Olfactory receptor gene evolution is unusually rapid across Tetrapoda and outpaces chemosensory phenotypic change

Laurel R. Yohe*, Matteo Fabbri, Michael Hanson and Bhart-Anjan S. Bhullar*

Department of Earth & Planetary Science, Peabody Museum of Natural History, Yale University, New Haven, CT, 06511, USA

*Address correspondence to Laurel Yohe and Bhart-Anjan Bhullar. E-mails: laurel.yohe@yale.edu (L.Y.); bhart-anjan.bhullar@yale.edu (B.-A. B.)

Handling editor: Martha Muñoz

Received on 4 May 2020; accepted on 28 August 2020

Abstract

Chemosensation is the most ubiquitous sense in animals, enacted by the products of complex gene families that detect environmental chemical cues and larger-scale sensory structures that process these cues. While there is a general conception that olfactory receptor (OR) genes evolve rapidly, the universality of this phenomenon across vertebrates, and its magnitude, are unclear. The supposed correlation between molecular rates of chemosensory evolution and phenotypic diversity of chemosensory systems is largely untested. We combine comparative genomics and sensory morphology to test whether OR genes and olfactory phenotypic traits evolve at faster rates than other genes or traits. Using published genomes, we identified ORs in 21 tetrapods, including amphibians, reptiles, birds, and mammals and compared their rates of evolution to those of orthologous non-OR protein-coding genes. We found that, for all clades investigated, most OR genes evolve nearly an order of magnitude faster than other protein-coding genes, with many OR genes showing signatures of diversifying selection across nearly all taxa in this study. This rapid rate of evolution suggests that chemoreceptor genes are in “evolutionary overdrive,” perhaps evolving in response to the ever-changing chemical space of the environment. To obtain complementary morphological data, we stained whole fixed specimens with iodine, μCT-scanned the specimens, and digitally segmented chemosensory and nonchemosensory brain regions. We then estimated phenotypic variation within traits and among tetrapods. While we found considerable variation in chemosensory structures, they were no more diverse than nonchemosensory regions. We suggest chemoreceptor genes evolve quickly in reflection of an ever-changing chemical space, whereas chemosensory phenotypes and processing regions are more conserved because they use a standardized or constrained architecture to receive and process a range of chemical cues.

Key words: chemosensation, diversifying selection, olfaction, olfactory bulb, olfactory receptor, tetrapod
the planet explains why chemosensation is so ubiquitous and so important to animals (indeed, to all life forms). However, the overwhelming complexity of chemical backgrounds, chemical signals, and chemoreceptors causes olfaction to remain one of the most poorly understood senses (Hayden and Teeling 2014). Detecting adaptive signatures and gaining a functional understanding of the molecular and phenotypic basis of chemosensation are monumental challenges in comparative biology. With the advent of improved sequencing approaches and morphological imaging techniques, exploration of the convoluted “tangled bank” of chemosensory diversity is becoming tractable.

Chemosensation in the tetrapod nose is performed by two systems: the main olfactory and the accessory vomeronasal. The main olfactory system is considered to be devoted primarily to the detection of chemical cues related to diet and environment (Jørgensen 2000; Fleischer et al. 2018), while vomeronasal olfaction is associated with social chemical signaling (Liberles 2014; Stowers and Kuo 2014). It has become increasingly clear that these systems are not mutually exclusive (Suárez et al. 2012). The neural signaling detection mechanisms of both systems are similar: a chemical odorant molecule binds to a single-receptor-expressing neuron, triggers depolarization, and sends a signal to converge with others in the olfactory bulb, where integration and interpretation occur (Bear et al. 2016). The two systems mainly differ in the chemoreceptors they express. The primary receptors in the main olfactory system are encoded by genes within the Olfactory Receptor (OR) and trace-amine associated receptor (TAAR) gene families and those in vomeronasal olfaction by vomeronasal-specific receptors (e.g., V1Rs or V2Rs) (Grus 2008; Brykczyńska et al. 2013; Bear et al. 2016; Eyun et al. 2016). The focus of this study is on OR genes, owing to their homology across tetrapods (and beyond), but also because many of our findings are relevant to all chemoreceptor families.

Irrespective of gene family, chemoreceptors are encoded by genes that evolve via birth–death evolution (Nei and Rooney 2005). This process is one in which genes are constantly tandemly duplicating through time. Duplicate genes accumulate novel mutations that lead either to pseudogenization (lost function) or neofunctionalization (new function) (Pegueroles et al. 2013; Yohe, Liu, et al. 2019). In the case of chemosensory receptor genes, many duplicated receptors that accumulate neofunctional mutations evolve via diversifying selection, in which new mutations lead to new odorant detection profiles (Nei et al. 2008). The birth–death process is exceptional in ORs—duplications have led to hundreds, and sometimes thousands, of accumulated copies (Niimura 2009, 2012; Niimura et al. 2014). ORs are multigene family members that encode G-protein-coupled receptors expressed in chemosensory epithelial tissue to detect odorant molecules from the environment. ORs are short, intronless ~900 base pair (bp) genes that evolve primarily by tandem gene duplication (Young and Trask 2002; Young et al. 2002). They are the largest gene family in the mammalian genome and compose ~5% of the protein-coding genes (Nei et al. 2008). While most OR work has been performed on mammals (Hayden et al. 2010; Niimura et al. 2014), Classes I and II receptor genes are also present in reptiles (Brykczyńska et al. 2013), birds (Steiger et al. 2008; Khan et al. 2015), and amphibians (Ji et al. 2009), as well as some fish (Zhang and Firestein 2009; Bear et al. 2016). Identifying the ligand of receptors is notoriously difficult, as the relationship of odorant to receptor is not one-to-one, and activation of odor recognition is combinatorial (Malnic et al. 1999). As a result, only a handful of receptors have had their ligands identified, and evidence is almost exclusive to model organisms (Nara et al. 2011).

The morphological structure devoted to making sense of the labyrinth of thousands of the OR-expressing sensory neurons is the olfactory bulb. It does so with astounding precision (Zou et al. 2009). In the rostral most portion of the forebrain, the olfactory bulb is organized into spheroid synaptic concentrations called glomeruli. All neurons expressing the same OR (Monahan and Lomvardas 2015), no matter the distances among them in the epithelia, will coalesce into the same glomerulus (Zou et al. 2009). In other words, hundreds of thousands of neurons deliver their sensory input into mere hundreds of cells. The number of glomeruli is correlated with the number of distinct receptors (Bressel et al. 2016), and their expression varies synchronously throughout ontogeny (Hinds and McNelly 1981). The parallels are even documented in bone, such that the number of foramina through which these bundles of olfactory sensory neurons are threaded correlate with OR repertoire size in mammals (Bird et al. 2018).

Both the molecular and morphological effectors of olfactory detection are observed from cyclostomes to mammals (Baier and Korschning 1994; Saraiva et al. 2015), suggesting over 450 million years of evolutionary conservation. Simultaneously, the individual OR genes are some of the fastest evolving within the genome. However, because the number of odor-encoding cells in the olfactory bulb is inherently governed by the receptors that their receiving sensory neurons express, we might expect synchronous patterns of evolution throughout tetrapod diversification. Here we compare the rates of evolution of OR gene repertoires and the morphological disparity of different brain regions in every major tetrapod clade. We aim in particular to quantify evolutionary rates of OR genes beyond mammals to test whether rapid evolution of OR genes is a ubiquitous tetrapod phenomenon. We compare these results to phenotypic evolution of sensory brain regions to test whether olfactory bulb morphology is evolving differently from that of other brain regions and whether olfactory bulb disparity is greater than that expected under Brownian motion. Given the tight link between OR gene repertoire and cellular morphology of the olfactory bulb, it might be predicted that rates of OR gene evolution will show significantly higher diversity than non-OR genes, and morphological disparity of the olfactory bulb should be significantly greater than that of other brain regions throughout tetrapod evolution.

**Materials and Methods**

**Approach**

We compared the ORs from 21 taxa for which genomes and soft-tissue μCT-scans were available. From the genomes, we identified ORs and chemosensory-related genes. To test whether OR genes were evolving faster than nonolfactory genes on the one hand and simulated genes on the other, we inferred gene trees and estimated rates of evolution for (1) each subfamily of ORs across species including all duplicate copies; (2) orthologs of non-OR genes; and (3) simulated genes evolved under different selection pressures across the tetrapod tree. We then used soft tissue μCT-scans of complementary specimens and reconstructed brain regions associated with sensory and nonsensory function. We modeled the disparity of the structures through time to observe whether their patterns of change were significantly different from those expected under Brownian motion.
**Genome data**

The following genomes were sampled: axolotl (Ambystoma mexicanum: GCA_002915635.2), 2-lined caecilian (Rhinatremra biwattum: GCF_901001135.1), common snapping turtle (Chelydra serpentina: GCA_007922165.1), American alligator (Alligator mississippiensis: GCF_00028125.3_ASM28125v4), saltwater crocodile (Crocodylus porosus: GCF_001723895.1), Chilean tinamou (Nothoprocta perdicaria: GCF_003342845.1), greater rhea (Rhea americana: GCA_003343005.1), red jungle fowl (Gallus gallus: GCF_000002315.6), Japanese quail (Coturnix japonica: GCF_001577835.1), great cormorant (Phalacrocorax carbo: GCF_000708925.1_ASM70892v1), Okinawa rail (Gallirallus okinawai: GCA_002003005.1), zebra finch (Taeniopygia guttata: GCF_003957565.1), tuataras (Sphenodon punctatus: GCA_003113815.1_ASM311381v1), red corn snake (Pantherophis guttatus: GCA_001185365.1), green anole (Anolis carolinensis: GCF_000090745.1), ocelot gekko (Paroedura picta: GCA_003118565.1), platypus (Ornithorhynchus anatinus: GCF_004115215.1), gray short-tailed opossum (Monodelphis domestica: GCF_000002295.2), house mouse (Mus musculus: GCF_000001635.26), and common vampire bat (Desmodus rotundus: GCA_002940915.2).

**OR identification**

While there are several chemosensory gene families present in the genome, the OR Classes I and II genes are well known to be shared among vertebrates (Young and Trask 2002; Hayden et al. 2010) and are the focus of this study. Protein sequences of ORs from mammals (Hayden et al. 2010), as well as published sequences from squamates and birds (Steiger et al. 2008, 2009; Khan et al. 2015) were used as queries and were blasted using the protein query-translated subject BLAST version 2.10.0+ (tblastn) program (Altschul et al. 1990; Gerts et al. 2006). Hits >100 bps and a score of at least 0.2 were converted to a gff format using the blast2gff.py script within the genomeGTFtools toolkit (Mills et al. 2018). Hits were then pulled out of the genome using the getfasta program within bedtools version 2.29.2 (Quinlan 2014). Duplicate hits and contaiements were removed using the dedupe.sh script within BBTools bioinformatics suite (https://sourceforge.net/projects/bbmap/). Identified receptor genes were then annotated according to previously published methodology (Yohe et al. 2019). Genes with <650 bp open reading frames or genes with premature stop codons were filtered as pseudogenes. Within each of these two families are several subfamilies that receptors are binned in based on their conserved motifs of their protein-coding regions. Class I genes can be classified as either OR51, OR52, OR53, or OR56, and Class II genes can be classified as either OR13/37, OR2/13, OR4, OR5/89, OR6, OR10, OR11, OR12, or OR14. In brief, receptors were identified using the ORA version 1.9.1 (Hayden et al. 2010), a Bisperl (version 1.6.924) program that makes profile motifs of aligned amino acid sequences collected from previously published ORs. It implements HMMER version 3.1b (Eddy 2011), which uses hidden Markov models to identify and classify the gene sequences with significant hits to the sequence motif profiles. This pipeline is one of the 2 major ways that OR sequences are identified in the genome, has been used in many studies, and has been shown to be robust against false positives (Hayden et al. 2010).

**Non-olfactory gene identification**

To compare rates of evolution of other parts of the protein-coding genome to those of ORs, we selected 50 random loci to identify across our targeted set of species. Random genes were selected from the random gene set generator of the M. musculus genome (http://www.molbiotools.com/randomgenesetgenerator.html). If the gene was present as an ortholog in amphibians, birds, and reptiles, then we identified the corresponding RefSeq from the NCBI Ortholog catalog. Non-OR genes included a selection of kinases, opsins, transcription factors, among others. A list of the selected gene subset is listed in the Supplementary Table S1. Open reading frames were identified using the getorf function of EMBOSS version 6.6.0.0 (Rice et al. 2000).

**Alignment and phylogenetic inference**

For each olfactory subfamily and non-OR gene set, genes were aligned using transAlign (Bininda-Emonds 2005) that implements the FFT-NS-2 algorithm MAFFT version 7.388 for the protein alignments (Katoh and Standley 2013). A BLOSUM62 matrix was used with a gap open penalty of 1.53 and an offset value of 0.123. Sequences were inspected for misalignment and stop codons were removed from the OR alignments. ModelOMatic version 1.01 (Whelan et al. 2015) was used to estimate the best-fit codon model and nucleotide model. Gene trees were inferred using IQ-TREE version 1.6.11 (Nguyen et al. 2015).

**Species tree**

An ultrametric species tree was essential for the molecular evolution simulations and for the phenotype analyses. A species tree was grafted from several published phylogenetic trees. Using first a general vertebrate tree (Uyeda et al. 2017) as a backbone, an amphibian tree (Jetz and Pyron 2018), a squamate tree (Tonini et al. 2016), a turtle tree (Pereira et al. 2017) that contained a grafted bird tree (Kimball et al. 2019) and both A. mississippiensis and C. porosus binded on at divergence times from timetree.org (Kumar et al. 2017), and a mammal tree (Upham et al. 2019) were carefully grafted using the ape version 5.3 (Paradis et al. 2004) package in R version 3.6.1 (Team RC 2016). The sumtrees.py script implemented through DendroPy version 4.0.3 (Sukumaran and Holder 2010) was used to summarize the distribution of trees from vertlife.org. The tree was trimmed to match the data using the treedata() function in geiger version 2.0.6.2 (Harmon et al. 2008).

**Molecular simulations**

To make predictions for genes evolving under purifying or diversifying selection for this set of taxa, we simulated genes mimicking the codon frequencies and sequence length of ORs. In molecular evolution at the species level (i.e., assuming the substitution is fixed within the lineage), the strength of selection is characterized by the Ω statistic, representing the ratio of rates of codon substitutions to nonamino acid-changing nucleotide substitutions (Mugal et al. 2014). Because codon substitutions change the amino acid and may have functional implications to protein function, under purifying selection, the rate of codon substitution is expected to be low (Ω < 1) relative to nucleotide substitutions that do not change the protein. Under diversifying or positive selection, selectively advantageous protein-coding changes may rapidly fix (Ω > 1). Using the evolveNSbranchsites evolve program in paml version 4.8 (Yang 2007), we simulated 100 alignments of 900 bp each and “evolved” each sequence along the grafted species tree using codon frequencies and the estimated transition/transversion ratio (2.74592) observed from the OR56 gene family. Three evolutionary scenarios were modeled: (1) genes under strong purifying selection, in which the
entire alignment (i.e., single-site class) is evolving at a rate of $\omega = 0.1$, where the rates of codon-changing substitutions are much lower than synonymous substitutions; (2) genes under weak purifying selection, in which the entire alignment is evolving at a rate of $\omega = 0.01$, where the rates of codon-changing substitutions are an order of magnitude higher than Scenario 1; and genes evolving under diversifying selection (i.e., positive selection), in which the 99% of the alignment evolves under strong purifying selection ($\omega = 0.01$), and 1% of the codon sites are under positive selection ($\omega = 2.5$). $\omega$ is a useful statistic at long time scales, but as divergence time approaches $\infty$, $\omega$ values approach zero (Mugal et al. 2014). Thus, at our long time scales (~400 million years), we inferred the branch lengths of a codon substitution gene tree and compared these lengths to those of a nucleotide substitution gene tree, which contains both codon and non amino acid-changing substitutions. These values were calculated for both simulations and empirical data to provide comparable measures of evolutionary change for both simulations and observed data. A codon substitution gene tree and a nucleotide substitution gene tree were estimated using IQ-TREE under Codon+F3X4 and GTR+gamma models, respectively, for all simulations. Cumulative branch lengths were read into R and plotted with the empirical data.

**Comparisons of rates of molecular evolution**

To determine whether ORs were evolving at faster rates than non-OR genes and to gauge the comparable strength of selection observed from the simulations, we compared rates of nucleotide substitution to rates of codon substitution. To quantify the rates of nucleotide and codon substitution, we measured the total amount of accumulated change per gene for each of the 2 tree types by quantifying the cumulative branch lengths of each gene. To calculate the cumulative branch lengths, the diagonals of the eigenvectors of the variance–covariance matrix of each tree were quantified, which measures the height of each node (i.e., gene) that represents the rate of substitution per gene (Yohe and Dávalos 2018). These values were extracted for all observed genes and gene trees inferred from the simulated alignments. To understand the strength of selection for each of the observed groups, we tested whether slopes differed among the observed ORs (Classes I and II), non-OR genes, and the three simulation scenarios. If the slopes did not significantly differ, then that particular evolutionary scenario was determined to be a good fit for a majority of the genes within that class. To quantify differences among rates observed for we used a linear regression formula to quantify the slope of the following model:

$$\text{codon, } \sim \text{nucleotide, class,}$$

where codon is the codon gene tree branch length and nucleotide is the nucleotide gene tree branch length for each gene (or simulated gene) $i$. We tested for whether there was a difference in slopes among $j$ “classes” using class as the interaction term. Classes included Class I ORs, Class II ORs, non-OR genes, simulated genes under strong purifying selection, weak purifying selection, and diversifying selection ($j = 6$). Models were implemented using the lsmeans package in R (Lenth 2016). $P$-values were adjusted for multiple comparisons using the Tukey test. Candidate genes under diversifying selection were determined if the ratio of the branch lengths of the codon gene trees to the branch lengths of the nucleotide gene trees fell within 1 standard deviation of the mean of the ratios obtained from the gene trees inferred from diversifying selection simulations.

**Specimen staining and soft tissue μCT-scanning**

Most taxa in our dataset were museum specimens fixed in 10% formalin and stored in 70% ethanol. For new soft-tissue data generated within this study, specimens were stained in solutions of either 5% phosphomolybdic acid (H3PMo12O40, a.k.a., “PMA”) or 10% I2KI (Lugol’s iodine) for 2–52 weeks, depending on the stain and specimen size. Specimens were scanned using the ultra-high-resolution Nikon H225 ST μCT-scanner at Yale University or the X-Tek HMXST Micro-CT imaging system at Harvard University. Additional μCT-scans of soft tissue were obtained from MorphoSource or from previously published studies (Fabbri et al. 2017). Supplementary Table S2 lists all specimens, details for each specimen, stains used for the respective specimen, and parameters under which the specimen was scanned. Exact species matches to their respective genome were available for each taxon, with the exception of the Okinawa rail, tinamou, and the great cormorant. As substitutes, we used *Nothoprocta pentlandii* (Andean tinamou), *Porzana carolina* (sora), and *Phalacrocorax auritus* (double-crested cormorant), respectively. Raw scan data were reconstructed using on-site Nikon reconstruction software and imported into VGStudio Max version 3.3 for segmentation (Volume Graphics GmbH 2014). For each scan, the following brain and sensory structures were segmented (Figure 1): olfactory bulb, optic nerves, labyrinth/semicircular canal, thalamus, cerebrum, medulla, midbrain, and cerebellum. We binned the following structures into “sensory” structures: olfactory bulb, optic nerves, and semicircular canal as these structures receive primary sensory input. The remaining segmented structures were considered “nonsensory” as they are either not involved in sensory processing or are involved at a secondary stage. Brain regions were segmented following best practices previously established by the field (Balanoff et al. 2016). Segmented regions of interest were converted into surface files and volumes of each surface were extracted within VGStudio. While the absolute values of the volumes may be susceptible to segmenting error and tissue defects (e.g., shrinkage Hedrick et al. 2018), biases should be similar across specimens. Moreover, we visually confirmed that brains in the specimens used filled the expected amount of the endocranial space.

**Phenotypic statistical analyses**

To infer the phenotypic disparity of different brain regions through time, we used the extracted volumes of each segmented brain region per species and scaled it by the total volume of all segmented brain regions for size. We calculated the disparity of the entire brain, each brain region scaled by total brain volume, and each sensory and nonsensory module using the dtt() function in the geiger package in R. The calculated morphological disparity index (MDI) is a measure computed from the average squared Euclidean pairwise distances between species (Harmon et al. 2003; Slater et al. 2010). Values less than zero indicate lower trait disparity within clades than expected under Brownian motion, and vice versa for positive MDI values. We performed 10,000 simulations to represent a null distribution of disparity of a trait evolving via Brownian motion for this group of taxa (Slater et al. 2010). *Coturnix japonica* and *C. serpenina* were removed from the morphological analyses, as these specimens were too young to reliably infer comparative volumes. We also estimated rates of trait evolution and tested whether a Brownian motion model of evolution or a model incorporating Pagel’s $\lambda$ was a better fit for each respective trait using the momot package in R (Puttick et al. 2020). Pagel’s $\lambda$ is a measure of phylogenetic signal that falls between 0 and 1; when $\lambda$ is 1, the trait evolving under Brownian motion and when $\lambda$ is 0, there is no phylogenetic signal and the trait
may be evolving under a different process than expected under Brownian motion.

Results

OR identification

We observed substantial variation in the number of intact ORs across tetrapods (Figure 1). Numbers of copies for both Classes I and II ORs varied by orders of magnitude, with the snapping turtle having the largest number of Class I ORs \((n = 789)\) and the anole having only a single intact copy. For Class II ORs, the caecilian \((n = 1,552)\) had the largest number, while the zebra finch had the smallest \((n = 7)\). Average GC content for alignment of different OR subfamilies ranged from 44.7 to 53.9\%. Average GC content for non-OR genes ranged from 41.0 to 64.5\%. Supplementary Table S3 shows the results for number of recovered genes per subfamily, alignment statistics, GC-content, and estimated best-fit models of evolution.

Rates of molecular evolution

Class II OR genes evolve at order of magnitude higher rates than non-OR genes, and both Classes I and II OR genes have numerous genes that overlap with the predicted scenarios consistent with diversifying selection (Figure 2). Figure 2A shows the outcomes of the three simulated evolutionary scenarios compared to those estimated for both ORs and non-OR genes. The linear model resulted in an intercept of 1.31 (standard error: 0.37). For the simulated evolutionary scenarios diversifying selection yielded the steepest slope (mean: 18.81; 95\% confidence intervals: [18.57–19.05]), strong purifying selection (2.58 [2.41–2.76]) the lowest, and weak purifying selection in between (4.09 [3.83–4.35]). All three slopes differed from each other significantly (Figure 2B and Table 1), confirming that diversifying selection currently simulates high rates of codon substitution relative to nucleotide substitution and can be used for comparisons to empirical data. Among the classes of genes in the genome, Class II ORs had the highest slope (4.1 [3.85–4.35]) and differed significantly from Class I and non-OR genes (Table 1). The slope of Class I genes (1.92 [1.11–2.74]) did not differ from that of non-OR genes (2.12 [1.45–2.78]; Table 1), but some outliers still reflect rates of diversifying selection. Figure 2B compares the slopes of different observed scenarios to the slopes of the simulated scenarios. For Class II OR genes (interaction term: 0.01; \(t\)-ratio = 0.032; \(P\)-value = 1; Table 1), the slope did not significantly differ from that of simulated weak purifying selection (\(\omega = 0.1\)). For both Class I OR and non-OR genes, strong purifying selection (\(\omega = 0.01\)) was the best fit for each class.

Candidate genes under diversifying selection

Of the 11,164 genes observed in the genome (both OR and non-OR), 1,158 of these genes had high enough ratio of rates of codon to nucleotide substitutions to be classified as candidates for genes experiencing diversifying selection (Figure 2C). Only two of these genes were non-OR genes (Celf2 in M. musculus [NM_001110228.1] and Tspan6 in O. anatinus [XM_029067969.1]). The remaining identified genes were ORs. With the exception of P. carolina and
M. domestica, diversifying selection in ORs was identified in every taxon, though some taxa had higher proportions of OR genes under diversifying versus purifying selection (Table 2 and Figure 2C). Greater than 10% of the OR repertoires may be experiencing diversifying selection in over half the taxa analyzed, with 4 taxa having >20% of their repertoires experiencing diversifying selection (Table 2). A total list of gene candidates is available as a Supplementary File.

Phenotypic disparity
Brain volumes for total brain and brain regions are available in Supplementary Table S4. For brain and brain region volumes, MDI values did not differ significantly from what was expected under Brownian motion evolution (Table 3). Disparity among total brain volumes and associated nonsensory structures was greatest early in diversification of tetrapods, while olfactory bulb disparity steadily increased through time, though no trend was different than what was observed in Brownian motion processes. Supplementary Figures S1 and S2 show the outcomes for each brain region and associated simulations. When estimating the rates of evolution, only total brain volumes and the midbrain demonstrated significant differences from Brownian motion (Table 3). The asterisk indicates estimates for Pagel’s $\lambda$ were closer to 0 for total brain and the midbrain, while they were close or at 1 for all other brain regions, including the olfactory bulb (Table 3).
Interactions among observed genes

| Contrast                          | Estimate | SE     | t-ratio | P-value |
|-----------------------------------|----------|--------|---------|---------|
| Class I–II-Gene                  | −2.17    | 0.43   | −5.01   | <0.001  |
| Class I–Non-OR                   | −0.19    | 0.54   | −0.36   | 0.99    |
| Class II–Non-OR                  | 1.98     | 0.36   | 5.45    | <0.001  |

Interactions among simulations

| Contrast                          | Estimate | SE     | t-ratio | P-value |
|-----------------------------------|----------|--------|---------|---------|
| Purifying<sub>OR</sub> = 0.01 to Purifying<sub>OR</sub> = 0.1 | −1.51    | 0.16   | −9.40   | <0.001  |
| Purifying<sub>OR</sub> = 0.01 to Diversifying<sub>OR</sub> = 2.5 | 16.22    | 0.15   | 105.45  | <0.001  |
| Purifying<sub>OR</sub> = 0.1 to Diversifying<sub>OR</sub> = 2.5 | 14.72    | 0.18   | 81.27   | <0.001  |

P-values were corrected for multiple comparisons. Purifying<sub>OR</sub> = 0.01 refers to strong purifying selection, purifying<sub>OR</sub> = 0.1 is weak purifying selection, and diversifying<sub>OR</sub> = 2.5 is diversifying or positive selection.

Table 2. Number of OR genes identified as candidates under diversifying selection and the relative proportion to the total number of intact OR genes in the respective genome

| Species           | Total diversifying candidates | Total intact ORs | Proportion |
|-------------------|-------------------------------|------------------|------------|
| Amblystoma mexicanum | 3                             | 47               | 0.06       |
| Rhinotraena buivittatum | 219                        | 1,758            | 0.12       |
| Ornithorhynchus anatina  | 22                          | 484              | 0.05       |
| Monodelphis domestica     | 0                            | 1,827            | 0          |
| Desmodus rotundus       | 11                           | 424              | 0.03       |
| Mus musculus            | 43                           | 1,762            | 0.02       |
| Sphenodon punctatus     | 101                          | 485              | 0.21       |
| Paroedura picta         | 108                          | 659              | 0.16       |
| Pantheropis guttatus    | 55                           | 439              | 0.13       |
| Anolis carolinensis      | 15                           | 117              | 0.13       |
| Cheyldra serpentina     | 360                          | 1,621            | 0.22       |
| Alligator mississippiensis | 150                     | 777              | 0.19       |
| Crocodylus porosus      | 2                            | 21               | 0.10       |
| Rhea americana          | 19                           | 89               | 0.21       |
| Notobroca perdicaria     | 16                           | 77               | 0.21       |
| Coturnix japonica       | 2                            | 36               | 0.06       |
| Gallus gallus           | 7                            | 69               | 0.10       |
| Porzana carolina        | 0                            | 74               | 0          |
| Taeniopygia guttata      | 1                            | 11               | 0.09       |
| Phalacrocorax carbo      | 5                            | 28               | 0.18       |
| Gavia stellata          | 7                            | 69               | 0.10       |

Discussion

We investigated the extent to which diversifying evolution of the olfactory system occurs throughout Tetrapoda. We characterized both the strength of selection in ORs—which constitute the largest gene family in the genome—and the morphological disparity of sensory and nonsensory brain regions to test whether OR genes and phenotypes display similar evolutionary trends. Our research revealed three key discoveries about olfactory systems: (1) OR genes are evolving at some of the fastest rates in the tetrapod genome; (2) olfactory bulbs evolve at similar rates to other sensory and nonsensory brain regions and do not differ from Brownian motion; and (3) olfactory bulb and OR gene evolution are decoupled.

We quantitatively demonstrate that most OR genes evolve at an order of magnitude higher rate than non-OR genes (Table 1 and Figure 2), and diversifying rates are observed ubiquitously across taxa (Figure 2C) and across multiple OR subfamilies (Figure 2D). Elevated rates of codon substitutions in chemosensory receptor genes have been incidentally reported in various studies focused on individual vertebrate genomes (e.g., Green et al. 2014; Lin et al. 2016; Mason et al. 2016; Li et al. 2018). High rates of evolution have also been reported within focused studies of chemosensory receptor genes of a specific clade (e.g., Glusman et al. 2000; Young et al. 2002; Niimura and Nei 2005; Yoder et al. 2014). However, our analysis is the first large-scale study of major vertebrate clades. We suggest the increased rate of evolution, especially in Class II genes (Table 2, Figure 2B,C), is due to the birth–death processes underlying OR gene evolution. Previous studies in mammals have found that Class II genes are under weaker evolutionary constraint and that this trend is attributed to increased rates of gene retention after duplication (Niimura et al. 2014). We also specifically propose that Class II OR subfamily 5/8/9 and OR 6 are evolving at particularly high rates in a number of divergent taxa, not just in mammals (Figure 2D). Novel duplications provide the substrate for the fixation of functional mutations (Niimura 2012), and the high rate of codon substitution relative to nucleotide substitution may indeed promote diversification of potential to bind to new odorant ligands. Simultaneously, rapid evolution puts receptors in a rather precarious position of loss of function. Previous studies have described chemosensory receptor gene families “on the verge of a functional breakdown” (Yoder and Larsen 2014). The rates of evolution are so high within these genes that if positive selection relaxes even slightly, widespread pseudogenization may occur (Yoder and Larsen 2014; Yoder et al. 2014; Hummict et al. 2019). The order-of-magnitude increases in rates of molecular evolution and rates equivalent to diversifying selection of ORs that we found suggest ongoing turnover and rapid evolution over the course of 400 million years of tetrapod diversification.

Our calculated rates of molecular OR evolution stand in stark contrast to our results from olfactory morphology. Despite the alloometric independence of olfactory bulb evolution (Finlay and Darlington 1995), we found that, relative to other brain regions, disparity in olfactory bulb volume does not vary significantly on the timescale of tetrapod diversification (Figure 3 and Table 3). The olfactory bulb has been shown to evolve differently within different clades and is often correlated with “olfactory ability” or some other physiological characteristic, yet the inferred patterns are inconsistent. Olfactory bulb size is smaller in aquatic versus terrestrial mammals (Gittleman 1991), but is larger in aquatic-foraging birds (Corfield et al. 2015). Nocturnality in mammals seems to predict olfactory bulb size, but the direction of the trend depends on the “ordinal-level” clade (Barton et al. 1995). There are also many instances in which olfactory bulb morphology remains highly conserved: the olfactory bulb and medulla, for example, showed among the smallest amounts of evolutionary change in 40 million years of anthropoid diversification (Smaers and Soligo 2013). With a few minor exceptions, neural projections from the olfactory bulb are highly conserved between turtles and pigeons (Reiner and Karten 1985). Indeed, the basic neural mechanism from receptor to
### Table 3. Parameters of morphological evolution models

| Brain region | MDI P-value | BMV | Root | LRT P-value | AICc |
|--------------|-------------|-----|------|-------------|------|
| Total brain  | 0.293   0.83 | 59.840 | 2.627 | 1e-8 | 0.03 | 7.18 |
| Sensory      | -0.078  0.24 | -  | -  | -  | -  |
| Non-sensory  | 0.036   0.50 | -  | -  | -  | -  |
| Olfactory bulb | 0.003  0.41 | 1.4e-5 | 0.11 | 1  | 1  | -2.85 |
| Semicircular canal | -0.141  0.16 | 2.8e-5 | 0.14 | 1  | 1  | -2.85 |
| Optic nerves | 0.303   0.84 | 1.7e-6 | 0.03 | 1  | 1  | -2.85 |
| Cerebrum     | 0.009   0.42 | 1.0e-4 | 0.37 | 0.73 | 0.38 | -1.33 |
| Thalamus     | 0.325   0.85 | 2.16e-6 | 0.05 | 0.33 | 0.21 | 0.32 |
| Cerebellum   | -0.083  0.37 | 1.34e-5 | 0.07 | 0.85 | 0.55 | -2.12 |
| Midbrain     | 0.206   0.73 | 1.7e-5 | 0.12 | 0.27 | 0.03 | 6.25 |
| Medulla      | 0.047   0.49 | 8.8e-6 | 0.13 | 1  | 1  | -2.85 |

The first 2 columns with continuous values correspond to the disparity through time analyses, MDIs for each brain region or group of brain regions and associated P-values that demonstrate differences from the mean of 10,000 simulations of Brownian motion trait evolution. BMV is the Brownian motion covariance and root is the starting value of the trait before any change.

Figure 3. Disparity of brain volumes through time. The mustard line is the volume of the entire brain and sensory regions, blue is the olfactory bulb scaled by total brain size, and red is the regions of the brain not associated with major primary sensory input: cerebrum, thalamus, midbrain, cerebellum, and medulla. The gray region of the figure is the confidence intervals from simulations modeling the total brain volume disparity as expected under Brownian motion and the dashed line is the mean value obtained from these simulations.

The morphological and genomic disassociation of the ORs and olfactory bulbs may be a unique phenomenon of chemosensation relative to other sensory systems (Bear et al. 2016). Olfactory bulb glomeruli has been maintained since the fish-mammal common ancestor (Saraiva et al. 2015). Thus, we conclude evolutionary change of brain sensory organization is consistent with that expected under Brownian motion, especially compared to the exceptional rates of change of molecular OR repertoires. It is also important to note our dataset only contains extant taxa, which may lead to some bias of the extremes observed in living taxa. We caution over-ambitious interpretation of the results and look forward to future analyses that incorporate fossil endocasts to further test our hypothesis.

The authors want to acknowledge Brandon Mercado for his assistance with μCT-scanning, the Harvard Center for Nanoscale Systems, and Greg Watkins-Colwell and Kristof Zyskowski for assistance with Yale Peabody Museum specimens.

### Acknowledgments

The authors want to acknowledge Brandon Mercado for his assistance with μCT-scanning, the Harvard Center for Nanoscale Systems, and Greg Watkins-Colwell and Kristof Zyskowski for assistance with Yale Peabody Museum specimens.

### Funding

L.R.Y. was supported by the National Science Foundation Postdoctoral Research Fellowship in Biology (NSF-DBI 1812035) and the Linnean Society of London and the Systematics Association Systematics Research Fund. M.H.
was supported by the Yale Institute for Biospheric Studies Small Grants Program Doctoral Pilot Award. B.A.S.B. was supported by Yale University and the Yale Institute for Biospheric Studies.

Author Contributions
L.R.Y. designed the study, collected and analyzed all molecular data, assisted with μCT-scanning and segmenting, and wrote the manuscript. M.F. segmented the μCT scan datasets, collected the volumetric data, and edited the manuscript. M.H. assisted with obtaining and staining specimens, μCT-scanning, segmenting, and editing the manuscript. B.A.S.B. helped to conceive the study, developed staining and μCT scanning protocols, acquired, stained, and scanned specimens, segmented some datasets, and edited the manuscript.

Supplementary Material
Supplementary material can be found at https://academic.oup.com/cz.

References
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. J Mol Biol 215:403–410.
Baier H, Korsching S, 1994. Olfactory glomeruli in the zebrafish are identifiable across animals. J Neurosci 14:219–230.
Balanoff AM, Bever GS, Collerton MW, Clarke JA, Field DJ et al., 2016. Best practices for digitally constructing endocasts: examples from birds and their dinosaurian relatives. J Anat 229:173–190.
Barton RA, Purvis A, Harvey PH, 1995. Evolutionary radiation of visual and olfactory brain systems in primates, bats and insectivores. Philos Trans R Soc London Ser B-Biological Sci 348:381–392.
Bear DM, Lassance JM, Hoekstra HE, Datta SR, 2016. The evolving neural and genetic architecture of vertebrate olfaction. Curr Biol 26: R1039–R1049.
Brenida-Emonds ORP, 2003. transAlign: using amino acids to facilitate the multiple alignment of protein-coding DNA sequences. BMC Bioinformatics 6:156.
Bird DJ, Murphy WJ, Fox-Rosales L, Hamid I, Eagle RA et al., 2018. Olfaction written in bone: cribiform plate size parallels olfactory receptor gene repertoires in mammals. Proc R Soc B Biol Sci 285:20180100.
Bressel OC, Khan M, Mombarts P, 2016. Linear correlation between the number of olfactory sensory neurons expressing a given mouse odorant receptor gene and the total volume of the corresponding glomeruli in the olfactory bulb. J Comp Neurol 524:199–209.
Brykczynska U, Tzika AC, Rodriguez I, Milinkovitch MC, 2013. Contrasted receptor gene repertoires in Mammalia. Genome Biol Evol 5:389–401.
Chell A, Simon I, Cedar H, Axel R, 1994. Allelic inactivation regulates olfactory receptor gene expression. Cell 78:823–834.
Corfield JR, Price K, Iwanuk AN, Gutiérrez-Ibáñez C, 2015. Diversity in olfactory bulb size in birds reflects allometry, ecology, and phylogeny. Front Neuroanat 9:1–16.
Eddy SR, 2011. Accelerated profile HMM searches.PLoS Comput Biol 7: e1002195.
Eyub Sil, Moriyama H, Hoffmann FG, Moriyama EN, 2016. Molecular evolution and functional divergence of trace amine-associated receptors. PLoS ONE 11:1–24.
Fabbri M, Mongiadino Koch N, Pritchard AC, Hanson M, Hoffman E et al., 2017. The skull roof tracks the brain during the evolution and development of reptiles including birds. Nat Ecol Evol 1:1543–1550.
Finlay BL, Darlington RB, 1995. Linked regularities in the development and evolution of mammalian brains. Science 268:1578–1584.
Fleischer J, Pregitzer P, Breer H, Krieger J, 2018. Access to the odor world: olfactory receptors and their role for signal transduction in insects. Cell Mol Life Sci 75:485–508.
Garrett EC, Steiper ME, 2014. Strong links between genomic and anatomical diversity in both mammalian olfactory chemosensory systems. Proc R Soc B Biol Sci 281:20132828.
Gerts EM, Yu YK, Agarwala R, Schäffer AA, Altschul SF, 2006. Composition-based statistics and translated nucleotide searches: improving the TBLASTN module of BLAST. BMC Biol 4:1–14.
Gittleman JL, 1991. Carnivore olfactory bulb size: allometry, phylogeny and ecology. J Zool 225:253–272.
Glusman G, Bahar A, Sharon D, Pilpel Y, White J et al., 2000. The olfactory receptor gene superfamily: data mining, classification, and nomenclature. Mamm Genome 11:1016–1023.
Green RE, Braun EL, Armstrong J, Carl D, Nguyen N et al., 2014. Three crocodilian genomes reveal ancestral patterns of evolution among archosaurs. Science 346:1254449.
Grus WE, 2008. Evolution of the vomeronasal system viewed through system-specific genes.
Harmon LJ, Larson A, Losos JB, 2003. Tempo and mode of evolutionary radiation in iguanian lizards: a phylogenetic comparative study. Science 301:1–28.
Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W, 2008. GEIGER: investigating evolutionary radiations. Bioinformatics 24:129–131.
Hayden S, Bekert M, Crider TA, Mariani S, Murphy WJ et al., 2010. Ecological adaptation determines functional mammalian olfactory subgenomes. Genome Res 20:1–9.
Hayden S, Teeling EC, 2014. The molecular biology of vertebrate olfaction. Anat Rec 297:2216–2226.
Hedrick BP, Yohe L, Vander LA, Dávalos LM, Sears K et al., 2018. Assessing soft-tissue shrinkage estimates in museum specimens imaged with diffusible iodine-based contrast-enhanced computed tomography (diceCT). Microsc Microanal 24:284–291.
Hinds JW, McNelly NA, 1981. Aging in the rat olfactory system: correlation of changes in the olfactory epithelium and olfactory bulb. J Comp Neurol 203:441–453.
Huigol D, Uiten S, Shimogori T, Saha B, Roy A et al., 2013. Dual origins of the mammalian accessory olfactory bulb revealed by an evolutionarily conserved migratory stream. Nat Neurosci 16:157–165.
Hummcutt KE, Tiley GP, Williams RC, Larsen PA, Blanco MB et al., 2019. Comparative genomic analysis of the pheromone receptor class 1 family (V1R) reveals extreme complexity in mouse leumrs (Genus, Microcebus) and a chromosomal hotspot across mammals. Genome Biol Evol 12:3562–3579.
Jetz W, Pyron RA, 2018. The interplay of past diversification and evolutionary isolation with present imperilment across the amphibian tree of life. Nat Ecol Evol 2:850–858.
Ji Y, Zhang Z, Hu Y, 2009. The repertoire of G-protein-coupled receptors in Xenopus tropicalis. BMC Genomics 10:12.
Jorgensen CB, 2000. Amphibian respiration and olfaction and their relationships: from Robert Townson (1794) to the present. Biol Rev 75:297–345.
Katoh K, Standley DM, 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780.
Khan I, Yang Z, Maldonado E, Li C, Zhang G et al., 2015. Olfactory receptor subgenomes linked with broad ecological adaptations in Sauropsida. Mol Biol Evol 32:1–12.
Kimball RT, Oliveros CH, Wang N, White ND, Barker FK et al., 2019. A phylogenomic supertree of birds. Diversity 14:109.
Kumar S, Stecher G, Suleski M, Hedges SB, 2017. TimeTree: a resource for timelines, timetrees and divergence times. Mol Biol Evol 34:1812–1819.
Lenth RV, 2016. Least-squares means: the R package lsmeans. J Stat Softw 69:1–28.
Liberles SD, 2014. Mammalian pheromones. Annu Rev Physiol 76:151–175.
Lin Q, Fan S, Zhang Y, Xu M, Zhang H et al., 2016. The seahorse genome and the evolution of its specialized morphology. Nature 540:395–399.
Malnic B, Hirono J, Sato T, Buck LB, 1999. Combinatorial receptor codes for odors. Cell 96:713–723.
Maresh A, Rodriguez Gil D, Whitman MC, Greer CA, 2008. Principles of glomerular organization in the human olfactory bulb - Implications for odor processing. PLoS ONE 3:e2640.
Mason VC, Li G, Minx P, Schmitz J, Churakov G et al., 2016. Genomic analysis reveals hidden biodiversity within colugos, the sister group to primates. *Sci Adv* 2:e1600633.

Meister M, 2015. On the dimensionality of odor space. *eLife* 4:1–12.

Mills DB, Francis WR, Vargas S, Larsen M, Elemans CPH et al., 2018. The last common ancestor of animals lacked the HIF pathway and resided in low-oxygen environments. *eLife* 7:e31176.

Monahan K, Lomvardas S, 2015. Monoallelic expression of olfactory receptors. *Annu Rev Cell Dev Biol* 31:721–740.

Mugal CF, Wolf JB, Kaj I, 2014. Why time matters: codon evolution and the temporal dynamics of dN/dS. *Mol Biol Evol* 31:212–233.

Nara K, Saraiva LR, Ye X, Buck LB, 2011. A large-scale analysis of odor coding in the olfactory epithelium. *J Neurosci* 31:9179–9191.

Nei M, Nimmer M, Nozawa M, 2008. The evolution of animal chemosensory receptor gene repertoires: roles of chance and necessity. *Nat Rev Genet* 9:951–963.

Nei M, Rooney AP, 2005. Converged and birth-and-death evolution of multi-gene families. *Annu Rev Genet* 39:121–152.

Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ, 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 32:268–274.

Nimmer M, 2009. Evolutionary dynamics of olfactory receptor genes in chordates: interaction between environments and genomic contents. *Hum Genomics* 4:107–118.

Nimmer M, 2012. Olfactory receptor multigene family in vertebrates: from the viewpoint of evolutionary genomics. *Carr Genomics* 13:103–114.

Nimmer M, Matsui A, Tsuchiya K, 2014. Extreme expansion of the olfactory receptor gene repertoire in African elephants and evolutionary dynamics of orthologous gene groups in 13 placental mammals. *Genome Res* 24:1485–1496.

Nimmer M, Nei M, 2005. Comparative evolutionary analysis of olfactory receptor gene clusters between humans and mice. *Gene* 346:13–21.

Paradis E, Claude J, Strimmer K, 2004. APE: analyses of phylogenetics and evolution in R. *Bioinformatics* 20:289–290.

Pelegueroes C, Laurie S, Abia MM, 2013. Accelerated evolution after gene duplication: a time-dependent process affecting just one copy. *Mol Biol Evol* 30:1830–1842.

Pereira AG, Sterli J, Moreira FRR, Schrago CG, 2017. Multilocus phylogeny and biogeographical biogeography clarify the evolutionary history of major lineages of turtles. *Mol Phylogenet Evol* 113:59–66.

Puttick MN, Ingram T, Clarke M, Thomas GH, 2020. MOTMOT: models of trait macroevolution on trees (an update). *Methods Ecol Evol* 11:464–471.

Quinlan AR, 2014. BEDTools: the Swiss-army tool for genome feature analysis. *Carr Protoc Bioinforma* 47:11–12.

Reiner A, Karten HJ, 1983. Comparison of olfactory bulb projections in pigeons and turtles. *Brain Behav Evol* 27:11–27.

Rice P, Longden I, Bleasby A, 2000. EMBOSS: the European molecular biology open software suite. *Trends Genet* 16:276–277.

Rodriguez I, 2013. Singular expression of olfactory receptor genes. *Cell* 155:274–277.

Rouayr A, Parry V, Preiern J, Blecic J, Renou M, 2011. A general odorant background affects the coding of phenomere stimulus intermittency in specialist olfactory receptor neurons. *PLoS One* 6:e26443.

Saraiva LR, Ahuja G, Iyvind C, Syed AS, Marioni JC et al., 2015. Molecular and neuronal homology between the olfactory systems of zebrafish and mouse. *Rep Sci* 5:16.

Slater GJ, Price SA, Santini F, Alfaro ME, 2010. Diversity versus disparity and the radiation of modern cetaceans. *Proc R Soc B Biol Sci* 277:3097–3104.

Smaers JB, Solgo C, 2013. Brain reorganization, not relative brain size, primarily characterizes anthropoid brain evolution. *Proc R Soc B Biol Sci* 280:20130269.