olfactory receptor genes. *Proc. Natl Acad. Sci. USA*, **100**, 3324–3327.
39. Gilad, Y., Wiebe, V., Przeworski, M., Lancet, D. and Paabo, S. (2004) Loss of olfactory receptor genes coincides with the acquisition of full trichromatic vision in primates. *PLoS Biol.*, **2**, 120–125.
40. Niimura, Y. and Nei, M. (2003) Evolution of olfactory receptor genes in the human genome. *Proc. Natl Acad. Sci. USA*, **100**, 12235–12240.
41. Gimelbrant, A.A., Skaletsky, H. and Chess, A. (2004) Selective pressures on the olfactory receptor repertoire since the human–chimpanzee divergence. *Proc. Natl Acad. Sci. USA*, **101**, 9019–9022.
42. Zhang, X., Rodriguez, I., Mombaerts, P. and Firestein, S. (2004) Odorant and vomeronasal receptor genes in two mouse genome assemblies. *Genomics*, **83**, 802–811.
43. Zhang, J. and Webb, D.M. (2003) Evolutionary deterioration of the vomeronasal pheromone transduction pathway in catarrhine primates. *Proc. Natl Acad. Sci. USA*, **100**, 8337–8341.
44. Liman, E.R. and Innan, H. (2003) Relaxed selective pressure on an essential component of pheromone transduction in primate evolution. *Proc. Natl Acad. Sci. USA*, **100**, 3328–3332.
45. Surridge, A.K., Osorio, D. and Mundy, N.I. (2003) Evolution and selection of trichromatic vision in primates. *Trends Ecol. Evol.*, **18**, 198–205.
46. Nance, W.E. and Kearsey, M.J. (2004) Relevance of connexin deafness (DFNB1) to human evolution. *Am. J. Hum. Genet.*, **74**, 1081–1087.
47. Zhang, J., Rosenberg, H.F. and Nei, M. (1998) Positive Darwinian selection after gene duplication in primate ribonuclease genes. *Proc. Natl Acad. Sci. USA*, **95**, 3708–3713.
48. Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X. and Rozas, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.
49. Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.
50. Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.
51. Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**, 1244–1245.
52. Britten, R.J. (2002) Divergence between samples of chimpanzee and human DNA sequences is 5%, counting indels. *Proc. Natl Acad. Sci. USA*, **99**, 13633–13635.
53. Podlaha, O. and Zhang, J. (2003) Positive selection on protein-length in the evolution of a primate sperm ion channel. *Proc. Natl Acad. Sci. USA*, **100**, 12241–12246.