In Brief: Based on the analysis of taste receptor genes, Zhao et al. show that penguins have lost sweet, umami and bitter taste.

Supplemental Information

Supplemental Information including supplemental results, discussion and experimental procedures and one figure and one table can be found with this article online at *bxs.

Supplemental Information

Document S1. Experimental Procedures and One figure and one table

Supplemental Information: ***Molecular evidence for the loss of three basic tastes in penguins***

Huabin Zhao, Jianwen Li, and Jianzhi Zhang

SUPPLEMENTAL BACKGROUND

Of the five basic tastes of vertebrates, sweet and umami tastes promote the consumption of nutrition by recognizing sugars and amino acids in food resources, respectively, while bitter and sour tastes tend to be associated with toxins and spoiled food and thus help avoid ingesting harmful compounds. The salty taste results in either consumption or rejection of a potential food item depending on an individual's physical need of salt [S1].

Prior to the identification of the taste receptor genes, our knowledge of animal taste sensations were based exclusively on behavioral studies, which are feasible in only a small number of species. As a result, our understanding of the animal taste variation and its ecological and evolutionary basis was limited. The elucidation of the molecular foundation of taste in the last 15 years allows inferring taste senses of a vertebrate by examining its taste receptor genes. Recent applications of this approach suggest previously unsuspected variations in mammalian taste senses [S2-7]. Compared with mammals, birds are thought to be poor tasters, because of their low numbers of taste buds, lack of mastication, and low saliva secretion [S8-10]. As a result, in spite of a huge dietary diversity among birds [S11], the avian taste system and taste receptor genes have not been well characterized [S8]. Preliminary surveys revealed a general reduction in the number of avian taste receptor genes. For example, the sweet taste receptor gene *Tas1r2* is absent from all bird genomes examined so far [S5, 12, 13] and the Tas2r bitter receptor gene repertoire is diminutive in birds compared with mammals [S14]. However, recent work also showed that all chicken and turkey Tas2rs are broadly tuned receptors that allow the recognition of a wide range of bitter compounds, suggesting that the low number of *Tas2r*s in birds could be compensated by their broad tuning [S15]. It was reported that the ratio between the taste bud number and food load in one bite is likely greater in chicken than in most mammals, suggesting that the avian taste ability is better than commonly appreciated [S8]. Notably, the *Tas2r* gene repertoire has expanded to at least 18 intact members in white-throated sparrow, potentially associated with the radiation of the sparrow subfamily into the New World [S16]. The ancestral umami receptor Tas1r1 was recently reported to have been converted to detecting sugar in the evolution of hummingbirds, which may have facilitated their massive radiation in a nectarivorous niche [S12]. Apparently, the avian taste system has a tremendous variation, especially among species facing different ecological niches.

To further explore the variation in the bird taste system, we choose to study penguins, aquatic flightless birds whose geographic distribution includes the coldest niche on Earth, the Antarctic. Penguins form the order Sphenisciformes, with 18 extant species. They originated in the Antarctica some 40 million years ago [S17] and subsequently dispersed throughout the southern hemisphere, with eight species still living in the Antarctic and sub-Antarctic regions [S18].

SUPPLEMENTAL RESULTS

Sweet taste receptor gene *Tas1r2* is missing in all bird genomes

We started by analyzing high-coverage genome sequences of Adelie penguin (*Pygoscelis adeliae*), emperor penguin (*Aptenodytes forsteri*), and little egret (*Egretta garzetta*) [S19], and 13 publically available genome sequences from non-penguin birds (Fig. 1). Both Adelie and emperor penguins inhabit the Antarctica [S18], while the little egret, belonging to Ciconiiformes, represents a relatively close outgroup of penguins. Using each exon of mouse *Tas1r2* (GenBank accession no. NM_031873) as a query, we undertook BLAST searches to identify *Tas1r2* that encodes the specific subunit of the sweet taste receptor in each of the 16 bird genomes. We could not detect *Tas1r2* in any bird genome, including the two penguins (Fig. 1), although this gene was identified in the genomes of mammals, reptiles, and other vertebrates using similar approaches [S5, 12, 13]. In most mammalian genome sequences surveyed, *Pax7* and *Aldh4a1* are adjacent to *Tas1r2* [S5], but these two genes are now adjacent to each other in the chicken genome [S13]. Using a similar BLAST analysis, we found that *Pax7* and *Aldh4a1* are syntenic in the two penguins and all other birds, suggesting a true loss of *Tas1r2* in all bird genomes rather than incomplete sequencing.

Penguin-specific loss of umami taste receptor gene *Tas1r1*

Using each exon of the chicken *Tas1r1* (GenBank accession no. XM_425740) as a query, we searched for the umami taste receptor-specific gene *Tas1r1* in the 16 bird genomes. In contrast to *Tas1r2*, we could identify *Tas1r1* in both penguins, but in both species it is a pseudogene characterized by a common 2-bp deletion that results in premature stop codons (Fig. S1a). However, *Tas1r1* is intact in all non-penguin bird genomes examined (Fig. 1), suggesting that the pseudogenization of *Tas1r1* is penguin-specific. To confirm this result, we examined three additional penguin species, one species in Gaviiformes, and seven species in Procellariiformes (tubenose seabirds); the latter order is the closest outgroup of penguins [S20]. From these 11 birds with no genome sequences, we attempted to amplify exon 3 (~770 bp) and exon 6 (~600 bp) of *Tas1r1*; together they account for ~60% of the coding region and most of the functional domains. We could amplify and sequence exon 3 from two of the three penguins and seven of the eight outgroup species, and could amplify and sequence exon 6 from only two of the outgroup species. While all amplified sequences from all outgroup species have intact open reading frames (ORFs), the amplified sequences from penguins contain premature stop codons (Fig. S1f). In fact, all four penguins with exon 3 sequences share the aforementioned 2-bp deletion (Fig. S1a), which is predicted to result in the loss of most of the extracellular domain and all transmembrane domains of the receptor. Because these four penguins represent all basal lineages of extant penguins [S17], our finding indicates that the pseudogenization of *Tas1r1* occurred in the common ancestor of all extant penguins since its divergence from tubenose seabirds.

Penguin-specific loss of *Tas1r3*

We next examined *Tas1r3*, which encodes a subunit in both the sweet and umami taste receptors. We found that *Tas1r3* is intact in each non-penguin bird genome examined, but failed to detect this gene in the two penguin genomes. We confirmed that *Gltpd1* and *Dvl1*, which are adjacent to *Tas1r3* in the mouse, chicken, and zebra finch genomes, are adjacent to each other in the two penguin genomes. We attempted to amplify and sequence exon 3 and exon 6 of *Tas1r3*, covering ~70% of its coding region, in the 11 species mentioned above. We could amplify and sequence exon 3 in 5 of the outgroup species and exon 6 in all 8 outgroup species; these segments of the ORF are intact in all of these sequences (Fig. S1f). By contrast, neither exon

was amplifiable in the three penguins (Fig. S1f). These results strongly suggest that *Tas1r3* was lost in the common ancestor of all extant penguins after its divergence from tubenose seabirds (Fig. 1).

Penguin-specific losses of *Tas2r* bitter taste receptor genes

Following a previous study [S13], we used all published bitter taste receptor genes (*Tas2r*s) from mammals and birds as queries to conduct TBLASTN searches in each of the 16 bird genomes. The identified genes were classified into three categories: (i) intact genes, referring to those with a complete and intact ORF; (ii) partial genes, referring to those with a partial coding region resulting from incomplete genome sequencing; and (iii) pseudogenes, which have a disrupted ORF due to premature stop codons or frame-shifting mutations. We detected 1 to 7 intact *Tas2r*s from each of the 14 non-penguin bird genomes (Fig. S1g), suggesting that these birds all possess a sense of bitter taste. The total number of the three categories of *Tas2r*s ranges from 2 to 12 (Fig. S1g). Of note, the little egret has two intact *Tas2rs* and one pseudogene. Strikingly, we did not find any intact or partial *Tas2r* in the two penguin genomes. Instead, we found three *Tas2r* pseudogenes in each penguin (Fig. S1g). A phylogenetic analysis of the nine *Tas2r* sequences from the two penguins and the little egret suggests that they are three one-to-one-to-one orthologs. We aligned the only pseudogene (*Tas2r3*) from the little egret with its orthologs from the two penguins, and observed multiple shared premature stop codons (Fig. S1b), suggesting that this gene was pseudogenized before the divergence between penguins and the little egret.

We next asked when the remaining two *Tas2r* pseudogenes (*Tas2r1* and *Tas2r2*) found in each of the two penguin genomes became pseudogenized. To this end, we attempted to amplify and sequence these two genes from the three additional penguins aforementioned. We found these genes either pseudogenized or unamplifiable in each species (Fig. S1c,d,f). All five penguins examined share a premature stop codon in *Tas2r1* (Fig. S1c, f) that would render most transmembrane domains of the receptor truncated. A common premature stop codon in *Tas2r2* exists in three penguins (Fig. S1d), but this region is missing in the Adelie penguin genome and cannot be amplified from the chinstrap penguin. Given the phylogenetic relationships among the five penguins (Fig. 1), it is most likely that the shared premature stop codon already existed in the common ancestor of all five penguins and that this region was subsequently deleted in the common ancestor of Adelie and chinstrap penguins. Because the premature stop codon would lead to the loss of most functional domains of Tas2r2, we infer that the gene was no longer functional in the common ancestor of all five examined penguins. In the eight outgroup species where we attempted to amplify *Tas2r1* and *Tas2r2*, with one exception, all amplified sequences of the two genes have intact ORFs (Fig. 1; Fig. S1f). The exception is the loon, where both *Tas2r1* and *Tas2r2* are also pseudogenized, apparently independently from the pseudogenization events in penguins (Fig. 1; Fig. S1f). Because the examined penguins represent all basal penguin lineages, our data strongly suggest that the bitter taste function was lost in the common ancestor of all extant penguins after its separation from tubenose seabirds, as a result of the pseudogenization of *Tas2r1* and *Tas2r2*.

Conservation of sour and salty taste receptor genes in penguins

Pkd2l1, a polycystic-kidney-disease-like ion channel, is believed to function as a sour taste receptor [S1], although additional sour receptors likely exist [S21]. The Pkd2l1 gene was detected in each of the 16 bird genomes examined. With one exception, all bird *Pkd2l1* genes

possess an intact ORF ranging between 2,361 and 2,415 bp. The exception is the macaw *Pkd2l1*, which has a partial coding region (1,743 bp) with a start codon but no stop codon, due to incomplete genome sequencing. We calculated nonsynonymous (d_N) and synonymous (d_S) nucleotide distances, and found that the d_N/d_S ratio for *Pkd2l1* is significantly lower than 1 in all pairwise comparisons among the 16 sequences (mean = 0.14, $P < 0.01$, *Z* test). These results strongly suggest that *Pkd2l1* is evolutionarily conserved in all birds examined (Fig. 1).

The three genes *Scnn1a*, *Scnn1b*, and *Scnn1g* encode three subunits of the epithelial sodium channel ENaC, believed to be a salty taste receptor in mammals [S1]. We were able to identify all three genes in each of the 16 avian genomes, and all of the genes are either intact or nearly complete with no premature stop codon detected. Consistently, the average pairwise d_N/d_S ratio for each of the three genes is significantly lower than 1 ($P < 0.01$, Z test).

These results suggest that penguins perceive sour and salty tastes (Fig. 1), but because the receptor genes studied may have other functions and because not all receptors for the two tastes are known, a behavioral test is needed to verify this prediction.

Relaxed selective constraints on umami, sweet, and bitter transduction genes in penguins

In addition to taste receptors, other components of taste signal transduction may also shed light on the evolution of taste perception. Two proteins, Trpm5 (transient receptor potential cation channel subfamily M member 5) and Calhm1 (calcium homeostasis modulator 1), are of particular interest because both are indispensable for umami, sweet, and bitter taste transductions [S22, 23]. Mice deficient for *Trpm5* or *Calhm1* have abolished or severely impaired bitter, umami, and sweet tastes, but have normal sour and salty tastes [S22-24]. Furthermore, these two genes are pseudogenes in at least some whales lacking functional receptor genes for umami, sweet, and bitter tastes [S6]. Plcβ2 (phospholipase C, beta 2) is another protein indispensable for the transduction of the three tastes [S23]. However, this protein also plays roles in the immune system [S25, 26]. In fact, *Plcβ2* was never found to be a pseudogene in any whale lacking the receptor genes for the three tastes [S6]. We thus focused on *Trpm5* and *Calhm1*.

We identified all 24 complete exons of *Trpm5* in 14 non-penguin birds and two penguins. After aligning the sequences, we found neither frame-shifting mutations nor premature stop codons. To investigate the possibility of relaxation of purifying selection in penguins, we analyzed two data sets, and estimated d_N/d_S (termed ω) using a likelihood method (see Supplementary Experimental Procedures). In Dataset I, we included the inferred sequence of the common ancestor of Adelie and emperor penguins, as well as the sequences from the 14 non-penguins. We assumed that all branches have the same ω_0 (model A in Table S1) and estimated that $\omega_0 = 0.197$, indicative of overall purifying selection acting on the gene in birds. When the exterior branch leading to the ancestral sequence of penguins was allowed a separate ω (model B), we found the ω of this branch ($\omega_2 = 0.496$) greater than that of other branches ($\omega_1 = 0.192$) and the likelihood significantly improved compared with that under model A ($P = 0.0037$; Table S1). This result supports the hypothesis that purifying selection on *Trpm5* has been relaxed in the common ancestor of penguins. However, model C, which is identical to model B except that ω_2 is fixed at 1, has a significantly lower likelihood than model B ($P = 0.037$; Table S1), suggesting that the purifying selection is not completely relaxed in the ancestral penguin branch. In Dataset II, we analyzed all 16 bird *Trpm5* sequences. After comparing models D with E (Table S1), we did not observe a significant difference in ω between the ancestral branch of the two penguins and the branches connecting the two penguins ($P > 0.05$), suggesting similar levels of relaxed selection on *Trpm5* before and after the radiation of extant penguins. However, the

relaxation is incomplete, because model D has a significantly higher likelihood than model F, in which the penguin ancestral branch as well as the branches connecting the two penguins all have a fixed ω of 1 (Table S1).

We similarly analyzed *Calhm1*. This gene consists of two coding exons, both of which were identified in each of the 16 avian genomes analyzed. In exon 1 of *Calhm1*, we observed a 2-bp ORF-disrupting insertion in emperor penguin and a nonsense substitution in Adelie penguin, (Fig. S1e). By contrast, in each of the 14 non-penguin birds, *Calhm1* has an intact ORF. To date the pseudogenization of penguin *Calhm1*, we sequenced both exons of *Calhm1* in the three additional penguins mentioned and three of the eight tubenose seabirds mentioned (Antarctic petrel, Northern fulmar, and streaked shearwater). King penguin has the same 2-bp insertion as in emperor penguin (Fig. S1e), but no ORF-disrupting mutations were found in chinstrap and rockhopper penguins (Fig. S1e). The three tubenose seabirds all have intact *Calhm1* genes (Fig. S1e). We next estimated ω values for the gene using the six birds newly sequenced and the 16 birds with genome sequences. After removing the insertion and premature stop codon, we did not find the penguin ancestor to be significantly different from other birds in ω ($P > 0.80$ by comparing models H and G; Table S1). We then tested relaxed selection after the divergence of penguins by comparing models J and I (Table S1). Indeed, we found a significantly higher ω for the branches connecting the five penguins than all other branches in the tree ($P = 3.4\times10^{-6}$; Table S1). These results suggest that the relaxation of functional constraints on *Calhm1* became apparent after the divergence among penguins, although the possibility that the relaxation started in the common ancestor of penguins could not be excluded.

SUPPLEMENTAL DISCUSSION

In this work, we studied taste receptor genes responsible for five basic tastes in penguins and related birds. By examining the genome sequences of emperor and Adelie penguins, we discovered that these penguins lack functional genes for the receptors of the sweet, umami, and bitter tastes, but have intact candidate genes for sour and salty tastes. Note, the possibility of a conversion of the umami receptor Tas1r1-Tas1r3 for sweet detection, reported to have occurred in hummingbirds [S12], does not exist in penguins, because penguins lack the gene (*Tas1r3*) that is indispensable for both umami and sweet tastes. By investigating the genome sequences of 14 other birds and by sequencing the relevant taste receptor genes in three additional penguins and eight species closely related to penguins, we determined that the loss of umami and bitter tastes occurred in the common ancestor of all extant penguins since the divergence from its sister lineage of tubenose seabirds and that the sweet taste was lost much earlier. Consistently, we found that two key genes involved in the signal transduction of the three tastes have undergone functional relaxation (*Trpm5* and *Calhm1*) or even pseudogenization (*Calhm1*) in penguins. Taken together, these results strongly suggest that the sweet, umami, and bitter tastes are absent in all penguins. Although behavioral tests of penguin tastes are lacking, anatomical observations [S27] are consistent with our molecular evidence of a much reduced taste function in penguins.

Why are the sweet, umami, and bitter tastes, especially the latter two, dispensable in penguins? There are several possibilities. The fact that penguins swallow food whole and the structure and function of their tongue [S27] suggest that they need no taste perception, but it is unknown whether these traits are a cause or consequence of their major taste loss. In particular, given that penguins are carnivorous, it seems unlikely that having umami taste would not be advantageous. Of note, the sweet, umami, and bitter tastes rely on the same ion channel, Trpm5,

for signal transduction [S23]. Intriguingly, Trpm5 activity is temperature-sensitive, with lower activities at lower temperatures [S28]. For example, the sweet sensitivity in mice decreases by 3 to 10 fold when the temperature of the sugar water tested drops from 35 to 15°C [S28]. In humans, the bitter taste (but not the sour taste) was also reported to decrease when the temperature drops [S29]. The mean annual temperature of the interior of the Antarctic is -57 °C. Although the coast is warmer, it is still extremely cold. For instance, monthly means at McMurdo Station range from -26 °C in August to -3 °C in January (http://www.coolantarctica.com/). Thus, the working temperature of Trpm5 in ancestral penguins' taste buds was likely close to 0°C. It is probable that Trpm5 is effectively nonfunctional at this temperature, rendering the umami and bitter tastes that rely on this channel unusable (while the sweet taste had long been lost). This hypothesis can be tested in the future by measuring the sensitivities of penguin Trpm5 at relevant temperatures. If our hypothesis is true, one wonders why penguin Trpm5 was not able to adapt to very low temperatures. There are two possibilities. First, the structure of Trpm5 is such that there may be no mutations within a reasonable population size and time frame that could bring Trpm5 functional at such low temperatures. Because the temperature at which a Trp protein is sensitive varies greatly among members of the Trp family [S30], it seems unlikely that no mutation could make Trpm5 sensitive at low temperatures. Furthermore, this hypothesis cannot explain why *Trpm5* remains intact in penguins while *Calhm1* and all relevant taste receptor genes have been pseudogenized. In any case, this hypothesis may be tested by using *in vitro* selection experiments [S31] to examine the possibility and number of mutations needed to make Trpm5 sensitive near 0°C. Second, Trpm5 may have another important physiological function in the body of penguins. Because penguins are warm-blooded, this second function, if it exists, would require Trpm5 to be sensitive at penguin's body temperature (e.g., 39°C in emperor penguins). Due to antagonistic pleiotropy [S32], it may be impossible for the same Trpm5 to function at 39°C as well as near 0°C, given its temperature-dependent property. The antagonistic pleiotropy hypothesis would explain why the ORF of penguin *Trpm5* is still maintained and purifying selection has been relaxed only partially. The possibility that Trpm5 has another function is supported by the finding in mice that Trpm5 regulates glucose-mediated insulin secretion in beta cells [S33, 34]. It appears that this regulation in mice is only partially dependent on Tas1r2 [S33, 35], suggesting that Trpm5's role in insulin secretion may still exist in penguins despite its loss of *Tas1r2*. In addition, mouse *Trpm5* functions in the signal transduction of volatile pheromones detected by the main olfactory epithelium [S36]. These considerations together suggest that the unresolvable antagonistic pleiotropy of *Trpm5* imposed by the extremely cold Antarctic may be responsible for the major taste loss in penguins. Note that the pseudogenization of *Trpm5* in some whales [S6] is not necessarily contradictory to the notion that *Trpm5* has non-taste functions, because the non-taste functions of *Trpm5* may be useless to whales. For instance, the fully aquatic whales have degenerated olfaction [S37], whereas olfaction is critical to penguins for prey and kin recognition [S38, 39]. Thus, transducing volatile pheromone signals by Trpm5 may be unnecessary for whales but important for penguins. The Trpm5 hypothesis predicts that all Antarctic and Arctic vertebrates that have inhabited these cold areas for long enough time should lack the sweet, umami, and bitter tastes, which could be tested in the future.

It is noteworthy that although penguins are not limited to the Antarctic, they originated from the Antarctic [S17]. Recent molecular dating [S40] suggested that the extant penguins radiated ~20 million years ago, after the formation of large ice sheets in the Antarctic 34 to 25 million years ago. If ancestral penguins had lost the receptor genes for the three tastes while in

the Antarctic, the genes and the tastes cannot be regained even when some penguins migrated out of the Antarctic. Considering such historical contingencies is important when making sense of the relationship between the feeding ecology and taste ability among species.

It is noteworthy that Tas1rs and Tas2rs have been proposed to possess non-taste functions [S35, 41]. The fact that they are dispensable in penguins suggests that these functions, discovered mostly in humans and/or mice, are physiologically unimportant in penguins.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Analysis of avian genome sequences

To identify a gene with multiple exons, we used mouse and/or chicken genes as queries to conduct BLAST searches [S42] against the whole genome sequences of 16 birds (Fig. 1). Genome data for the two penguins and the little egret were published elsewhere [S19], the swift genome data were retrieved from GigaDB (http://gigadb.org/), and the draft genomes of the remaining 12 birds were obtained from GenBank with the following assembly IDs: zebra finch (GCA_000 151 805.2), ground finch (GCA_000 277 835.1), ground tit (GCA_000 331 425.1), flycatcher (GCA_000 247 815.2), Amazon parrot (GCA_000 332 375.1), macaw (GCA_000 400 695.1), falcon (GCA_000 337 955.1), hummingbird (GCA_000 699 085.1), dove (GCA_000 337 935.1), chicken (GCA_000 002 315.2), turkey (GCA_000 146 605.2), and mallard (GCA_000 355 885.1). To determine the exon/intron structures, we used BLAST2 and GeneWise [S43] programs to predict the coding regions in the targeted genomic fragments following manual compilation. We used this approach to identify all genes of interest except the single-exon genes *Tas2r*s, which were identified following a previous study [S13]. To ensure the accuracy of gene prediction, all identified genes were used to conduct BLASTP searches against the GenBank and confirmed by their best hits, which must be the known genes of interest [S13].

Polymerase chain reaction (PCR) and DNA sequencing

To amplify *Tas1r1*, *Tas1r3*, *Tas2r1*, *Tas2r2*, and *Calhm1* from three additional penguins, seven tubenose seabirds, and one red-throated loon (Fig. 1), we designed a suite of primers based on the intact forms of relevant genes from the little egret, which is the most closely related species to penguins with available genome data. Two to four pairs of primers were designed to amplify each segment of the targeted genes. The primer sequences for each gene segment are: *Tas1r1* exon 3 (T1E3F1: AGATTAGCTACGAAGCCTC, T1E3R1: GGATAGACTGTGCCTTTGC; T1E3F2: ACCCCTCGTTCCTGCGCACC,T1E3R2: CGAGGCACAGCCCAGCAGGT), *Tas1r1* exon 6 (T1E6F1: AGCGAAGCTTGCTTCAACCG, T1E6R1: AGTGCTGCGAGCGTGCCCA; T1E6F2: CTGTCCTCCTCCTGCTGCTCA; T1E6R2: CTCAGGTGGAGCAGCAGGC; T1E6F3: GGAGCGAAGCTTGCTTCAACCG, T1E6R3: GCGCGTGTACTCCTGGAT), *Tas1r3* exon 3 (T3E3F1: AGGTCAGCTATGGAGCCAG, T3E3R1: ACCCCAGCGCTCTGTGCAG; T3E3F2: GAGTTTGGATGGAACTGGAT, T3E3R2: GCGCGTAAGCCACGCTGTA), *Tas1r3* exon 6 (T3E6F1:AGACAGCTCCACCTGCACTCC, T3E6R1: AAATAATCCACTGTGTTCA; T3E6F2: ACAGCACCAGCTGCTCCCC, T3E6R2: GTTTATTGCCTGTCCTGCTCTGG; T3E6F3: TCCCTGCCTAGAGCATCAGT, T3E6R3: AGAGTCCTCCTCTGGCTCCT; T3E6F3: TCCCTGCCTAGAGCATCAGT, T3E6R4: TTTATTGAGAGTCCTCCTCTGG); *Tas2r1* (PET2R1F1: ATGGATGCTTGTTACTCTCA, PET2R1R1: CTACCTCACGCAAACTTTACAC; PET2R1F2: ATTTAATGCCACTTCATAC, PET2R1R2: ACGCAAACTTTACACTTC; PET2R1F3:

GGATGCTTGTTACTCTCAAGATAAAT, PET2R1R3: ACACTTCACACAGGGCAGAG); *Tas2r2* (PET2R2F3: AATAAGCTTTGTAGCTATTGAAGT, PET2R2R3: TGAGGCATAAAGTCATATTTTCCA; PET2R2F4: TTGTTGGTTTTATTGGAAATGG, PET2R2R3: TGAGGCATAAAGTCATATTTTCCA). Avian tissues were acquired from the Museum of Zoology, University of Michigan. The genomic DNAs were purified using the Qiagen DNeasy kit. PCRs were conducted in a 30μl reaction mixture, which contained 0.5μl genomic DNA (10 ng/μl), 15μl of 2×PCR solution (Takara Premix Taq^{TM}), and 1μl of each primer (10 μM). All PCRs were carried out on a BioRad T100 Thermal Cycler, with the following cycling parameters: an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at a temperature gradient from 50°C to 55°C for 30 s, extension at 72°C for 60 s, and a final elongation at 72°C for 5 min. All PCR products were purified and sequenced directly in both forward and reverse directions by Sanger sequencing. Newly generated sequences were submitted to GenBank under accessions KP121467-KP121504.

Evolutionary analysis

The DNA sequences were aligned based on protein sequence alignment by MUSCLE [S44] with manual adjustments. We applied the modified Nei-Gojobori method [S45] to calculate d_S and d_N and their variances between pairs of DNA sequences. We also analyzed the variation in ω (nonsynonymous to synonymous rate ratio) along a bird phylogeny (Fig. 1) as well as the action of purifying selection using PAML [S46]. A likelihood ratio test was employed to compare nested models. We inferred ancestral gene sequences using PAML [S46].

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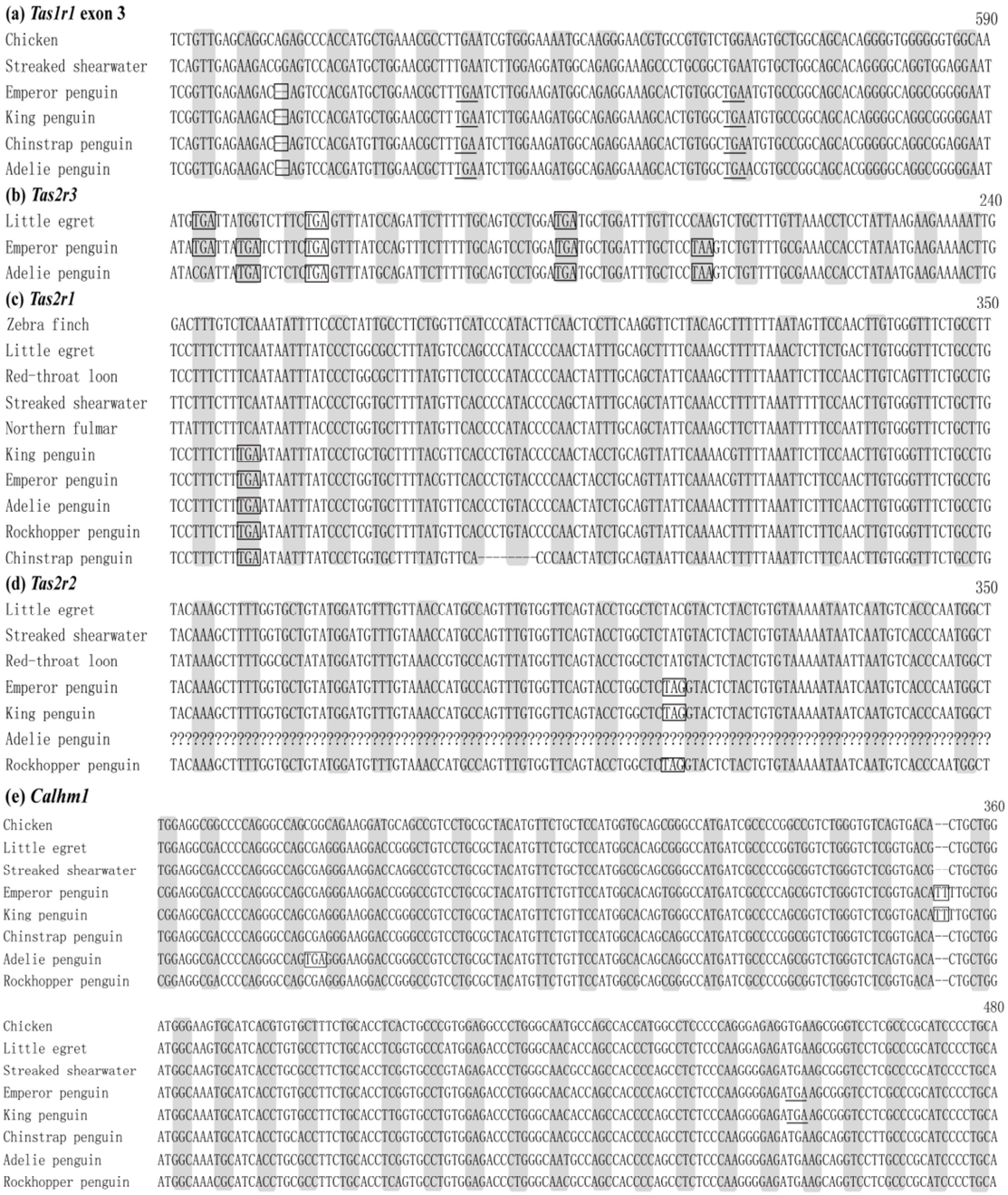

(f) Taxa and genes examined

	Tas1r1 Exon 3	*Tas1r1* Exon 6	*Tas1r3* Exon 3	*Tas1r3* Exon 6	*Tas2r1*	*Tas2r2*
Adelie penguin	S		M	M	S	
Chinstrap penguin	S				S	
Rockhopper penguin					S	S
Emperor penguin	S		M	M	S	S
King penguin	S				S	S
Antarctic petrel	+			+		+
Northern fulmar	+			+	+	+
Manx shearwater	+		+	+		+
Streaked shearwater	+	+	+	+	+	+
Bonin islands petrel	+			+		+
Leach's storm petrel			+	+		
Black-footed albatross	+	+	+	+		+
Little egret	+	+	+	+	+	+
Red-throated loon	+		+	+	S*	S*

(g) Numbers of Tas2rs in avian genomes

	Intact gene	Partial gene	Pseudo-gene	Total
Adelie penguin	0	0	3	3
Emperor penguin	0	0	3	3
Little egret	2	0	1	3
Zebra finch	7	0	1	8
Ground finch	6	3	2	11
Ground tit	4	3	1	8
Flycatcher	5	0	4	9
Amazon parrot	2	0	0	2
Macaw	2	0	0	2
Falcon	2	0	0	2
Hummingbird	6	4	2	12
Swift	4	2	0	6
Dove	1	0	0	1
Chicken	3	0	0	3
Turkey	2	0	2	4
Mallard	2	2	0	4

Fig. S1. Taste receptor and transduction genes in penguins and selected outgroups. (**a**) Alignment of *Tas1r1* exon 3. (**b**) Alignment of *Tas2r3*. (**c**) Alignment of *Tas2r1*. (**d**) Alignment of *Tas2r2*. (**e**) Alignment of *Calhm1*. In all alignments, frame-shifting mutations and nonsense mutations are boxed, whereas premature stop codons resulting from frame-shifting mutations are underlined. The correct reading frames in functional receptors are indicated by shading. Dashes denote alignment gaps, whereas question marks indicate unknown nucleotides due to incomplete genome sequencing. Numbers after the alignments indicate alignment positions following the first sequences. **(f)** Taxa and genes examined. "+" indicates intact sequence, "M" represents missing data from the genome sequence, "S" stands for pseudogenization by shared premature stop codons, "S^*" indicates unshared premature stop codons, and genes or exons without any sign were not sequenced. The lengths of *Tas1r1* exon 3, *Tas1r1* exon 6, *Tas1r3* exon 3, *Tas1r3* exon 6, *Tas2r1*, and *Tas2r2* that we sequenced in birds are approximately 770, 600, 730, 850, 940, and 630 nucleotides, respectively. The bird phylogeny follows [S20]. (**e**) Numbers of Tas2r bitter taste receptor genes and pseudogenes in avian genomes examined in this study. An intact gene has the complete coding region and an intact open reading frame; a partial gene has an intact open reading frame but with partial coding region resulting from incomplete genome sequencing; and a pseudogene has a disrupted open reading frame due to a premature stop codon or fame-shifting mutation. The bird phylogeny follows [S20]. See also Fig. 1.

Table S1. Likelihood ratio tests of selection in the evolution of avian taste transduction genes *Trpm5* and *Calhm1*. Significant *P* values (<0.05) are italicized. See also Fig. 1.

Datasets and models	**ω (d_N/d_S)**	**Comparisons**	***P* values**
Data set I: 15 *Trpm5* sequences (14 non-penguins plus the ancestral sequence of two penguins)			
A. All branches have the same ω_0	ω_0=0.197		
B. Ancestral branch of two penguins has ω_2 and other branches have ω_1	ω_1=0.192, ω_2=0.496	B vs. A	*0.0037*
C. Ancestral branch of two penguins has a fixed ω_2=1; other branches have ω_1	ω_1=0.193, ω_2=1	B vs. C	*0.0368*
Data set II: 16 *Trpm5* sequences (14 non-penguins plus two penguins)			
D. Ancestral branch of two penguins and branches connecting two penguins have ω_2, whereas other branches have ω_1	ω_1=0.194, ω_2=0.321		
E. Ancestral branch of two penguins has ω_3, branches connecting two penguins have ω_2, and other branches have ω_1	ω_1=0.194, ω_2=0.197, ω_3=0.489	E vs. D	0.0563
F. Ancestral branch of two penguins and branches connecting two penguins have a fixed ω_2=1; other branches have ω_1,	ω_1=0.195, ω_2=1	F vs. D	1.6×10^{-6}
Data set III: 18 *Calhm1* sequences (17 non-penguins plus the ancestral sequence of five penguins)			
G. All branches have the same ω_0	ω_0= 0.098		
H. Ancestral branch of five penguins has ω_2, and other branches have ω_1	ω_1=0.097, ω_2= 0.115	H vs. G	0.8007
Data set IV: 22 *Calhm1* sequences (17 non-penguins plus five penguins)			
I. All branches have the same ω_0	ω_0=0.111		
J. Branches connecting five penguins have ω_2, and other branches have ω_1	ω_1=0.098, ω_2=0.322	J vs. I	3.4×10^{-6}