# Tuning Properties of Avian and Frog Bitter Taste Receptors Dynamically Fit Gene Repertoire sizes

Maik Behrens,*[1] Sigrun I. Korsching,[2] and Wolfgang Meyerhof[1]

[1]Department of Molecular Genetics, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany

[2]Institute of Genetics, University at Cologne, Cologne, Germany

***Corresponding author:** E-mail: behrens@dife.de.

**Associate editor**: Jianzhi Zhang

## Abstract

**Bitter taste perception in vertebrates relies on a variable number of bitter taste receptor (*Tas2r*) genes, ranging from only three functional genes in chicken to as many as approximately 50 in frogs. Humans possess a medium-sized *Tas2r* repertoire encoding three broadly and several narrowly tuned receptors plus receptors with intermediate tuning properties. Such tuning information is not available for bitter taste receptors of other vertebrate species. In particular it is not known, whether a small *Tas2r* repertoire may be compensated for by broad tuning of these receptors, and on the other side, whether a large repertoire might entail a preponderance of narrowly tuned receptors. To elucidate this question, we cloned all three chicken Tas2rs, the two turkey Tas2rs, three zebra finch Tas2rs, and six Tas2rs of the Western clawed frog representative of major branches of the phylogenetic tree, and screened them with 46 different bitter compounds. All chicken and turkey Tas2rs were broadly tuned, the zebra finch Tas2rs were narrowly tuned, and frog Tas2rs ranged from broadly to narrowly tuned receptors. We conclude that a low number of functional *Tas2r* genes does not imply a reduced importance of bitter taste per se, as it can be compensated by large tuning width. A high number of functional *Tas2r* genes appears to allow the evolution of specialized receptors, possibly for toxins with species-specific relevance. In sum, we show that variability in tuning breadth, overlapping agonist profiles, and staggered effective agonist concentration ranges are shared features of human and other vertebrate Tas2rs.**

***Key words:*** **bitter taste receptor, heterologous expression, evolution, G protein-coupled receptor.**

Article

## Introduction

Bitter taste perception in vertebrates is important to avoid the involuntary ingestion of potentially harmful substances. The detection of a vast array of structurally diverse bitter compounds present in nature is facilitated by the taste 2 receptor genes (Tas2r, in human TAS2R) (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000). Although many vertebrate species, including humans, possess average numbers of *Tas2r* genes, some species fall below or exceed this number by far (Dong et al. 2009). At the extremes, species can have as few as two or three functional *Tas2r* genes in turkey and chicken, respectively, and as many as approximately 50 *Tas2r* genes in frogs (Shi and Zhang 2006; Dong et al. 2009). In humans, the TAS2R repertoire consists of three broadly tuned receptors and several receptors with a very limited range of agonists in addition to those receptors with intermediate tuning properties (Meyerhof et al. 2010). Although the human TAS2R repertoire has been extensively characterized, very few Tas2rs of other vertebrates have been deorphaned to date, one of zebrafish (Oike et al. 2007), two of mouse (Chandrashekar et al. 2000), one of rat (Bufe et al. 2002), and two primate Tas2rs (Wooding et al. 2006; Imai et al. 2012). The rodent receptors are rather narrowly tuned, and the primate receptors share the response properties of their human orthologs (Bufe et al. 2002). It is unknown, whether very broadly tuned receptors, such as TAS2R10 (Bufe et al. 2002; Meyerhof et al. 2010), -R14 (Behrens et al. 2004), and -R46 (Brockhoff et al. 2007) exist in nonhuman vertebrates. The presence of such broadly tuned receptors could, at least in part, compensate for a diminutive *Tas2r* repertoire. Conversely, a large *Tas2r* repertoire of narrowly tuned receptors might overall not enable the detection of more bitter compounds. If on the other hand, the breadth of tuning of *Tas2rs* is comparable between species, the total number of *Tas2r* genes could indicate the relative importance of bitter taste in different vertebrate species.

We have approached these questions by functional analysis of several *Tas2r* genes from four nonmammalian vertebrate species, chicken, turkey, zebra finch, and frog, with very small, small, and very large *Tas2r* repertoires, respectively. We cloned all three chicken (*Gallus gallus*) Tas2rs, the two turkey (*Meleagris gallopavo*) Tas2rs, three of seven zebra finch (*Taeniopygia guttata*) Tas2rs, and six representative Tas2rs of the Western clawed frog (*Xenopus tropicalis*). Tuning width of all cloned receptors was investigated by functional experiments using a large array of bitter compounds.

## Results

### Phylogenetic Analysis Shows Absence of Gene Expansions as Main Cause of the Diminutive Chicken and Turkey *Tas2r* Gene Repertoires and Maximal Divergence within the Frog *Tas2r* Repertoire

To elucidate the phylogenetic relationship of chicken, turkey, zebra finch, and frog *Tas2r* gene repertoires, we performed

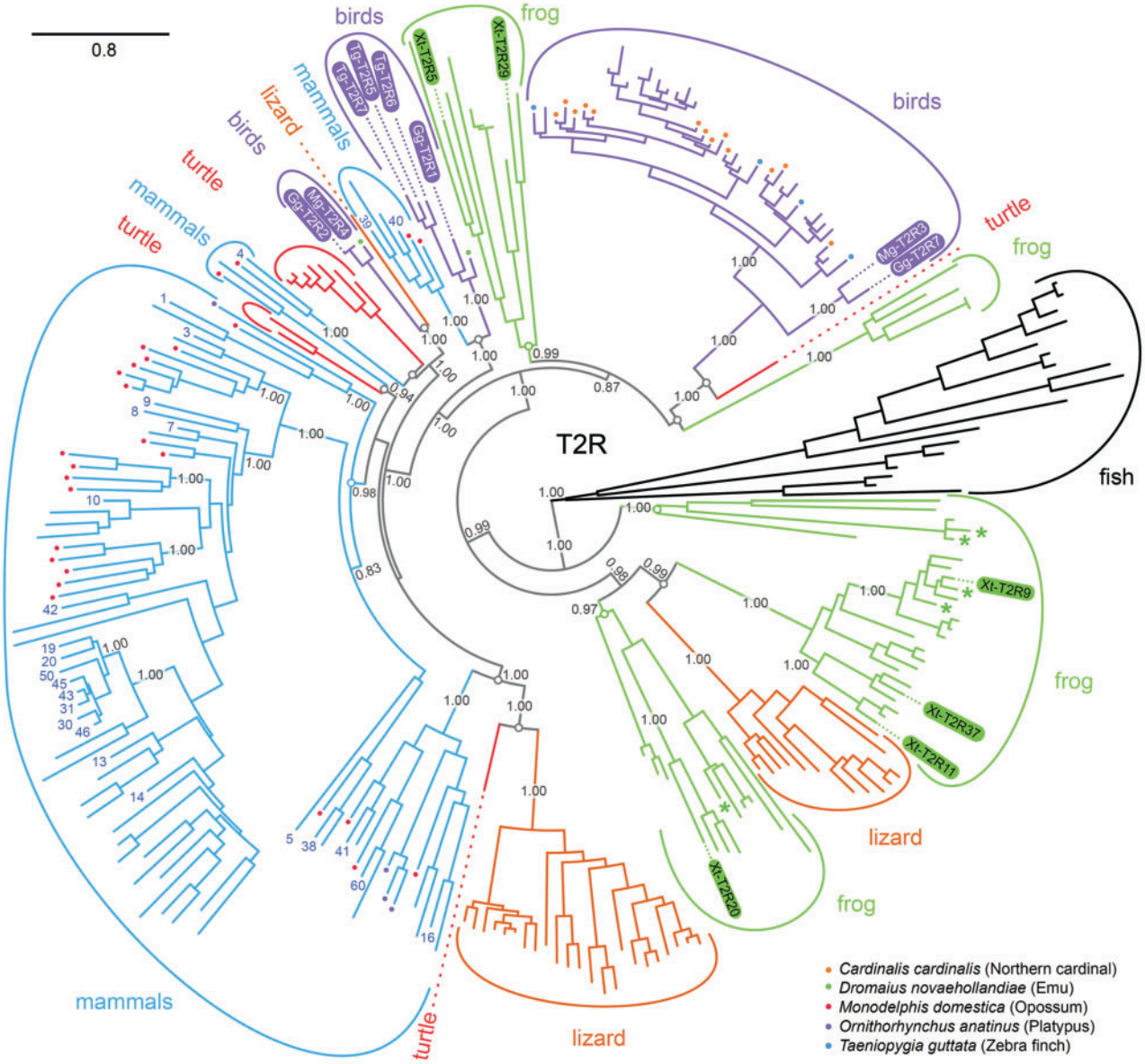


**Fig. 1.** Phylogenetic tree of *Tas2r* gene repertoires of four fish, one amphibian, two reptilian, seven avian, and four mammalian species. Tree branches for the different animal phyla are color coded, some of the individual species are indicated by colored dots, and human TAS2Rs are indicated by their gene numbers. Ancestral nodes are indicated. The tree was constructed using a modified maximum-likelihood method (PhyML-aLRT). Branch support of selected nodes is shown as *P* values (1.00 equals $P > 0.995$). Sequences were taken from Dong et al. (2009) (*Danio rerio*, zebrafish; *Takifugu rubripes*, fugu; *Gasteroceus aculeatus*, stickleback; *Tetraodon nigroviridis*, pufferfish; *Xenopus tropicalis*, Western clawed-frog; *Anolis carolinensis*, Carolina anole, a lizard; *Gallus gallus*, chicken; *Ornithorhynchus anatinus*, platypus; *Monodelphis domestica*, gray short-tailed opossum; *Mus musculus*, house mouse; *Homo sapiens*, humans; for accession numbers see supplementary table S1, Supplementary Material online), and Davis et al. (2010) (*Motacilla flava*, yellow wagtail; *Carduelis pinus*, Pine Siskin; *Cardinalis cardinalis*, Northern cardinal; for accession numbers see supplementary table S1, Supplementary Material online) or were newly identified in databank searches (5 additional *Xenopus tropicalis* genes, asterisks; 11 genes, *Pelodiscus sinensis*, Chinese softshell turtle; 2 genes, *Dromaius novaehollandiae*, emu; 2 genes, *Meleagris gallopavo*, wild turkey; 7 genes, *Taeniopygia guttata*, zebra finch; supplementary table S1, Supplementary Material online).

extensive BLAST searches in several avian and reptilian genomes using representative sequences from the closest species already described (Dong et al. 2009; Davis et al. 2010). In turkey, a close relative of chicken, we found an equally small repertoire of two intact genes and one pseudogene, and a somewhat larger family of seven genes in a passerine bird, *T. guttata* (Zebra finch) (fig. 1). In contrast, an earlier diverging reptilian species, *Pelodiscus sinensis* (Chinese softshell turtle), exhibited a medium-sized repertoire of 11 intact genes. A thorough search in the frog genome revealed five additional genes (supplementary table S1, Supplementary Material online) not described in Dong et al. (2009), bringing the total number of frog *Tas2r* genes to 54 intact genes.

A phylogenetic tree constructed with all *Tas2r* genes from four teleost fish, one amphibian, two reptile, seven bird, and four mammalian species shows the presence of 2–5 ancestral

nodes (ancestral *Tas2r* genes) per phylum (fig. 1), many of which are shared with 1–3 other phyla. In particular, all avian *Tas2r* gene families are derived from the same three ancestral genes, one shared with mammals, another with turtle and frog, and the third with turtle, lizard, and mammals. In comparison, frog has five clearly segregated ancestral nodes, corresponding to five ancestral genes (fig. 1). Thus, the observed large differences in repertoire sizes between species are mainly due to differing degree of later gene duplications within species. Gene death as inferred by the absence of a species in one of the ancestral nodes does occur, but seems to affect all species to a similar extent (fig. 1).

The three chicken *Tas2rs* are distributed over all three avian subfamilies. The receptor ggTas2r1 is part of a small subfamily related to human TAS2R39 and -R40, ggTas2r2 shows a common branch with lizard and turtle Tas2r subfamilies, and is related to human TAS2R4. The receptor ggTas2r7 is basal to a large bird-specific subfamily, which is monophyletic with a single turtle *Tas2r* gene and a small frog subfamily of five genes. The two turkey Tas2rs, mgTas2r3 and mgTas2r4, are orthologues of chicken Tas2r7 and Tas2r2, respectively. No functional ortholog of ggTas2r1 has been detected in the turkey genome. Three of the seven zebra finch *Tas2r* genes, *tgTas2r5*, *-r6*, and *–r7*, form a cluster with ggTas2r1, and the other four *Tas2r* genes are part of the bird-specific expansion mentioned above. *Xenopus tropicalis* (frog) shows both the largest divergence within subfamilies and the largest number of ancestral genes not monophyletic with other species, including the most ancient subfamily of Tas2r receptors. Interestingly, none of the five xtTas2r subfamilies is shared with the mammalian lineage. One subfamily is monophyletic with a lizard subfamily, and both show extended, but late gene expansion. The second subfamily is monophyletic with turtle and avian genes as mentioned above. For our functional analyses, we chose all three chicken Tas2rs, ggTas2r1, -r2, and -r7, the turkey Tas2rs, mgTas2r3 and –r4, the zebra finch Tas2rs, tgTas2r5, -r6, and –r7, as well as the frog xtTas2r9a, -r11 and –r37, which belong to the subfamily of frog Tas2rs forming a common branch with lizard receptors, the receptor xtTas2r20 of a frog-specific subfamily, and, finally, the receptors xtTas2r5 and –r29 of another frog-specific Tas2r-subfamily.

## Frog Tas2rs Range from Broadly to Narrowly Tuned, Zebra Finch Tas2rs Are Narrowly Tuned, Whereas All Chicken and Turkey Tas2rs Are Broadly Tuned

To identify agonists for the three chicken, two turkey, three zebra finch, and six frog receptors, we screened them with 46 natural or synthetic bitter compounds (supplementary table S2, Supplementary Material online). The substances were selected from a list of 104 chemicals used recently for the screening of human TAS2Rs (Meyerhof et al. 2010). The subgroup was chosen to represent diverse chemical classes and predominantly natural substances to elevate the chances for successful receptor deorphanization. Indeed, we identified agonists for all 14 receptors examined.

For the chicken receptor, ggTas2r1 ten agonists (five natural, five synthetic) were identified (fig. 2 and supplementary fig. S1, Supplementary Material online). Thus, this receptor recognized 22% of the compounds. Chicken receptor ggTas2r2 responded to eight compounds (six natural, two synthetic; 17%) and for receptor ggTas2r7 17 agonists (14 natural, 3 synthetic; 37%) were found. Combined, the three chicken receptors, representing the entire chicken Tas2r repertoire, responded to 23 substances (50%). Four of the substances, chloramphenicol, chlorpheniramine, diphenidol, and quinine sulfate activated all three receptors (supplementary table S2, Supplementary Material online), four substances activated two receptors, whereas 15 compounds led to responses of single receptors. Two of seven substances that did not activate any human TAS2R in a previous study (Meyerhof et al. 2010), that is, the alkaloid nicotine and the diterpene ginkgolide A, were shown as agonists of ggTas2r1 and -r7, respectively.

Similar to the results obtained for the chicken Tas2rs, both of the turkey Tas2rs responded to numerous bitter substances (fig. 3 and supplementary fig. S1, Supplementary Material online). The turkey receptor mgTas2r3, the ortholog of chicken ggTas2r7, recognized 15 agonists (12 natural, 3 synthetic; 33%), whereas mgTas2r4 was activated by eight compounds (five natural, three synthetic; 17%). Thus, the two functional turkey bitter taste receptors together recognized 19 of the tested 46 substances (41%). Four substances, chloramphenicol, diphenidol, parthenolide, and quinine sulfate, activated both of the turkey receptors (supplementary table S2, Supplementary Material online). Similar to the results obtained in the chicken Tas2r screening, ginkgolide A, a substance which did not activate any human bitter taste receptor in a previous screening was found as an agonist for one of the two turkey receptors.

In contrast to chicken and turkey Tas2rs the three zebra finch Tas2rs homologous to chicken Tas2r1 recognized fewer bitter substances (fig. 4 and supplementary fig. S1, Supplementary Material online). The zebra finch receptor tgTas2r5 was activated by five agonists (one natural, four synthetic; 11%). For receptor tgTas2r6 we identified only three agonists (two natural, one synthetic; 7%) and for receptor tgTas2r7 again five agonists were found (three natural, two synthetic; 11%). Because of a considerable overlap among the agonist spectra of the three receptors (supplementary table S2, Supplementary Material online), the combined activity of the three zebra finch receptors amounted to only nine substances (20%), which is slightly less than the number of agonists identified for the homologous chicken receptor ggTas2r1 (see above). A large fraction of the agonists for the zebra finch receptors represented synthetic bitter compounds (five substances) and the responses were in most cases small.

The frog receptor xtTas2r5 displayed a restricted agonist spectrum (fig. 5 and supplementary fig. S2, Supplementary Material online) recognizing only three natural bitter compounds (7%). Receptor xtTas2r9a responded to 15 test substances (12 natural, 3 synthetic; 33%). For xtTas2r11 we identified 14 agonists (nine natural, five synthetic; 30%) and xtTas2r20 was activated by four natural bitter substances (9%). Although xtTas2r29 displayed only small responses

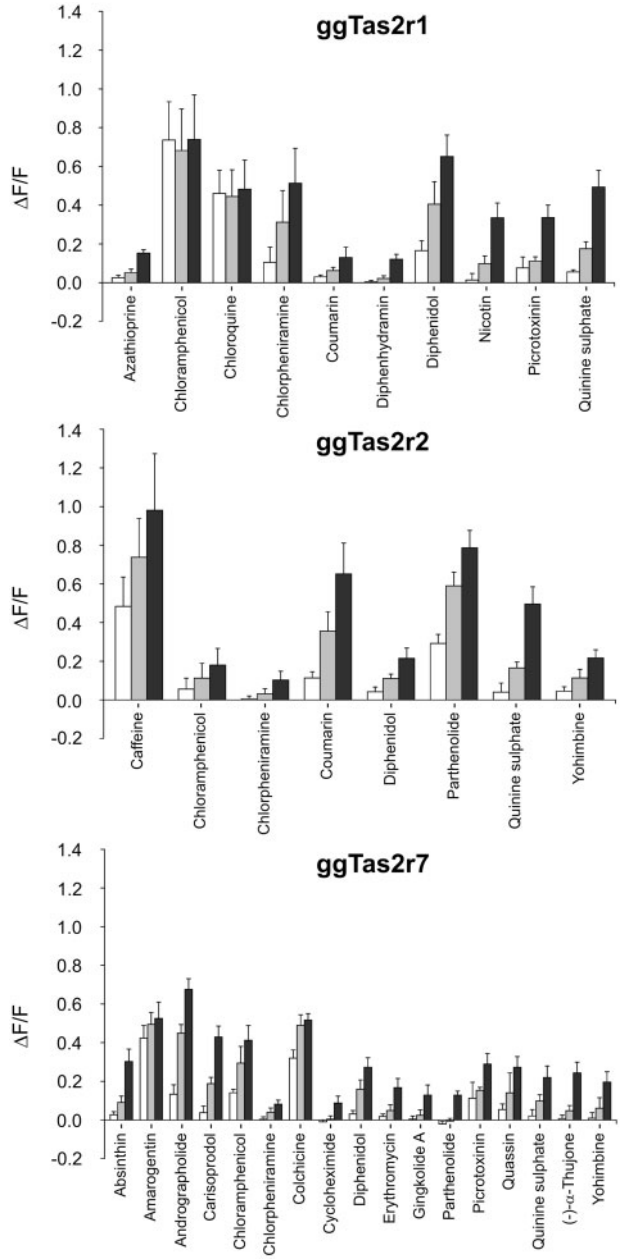


**Fig. 2.** Functional screening of chicken bitter taste receptors. The three chicken Tas2rs, ggTas2r1, -r2, and -r7, were transiently transfected in HEK 293T-G$\alpha$16gust44 cells and screened with in total 46 natural and synthetic bitter compounds by calcium imaging. Each compound resulting in the stimulation of a chicken Tas2r in a prescreening was tested in three different concentrations. The responses upon stimulation with the maximal concentration (cf. supplementary table S2, Supplementary Material online) not leading to unspecific cellular responses, are shown by black bars. In addition, compounds were tested in 1:3 (gray bars) and 1:10 (white bars) dilutions. The *y* axis shows the relative fluorescence changes ($\Delta$F/F), and the *x* axis is labeled with the activating bitter compounds.

upon stimulation by a single natural bitter substance (2%), xtTas2r37 recognized a broad panel of 14 substances (13 natural, 1 synthetic; 30%). The combined activity of the six investigated frog Tas2rs corresponding to approximately 11% of the *X. tropicalis* Tas2r repertoire of 54 receptors amounted to

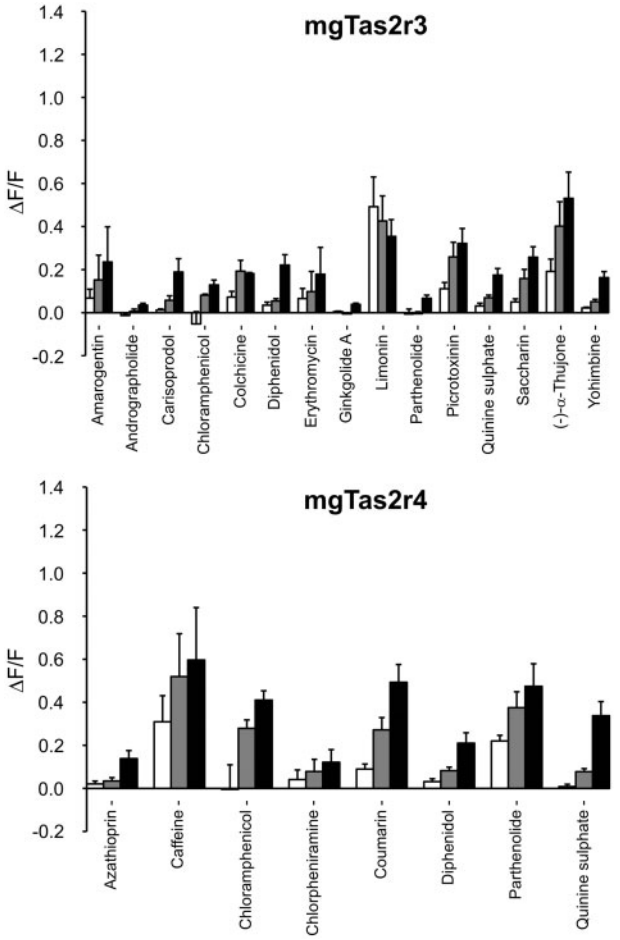


**Fig. 3.** Functional screening of turkey bitter taste receptors. The two turkey Tas2rs, mgTas2r3 and –r4, were transiently transfected in HEK 293T-G$\alpha$16gust44 cells and screened with in total 46 natural and synthetic bitter compounds by calcium imaging. Each compound resulting in the stimulation of a turkey Tas2r in a prescreening was tested in three different concentrations. The responses upon stimulation with the maximal concentration (cf. supplementary table S2, Supplementary Material online) not leading to unspecific cellular responses, are shown by black bars. In addition, compounds were tested in 1:3 (gray bars) and 1:10 (white bars) dilutions. The *y* axis shows the relative fluorescence changes ($\Delta$F/F), the *x* axis is labeled with the activating bitter compounds.

26 substances (57%) (supplementary table S2, Supplementary Material online). Similar to the results obtained for chicken receptors, ginkgolide A, not previously shown to activate human TAS2Rs activated two frog receptors, xtTas2r9a and -r37.

## Effective Concentration Ranges for the Detection of Bitter Compounds by Chicken and Frog *Tas2rs* Are Staggered

To determine the sensitivities of chicken and frog Tas2rs for selected bitter compounds we obtained dose-response relationships. The substances quinine and chloramphenicol activated all three ggTas2rs (fig. 6). Although quinine sulfate activated the three receptors in similar concentration ranges, receptor responses upon chloramphenicol application

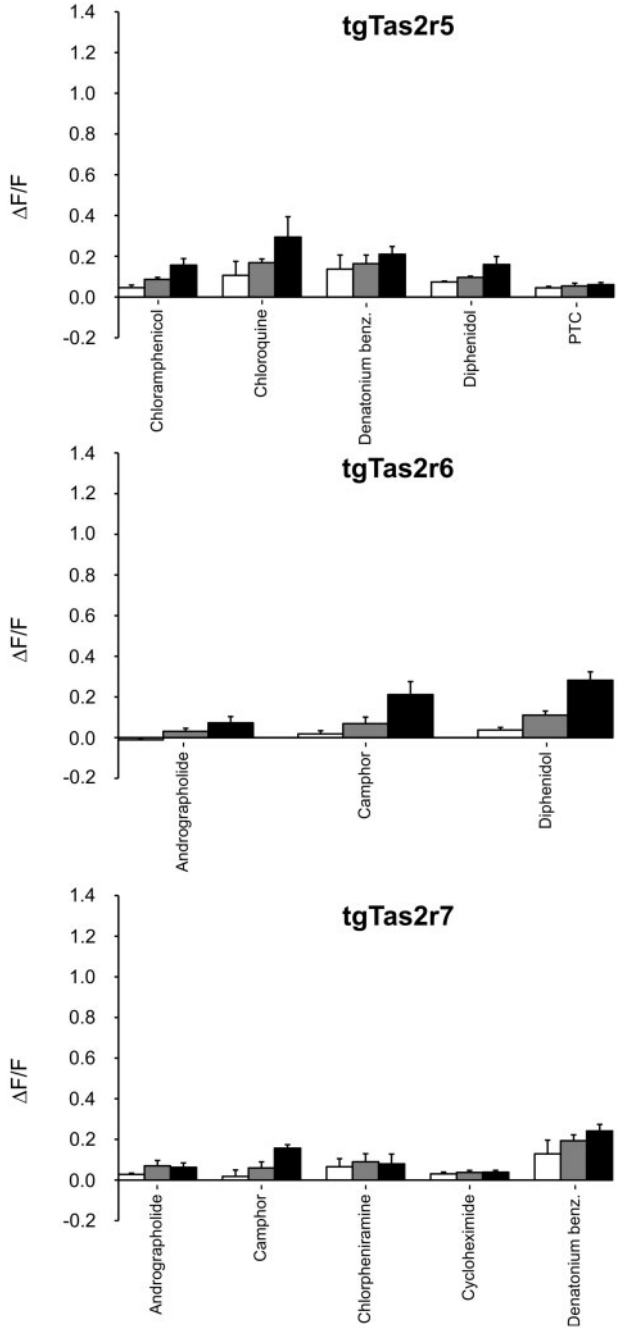


**Fig. 4.** Functional screening of zebra finch bitter taste receptors. The three zebra finch Tas2rs, tgTas2r5, -r6, and –r7, were transiently transfected in HEK 293T-G$\alpha$16gust44 cells and screened with in total 46 natural and synthetic bitter compounds by calcium imaging. Each compound resulting in the stimulation of a zebra finch Tas2r in a prescreening was tested in three different concentrations. The responses upon stimulation with the maximal concentration (cf. supplementary table S2, Supplementary Material online) not leading to unspecific cellular responses, are shown by black bars. In addition, compounds were tested in 1:3 (gray bars) and 1:10 (white bars) dilutions. The *y* axis shows the relative fluorescence changes ($\Delta$F/F), and the *x* axis is labeled with the activating bitter compounds.

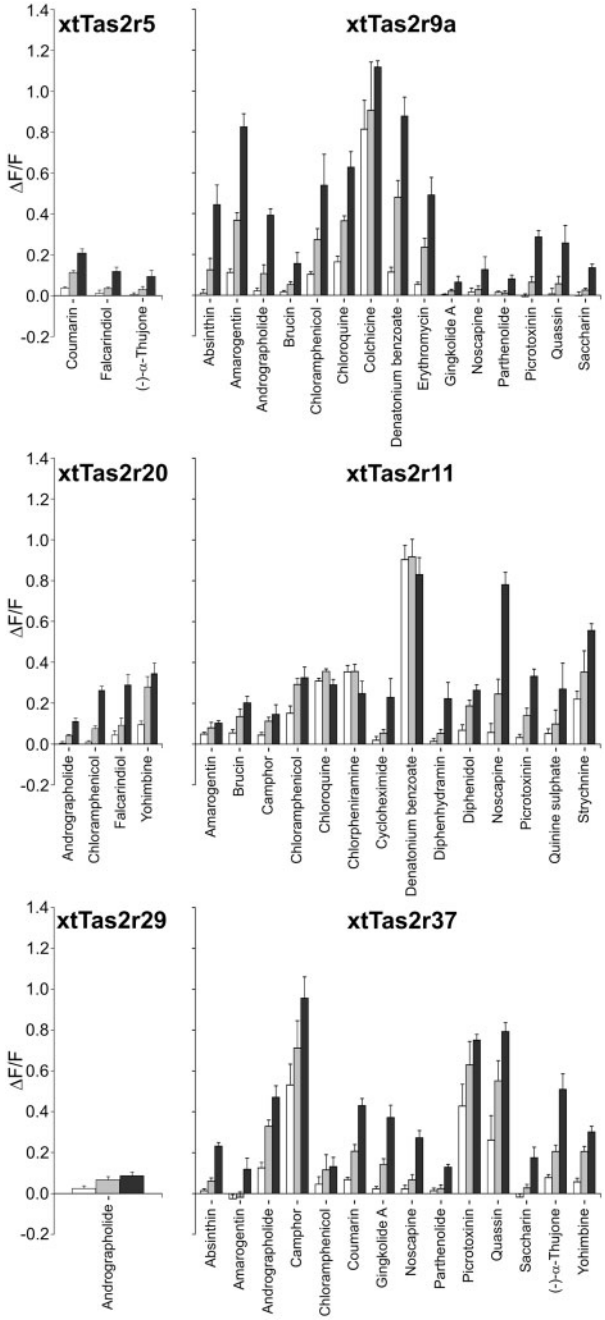


**Fig. 5.** Functional screening of frog bitter taste receptors. The six frog Tas2rs, xtTas2r5, -r9a, -r11, -r20, -r29, and -r37, were transiently transfected in HEK 293T-G$\alpha$16gust44 cells and screened with in total 46 natural and synthetic bitter compounds by calcium imaging. Each compound resulting in the stimulation of frog a Tas2r in a prescreening was tested in three different concentrations. The responses upon stimulation with the maximal concentration (cf. supplementary table S2, Supplementary Material online) not leading to unspecific cellular responses, are shown by black bars. In addition, compounds were tested in 1:3 (gray bars) and 1:10 (white bars) dilutions. The *y* axis shows the relative fluorescence changes ($\Delta$F/F), and the *x* axis is labeled with the activating bitter compounds.

deviated clearly with ggTas2r1 being the most sensitive and ggTas2r the least sensitive receptor. Coumarin activated ggTas2r2 and –r1 with different potency. Only a single receptor each responded to amarogentin (ggTas2r7) and nicotine (ggTas2r1), respectively. Signal saturation was not reached for the majority of receptor/compound combinations, and so only the half-effective concentration $EC_{50}$ of ggTas2r1 stimulated with nicotine was determined ($EC_{50} = 0.0288 \pm 0.0008$ mM).

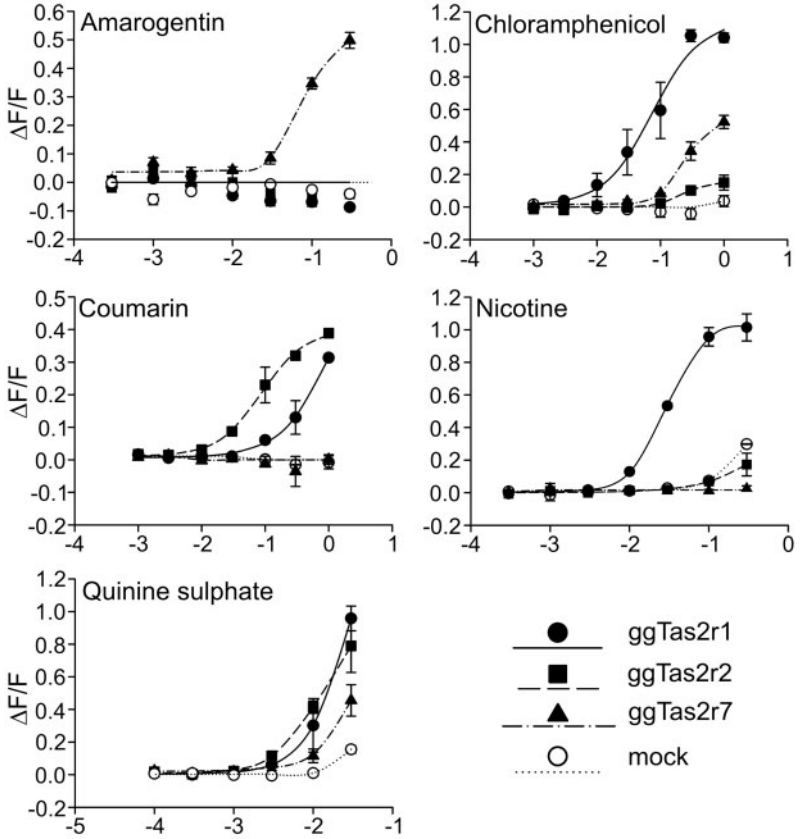


**Fig. 6.** Dose-response relationships of chicken bitter taste receptors. The three chicken Tas2rs were transiently transfected in HEK 293T-G$\alpha$16gust44 cells and stimulated with increasing concentrations of bitter compounds. The fluorescent changes (*y* axis; $\Delta$F/F) upon agonist stimulation of ggTas2r1 (black circles), ggTas2r2 (black squares), and ggTas2r7 (black triangles) were monitored and plotted against the logarithm of compound concentration (*x* axis; log mM). The curves obtained for cells transfected with empty vector (mock, empty circles) and stimulated with bitter compounds are shown to document artificial, receptor-independent responses.

Also the efficacies of the compounds differed depending on receptors and substances. Although ggTas2r1 exhibited maximal signal amplitudes of approximately 1.0 ($\Delta$F/F) upon stimulation with nicotine, chloramphenicol, and quinine sulfate, the maximally observed fluorescence changes for cells transfected with ggTas2r7 never exceeded approximately 0.5. The maximal signal amplitudes obtained for ggTas2r2 transfected cells showed intermediate values not exceeding 0.8. Hence, the most broadly tuned ggTas2r7 is the most sensitive (and indeed the sole) receptor only for amarogentin, for all other tested compounds the less broadly tuned receptors ggTas2r1 and -r2 were more sensitive and reached higher signal amplitudes. In general, individual substances activate different receptors in clearly separated concentration ranges.

The most potent compound activating frog Tas2rs with a threshold concentration of approximately 1 $\mu$M and an $EC_{50}$ concentration of 0.0051 $\pm$ 0.0009 mM is the synthetic antihistamine chlorpheniramine, which is exclusively recognized by xtTas2r11 (fig. 7). The same receptor is stimulated by the alkaloid noscapine and the synthetic denatonium benzoate ($EC_{50}$ concentration = 0.0191 $\pm$ 0.002 mM) with high potency, although higher concentrations of these two substances also activate additional frog Tas2rs. Similar to the results obtained for the chicken receptors, for each substance, a receptor responding most sensitively was identified. For camphor, picrotoxinin, quassin, and $\alpha$-thujone, the most sensitive receptor is xtTas2r37. As all of these substances are natural terpenoids, this receptor seems to be specialized for the detection of this compound class. This is further underscored by our screening results showing that of the 14 agonists identified for xtTas2r37, mine (absinthin, amarogentin, andrographolide, camphor, gingkolide A, parthenolide, picrotoxinin, quassin, and $\alpha$-thujone) represent terpenoids. The antibiotic chloramphenicol activates most potently xtTas2r11 as well as, with reduced potency, xtTas2r9a, -r20, and –r37. The toxic alkaloid colchicine is not only a potent agonist for xtTas2r9a ($EC_{50}$ concentration = 0.0962 $\pm$ 0.0142 mM), but also exhibits the highest efficacy of all tested compounds as visible from the maximal signal amplitudes. Hence, in contrast to our data obtained for chicken Tas2rs, the more broadly tuned frog receptors exhibited higher sensitivities and higher maximal signal amplitudes compared with the more narrowly tuned receptors.

## Broad Activation Spectrum of Individual Bitter Compounds Appears to Be a Partially Intrinsic Feature

Until now receptor assays mostly have been performed with human receptors and the ability of the tested agonists to activate TAS2Rs differed considerably. Although for some

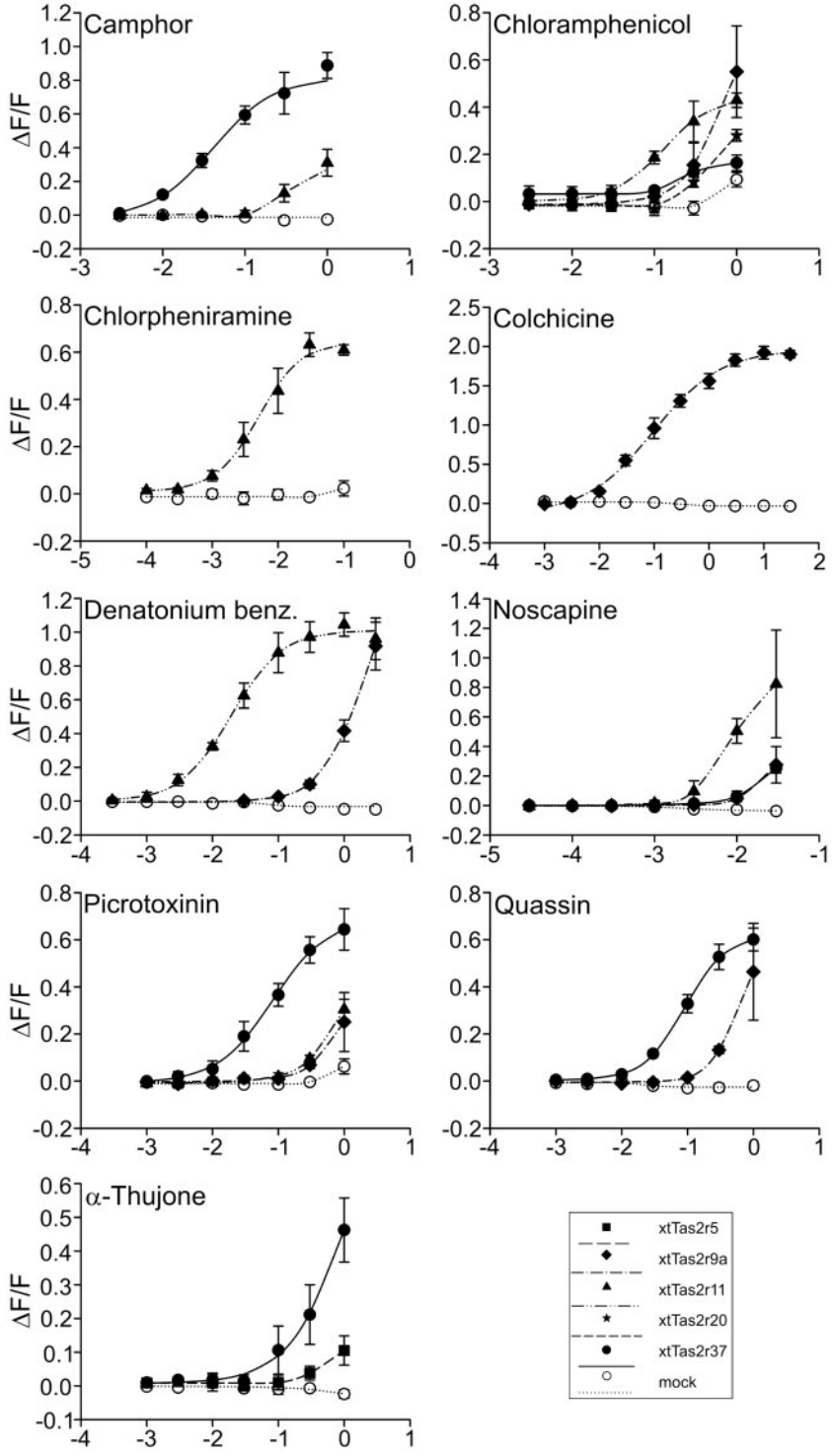


**Fig. 7.** Dose-response relationships of frog bitter taste receptors. Five of the six chicken Tas2rs used in this study were transiently transfected in HEK 293T-G$\alpha$16gust44 cells and stimulated with increasing concentrations of bitter compounds. The fluorescent changes ($y$ axis; $\Delta F/F$) upon agonist stimulation of xtTas2r5 (black squares), xtTas2r9a (black diamonds), xtTas2r11 (black triangles), xtTas2r20 (black stars), and xtTas2r37 (black circles) were monitored and plotted against the logarithm of compound concentration ($x$ axis; log mM). The curves obtained for cells transfected with empty vector (mock, empty circles) and stimulated with bitter compounds are shown to document artificial, receptor-independent responses.

substances a corresponding TAS2R has not been identified so far, other compounds stimulated up to 15 TAS2Rs (Meyerhof et al. 2010). It is unknown, to what extent the ability of a substance to activate numerous receptors is intrinsic to the substance, that is, would also result in the activation of numerous Tas2rs of different species, or, alternatively, dependent on species-specific Tas2r repertoires. Our results for frog, turkey, zebra finch, and chicken Tas2rs allow us to approach this question: All of the tested substances that were previously shown to activate a particularly high number of human

TAS2Rs, for example, diphenidol (15 of 25 TAS2Rs), quinine (9), denatonium benzoate, chlorpheniramine (8), amarogentin, parthenolide (7), chloramphenicol (6) activated also at least 4 (denatonium benzoate) and up to 10 (chloramphenicol) of the 14 frog, turkey, zebra finch, and chicken Tas2rs (supplementary table S2, Supplementary Material online). Of those compounds activating no (curcumin, emetine, gingkolide A, naringin, nicotine, ouabain, and solanin) or only one (acetaminophen, arbutin, cycloheximide, erythromycin, limonin, noscapin, phenylthiocarbamide [PTC], salicin) human TAS2R, the majority also activated no or few chicken, turkey, zebra finch, and frog receptors. However, some bitter compounds not activating any human TAS2R despite considerable previous screening efforts, indeed activated chicken, turkey, or frog receptors (Gingkolide A, ggTas2r7, mgTas2r3, xtTas2r9a, xtTas2r37; nicotine, ggTas2r1). Hence, the data suggest that the activation spectra of bitter substances may indeed partially depend on species-specific Tas2r repertoires, however, also reflect to some degree substance intrinsic features.

## Discussion

By cloning and functional characterization of all three chicken, all two turkey, three zebra finch, and six frog Tas2rs, we were able to identify bitter agonists for all of them. We chose two of these species, because they exhibit the smallest (chicken) and largest (frog) number of putatively functional *Tas2r* genes of all vertebrate genomes analyzed so far (Shi and Zhang 2006; Dong et al. 2009). The two turkey receptors are direct orthologs of two chicken receptors, allowing a comparison of the ligand spectra of one-to-one homologs. The three zebra finch receptors cluster with chicken *Tas2r1*, and thus allow to investigate the consequences of species-specific *Tas2r* gene expansions. An obvious question arising from this enormous range of *Tas2r* gene numbers in vertebrates is whether the *Tas2r* gene number is correlated with the relative importance of the bitter-tasting abilities of animals? Our recent screenings of all 25 putatively functional human TAS2Rs with more than 100 natural and synthetic compounds resulted in the identification of 83 substances activating 21 TAS2Rs (four receptors currently remain orphan) (Meyerhof et al. 2010; Thalmann et al. 2013). Thus, approximately 80% of the applied substances were recognized by at least one receptor. Our present screening of three chicken, two turkey, and six frog receptors with 46 of the above mentioned 104 chemicals resulted in the recognition of 50% (23 compounds) of the substances by chicken receptors, 41% (19 compounds) by turkey receptors, and 57% (26 compounds) by frog receptors. Hence, there is no obvious sign for a substantially reduced importance of bitter taste in chicken or turkey, two phasianid bird species evolutionary well separated since about 35–45 My (Ellegren 2007). An elevated importance for frogs' bitter-tasting abilities correlating with functional *Tas2r* gene numbers cannot be ruled out as we analyzed only 6 out of 54 receptors. However, these findings indicate that another important property of the three human TAS2Rs, TAS2R10 (Bufe et al. 2002), -R14 (Behrens et al. 2004), and −R46 (Brockhoff et al. 2007), namely their astonishingly broad tuning applies to other vertebrates' TAS2Rs. Indeed, all three chicken and the two turkey receptors recognize between 17% and 37% of the tested bitter stimuli identifying them as broadly tuned receptors which is also evident from their similarly shaped tuning curves (supplementary fig. S1, Supplementary Material online). Presumably, the low number of chicken and turkey *Tas2r* genes is compensated by large average tuning-breadth indicating that the bitter tasting abilities of these animal species are important for survival. This is in good agreement with data obtained for the three zebra finch *Tas2rs* that cluster together with chicken *Tas2r1* and hence, represent a case of species-specific *Tas2r* gene expansions (fig. 4 and supplementary fig. S1, Supplementary Material online). The combined activities of tgTas2r5, -r6, and −r7 accounted for the recognition of only nine bitter substances, whereas chicken Tas2r1 alone responded to ten substances. We have recently shown for TAS2R46 that even a very broadly tuned human receptor only possesses a single ligand-binding pocket accommodating all agonists (Brockhoff et al. 2010). Moreover, using another broadly tuned receptor, the TAS2R10, we found that broad tuning of TAS2Rs is achieved, at least in part, at the expense of potency for individual agonists (Born et al. 2013). The observation that the more narrowly tuned chicken receptors ggTas2r1 and −r2 display usually higher sensitivities for compounds compared with the broader tuned ggTas2r7 is in good agreement with this proposed mechanism (fig. 6).

The idea that the sizes of *Tas2r* gene repertoires may in general affect the average tuning properties, however, is not fully compatible with our data on frog receptors. Three of the six investigated receptors xtTas2r9a, -r11 and −r37, are broadly tuned receptors detecting approximately one-third of the tested bitter compounds. Hence, even species possessing a larger number of *Tas2r* genes than human rely to some extent on "generalist" receptors. On the other hand, the other three frog receptors display a very small number of agonists and hence, a large Tas2r repertoire may allow the development of more numerous "specialist" receptors. This hypothesis is supported by our data on the three zebra finch receptors that altogether do not recognize more agonists (in fact one less) than the single homologous chicken receptor ggTas2r1. As we found that these receptors, namely xtTas2r5, -r20, and -r29 as well as tgTas2r5, -r6, and −r7, exhibited generally lower maximal signal amplitudes in our assay (figs. 4 and 5 and supplementary figs. S1 and S2, Supplementary Material online), we cannot rule out that technical reasons prevented us from identifying additional weaker agonists and therefore we may underestimate their tuning width at present. However, the fact that we observed similar response magnitudes of receptors exhibiting high signal amplitudes and receptors with low signal amplitudes for common agonists indicates that this might not be the case. A good example for this is the activation of xtTas2r5 (fig. 5) and ggTas2r1 (fig. 2) by coumarin. The stimulation of both receptors with this substance resulted in similar response magnitudes arguing against a generally impaired functionality of xtTas2r5.

Another question which may arise in this context is whether the responses of the analyzed Tas2rs could be

impaired by the use of a heterospecific G protein-chimera. The data presented in this manuscript demonstrate functionality of chicken, turkey, zebra finch, and frog receptors in our assay system. Hence, one can conclude that our experiments do not suffer from a lack in G protein coupling among heterospecific G protein-coupled receptors and G proteins. In fact, it has been shown that bitter taste receptors couple to a variety of different G proteins such as $\alpha$-gustducin, $\alpha$-transducin, G$\alpha$i, and G$\alpha$o (Sainz et al. 2007). Moreover, it was shown that the integration of human TAS2R16 into the genome of mice is sufficient to transfer salicin-sensitivity to otherwise insensitive mice (Mueller et al. 2005). This does not only mean that human TAS2R16 is able to couple to the components of the mouse taste signaling cascade, but also that the in vitro response properties monitored in human embryonic kidney (HEK) cells (Bufe et al. 2002) are matching the in vivo responses. G proteins are highly conserved proteins, for example, human $\alpha$-gustducin (accession numbers NP_001095856) shows 82% and 81% amino acid sequence identity with chicken (NP_001254740) and *X. tropicalis* (NP_001135577) sequences, respectively, and even insect receptors have been shown to couple with this G protein-chimera in human HEK cells (Mayoral et al. 2010; Nouzova et al. 2012). Of course, some bitter taste receptors of species distantly related to humans may couple even better to homospecific G protein chimeras; however, if this would be beneficial in the context of a human cell line is uncertain.

The fact, that we previously identified three extremely broadly tuned human receptors (Meyerhof et al. 2010) and now three chicken, two turkey, as well as three frog generalist receptors raises the question whether this feature has been inherited from ancient broadly tuned Tas2rs. In fact, this may apply to the broadly tuned frog receptors (xtTas2r9a, -r11, -37), which all share a common branch with a subfamily of reptile receptors (fig. 1). However, the chicken and the orthologous turkey receptors as well as the broadly tuned human receptors occur interspersed with less broadly tuned receptors in the phylogenetic tree. A good example is provided by the three close homologs of chicken Tas2r1 in the zebra finch Tas2r family, which nevertheless all are in the specialist category. Moreover, we have recently shown that the binding modes for identical agonists, for example, for strychnine in TAS2R10 and -R46, can be different and thus should have evolved independently (Born et al. 2013). Thus, the tuning properties of Tas2rs may develop independently from common ancestral Tas2rs. As fish Tas2rs seem to respond to few bitter agonists (Oike et al. 2007) and thus can be considered as specialist receptors, the gain of tuning breadth may have accompanied the development of amphibians.

The chicken receptor ggTas2r1 and human TAS2R39 and −R40 share a common ancestral node (fig. 1) raising the question whether these receptors are activated by an overlapping agonist spectrum. Of the ten identified ggTas2r1 agonists five are shared with human TAS2R39 and three are shared with TAS2R40 (supplementary table S2, Supplementary Material online). Of the eight identified agonists of ggTas2r2, a receptor belonging to a different ancestral node, three are shared with TAS2R39 and two are shared with TAS2R40 (supplementary table S2, Supplementary Material online). This suggests that overlapping agonist spectra may occur among phylogenetically close as well as among more distantly related receptors. This is in good agreement with data from human TAS2Rs for which it has been shown that closely receptors may possess very different agonist spectra (Brockhoff et al. 2010), whereas distantly related receptors can exhibit somewhat overlapping agonist profiles (Born et al. 2013). Perhaps due to the closer phylogenetic relationship between chicken and turkey, the two pairs of orthologous receptors, ggTas2r2 and mgTas2r4 as well as ggTas2r7 and mgTas2r3, respectively, show similar agonist spectra. The turkey receptor mgTas2r4 does not only recognize the same number of agonists (8 of 46) compared with ggTas2r2 it shares seven of the bitter agonists with its chicken ortholog. Also mgTas2r3 shares 13 common agonists with its chicken counterpart ggTas2r7. Hence, functional conservation is evident with regards to tuning breadths and agonist profiles.

The question, why chicken and turkey have so few *Tas2r* genes, whereas frogs possess so many of them may be answered by different evolutionary origins and lifestyles. Frogs, originating from an aquatic environment, conquered terrestrial habitats, before, in case of *X. tropicalis*, again purely aquatic biotopes were occupied. Other frog species persistently inhabit both, aquatic and terrestrial habitats during their development from larval stages to adulthood. As bitter compounds can be water soluble as well as very hydrophobic compounds, frogs simply may have encountered (and possibly still encounter) a larger variety of bitter substances elevating the evolutionary pressure for a larger *Tas2r* gene repertoire. This hypothesis would be consistent with observations in the olfactory system of *X. laevis*, a close relative of *X. tropicalis*. In larval *X. laevis*, the olfactory organ expresses a considerable fraction of the V2R receptor repertoire (Syed et al. 2013), which normally is restricted to the vomeronasal organ. In the adult *Xenopus*, a split main olfactory system, with the lateral diverticulum for the detection of water-soluble stimuli, and the medial diverticulum for the detection of airborne odors, has been described (Altner 1962). The lateral diverticulum expresses a class of odorant receptors (ORs) related to fish ORs, whereas the medial diverticulum expresses a separate class of ORs that are closer related to mammalian ORs (Freitag et al. 1995). To our best knowledge, the bitter tasting abilities of *X. tropicalis* have not been tested experimentally; however, exquisite sensitivity of *X. laevis* gustatory receptors for strychnine, quinine, nicotine, PTC, naringin, and caffeine was demonstrated by nerve activity recordings (Yoshii et al. 1982). Although we found bitter taste receptors responding to strychnine and quinine in *X. tropicalis*, receptors activated by the other four compounds were not among those tested. The observed increases in the response amplitudes of xtTas2r11 in the tested concentration ranges (cf. fig. 5, 3, 10, 30 μM strychnine; 1, 3, 10 μM quinine sulfate) fits to the nerve response properties reported for both substances in the same concentration ranges in *X. laevis* gustatory receptors (Yoshii et al. 1982).

Due to their comparatively low taste bud number, low saliva production, and lacking mastication, it was believed

that birds possess only an inferior taste system (for a review see Roura et al. [2012]). Indeed, chicken do not possess a functional *Tas1r2* gene coding for the specific sweet taste receptor subunit (Lagerstrom et al. 2006) and only three functional Tas2rs. However, with respect to bitter taste it has been demonstrated that chicken can perceive 0.5–2 mM quinine-HCl depending on the number of taste buds found in different chicken breeds (Kudo et al. 2010) correlating well with our finding that all ggTas2rs are quinine responsive. On the other hand, chicken exhibit low or no avoidance of 0.5% (~11 mM) denatonium benzoate-coated beads (Richard and Davies 2000), again consistent with the absence of denatonium responsive ggTas2rs within the concentration limits ($\leq$ 3 mM) of our cellular test system. Obviously, the bitter detection system of chicken contains some "gaps" not covered by responsive Tas2rs. However, the chicken shares a low *Tas2r* gene number with other avian species such as turkey or emu. In fact, chicken and turkey belonging to the order of galliformes on the one hand, and the emu (casuariiformes) on the other hand belong to different but related bird clades (Hackett et al. 2008). Although chicken, turkey, and emu are all ground-feeding birds their regional distribution (Southeast Asia, North America, Australia) and habitats differ considerably. That in all these cases 2–3 Tas2rs suffice to assure the survival of the species is attesting to the enormous versatility of this gene family. For some birds, for example, the white-throated sparrow (*Zonotrichia albicollis*) with 18 putatively functional *Tas2r* genes (Davis et al. 2010), exhibiting *Tas2r* gene numbers similar to most mammalian species, the required *Tas2r* gene repertoire obviously required an expansion to guarantee survival. However, as shown for the species-specific expansion of zebra finch *Tas2r* genes homologous to chicken Tas2r1 in this report, an elevated number of Tas2r does not necessarily result in a larger spectrum of recognized bitter compounds.

It is important to note that bitter taste receptor gene expression is not limited to gustatory tissue in the oral cavity. An ever increasing number of reports suggest that bitter taste receptors fulfill a variety of physiological functions beyond the taste system (Behrens and Meyerhof 2011). Although some of these additional physiological functions may be directly related to the protection of organisms from noxious xenobiotics, for example, bitter taste receptor expression in the respiratory system (Finger and Kinnamon 2011), and hence point to similar defensive roles of bitter taste receptors in oral and extraoral tissues, the expression of bitter taste receptors in brain (Singh et al. 2011; Dehkordi et al. 2012) or the male reproductive system (Li 2013) could be related to different functions and therefore may include the recognition of endogenous ligands or endogenously modified agonists. It is currently impossible to infer the relative contribution of the latter processes to the overall selective pressure that shaped the bitter taste receptor gene repertoires of vertebrates.

If the number of *Tas2r* genes can be so different among vertebrates and, obviously, even a low *Tas2r* gene number is sufficient to guarantee the survival of species, what may then be the benefit of having "more than enough" *Tas2r* genes? A possible answer to this question may come from the observation of naturally occurring TAS2R inhibitors (Brockhoff et al. 2011). These compounds originate from the same plants as bitter agonists, but block the responses of a subset of TAS2Rs, whereas they activate others. It has been hypothesized that organisms having many *Tas2r* genes with overlapping agonist profiles are not subjected to a complete block of their receptors by the presence of bitter antagonists and hence, reduce the incidence of failing recognition of bitter toxins with potentially fatal consequences. This could make chickens and turkeys more prone than humans and frogs to such recognition failures, a hypothesis that should be investigated in the future.

## Materials and Methods

### Bitter Tastants

Absinthin and Parthenolide were gifts from G. Appendino, Novara, Italy. All other chemicals except for Amarogentin (ChromaDex), Limonin (Apin Chemicals), and Quassin (CPS Chemie) were purchased from Sigma-Aldrich.

### Database Mining and Construction of the *Tas2r* Phylogenetic Tree

Amino acid sequences of *Danio rerio*, zebrafish; *Takifugu rubripes*, fugu; *Gasteroceus aculeatus*, stickleback; *Tetraodon nigroviridis*, pufferfish; *X. tropicalis*, Western clawed-frog; *Anolis carolinensis*, Carolina anole, a lizard; *G. gallus*, chicken; *Ornithorhynchus anatinus*, platypus; *Monodelphis domestica*, gray short-tailed opossum; *Mus musculus*, house mouse; and *Homo sapiens*, humans were taken from (Dong et al. 2009) and confirmed in NCBI searches (for accession numbers see supplementary table S1, Supplementary Material online). Sequences for *Motacilla flava*, yellow wagtail; *Carduelis pinus*, Pine Siskin; and *C. cardinalis*, Northern cardinal were taken from (Davis et al. 2010) and likewise confirmed (for accession numbers see supplementary table S1, Supplementary Material online). Tas2r sequences for *P. sinensis*, Chinese softshell turtle; *Dromaius novaehollandiae*, emu; *M. gallopavo*, wild turkey; and *T. guttata*, zebra finch (supplementary table S1, Supplementary Material online) were obtained by recurrent BLAST searches using representative templates from neighboring species. Sequences were aligned by MAFFT algorithm (Katoh et al. 2002), and a phylogenic tree was constructed using a modified maximum-likelihood method (PhyML-aLRT) with subtree pruning and regrafting (SPR) setting for tree optimization and chi-square-based aLRT for branch support (Guindon et al. 2010). The tree was drawn using Treedyn (Chevenet et al. 2006). The teleost fish Tas2r sequences served as outgroup.

### Cloning of Chicken, Turkey, Zebra Finch, and Frog *Tas2r* cDNA

Genomic DNA of chicken (*G. gallus*) was purchased from AMS Biotechnology, genomic DNA from the Western clawed frog (*X. tropicalis*) was prepared from liver tissue using the peqGOLD blood DNA mini kit (peqLAB) according to the manufacturer's protocol. The Tas2r cDNAs of chicken and frog were amplified from genomic DNA using Pfu-DNA-

Polymerase (Promega) (Primers are listed in supplementary table S3, Supplementary Material online) and cloned into the vector pcDNA5FRT (Invitrogen), which was modified to result in the addition of an sst3-tag to the 5′-end and of a HSV-tag to the 3′-end of the receptor cDNA as reported before (e.g., Bufe et al. 2002; Behrens et al. 2004; Meyerhof et al. 2010). The cDNAs of turkey (*M. galloparvo*) Tas2r3 and Tas2r4 as well as zebra finch Tas2r5, Tas2r6, and Tas2r7 were synthesized by Eurofins MWG Operon and subcloned into the same vector described above. The integrity of constructs was confirmed by sequence analyses. The derived amino acid sequence obtained for *X. tropicalis* Tas2r9 deviated from the database entry in several positions and was therefore designated as xtTas2r9a (cf. supplementary table S1, Supplementary Material online).

### Screening of Bitter Compounds

The Tas2r constructs were transiently transfected in HEK 293T cells stably expressing the G protein chimera G$\alpha$16gust44 (Ueda et al. 2003) using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Cultivation of transfected cells and functional calcium imaging analyses were performed as described previously (e.g., Meyerhof et al. 2010). Test substances diluted in C1-buffer were applied to the cells in two different concentrations. The highest compound concentration used for the initial screening was derived from Meyerhof et al. (2010) representing the highest concentration not leading to artificial calcium signals. Additionally, a 10-fold lower concentration was applied. Calcium signals of cells transfected with either Tas2rs or empty vector (negative control) were recorded and compared. All receptor-compound combinations eliciting apparent receptor-dependent cellular responses were subjected to vigorous retesting. Retesting was done using three serial dilutions starting with the highest artifact-free concentration of each candidate agonist per identified responding receptor in at least two independent experiments performed in triplicates. Substances resulting in statistically significant ($P < 0.05$; *t*-test) higher changes in fluorescence in receptor transfected cells compared cells transfected with empty vector (mock controls) were considered as agonists.

### Recording and Calculation of Dose-Response Relations

For selected compound-receptor combinations dose-response relationships were monitored as detailed before (Meyerhof et al. 2010). Briefly, cells were seeded, transfected, and stimulated as described for the screening procedure. The averaged signal amplitudes were plotted against the logarithm of the compound concentrations. Using nonlinear regression analysis the $EC_{50}$-values were calculated using the equation $f(x) = (a-d)/1+((x/EC_{50})^{nh})+d$, with a = maximum, d = minimum, x = agonist concentration, nh = Hill-coefficient. Calculation and plotting was done using the SigmaPlot software (Systat Software Inc.).

## Supplementary Material

Supplementary figures S1 and S2 and tables S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

## Acknowledgments

The authors thank U. Reichelt, E. Schoeley-Pohl, U. Redel, and S. Hannemann for expert technical assistance. This work was supported in part by Deutsche Forschungsgemeinschaft (Ko1046/7-1 to S.I.K.).

## References

Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, Zuker CS. 2000. A novel family of mammalian taste receptors. *Cell* 100: 693–702.

Altner H. 1962. Untersuchungen über Leistungen und Bau der Nase des südafrikanischen Krallenfrosches *Xenopus laevis* (Daudin, 1803). *Z Vergl Physiol.* 45:272–306.

Behrens M, Brockhoff A, Kuhn C, Bufe B, Winnig M, Meyerhof W. 2004. The human taste receptor hTAS2R14 responds to a variety of different bitter compounds. *Biochem Biophys Res Commun.* 319: 479–485.

Behrens M, Meyerhof W. 2011. Gustatory and extragustatory functions of mammalian taste receptors. *Physiol Behav.* 105:4–13.

Born S, Levit A, Niv MY, Meyerhof W, Behrens M. 2013. The human bitter taste receptor TAS2R10 is tailored to accommodate numerous diverse ligands. *J Neurosci.* 33:201–213.

Brockhoff A, Behrens M, Massarotti A, Appendino G, Meyerhof W. 2007. Broad tuning of the human bitter taste receptor hTAS2R46 to various sesquiterpene lactones, clerodane and labdane diterpenoids, strychnine, and denatonium. *J Agric Food Chem.* 55:6236–6243.

Brockhoff A, Behrens M, Niv MY, Meyerhof W. 2010. Structural requirements of bitter taste receptor activation. *Proc Natl Acad Sci U S A.* 107:11110–11115.

Brockhoff A, Behrens M, Roudnitzky N, Appendino G, Avonto C, Meyerhof W. 2011. Receptor agonism and antagonism of dietary bitter compounds. *J Neurosci.* 31:14775–14782.

Bufe B, Hofmann T, Krautwurst D, Raguse JD, Meyerhof W. 2002. The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides. *Nat Genet.* 32:397–401.

Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, Ryba NJ. 2000. T2Rs function as bitter taste receptors. *Cell* 100: 703–711.

Chevenet F, Brun C, Banuls AL, Jacq B, Christen R. 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 7:439.

Davis JK, Lowman JJ, Thomas PJ, ten Hallers BF, Koriabine M, Huynh LY, Maney DL, de Jong PJ, Martin CL, Thomas JW. 2010. Evolution of a bitter taste receptor gene cluster in a New World sparrow. *Genome Biol Evol.* 2:358–370.

Dehkordi O, Rose JE, Fatemi M, Allard JS, Balan KV, Young JK, Fatima S, Millis RM, Jayam-Trouth A. 2012. Neuronal expression of bitter taste receptors and downstream signaling molecules in the rat brainstem. *Brain Res.* 1475:1–10.

Dong D, Jones G, Zhang S. 2009. Dynamic evolution of bitter taste receptor genes in vertebrates. *BMC Evol Biol.* 9:12.

Ellegren H. 2007. Molecular evolutionary genomics of birds. *Cytogenet Genome Res.* 117:120–130.

Finger TE, Kinnamon SC. 2011. Taste isn't just for taste buds anymore. *F1000 Biol Rep.* 3:20.

Freitag J, Krieger J, Strotmann J, Breer H. 1995. Two classes of olfactory receptors in *Xenopus laevis*. *Neuron* 15:1383–1392.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol.* 59:307–321.

Hackett SJ, Kimball RT, Reddy S, Bowie RC, Braun EL, Braun MJ, Chojnowski JL, Cox WA, Han KL, Harshman J, et al. 2008. A phylogenomic study of birds reveals their evolutionary history. *Science* 320:1763–1768.

Imai H, Suzuki N, Ishimaru Y, Sakurai T, Yin L, Pan W, Abe K, Misaka T, Hirai H. 2012. Functional diversity of bitter taste receptor TAS2R16 in primates. *Biol Lett.* 8:652–656.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30:3059–3066.

Kudo K, Shiraishi J, Nishimura S, Bungo T, Tabata S. 2010. The number of taste buds is related to bitter taste sensitivity in layer and broiler chickens. *Anim Sci J.* 81:240–244.

Lagerstrom MC, Hellstrom AR, Gloriam DE, Larsson TP, Schioth HB, Fredriksson R. 2006. The G protein-coupled receptor subset of the chicken genome. *PLoS Comput Biol.* 2:e54.

Li F. 2013. Taste perception: from the tongue to the testis. *Mol Hum Reprod.* 19:349–360.

Matsunami H, Montmayeur JP, Buck LB. 2000. A family of candidate taste receptors in human and mouse. *Nature* 404:601–604.

Mayoral JG, Nouzova M, Brockhoff A, Goodwin M, Hernandez-Martinez S, Richter D, Meyerhof W, Noriega FG. 2010. Allatostatin-C receptors in mosquitoes. *Peptides* 31:442–450.

Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufe B, Appendino G, Behrens M. 2010. The molecular receptive ranges of human TAS2R bitter taste receptors. *Chem Senses.* 35: 157–170.

Mueller KL, Hoon MA, Erlenbach I, Chandrashekar J, Zuker CS, Ryba NJ. 2005. The receptors and coding logic for bitter taste. *Nature* 434: 225–229.

Nouzova M, Brockhoff A, Mayoral JG, Goodwin M, Meyerhof W, Noriega FG. 2012. Functional characterization of an allatotropin receptor expressed in the corpora allata of mosquitoes. *Peptides* 34: 201–208.

Oike H, Nagai T, Furuyama A, Okada S, Aihara Y, Ishimaru Y, Marui T, Matsumoto I, Misaka T, Abe K. 2007. Characterization of ligands for fish taste receptors. *J Neurosci.* 27:5584–5592.

Richard S, Davies DC. 2000. Comparison of methyl anthranilate and denatonium benzoate as aversants for learning in chicks. *Physiol Behav.* 70:521–525.

Roura E, Baldwin M, Klasing K. 2012. The avian taste system: an update. Proceedings of the 23rd Annual Australian poultry science symposium; 2012. Sydney (Australia): Poultry Research Foundation.

Sainz E, Cavenagh MM, Gutierrez J, Battey JF, Northup JK, Sullivan SL. 2007. Functional characterization of human bitter taste receptors. *Biochem J.* 403:537–543.

Shi P, Zhang J. 2006. Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. *Mol Biol Evol.* 23:292–300.

Singh N, Vrontakis M, Parkinson F, Chelikani P. 2011. Functional bitter taste receptors are expressed in brain cells. *Biochem Biophys Res Commun.* 406:146–151.

Syed AS, Sansone A, Nadler W, Manzini I, Korsching SI. 2013. Ancestral amphibian v2rs are expressed in the main olfactory epithelium. *Proc Natl Acad Sci U S A.* 110:7714–7719.

Thalmann S, Behrens M, Meyerhof W. 2013. Major haplotypes of the human bitter taste receptor TAS2R41 encode functional receptors for chloramphenicol. *Biochem Biophys Res Commun.* 435:267–273.

Ueda T, Ugawa S, Yamamura H, Imaizumi Y, Shimada S. 2003. Functional interaction between T2R taste receptors and G-protein alpha subunits expressed in taste receptor cells. *J Neurosci.* 23:7376–7380.

Wooding S, Bufe B, Grassi C, Howard MT, Stone AC, Vazquez M, Dunn DM, Meyerhof W, Weiss RB, Bamshad MJ. 2006. Independent evolution of bitter-taste sensitivity in humans and chimpanzees. *Nature* 440:930–934.

Yoshii K, Yoshii C, Kobatake Y, Kurihara K. 1982. High sensitivity of *Xenopus* gustatory receptors to amino acids and bitter substances. *Am J Physiol.* 243:R42–R48.