Olfactory Receptor Subgenomes Linked with Broad Ecological Adaptations in Sauropsida

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Abstract

Olfactory receptors (ORs) govern a prime sensory function. Extant birds have distinct olfactory abilities, but the molecular mechanisms underlining diversification and specialization remain mostly unknown. We explored OR diversity in 48 phylogenetic and ecologically diverse birds and 2 reptiles (alligator and green sea turtle). OR subgenomes showed species- and lineage-specific variation related with ecological requirements. Overall 1,953 OR genes were identified in reptiles and 16,503 in birds. The two reptiles had larger OR gene repertoires (989 and 964 genes, respectively) than birds (182–688 genes). Overall, birds had more pseudogenes (7,855) than intact genes (1,944). The alligator had significantly more functional genes than sea turtle, likely because of distinct foraging habits. We found rapid species-specific expansion and positive selection in OR14 (detects hydrophobic compounds) in birds and in OR51 and OR52 (detect hydrophilic compounds) in sea turtle, suggestive of terrestrial and aquatic adaptations, respectively. Ecological partitioning among birds of prey, water birds, land birds, and vocal learners showed that diverse ecological factors determined olfactory ability and influenced corresponding olfactory-receptor subgenome. OR5/8/9 was expanded in predatory birds and alligator, suggesting adaptive specialization for carnivory. OR families 2/13, 51, and 52 were correlated with aquatic adaptations (water birds), OR families 6 and 10 were more pronounced in vocal-learning birds, whereas most specialized land birds had an expanded OR family 14. Olfactory bulb ratio (OBR) and OR gene repertoire were correlated. Birds that forage for prey (carnivores/piscivores) had relatively complex OBR and OR gene repertoires compared with modern birds, including passerines, perhaps due to highly developed cognitive capacities facilitating foraging innovations.

Key words: **olfactory receptors, adaptation, selection, birds.**

Article

Introduction

Olfactory receptors (ORs) are largely responsible for odor perception and detection of chemical cues, facilitating the differentiation of tens of thousands of unique odorants. This makes olfaction an important physiological function crucial to the survival of animals because of its role in recognizing suitable food, mates, offspring, territories, and the presence of predators or prey (Niimura and Nei 2006; Nei et al. 2008; Adipietro et al. 2012).

ORs are intron-less small-sized (1,000 bp) seven-transmembrane (TM) G-protein coupled receptors (GPCRs) with many characteristic conserved motifs (Buck and Axel 1991). The ligand binding sites responsible for detection of specific odor molecules are conserved among orthologs (same function between species) and variable among paralogs (neo or subfunctionalization). Most of these sites are located in the third and seventh TM domains (Man et al. 2004). OR gene expression occurs primarily in the main olfactory epithelium and to a lesser extent in the vomeronasal organ (Lévai et al. 2006), suggesting that they might have some overlapping functions (Baxi et al. 2006). The ectopic expression of OR genes in nonolfactory tissues (e.g., heart, lung, liver and testis) also implies that OR genes are likely to have additional functionalities (De la Cruz et al. 2009), for example, OR genes are also expressed in the testis, where they have a role in sperm chemotaxis (Spehr et al. 2003). Though the relationship between odors and ORs is not clear, it has been hypothesized that a combinatorial coding scheme might allow a

single OR to identify multiple odors and also permit different ORs to identify similar odors (Malnic et al. 1999).

Vertebrate and invertebrate OR genes have distinct evolutionary origins (Niimura 2009a). OR genes evolved independently multiple times during animal evolution (Niimura 2012), resulting in considerable differences in OR gene family repertoire (Bargmann 2006; Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008). In vertebrates, ORs are considered to be the largest multigene family (Niimura 2009b), with characteristic and dramatic variation among diverse species and lineages and ranging from a single intact gene in elephant sharks to more than 1,000 genes in mammals (~1,200 genes in rat and opossum and ~1,900 intact genes in elephant) (Zhang and Firestein 2002; Niimura and Nei 2005; Niimura 2009b).

The classification of OR genes is complex. The vertebrate OR gene family is divided into two types. Type I includes Class I (α, β, δ, ε, and ζ groups) and Class II (γ group) genes. There is only one group (η) of Type II OR genes (supplementary table S1, Supplementary Material online). The Type II OR genes have been lost in amniotes but are found in fishes and amphibians. Type I genes have diversified in fishes and amphibians and groups δ, ε, and ζ are unique to these two lineages (Niimura 2009b). The α and γ OR genes are tetrapod specific (bar one γ gene in the zebrafish). The β group is reported in both tetrapods and fishes. Based on genetic similarity, mammalian OR genes are grouped into 18 families. Class I families (51–56; supplementary table S2, Supplementary Material online) are postulated to bind to water-borne molecules and the 14 Class II families (OR1–14, supplementary table S2, Supplementary Material online) are hypothesized to bind mainly to airborne molecules (Glusman et al. 2000; Olender et al. 2004; Quignon et al. 2005; Hayden et al. 2010; Nguyen et al. 2012).

Ecological adaptation has been instrumental in structuring mammalian olfactory subgenomes (Hayden et al. 2010, 2014). Additional modifications would have occurred through gene duplication, positive selection, and gene conversion, leading to the formation of new gene families and potentially providing increased adaptive capacity (Steiger et al. 2010). Through time, this OR gene diversity would have facilitated the adaptation of vertebrates to varied ecological niches at both the broad evolutionary scale (e.g., among fishes, amphibians, reptiles, birds, and mammals) as well as among more-recently diverged species (Niimura 2009b).

In this study, we characterized the OR gene family repertoire of 48 avian and 2 reptilian genomes to assess how ecological conditions may have shaped patterns of diversification of olfactory abilities and to assess correlations among genetic patterns and olfactory ability, behavior and morphology including olfactory bulb size, feeding and activity habits, and patterns of cognitive ability such as vocal learning in birds.

Results and Discussion

Genome Coverage and the OR Subgenome

Representatives of the OR multigene family are intron-less approximately 1,000 bp genes, making whole-genome sequencing by far the best approach for an in-depth study of the evolutionary dynamics of this large multigene family (Steiger, Kuryshev, et al. 2009; Hayden et al. 2010; Young et al. 2010; Dehara et al. 2012; Hayden et al. 2014). Extensive BLAST (Basic Local Alignment Search Tool) searches for OR genes in 48 avian and 2 reptilian genomes confirmed that the 2 reptiles had larger OR repertoires (*Alligator mississippiensis*, American alligator, 989 genes, hereafter referred as alligator; and *Chelonia mydas*, green sea turtle, 964 genes, hereafter referred as sea turtle) than any of the avian species (which have 182–688 genes). Some bird species exhibited evidence of OR gene expansion, including the little egret (490 genes), parrot (484), chicken (675), hoatzin (467) and zebra finch (688), whereas others had reduced numbers of ORs, as for example the medium ground finch (182), rifleman (222) and manakin (227; supplementary table S3, Supplementary Material online). The number of OR genes identified in the chicken and zebra finch were significantly larger than those in other birds. This may be because these genomes were assembled using traditional long-read sequencing instead of the short-read sequencing employed in the other genomes. However, a scatter plot between the number of identified OR genes and sequencing depth (supplementary fig. S1 and table S3, Supplementary Material online) did not show a strong positive correlation, which suggests that the differences are due to real biological features that arose through avian OR evolution. There was no correlation between the normalized mapping depth of OR genes and sequencing depth (supplementary fig. S2, Supplementary Material online). OR gene repertoire sizes were probably slightly underestimated because of the collapse of the most-similar OR sequences and the short-reads sequencing may have more impact based on the read mapping result. However, as most of the avian genomes used here (46 out of 48) were sequenced using the same technology (Illumina), our data still provide reliable comparisons among birds OR repertoires. This is further validated by the detection of larger OR gene repertoire in the two reptilian genomes (Illumina) that were compared with the avian genomes.

OR Gene Family Phylogeny

Phylogenetic analyses were performed using all identified functional ORs from the avian and reptilian genomes, including the α, β, γ, δ, ε, ζ, and η groups (Niimura 2009b) and the 1–18 Class II and 51–56 Class I families from the Horde database http://genome.weizmann.ac.il/horde/ (last accessed April 11, 2014). The resulting phylogenetic inferences suggested that there are 11 OR gene families in birds and reptiles (1/3/7, 2/13, 4, 5/8/9, 6, 10, 11, 12, 14, 51, and 52), following nomenclature used in the grouping of mammalian OR families into 13 OR families (Hayden et al. 2010). The OR gene family patterns observed in birds and reptiles were supported with high bootstrap support values (more than 80% in 1,000 replicates) for most of the families, including 2/13, 4, 5/8/9, 6, 10, 11, 14 (γ-c clade; Steiger, Kuryshev, et al. 2009), 51, and 52 (fig. 1). However, OR families 1/3/7 and 12 had bootstrap support values of less than 65%. The intermixed OR families

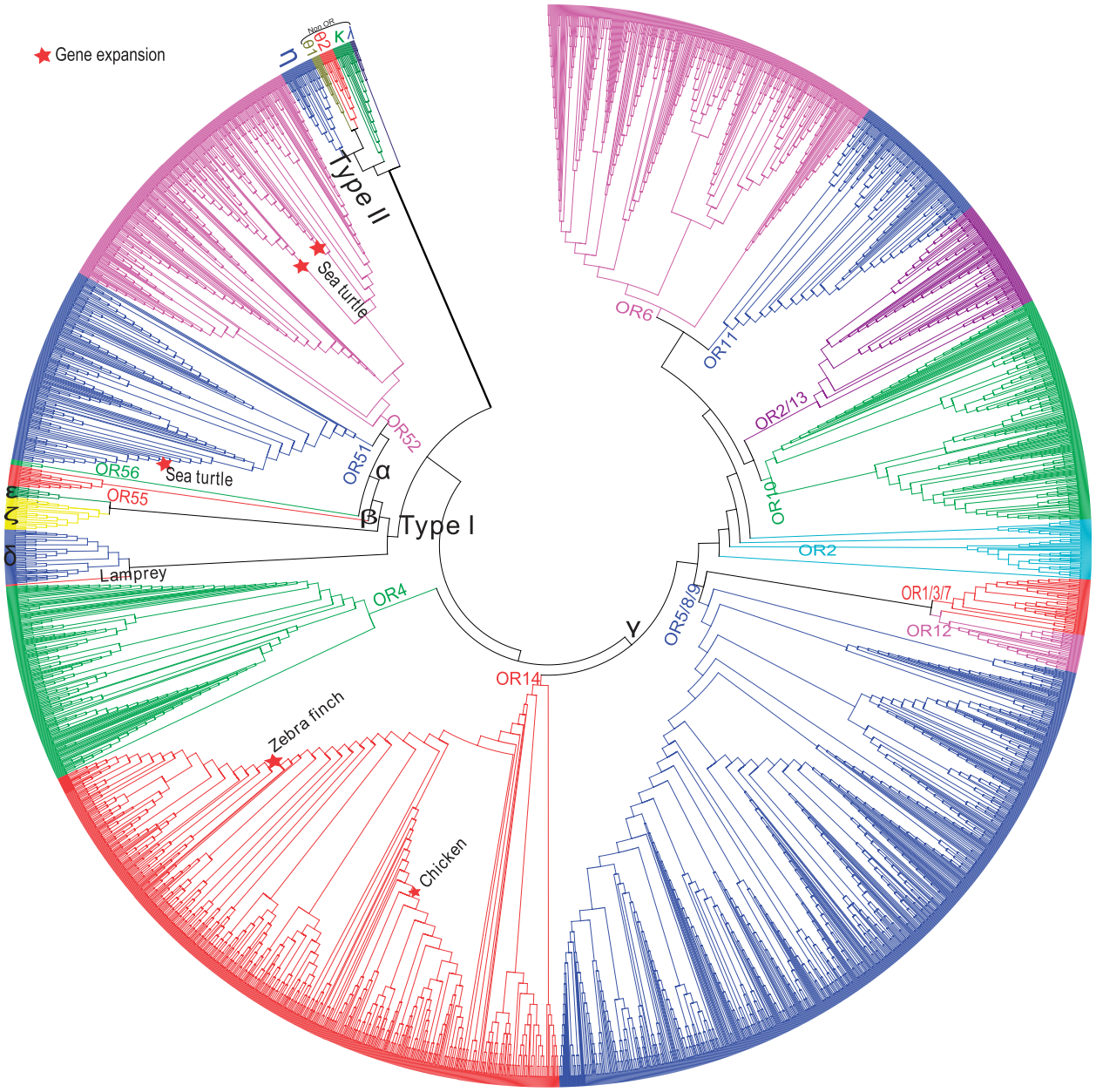

Fig. 1. Neighbor-joining phylogeny of the OR gene families including the functional OR genes identified in the 48 avian and 2 reptilian genomes ($n = 2{,}599$) together with vertebrate representative sequences from Niimura (2009b). The human and anolis OR genes, the "β" OR genes sequences from frog, mouse and opossum, the non-OR GPCR θ, κ and λ, the fish OR genes δ, ε, ζ and η, and the two river lamprey OR genes are from Niimura (2009b). All major clades had bootstrap values greater than 80% (1,000 replicates). The gene expansion is represented by a star.

in clades 1/3/7, 2/13, and 5/8/9 possibly reflect their functional redundancy and/or combinatorial coding (Malnic et al. 1999; De la Cruz et al. 2009).

Enhanced Role of OR Gene Loss (Pseudogenization) in Birds

Gene gain and gene loss is an essential evolution process that leads to numerous changes, including gene subfunctionalization, neofunctionlization, and pseudogenization. Extensive gene gains and losses often lead to changes in gene groups, for example, families, subfamilies, and classes (Niimura and Nei 2007). The impact of these forces is much more pronounced in large families like ORs, because of the broader range of evolutionary pressures that act upon them (supplementary fig. S3, Supplementary Material online). As previously suggested, the olfactory capacity of an organism can be determined by the number of functional and/or nonfunctional ORs (Steiger, Kuryshev, et al. 2009; Hayden et al. 2010; Dehara et al. 2012; Hayden et al. 2014).

The OR gene repertoire was divided into three major categories, including intact genes with normal start codons and stop codons and more than 650 bp in size, partial genes without start codon or stop codon or both, and pseudogenes with frame shift mutations and/or premature stop codons. In total,

16,503 OR genes were identified in the 48 bird genomes including 6,704 partial and 7,855 pseudo-ORs and 1,944 functional ORs. If partial genes were considered to be nonfunctional, then the total number of nonfunctional ORs increased to 14,599. Conversely, if partial genes were considered to be functional, the total number of functional OR genes would still be a low 8,648, highlighting the importance of gene loss in the evolution of the avian OR gene repertoire. OR comparisons among individual bird species resulted in similar patterns (supplementary table S3, Supplementary Material online).

The alligator and sea turtle had similar overall numbers of OR (989 and 964, respectively), but distinct ratios of functional and nonfunctional OR genes. The alligator had 405 functional OR genes compared with 205 in the sea turtle. If partial genes are assumed to be pseudogenes, there were 584 and 714 ORs in alligator and sea turtle, respectively, whereas if all the partial genes are considered to be functional, there were 638 and 459 genes for alligator and sea turtle, respectively (supplementary table S3, Supplementary Material online). These results suggest that gene loss in sea turtle was higher than in alligator, and that the sea turtle is less reliant on olfaction. These results also suggest that OR gene evolution is shaped by olfaction requirements stemming from different evolutionary ecological, behavioral, and physiological adaptations (Steiger, Kuryshev, et al. 2009; Hayden et al. 2010; Young et al. 2010; Dehara et al. 2012; Hayden et al. 2014). In aggregate, the complete characterization of OR genes into functional and nonfunctional genes suggests that patterns of gene gain and gene loss had an important role in shaping the OR gene repertoires, and thus, the olfactory abilities of different species and patterns among lineages.

OR Subgenomes Variation among Birds, Sea Turtle, and Alligator

To explore the evolutionary dynamics of OR genes in avian and reptilian genomes, we identified the complete repertoire of ORs gene families in these lineages. The relative percentage of functional genes in each OR gene family varied across avian species, ranging from 0 to 96% (fig. 2 and supplementary table S4, Supplementary Material online). The 48 avian genomes lacked OR1/3/7 genes and only 3 members were present in alligator and 1 in sea turtle. In addition, OR12 genes were relatively rare and thus may contribute relatively less to olfactory sensibility in these species.

In contrast, OR families 2/13, 5/8/9, 4, 6, 10, 11, 14, 51, and 52 appeared to have contributed significantly to the diversification of ORs among sauropsids. In particular, Class I families 51 and 52, which are predicted to be sensitive to hydrophilic compounds present in aquatic environments (Hayden et al. 2010), expanded dramatically and diversified in the sea turtle. The OR gene family 14 (γ-c clade; Steiger, Kuryshev, et al. 2009), a group of Class II genes associated with volatile compounds (Hayden et al. 2010), was most abundant in birds. The sea turtle and alligator also had comparatively large number of genes in Class II families OR 1-14, which could be related with their use of terrestrial habitats for breeding and other functions (Kishida et al. 2007). Among the reptiles studied to date, the sea turtle and alligator studied here had a highly developed OR family repertoire relative to other previously characterized squamata (i.e., lizard = 136 and python = 280, rat snake = 96; Kishida and Hikida 2010; Dehara et al. 2012).

Olfactory Bulb Ratio, ORs Gene Repertoire, and Olfactory Ability

Basal birds and early neornithines have larger olfactory bulbs relative to the more-derived neoavian clades. Larger olfactory bulb size has previously been suggested to improve olfaction and foraging or navigation skills, which may possibly have helped prevent mass extinction in the end-Cretaceous period (Zelenitsky et al. 2011). Several studies have also suggested that there is a correlation between olfactory bulb size and olfactory ability (Bang and Cobb 1968; Bang 1971; Zelenitsky et al. 2011), which may be a function of a greater number of mitral cells and glomeruli in the bulb and an increased number of OR genes (Wenzel and Meisami 1987; Steiger et al. 2008; Steiger, Fidler, et al. 2009).

In birds, the olfactory bulb ratio (OBR) is positively correlated with the total OR gene repertoire suggesting that the OR subgenomes can be used as a proxy for olfactory ability (Zelenitsky et al. 2011). In our data set, this positive correlation (supplementary table S5 and fig. S4, Supplementary Material online) was significant ($r = 0.66$, $P < 0.05$) after removing outlier birds such as the chicken, zebra finch, and budgerigar that showed evidence of species-specific recent rapid expansion and positive selection of OR14. These outliers are possible examples of how ecological adaptation can shape the composition and function of OR family genes and in turn, the olfactory ability of these birds.

As has been suggested previously by other authors, more-derived neoavian clades have decreased OBRs (Bang and Cobb 1968; Bang 1971; Zelenitsky et al. 2011) and we found that overall, passerine birds (excluding zebra finch) and psittaciformes (excluding budgerigar) have the least number of OR genes. This could be due to the increased cognitive abilities of birds in these orders such as the capacity for vocal learning, the cooperative displays and vocal repertoire of manakin (Trainer et al. 2002) and the examples of tool use in learned and innovated foraging techniques (Lefebvre et al. 1997; Timmermans et al. 2000; Lefebvre et al. 2002), which in turn may have resulted in a reduced role of olfaction (Zelenitsky et al. 2011).

The mean OBR for passeriforms and psittaciforms birds (mean = 8.2%, $n = 7$) was below the average of all birds excluding these two orders (mean = 19%; $P \leq 0.001$, supplementary table S5, Supplementary Material online). Similarly, there was a significant difference in the mean number of OR genes between passeriformes and psittaciformes (mean = 219) (excluding zebra finch and budgerigar) and other birds (mean = 342; $P \leq 0.001$, supplementary table S5, Supplementary Material online). The differences in gene patterns between the zebra finch and budgerigar could reflect distinct evolutionary pressures that drove the rapid diversification of the rapidly expanded paralogs of these species.

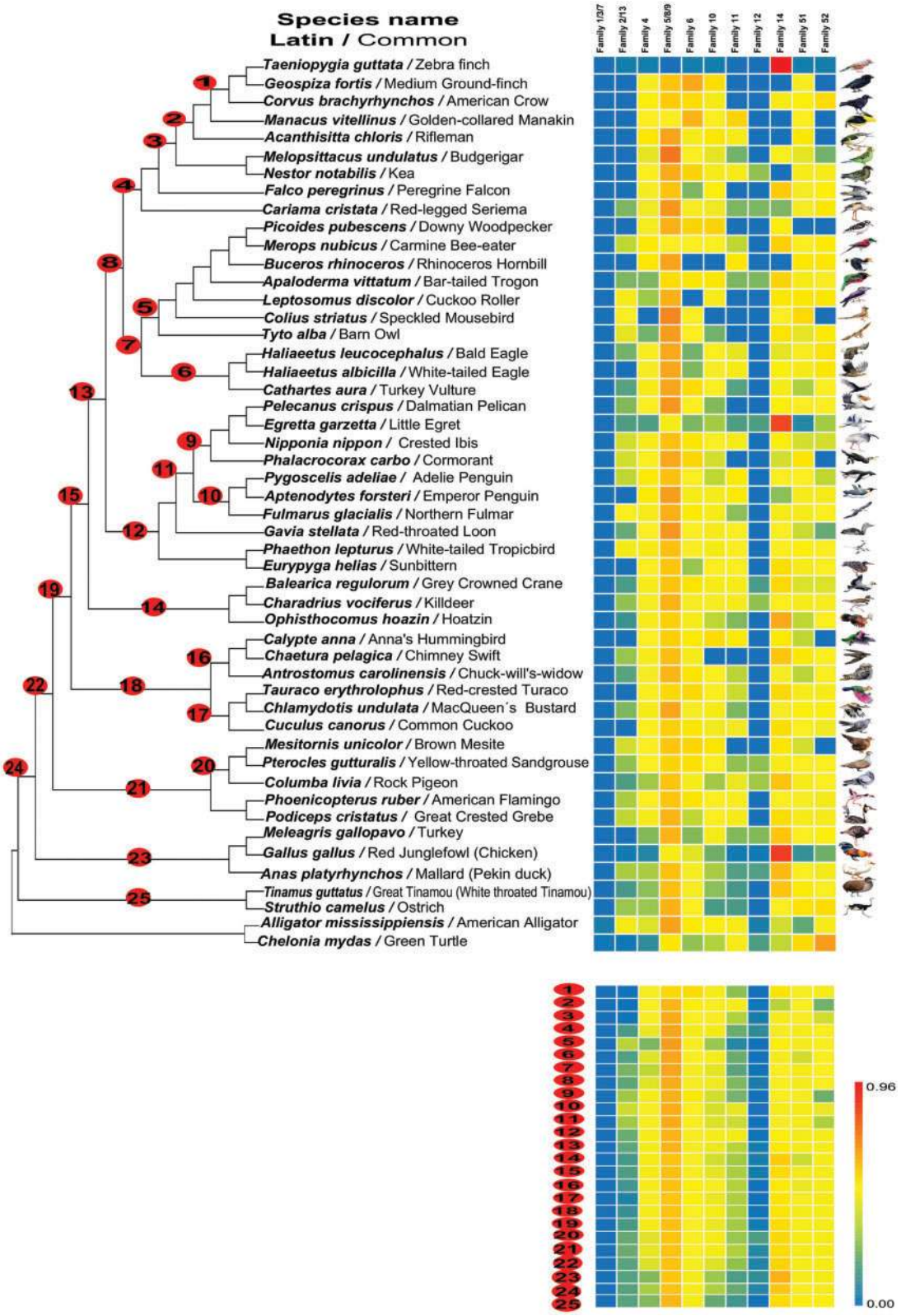

Fig. 2. Consensus phylogeny of the avian genomes following Jarvis et al. (2014) with alligator and sea turtle as outgroups showing a heat map reflecting the relative percentage of functional OR genes in each family (0–96%) of each species. The corresponding reconstructed ancestral states nodes in the tree are labeled 1–25.

Bird activity patterns also influenced olfaction patterns. For example, nocturnal species such as the night jar had an OBR of 23.8% and 352 OR genes and the barn owl had an OBR of 18.5% and 321 OR genes (which is closer to the average of 17% and 343 genes; supplementary table S5, Supplementary Material online). Comparisons of OBR and OR gene repertoire among water birds, birds of prey, vocal learners, and the remaining land birds suggest that birds with specialized

olfaction also have a broader OBR and OR subgenomes. In addition, birds with specialized foraging adaptations, including the carnivore birds of prey (Zelenitsky et al. 2011) and water birds, had very similar patterns of OBR and OR genes (birds of prey had an OBR of 21% and a mean number of OR genes of 336 and water birds had an OBR of 19% and mean number of OR genes of 356). In contrast, vocal-learning species had a relatively reduced OBR of 8.7% and a mean number of OR genes of 243, which is possibly linked with their more-highly developed cognitive abilities (Lefebvre et al. 1997, 2002; Emery 2006; Zelenitsky et al. 2011).

OR Subgenomes and Ecological Adaptation in Birds

The principal component analysis (PCA) and naïve Bayes assignment algorithm assigned most species into their respective ecogroups with only a few exceptions (duck, egret, zebra finch, and budgerigar), suggesting that ecological adaptation played a role in determining the configuration of the avian olfactory subgenome (figs. 3 and 4 and supplementary fig. S5, Supplementary Material online). The duck and egret have shared land-characteristics possibly because of their semiaquatic adaptations, whereas zebra finch and budgerigar had pattern more similar with land birds (e.g., zebra finch similar to land birds had expanded OR14 under positive selection).

OR families 51, 52, and 2/13 were most-closely associated with the aquatic birds group (fig. 4) and OR families 6 and 10 contributed the most to defining the vocal learner group of species. Gene family 5/8/9 was concentrated in the birds of prey, whereas most of the specialized land birds had an expanded number of genes from OR family 14 (γ-c clade). The two components explained more than 68% variance within data (supplementary table S6, Supplementary Material online, analysis of similarities [ANOSIM] $r = 0.58$, $P < 0.05$). Each of the ecogroups was significantly different (had unique patterns) relative to land birds (fig. 4). This further demonstrates that the relative percentage of functional OR genes in each OR gene family are correlated with the ecological adaptations of each species and are less a function of shared ancestry and phylogenetic relationships (supplementary figs. S6 and S7, Supplementary Material online).

The ecological partitioning of birds based on the proportion of functional OR gene family suggests a correlation with adaptive requirements. Birds with higher olfactory requisites such as birds of prey (carnivores/piscivores) that use olfaction for foraging (Zelenitsky et al. 2011) clustered together in the PCA, whereas vocal-learner birds, which rely more on cognitive ability and have reduced olfaction ability and reduced OBR, clustered separately. The water birds, with their specialized anatomical modifications for aquatic life (Bang and Cobb 1968; Bang 1971; Zelenitsky et al. 2011) and their use of olfaction in foraging, navigation and nest recognition (Bonadonna et al. 2003; Nevitt and Bonadonna 2005), also clustered together in the PCA (fig. 4).

The passerine birds (song birds) had an overall reduced repertoire of OR genes, which suggests a possible loss of functions. The presumed reduced dependence on olfaction in these vocal learners birds might possibly be compensated for by a highly developed cognitive ability (Emery 2006) that helped lead to true tool use and a high frequency of foraging innovations (Lefebvre et al. 1997; Timmermans et al. 2000; Lefebvre et al. 2002). The zebra finch had a comparatively large number of OR gene and a notable species-specific expansion of OR14 influenced by positive selection.

Overall, OR families 2/13, 51, and 52 were more common in aquatic lineages and families 6 and 10 were more determinant in vocal learners (fig. 4). Birds of prey had a comparatively high percentage of OR families 5/8/9. These were also the largest OR families observed in alligator, which like birds, depend heavily on pursuit, hunting or scavenging for prey. Similar ecological partitioning of gene characteristics was also apparent with the sea turtle and alligator. The sea turtle grouped with aquatic birds and the alligator (a semiaquatic species) was more closely aligned with aquatic prey birds (carnivory, supplementary fig. S8, Supplementary Material online).

A few species had unique patterns, and they did not cluster together with the other species from their purported ecological cluster. This was most noticeably in species that had an expanding OR14 repertoire, such as the zebra finch and budgerigar. Similarly the egret and duck grouped into land birds instead of aquatic birds (fig. 4) possibly because of their semiaquatic requirements combined with their dependency on land environments. The Psittaciformes (budgerigar) have highly developed vision together with a large OR gene repertoire and it has been suggested that certain parrot species use olfactory cues to forage (Hagelin et al. 2003; Roper 2003).

Reconstruction of the ancestral states at 25 nodes (figs. 2 and 3), using PCA and naïve Bayes assignment tests generally matched the ecological grouping of birds (supplementary figs. S5 and S8, Supplementary Material online; e.g., nodes A1 and A2 grouped in vocal learners).

OR Adaptive Evolution: Positive Selection Hotspots in OR Ligand Binding Domains

To detect evidence of positive selection, we used Single Likelihood Ancestral Counting (SLAC), Fixed Effects Likelihood (FEL), Random Effects Likelihood (REL), Fast Unconstrained Bayesian AppRoximation (FUBAR), and Mixed Effects Model of Evolution (MEME) (Pond and Frost 2005a; Delport et al. 2010; Murrell et al. 2013), as well as an integrative approach that considered multiple phylogenies based on the inferred potential breakpoints. This approach is generally more reliable compared with PAML, which depends on a single phylogeny and may lead to more false positives, especially when recombination and gene conversion rates are high (Steiger et al. 2010). Using both individual and integrative approaches, we identified signals of positive selection in the expanded OR family 14 in birds (eight bird species) and in OR family 51 and 52 in the sea turtle (supplementary table S7, Supplementary Material online), suggestive that positive selection is playing a role in the functional diversification and ecological adaptation of the OR genes.

The alignment-wide test for positive selection using the PARRIS method, a robust inference of positive selection from

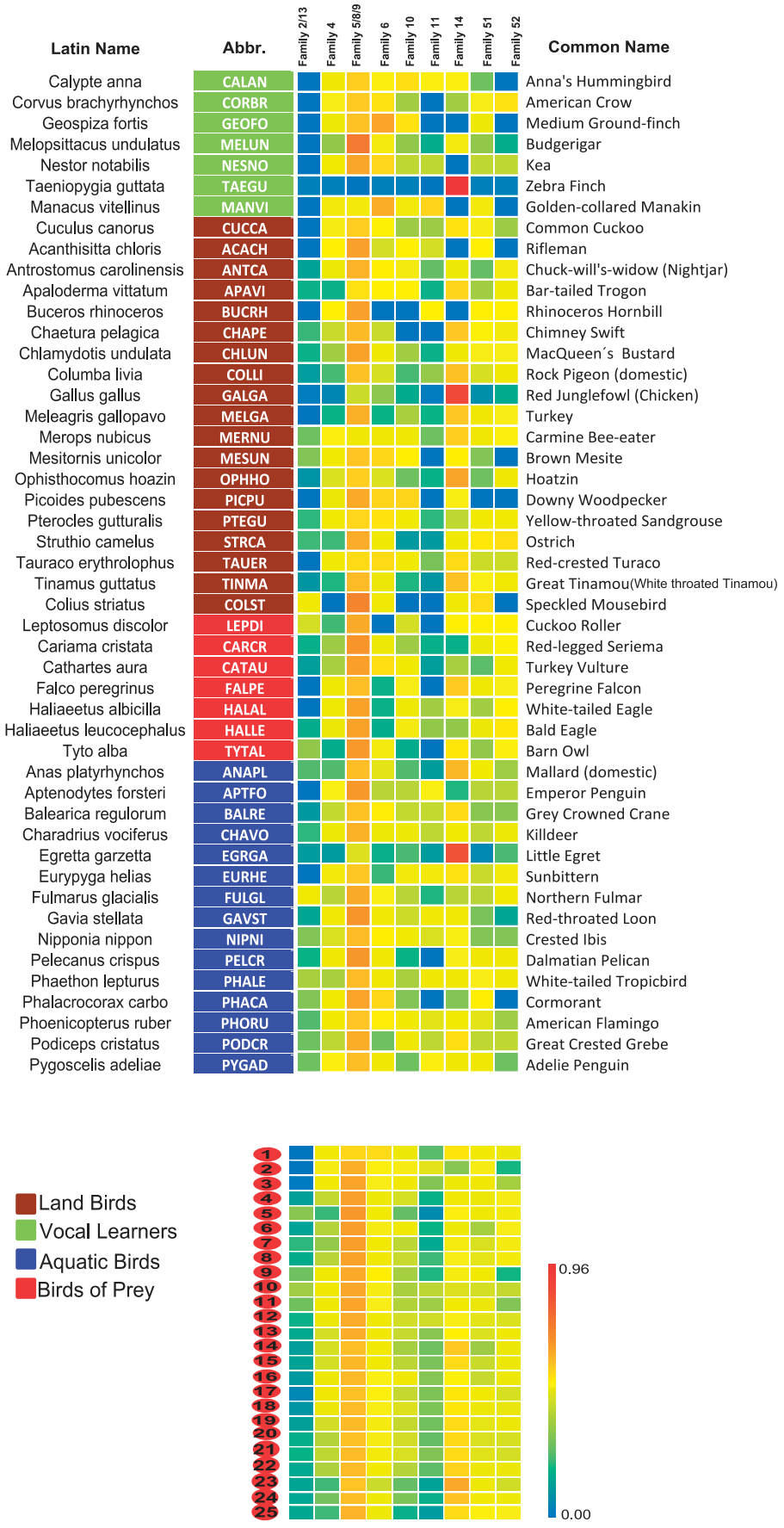

Fig. 3. Heat map partition of informative OR gene families considering the broad ecological traits groups in birds (land birds, water birds, vocal learners, and birds of prey). The four outlier birds, ANAPL (duck), EGRGA (egret), TAEGU (zebra finch) and MELUN (budgerigar), were grouped with land birds in our analysis. For convenience, the MANVI (manakin) was included in the vocal learner group, due to its potential courtship dance learning behavior (Trainer et al. 2002). The LEPDO (cuckoo roller) was included as prey bird.

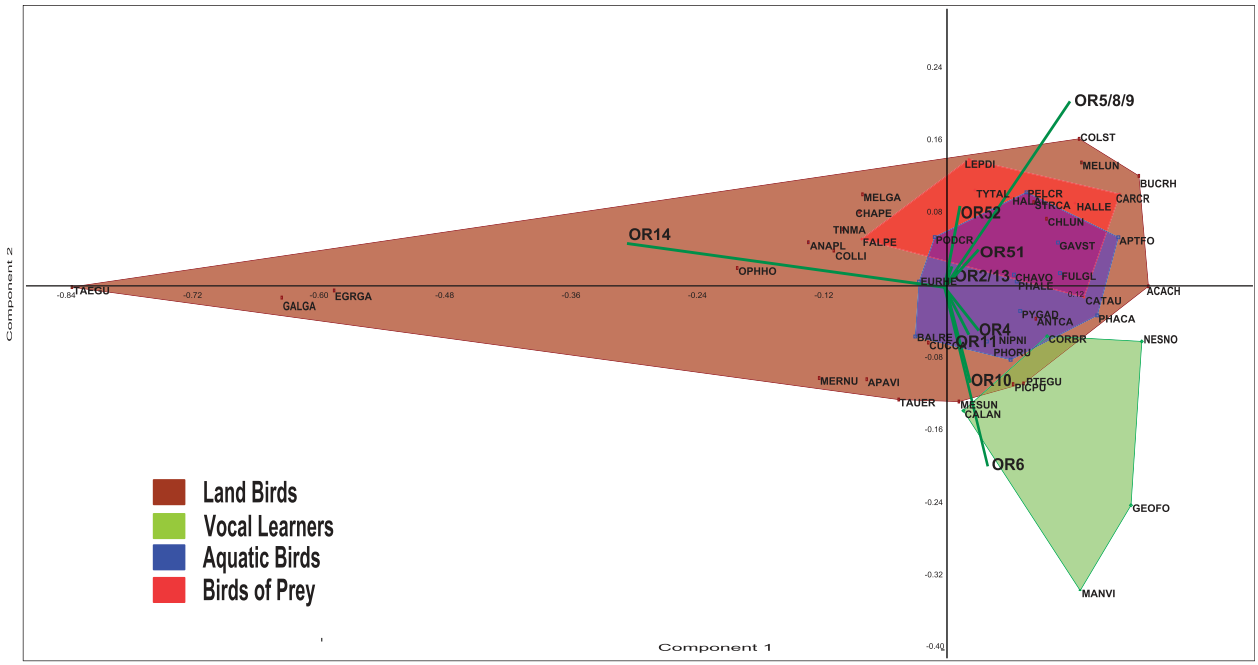

Fig. 4. PCA scatterplots showing the partitioning of ecological traits groups (land birds, water birds, vocal learners, and birds of prey); see legend of figure 3 for further details) and the OR gene families contribution for each group. The two components explained more than 68% variance within data (ANOSIM $r = 0.58$, $P < 0.05$).

recombining coding sequences (Scheffler et al. 2006) was significant with a P valve < 0.001 for OR14 family in chicken, zebra finch, and little egret and for OR52 in sea turtle (supplementary table S8, Supplementary Material online). Sites with evidence of positive selection that were identified with two or more methods and in two or more bird species were plotted against the chicken OR14 protein sequence (supplementary fig. S9, Supplementary Material online) clearly demonstrating that the majority of these positive-selected sites is restricted to the protein TM domains (Steiger et al. 2010). Most of the sites found in the sea turtle OR51 (supplementary table S9*a*, Supplementary Material online) and OR52 (supplementary table S9*b*, Supplementary Material online) were also located in TM domains. These positive-selected sites provide additional evidence of the important role that ecological adaptation has had in the evolution of olfactory capabilities.

Some of these families, such as family 14 in birds and family 51 and 52 in the sea turtle, showed evidence of extensive gene expansion and members of these expanded families had species-specific clustering in the phylogeny due to events of gene conversion and recombination detected by the analysis of putative recombination breakpoints (Pond et al. 2006; Steiger et al. 2008; supplementary table S10, Supplementary Material online).

Conclusions

Differences in the olfactory abilities among birds reflect diverse specialized functions, such as foraging, orientation/navigation, homing, nesting, activity pattern, and individual recognition (Cobb 1959; Cobb 1960; Bang 1965; Bang and Cobb 1968; Bang 1971; Bang and Wenzel 1985; Waldvogel 1989; Healy and Guilford 1990; Papi 1991; Culik 2001; Wallraff 2001; Bonadonna and Nevitt 2004; Van Buskirk and Nevitt 2008; Zelenitsky et al. 2011). These complex behaviors depend on multiple modes of perception, and the observed differences in OR subgenomes described here are possibly interrelated with other sensorial abilities, including vision and vocalizations (Martin et al. 2004; Nevitt et al. 2004; Partan and Marler 2005; Hagelin and Jones 2007). For example, giant petrels use multimodal cues of odor and vision for foraging. Similarly, the Psittaciformes (parrots), typically assumed to be highly reliant on visual cues, are thought to use also olfactory cues when foraging (Hagelin et al. 2003; Roper 2003).

Differences in rearing environment could lead to differences in sensory function, as birds that nest in dark locations may depend more on olfaction, whereas birds exposed to light may use visual, aural, and olfactory cues (Hagelin and Jones 2007; Van Buskirk and Nevitt 2007). Olfactory memories, such as olfactory imprinting, also help in prey avoidance and self-recognition (Burne and Rogers 1995; Sneddon et al. 1998; Cunningham et al. 2003). Our study analyzed the OR repertoire from 48 birds and 2 reptiles with in-depth characterization of gene gain, gene loss (functional and nonfunctional genes), and differential evolution of OR families. We found gene loss to be an important driving force in OR diversification among birds with the detection of a high proportion of pseudogenes in the avian lineage. This contrasting trend is also observed between the OR repertoires of the two reptilian genomes, with gene loss being more prominent in sea turtle than alligator, but with concurrent differential evolution of OR gene families in the two genomes. The drastic expansion of OR51 and OR52 in sea turtle and the expansion of OR14 in the bird lineage strongly support the role of OR51

and OR52 in the detection of hydrophilic compounds and OR14 in hydrophobic volant compounds.

The rapid expansion of those OR gene families was followed by positive selection favoring gene diversification leading to differences in olfactory ability linked with adaptations to different environmental requirements. The relative size of the olfactory bulb in birds correlates well with ecological adaptations, including habitat association (e.g., water birds), type of nesting strategy, and diet (Cobb 1959; Bang 1971; Zelenitsky et al. 2011). For example, birds of prey, including vultures and seabirds, hunt and recognize food by smell, and have relatively large olfactory bulbs (Cobb 1959; Stager 1964; Bang and Cobb 1968; Bang 1971; Zelenitsky et al. 2011), whereas the Passeriformes (song birds) that rely more on cognitive abilities helpful in tool making (Emery 2006), vocal learning (Lefebvre et al. 2002) and feeding innovations (Lefebvre et al. 1997; Timmermans et al. 2000) have reduced olfactory bulb sizes (Cobb 1959; Bang and Cobb 1968; Wenzel 1971; Zelenitsky et al. 2011). The relatively large olfactory bulbs observed in the earliest neornithines relative to basal birds possibly reflect adaptations that improved foraging and/or navigation skills that helped these ancestral birds to adapt, and thus to survive the end-Cretaceous mass extinction (Zelenitsky et al. 2011).

Earlier studies have highlighted the positive correlation between OBR and olfactory ability. Here, we estimated the relationship between the OBR and the OR genes repertoire of 48 birds species and found a positive correlation ($r = 0.66$). This finding provides evidence that olfactory ability is determined by the repertoire of OR genes. We demonstrated that birds of prey (carnivorous/piscivorous) had the largest OR gene repertoire, whereas passerine birds (vocal learners) had the least number of OR genes. The PCA analysis showed that the ecological partitioning of vocal learners, birds of prey, water birds, and land birds strongly influences olfactory ability and that differences in the OR subgenome (e.g., OR51, OR52, and OR2/13) contribute toward aquatic olfactory adaptation, which are further supported by anatomical specializations (Cobb 1959; Bang 1965; Bang and Cobb 1968; Bang 1971; Zelenitsky et al. 2011). In addition, OR6 and OR10 were prominent in vocal learners, OR5/8/9 was linked with foraging behavior of prey birds, and most of the specialized land birds had an expanded number OR14 (γ-c clade). The comparison of our results with Hayden et al. (2010) suggested that OR2/13, together with OR51 and OR52, is important in aquatic adaptation in both mammals and birds. Similarly OR12, OR55, and OR56 contributed the least to olfactory ability in both birds and mammals. In contrast, we observed that OR14, which is expanded in the bird lineage, is less useful in mammals as a measure of the relative importance of OR genes. However, overall, the role of ecological adaptation in shaping the OR subgenome is consistent in both birds and mammals.

The diverse olfactory ability seen across animals was shaped by varied adaptive requirements, as was highlighted by differences among birds and reptiles. Although birds are often thought to be less dependent on olfaction, we provide evidence supporting the premise that a wide range of avian olfactory abilities are linked with different uses of olfaction in crucial behaviors (e.g., foraging, homing, and navigation), and that the crucial roles of these genes are reflected in the genetic architecture of their OR subgenomes.

Materials and Methods

Annotation of OR Genes in Bird Genomes

To identify the OR genes in bird genomes, we downloaded the known amino acid sequences of OR genes excluding non-OR theta genes, from anole lizard, chicken, and zebra finch (Steiger, Kuryshev, et al. 2009). We have followed the procedure described by Steiger, Kuryshev et al. (2009). Overall we got putative OR genes from 48 avian genomes (Jarvis et al. 2014; Zhang et al. 2014) and two reptiles, alligator and green sea turtle

First, TBLASTN searches with an *E*-value cut-off of 10 were conducted to identify candidate OR loci. Then, the results of TBLASTN were clustered together according to the locations of BLAST hits in the genome. For a given locus, the best hit with smallest *E* value and with length of $\geq$150 bp was retained for subsequent analysis. For the candidates lacking start/stop codons, we searched 90 bp upstream to find start codons and 90 bp downstream to find stop codons.

Second, RepeatProteinMask was adopted to distinguish OR genes from non-OR GPCRs. The above known full-length OR sequences from Steiger, Kuryshev, et al. (2009) and 328 non-OR GPCR sequences from Lagerstrom et al. (2006) were merged together as the library to run RepeatProteinMask for each genome. Based on the results of RepeatProteinMask, the candidate loci from the TBLASTN step that matched non-OR GPCR regions (overlapping length/candidate length $>$ 50%) were filtered out. The remaining OR candidates can be classified into three categories: Intact genes with normal start codons and stop codons and more than 650 bp in size thus can code for seven TM domains, partial genes without start codon or stop codon or both, and pseudogenes with frame shift mutations and/or premature stop codons.

We investigated the relationship between the normalized mapping depths of OR genes and sequencing depths. After mapping the reads back to the assemblies (~30$\times$ coverage reads for each bird; for chicken and zebra finch, we mapped the available Illumina reads), we calculated the mapping depth for each OR gene locus and normalized them by dividing the genome average mapping depth.

OR Assignments of Group, Families, and Subfamilies

To assign all functional genes to their respective OR families, we performed HMMER searches against a local database consisting of protein profiles of all known OR families present in HORDE database (OR1-14 and OR51-56) and other known OR groups from river lamprey, zebrafish, and frog (Freitag et al. 1999; Niimura 2009b) thereby covering all known ORs (α–η) from all major vertebrate groups. The sensitive search against the database allowed us to assign each OR gene based on best similarity to the closest known OR gene profiles with high confidence. The accuracy of assignment was tested, by

assigning known human and lizard ORs against the database with each known OR being correctly assigned to their respective family.

Avian Phylogeny of ORs

The amino acid sequences of all intact functional OR genes ≥650 bp found in this study were aligned using MUSCLE (Edgar 2004) and the alignment was manually corrected and used to construct a Neighbor-Joining tree in MEGA5 (Tamura et al. 2011) with Poisson correction method and 1,000 replicates (Felsenstein 1985). We used all available previously described representative ORs families (OR1-14 and OR51-56) and groups (α–η) from zebrafish, river lamprey, frog, and human (Niimura 2009b), which improved the resolution of the OR gene family tree.

Positive Selection

The ratio of nonsynonymous and synonymous mutations ($\omega = dN/dS$) provides an estimate of changes that are advantageous, reflecting positive selection ($\omega > 1$), neutral ($\omega = 1$), or disadvantageous, reflecting negative selection ($\omega < 1$) (Yang 1997). Because of gene conversion and recombination, no single tree can represent a correct phylogeny, and methods such as PAML, which are based on single phylogeny, can give false positives. Therefore, we used five different individual methods along with an integrated approach to allow use of multiple phylogenies based on inferred potential breakpoints and thus obtain ostensibly more-accurate signals of positive selection. All these methods are implemented in Datamonkey web server http://www.datamonkey.org (last accessed March 10, 2014) (Pond and Frost 2005a) and also in the HyPhy package (Pond et al. 2005). These includes SLAC, FEL, REL (Pond and Frost 2005b), MEME (Murrell et al. 2012), FUBAR (Murrell et al. 2013), and integrative approach. SLAC model uses ancestral sequences reconstruction. FEL calculates site-by-site *dN*/*dS* without assuming a prior distribution. REL assumes a prior distribution across site. FUBAR ensures robustness against model misspecification. MEME is the most appropriate to detect episodic diversifying selection affecting individual codon sites. The integrative approach incorporates all sites detected by SLAC, FEL, REL, FUBAR, and MEME. The sites detected by two different methods can be supportive of positive selection. Combined with the PARRIS method, our approach provides a robust inference of positive selection in recombining coding sequences by allowing for variable tree topologies and branch lengths across detected recombination breakpoints and variable synonymous substitution rates across sites. These methods make use of multiple phylogenies resulting from each recombinant fragment and thus are less prone to false positives. All these methods were used with default settings.

Principal Component Analysis and Analysis of Similarities

The proportion of OR functional genes was used for PCA. PCA was conducted to assess the degree of correlation of specific OR gene families with the four avian ecological groups (fig. 4 and supplementary fig. S5, Supplementary Material online). PCA analysis of all functional genes was carried out using PAST v1.89 (Hammer et al. 2001). The covariance matrix was used to assess patterns of variation in OR family distribution in different bird groups based on their shared traits (namely land birds, water birds, vocal learners, and birds of prey). The significance of these groupings was tested using a nonparametric test for ANOSIM (Clarke 1993) between groups using Euclidean distances and derivations of *R* statistics. The observed values were compared with 95% confidence interval of a simulated distribution.

Ancestral State Reconstruction

The ancestral state construction of OR gene repertoire for nodes 1–25 in figure 2 was carried out using Mesquite v2.75 (Maddison WP and Maddison DR 2011) using the consensus avian phylogeny from Jarvis et al. (2014) and Zhang et al. (2014). The parsimony method using continuous character was used to estimate the ancestral OR familial distribution at each node. The OR family distribution at each ancestral node was determined based on the assignment test.

Bayesian Assignments

Naïve Bayes assignment is a machine learning algorithm implemented in the WEKA package (Whitten and Frank 2005). It uses independent assumptions to determine how best to categorize a data set based on the expressed variation (here based on OR familial distribution and ecological trait categories including land birds, water birds, vocal learners, and birds of prey). This training data set is then used to assign each species to a respective ecological group based on OR family distribution. The species to be assigned (the target species) is removed from the training set and subsequently assigned.

Supplementary Material

Supplementary figures S1–S9 and tables S1–S10 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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