A Cluster of Olfactory Receptor Genes Linked to Frugivory in Bats

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Abstract

Diversity of the mammalian olfactory receptor (OR) repertoire has been globally reshaped by niche specialization. However, little is known about the variability of the OR repertoire at a shallower evolutionary timeframe. The vast bat radiation exhibits an extraordinary variety of trophic and sensory specializations. Unlike other mammals, bats possess a unique and diverse *OR* gene repertoire. We elucidated whether the evolution of the *OR* gene repertoire can be linked to ecological niche specializations, such as sensory modalities and diet. The *OR* gene repertoires of 27 bat species spanning the chiropteran radiation were amplified and sequenced. For each species, intact and nonfunctional genes were assessed, and the *OR* gene abundances in each gene family were analyzed and compared. We identified a unique *OR* pattern linked to the frugivorous diet of New World fruit-eating bats and a similar convergent pattern in the Old World fruit-eating bats. Our results show a strong association between niche specialization and *OR* repertoire diversity even at a shallow evolutionary timeframe.

Key words: **Chiroptera, Phyllostomidae, olfaction, niche specialization.**

Introduction

Among vertebrates, olfaction is used to varying degrees in all aspects of life including detection of food, predator avoidance, and social communication. The olfactory receptor (OR) gene repertoire is the largest gene family within the mammalian genome, with approximately 1,000 functional *OR* genes, each coding for an individual OR, and each expressed consecutively in the olfactory epithelium cells (Buck and Axel 1991; Niimura and Nei 2007; Keller and Vosshall 2008). Odor perception is conferred by the binding of odors to ORs, which initiates a signaling cascade mediated by a G-protein coupled receptor to the olfactory bulb in the brain (Mombaerts 1999; Ronnett and Moon 2002). Phylogenetic analyses of nucleotide sequences of mammalian *OR* genes supports 13 monophyletic groups (OR 1/3/7, OR 2/13, OR 4, OR 5/8/9, OR 6, OR 10, OR 11, OR 12, OR 14, OR 51, OR 52, OR 55, OR 56; Warren et al. 2008; Hayden et al. 2010). Although the *OR* gene superfamily constitutes 3–6% of mammalian genes and is well annotated in the finished human and mouse genomes, we still do not fully understand which odorants bind to which receptors, and how this complex process translates into interpreting a particular smell (Krautwurst and Kotthoff 2013). Combining functional assays with comparative genomics, studies have begun to elucidate the relationships between odor and receptor and OR orthologs between species (Krautwurst et al. 1998; Shirokova et al. 2005; Schmiedeberg et al. 2007; Adipietro et al. 2012). However, the scope of studies to date has been restricted to a few *OR* genes.

Hayden et al. (2010) showed evidence for ecological niche specialization linked to the evolution of the *OR* gene repertoire across all major clades of eutherian mammals. In that study, bats displayed a highly diverse *OR* gene repertoire that was distinct from all other studied mammals. With approximately 1,100 species (20% of the extant mammalian diversity) and a wide variety of niches, sensory modes, and dietary specializations (Simmons 2005; Teeling et al. 2005; Wilson and Reeder 2005), the chiropteran radiation provides an opportunity to investigate the ecological drivers of this unique and diverse *OR* genomic repertoire. Bats exhibit diversity along three dimensions: 1) laryngeal echolocation; 2) functionality of the vomeronasal organ (VNO); and 3) feeding specialization (fig. 1). Bats have evolved the ability to use sophisticated laryngeal echolocation or "biosonar" enabling them to develop an acoustic image of their environment and ultimately orient themselves in complete darkness (Jones et al. 2005). This unique emission and auditory capability shows great

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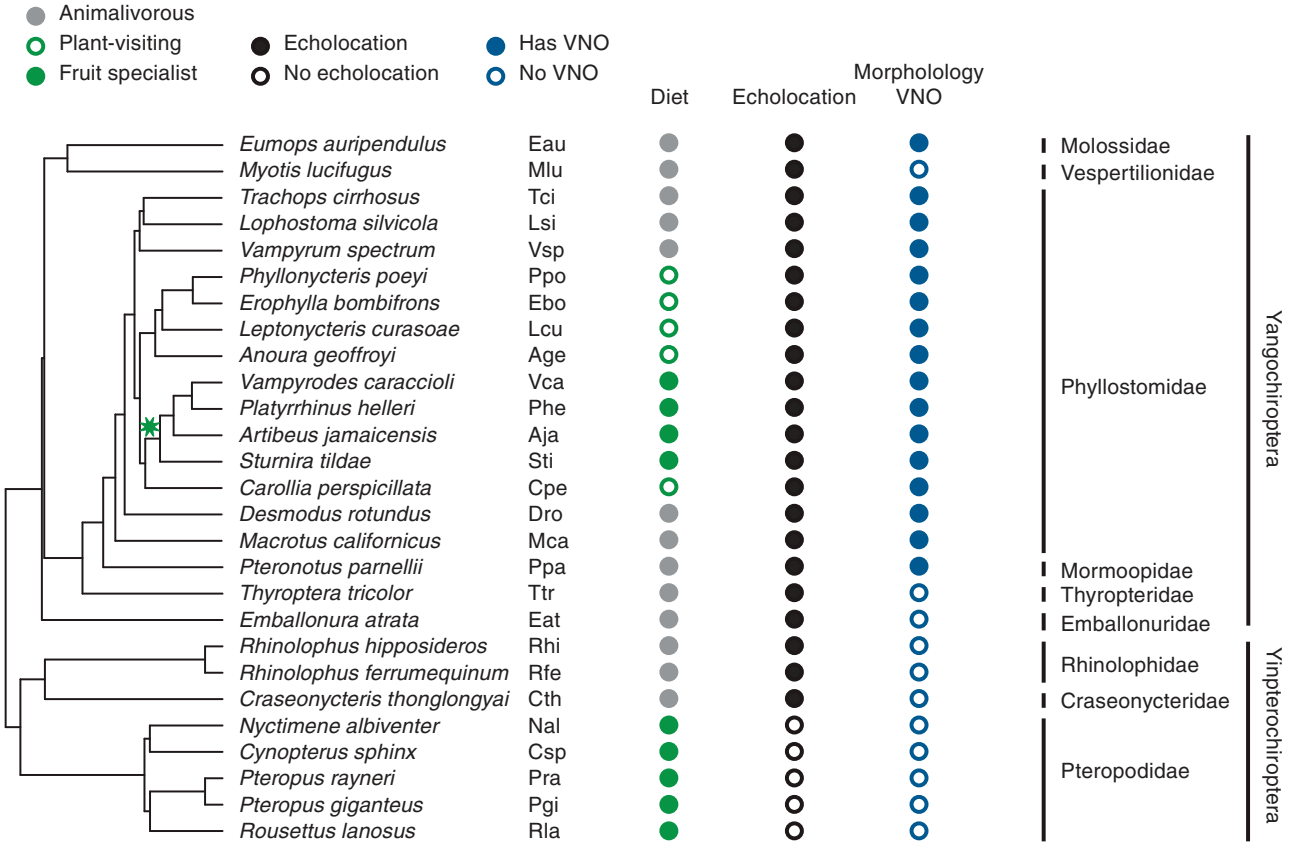

Fig. 1. Phylogenetic tree depicting the evolutionary relationships and systematic classifications used, based on Teeling et al. (2005) and Dumont et al. (2012). Dietary niche, echolocation capabilities, and presence of a VNO are depicted for each species. The plant visiting diet category includes nectarivorous and frugivorous bat species. Species abbreviations used throughout the text are highlighted right of the species name. The subfamily Stenodermatinae is depicted by a green star.

sensory plasticity among families of echolocating bats and appears to be shaped more by ecological adaptation than by shared ancestry (Teeling et al. 2005; Jones and Teeling 2006). Not all bats rely on laryngeal echolocation. The family Pteropodidae (fig. 1) comprises nonlaryngeal echolocators that rely on vision and olfaction instead of sound to orient (Zhao et al. 2009). The wide variety of sensory specializations and modalities in bats could explain the variety and distinctness of the bat OR repertoire if reflected in the *OR* gene diversity. Finally, the VNO is involved in chemosensation, housing ORs along with vomeronasal receptors (VNRs) for the detection of pheromones (Keller and Vosshall 2008). Aside from primates, bats are the only mammalian group that exhibits great variation in the VNO (Wible and Bhatnagar 1996; Zhang and Webb 2003), with some species having a nonfunctional VNO (Zhao et al. 2011; fig. 1). It is possible that variation in the chiropteran VNO results from a sensory reallocation between olfaction and vomeronasal function for chemosensation, in which VNO function is taken up by ORs, driving the genomic diversity observed in bat ORs.

Bats arguably encompass the entire range of vertebrate diets, including insects, small vertebrates, fish, blood, nectar, fruit, and pollen (Nowak and Walker 1994). The majority of bats are insectivorous, yet dedicated frugivory has evolved at least twice: once within the suborder Yinpterochiroptera, family Pteropodidae and once within the suborder Yangochiroptera, family Phyllostomidae, subfamily Stenodermatinae (Voigt et al. 2011; see Teeling et al. [2005] for classification; fig. 1). In both radiations, this dietary specialization has opened new ecological opportunities and allowed for great increased taxonomic and ecological diversification (Almeida et al. 2005; Voigt et al. 2011; Dumont et al. 2012).

Access to fruits may depend, at least in part, on sensory evolution. In behavioral studies, it has been shown that bats can use olfactory cues to locate fruits (Kalko and Condon 1998; Korine and Kalko 2005). Morphological studies have shown significant differences in the thickness of the olfactory epithelium in insectivorous Yangochiroptera and the frugivorous Pteropodidae (Neuweiler 2000). The variation in the olfactory epithelium is reflected in olfactory bulb size: Frugivorous bats within both Yinpterochiropera and Yangochiroptera have relatively larger olfactory bulbs than nonfruit eating bats (Reep and Bhatnagar 2000; Jones and MacLarnon 2004). Comparisons of folivorous, generalist, and frugivorous lemurs have demonstrated a dominant use of olfaction over vision by frugivorous lemurs (Rushmore et al. 2012), and this may also be the case for bats. This morphological and behavioral background suggests olfaction is linked to dietary specialization, and we

investigated this relationship in the evolution of the *OR* gene repertoire in bats.

Here, we hypothesize that the importance of olfaction in bats varies depending on the sensory or ecological niche to which species have adapted. Extant mammals and their associated OR diversity have evolved for 217–236 My (Springer and Murphy 2007; Meredith et al. 2011), and at this phylogenetic depth, there is evidence for *OR* adaptation to specific ecological niches (Hayden et al. 2010). In contrast, bats originated approximately 64 Ma (Zhao et al. 2009). By focusing on the diversity in *OR* gene repertoire of the chiropteran radiation, we investigated whether the effects of ecological niche specialization observed broadly among mammals (Hayden et al. 2010) are associated with OR diversity over a more recent time frame, enabling us to better "fine-scale" our understanding between *OR* evolution and ecological niche specialization. We generated and compared the *OR* gene repertoire across 27 bat species spanning the entire chiropteran phylogeny to investigate whether the evolution of the *OR* gene repertoire in bats was linked to sensory and ecological specialization and to identify which gene families are important in each ecological niche. Our results suggest that the *OR* gene repertoire of bats has been linked to the independent evolution of frugivory in two radiations and highlight the importance of two *OR* gene families in frugivory.

Results

A total of 3,401 new *OR* gene copies (supplementary table S1, Supplementary Material online) from 16 species of bats were successfully amplified and sequenced. These data were supplemented with raw sequence data from an additional 11 species of bats previously published (Hayden et al. 2010). In total, 5,517 *OR* gene copies from 27 bat species were examined (National Center for Biotechnology Information accession numbers: KC928445–KC933933). The actual number of DNA sequences amplified per species ranged from 152 to 450, and the resulting number of genes (assembled at 99% sequence similarity) sampled per species varied from 100 to 390 *OR* genes (supplementary table S1, Supplementary Material online).

Principal Component Analyses

There was great variability between the *OR* gene repertoires of all bat species studied (fig. 2; supplementary tables S2 and S3, Supplementary Material online). The first two principal components (PCs) explained more than 84% of the variance in *OR* gene repertoires (fig. 2; supplementary tables S4 and S5, Supplementary Material online). There were no significant differences between groups in the first PCs of all *OR* genes, functional, or pseudogene subsets as well as the proportion of pseudogenes (table 1). Analyses across all bat species studied revealed no significant differences between echolocating and nonecholocating bats in the first PCs of all *OR* genes, functional, or pseudogene subsets (P value ≥ 0.50; table 1). The effect of having a functional VNO or accessory olfactory organ was also tested, but again, this did not explain the variability in *OR* gene repertoire between species (P value ≥ 0.29; table 1). Neither was there any significant difference between Yinpterochiroptera and Yangochiroptera (P value ≥ 0.07; table 1), suggesting the suborders have not evolved distinctive *OR* profiles. Analyses across all bat species studied revealed no significant differences between frugivorous and nonfrugivorous bats across the entire chiropteran radiation (P value ≥ 0.50).

In contrast, comparisons of *OR* gene diversity in frugivorous versus nonfrugivorous phyllostomids were consistently significant for the first PC with the best models of trait evolution (table 2; supplementary table S6, Supplementary Material online). Within the Phyllostomidae, PC analyses show frugivores grouping away from other phyllostomids, the difference in the *OR* gene repertoire is significant when analyzing all *OR* genes together, functional, or pseudogenes separately (P value ≤ 0.05, fig. 2 and table 2). The PC analyses distinguished which *OR* gene families were driving the differences in *OR* gene repertoire between species. Gene family OR 1/3/7 and, to a lesser extent, family OR 2/13 appear to be important for frugivorous phyllostomids, whereas family OR 5/8/9 appears to be important for other phyllostomids. This pattern in phyllostomids is consistent when data are analyzed as a whole or split into functional and pseudogenes (fig. 2) and is clearly depicted in the heat map (fig. 4).

The link between OR gene variation and frugivory is also evident in Yinpterochiroptera. Pseudogenes and, to a lesser extent, combined functional and pseudogenized diversity show frugivores grouping together within Yinpterochiroptera (fig. 3; supplementary table S7, Supplementary Material online). As in phyllostomids, the main axis of differentiation for frugivorous yinpterochiropterans for combined genes showed increases in subfamilies OR 1/3/7 and OR 2/13. In contrast, differences in pseudogenes involved changes in proportion of pseudogenes in OR subfamilies 11 and 52. Differences in *OR* gene repertoire in the Yinpterochiroptera were significant for the second PC of pseudogenized genes (P value = 0.05, table 2) but not for combined or functional genes. This suggests that pseudogenization has been more important in the differentiation of *OR* gene repertoires in Yinpterochiroptera. An increase in the proportion of genes in families OR 1/3/7 and OR 2/13, and a loss of genes in family OR 5/8/9, coincides with frugivory in both, all genes, and functional gene data sets. The molecular evolutionary mechanisms through which these patterns have evolved differ between yinpterochiropterans and phyllostomids.

We conducted power analyses to investigate how many observations would be required to significantly distinguish *OR* diversity among functional and all genes data sets within Yinpterochiroptera. Table 3 displays sample sizes needed per group (frugivores versus nonfrugivore) given the differences in our results and a probability of falsely failing to reject the null (β) of 20%. Functional OR genes are indistinguishable, and over 281 independent observations would be required to generate significant results on either the first or second PC. In contrast, the data sets including all OR genes would require only 19 observations to differentiate between feeding groups. Given that significant results were obtained for pseudogenic

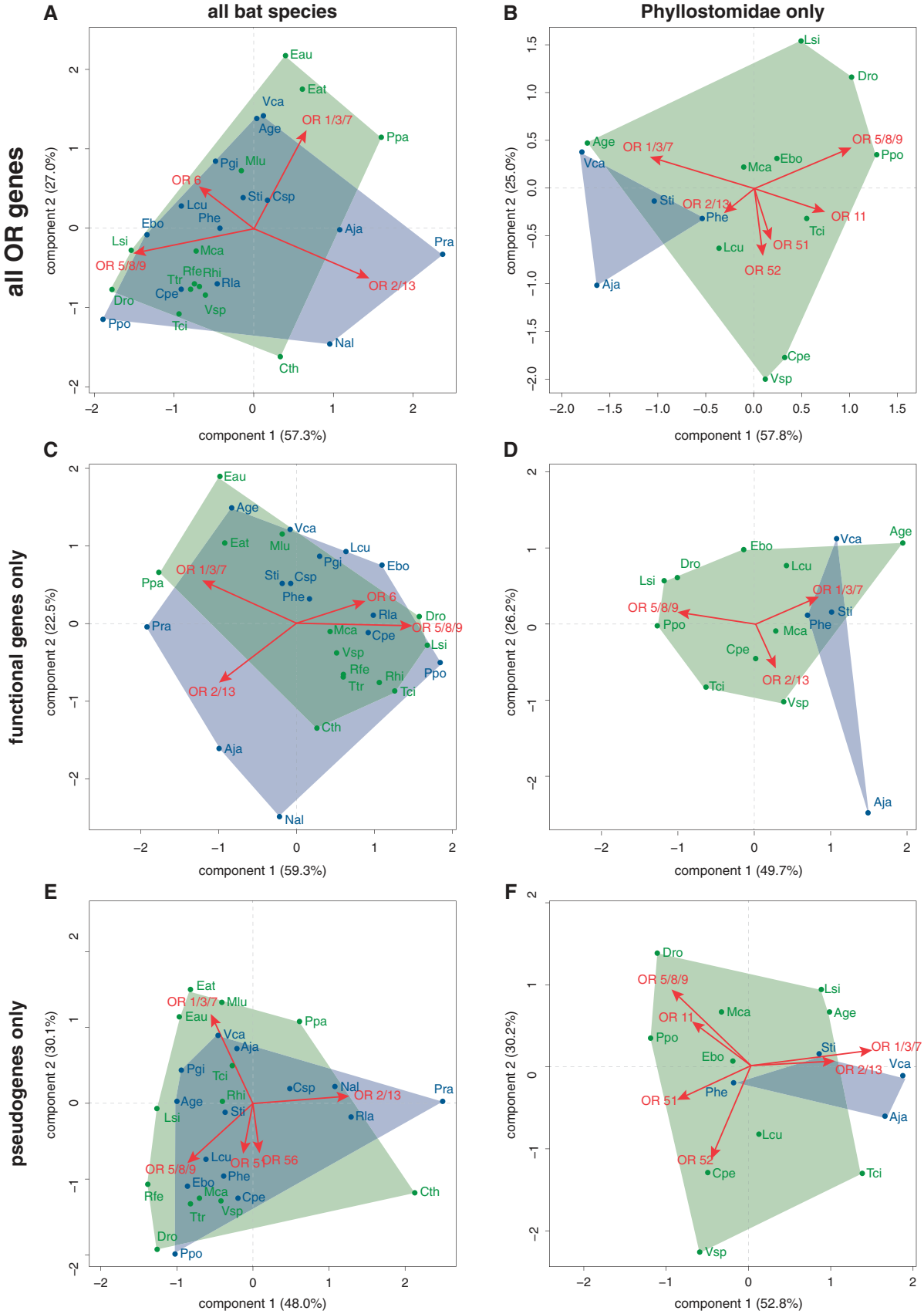

FIG. 2. PCA scatter plots showing PCs (1 and 2). (*A*) and (*B*) PCs generated from all *OR* genes; (*C*) and (D) the functional *OR* gene subset; (*E*) and (*F*) the pseudogene subset. (*A*), (*C*), and (*E*) include all bat species studied, whereas (*B*), (D), and (*F*) represent the Phyllostomidae. Blue polygons represent frugivorous species and green polygons represent nonfrugivorous species. Red arrows represent the influence of particular *OR* gene families on the positioning of each species on the plot. The variance explained by each PC is indicated in brackets. Species name abbreviations follow figure 1.

Table 1. Results of PGLS Models of PCs of OR Gene Diversity and Proportion of Pseudogenes as Functions of Biological Predictors.

Response Variable	Predictor Variable	Lambda	Coefficient	Std. Err. Coefficient	*t* Value	*P*
All genes PC1	Diet	0.20	0.03	0.06	0.60	0.55
All genes PC2	Diet	0.67	0.04	0.06	0.69	0.50
Functional genes PC1	Diet	0.00	−0.03	0.05	−0.57	0.58
Functional genes PC2	Diet	0.00	0.01	0.04	0.40	0.70
Pseudogenes PC1	Diet	0.00	−0.03	0.05	−0.57	0.58
Pseudogenes PC2	Diet	0.00	0.01	0.04	0.40	0.70
Proportion pseudogene	Diet	0.00	0.05	0.03	1.54	0.14
All genes PC1	Echolocation	0.00	−0.13	0.06	−2.00	0.06
All genes PC2	Echolocation	0.59	0.03	0.11	0.28	0.78
Functional genes PC1	Echolocation	0.00	0.07	0.07	1.08	0.29
Functional genes PC2	Echolocation	0.00	0.03	0.05	0.68	0.50
Pseudogenes PC1	Echolocation	0.00	0.07	0.07	1.08	0.29
Pseudogenes PC2	Echolocation	0.00	0.03	0.05	0.68	0.50
Proportion pseudogene	Echolocation	0.00	−0.04	0.04	−1.00	0.33
All genes PC1	Vomeronasal	0.00	−0.08	0.05	−1.58	0.13
All genes PC2	Vomeronasal	0.62	0.06	0.08	0.79	0.44
Functional genes PC1	Vomeronasal	0.00	0.04	0.05	0.81	0.42
Functional genes PC2	Vomeronasal	0.00	0.04	0.04	1.19	0.25
Pseudogenes PC1	Vomeronasal	0.00	0.04	0.05	0.81	0.42
Pseudogenes PC2	Vomeronasal	0.00	0.04	0.04	1.19	0.25
Proportion pseudogene	Vomeronasal	0.00	−0.02	0.04	−0.47	0.64
All genes PC1	Yangochiroptera	0.00	−0.08	0.06	−1.49	0.15
All genes PC2	Yangochiroptera	0.41	0.14	0.08	1.80	0.08
Functional genes PC1	Yangochiroptera	0.00	0.02	0.06	0.38	0.71
Functional genes PC2	Yangochiroptera	0.00	0.07	0.04	1.92	0.07
Pseudogenes PC1	Yangochiroptera	0.00	0.02	0.06	0.38	0.71
Pseudogenes PC2	Yangochiroptera	0.00	0.07	0.04	1.92	0.07
Proportion pseudogene	Yangochiroptera	0.00	−0.03	0.04	−0.70	0.49

NOTE.—All predictor variables were coded as binaries, with one corresponding to a plant-based diet for diet, having laryngeal echolocation for echolocation, having a VNO, and belonging to the suborder Yangochiroptera. The coefficient estimates the effect of these factors on the response variable. All best-fit models estimated the lambda parameter using maximum likelihood. See supplementary table S8, Supplementary Material online, for BM models and AICc values.

Table 2. Results of Best-Fit PGLS Models of PCs of OR Gene Diversity and Proportion of Pseudogenes as a Function of Diet for Yinpterochiroptera and Phyllostomidae.

Taxonomic Group	Response Variable	Coefficient	Std. Err. Coefficient	*t* Value	*P*	w_i
Yinpterochiroptera	All genes PC1	0.09	0.11	0.82	0.44	1.00
Yinpterochiroptera	All genes PC2	0.12	0.06	2.14	0.08	0.90
Yinpterochiroptera	Functional genes PC1	−0.10	0.09	−1.11	0.31	1.00
Yinpterochiroptera	Functional genes PC2	0.08	0.07	1.04	0.34	0.96
Yinpterochiroptera	Pseudogenes PC1	−0.14	0.21	−0.70	0.51	1.00
Yinpterochiroptera	Pseudogenes PC2	0.16	0.06	2.50	0.05	1.00
Yinpterochiroptera	Proportion pseudogenes	0.05	0.05	0.88	0.41	1.00
Phyllostomidae	All genes PC1	−0.18	0.06	−3.02	0.01	1.00
Phyllostomidae	All genes PC2	−0.02	0.05	−0.34	0.74	0.80
Phyllostomidae	Functional genes PC1	0.15	0.06	2.38	0.04	0.99
Phyllostomidae	Functional genes PC2	−0.03	0.05	−0.71	0.49	1.00
Phyllostomidae	Pseudogenes PC1	0.21	0.10	2.14	0.05	1.00
Phyllostomidae	Pseudogenes PC2	0.03	0.13	0.25	0.81	0.50
Phyllostomidae	Proportion pseudogenes	0.06	0.06	1.07	0.30	1.00

NOTE.—The coefficient estimates the effect of a plant-based diet on the response variable. All best-fit models estimated the lambda parameter using maximum likelihood. See supplementary table S9, Supplementary Material online, for BM models, log-likelihood, and AICc values.

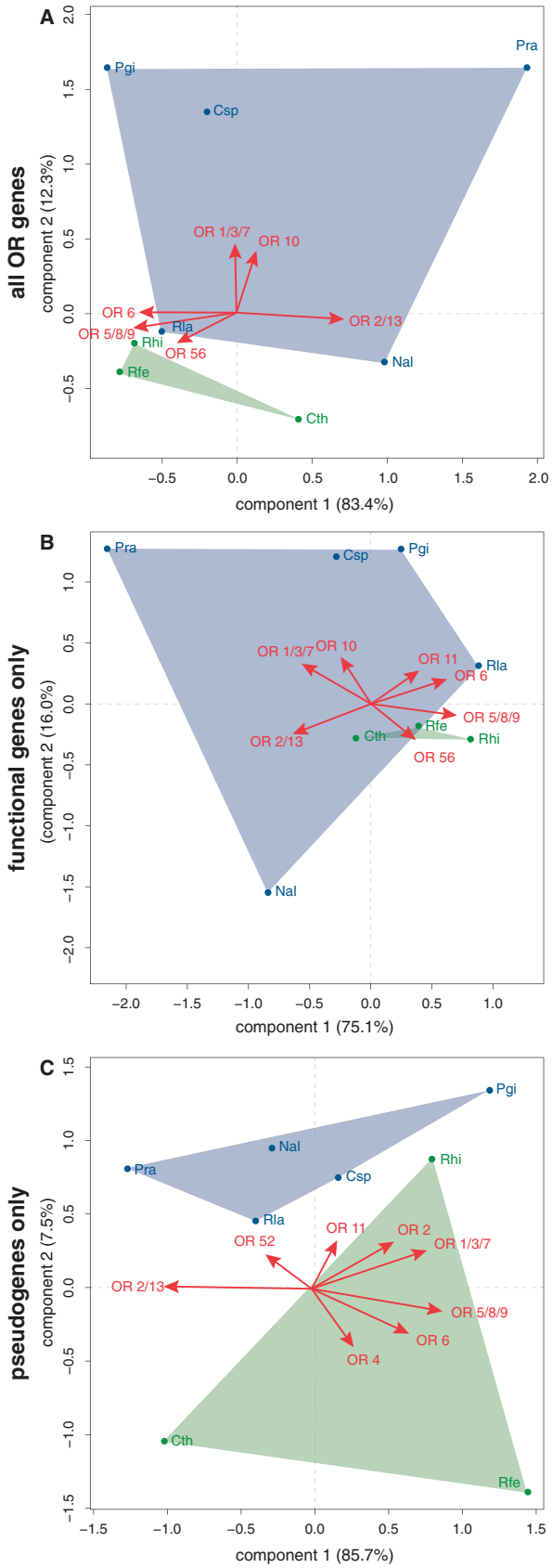

Fig. 3. PCA scatter plots showing PCs (1 and 2) for yinpterochiropteran species only. (*A*) All *OR* genes; (*B*) functional *OR* genes; and (*C*) pseudogenes only. Blue polygons represent frugivorous species and green polygons represent nonfrugivorous species. Species name abbreviations follow figure 1.

OR diversity, the power analyses suggest that important differences in *OR* gene repertoires between dietary specializations in Yinpterochiroptera involve the pseudogenization of certain genes.

Percentage Pseudogenes

There was a considerable amount of variance in the percentage of the bat *OR* gene repertoire that has been pseudogenized. Values ranged from 8% in *Myotis lucifugus* (Little brown bat) to 41% in *Pteropus rayneri* (Solomons flying fox; supplementary table S3, Supplementary Material online). Within phyllostomids values ranged from 9% in *Trachops cirrhosus* (fringe-lipped bat) to 37% in *Vampyrodes caraccioli* (Great stripe-faced bat; supplementary table S3, Supplementary Material online). This considerable variance could not be explained by any of the ecological niche or phylogenetic influences studied here (supplementary table S3, Supplementary Material online). Similarly, after accounting for phylogeny, none of the ecological factors significantly explain the overall percentage of *OR* pseudogenes (tables 1 and 2).

Discussion

Sensory Trade-Offs between Echolocation and Olfaction

This study showed no evidence of a sensory trade-off between echolocation and olfaction. Echolocating bats did not have an *OR* gene repertoire that was significantly different from nonecholocating bats, and the variability in levels of *OR* pseudogenes could not be explained by echolocation capabilities of the species studied. Although there was a high diversity within the *OR* gene repertoire of bats, this diversity was not explained by a gain or loss of echolocation capabilities. This could indicate that more than one sensory signal is at play in this data, and perhaps vision plays a larger role in bat sensory perception than previously considered. Sensory trade-offs between vision and echolocation have been shown at the molecular level with the loss of function in the vision genes *SWS1*, *Gja10*, and *Rbp3* coinciding with the origin of high duty cycle echolocation (Zhao et al. 2009; Shen et al. 2013). It is plausible that a combination of vision and olfaction is being "traded" for echolocation, resulting in the differences in olfactory bulb and visual brain component sizes in the brains of echolocating and nonecholocating bats (Reep and Bhatnagar 2000; Jones and MacLarnon 2004).

Similarly, there was little evidence for a sensory trade-off between the VNO and the OR gene repertoire. This result was surprising, as the VNO complex is a chemosensory organ thought to be involved in pheromone detection (Halpern and Martinez-Marcos 2003; Young et al. 2010). ORs were considered to detect general odors and VNRs to detect a particular type or subset of water-borne molecules, such as pheromones, but recent studies have shown that these systems are not so easily split. Pheromones have been shown to stimulate olfactory neurons as well as vomeronasal neurons (Shepherd 2006; Ma 2007), and the VNO is known to express *OR* genes (Keller and Vosshall 2008). Our results indicate that the *OR* gene repertoire and the VNR gene repertoire are not

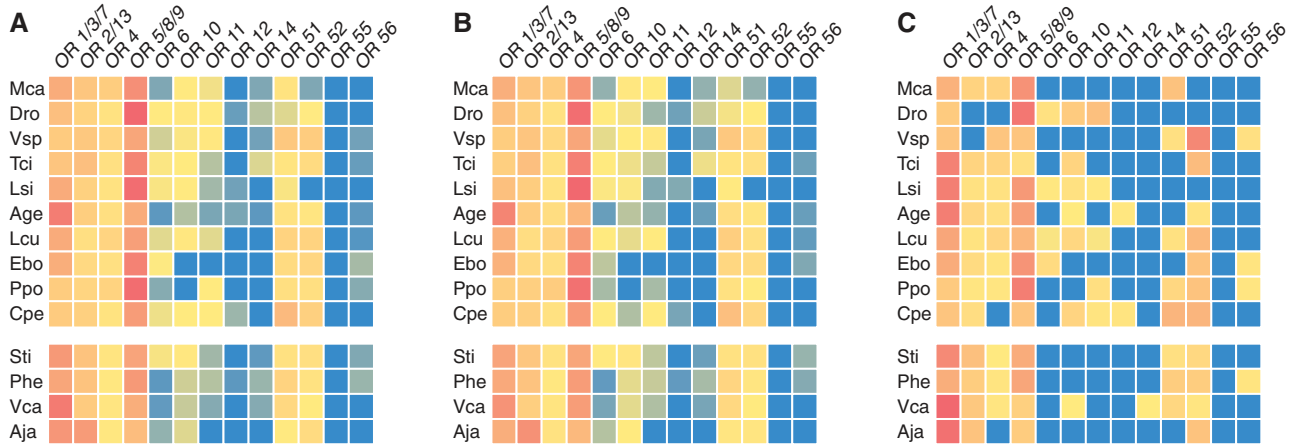

FIG. 4. Heat maps illustrating the normalized level of *OR* genes in each *OR* gene family within the Phyllostomidae. Blue represents a low level of *OR* genes, whereas red represents a high level. (*A*) The data generated from all genes; (*B*) data generated from the functional *OR* gene subset; and (*C*) data generated from the pseudogene subset. Species names are abbreviated according to figure 1. Species are separated into nonfrugivorous above and frugivorous below to illustrate the increased level of functional genes in OR 5/8/9 within nonfrugivorous and the same for OR 1/3/7 within frugivorous for all gene and functional gene subsets.

Table 3. Power Analysis of Phylogenetic PCs Derived from OR Genes in the Yinpterochiroptera.

Data	f^2	$N_{estimate}$ ($\beta = 0.2$)
All PC1	**0.046**	**208**
All PC2	**0.514**	**19**
Functional PC1	**0.034**	**281**
Functional PC2	**0.011**	**839**

NOTE.—Effect sizes (f^2) were calculated based on PGLS models. $N_{estimates}$ of the number of independent observations were rounded up to the nearest integer.

tightly linked and must be more independent than previously thought. A recent study investigating vomeronasal function across bats suggested widespread loss of function in the *TRPC2* ion channel gene crucial for vomeronasal signal transduction in yangochiropteran species. Interestingly, three of the four yinpterochiropteran species studied displayed functional *TRPC2* genes, and two of these were also examined in our study (Zhao et al. 2011). Combined with our results, the findings of Zhao et al. (2011) suggest the link between vomeronasal chemosensation and olfaction is more complex than captured by a simple classification of presence or absence of a functional VNO. In this study, we classified a functional VNO based on morphology. If the array of VNR genes were examined instead of the presence or absence of a functional VNO from morphological data alone, a relationship between these two modes of chemosensation might be elucidated.

Links between Frugivory and Olfaction

Bats originated approximately 65 Ma followed closely by the split of the suborders Yinpterochiroptera and Yangochiroptera approximately 58 Ma (Teeling et al. 2005). The crown Pteropodidae evolved approximately 16 Ma in the Old World (Teeling 2009; Meredith et al. 2011; Teeling et al. 2012). The frugivorous subfamily Stenodermatinae originated approximately 14 Ma (Datzmann et al. 2010), and the family Phyllostomidae is confined to the New World (Teeling et al. 2005; Springer et al. 2011; fig. 1). Given their divergent positions within the chiropteran phylogeny and disparate geographic origins, frugivory has independently evolved within a similar time frame in both Old and New World bat radiations.

Here, we show that *OR* gene families OR 1/3/7 and OR 2/13 are associated with the *OR* gene repertoire of frugivorous bats across two large radiations: the Yangochiroptera, including New World fruit bats in the family Phyllostomidae, and the Yinpterochiroptera, including Old World fruit bats and the family Pteropodidae (figs. 2–4).

Adaptation to frugivory within Stenodermatinae (fig. 1) appears to have occurred rapidly after the evolution of a new cranial phenotype that allowed comparatively small bats to feed on relatively hard canopy fruits (Dumont et al. 2012; Santana et al. 2012). It is possible that the relatively fast adaptation to dedicated frugivory resulted in the dramatically different *OR* gene repertoire that we see in the Phyllostomidae. We found shifts associated with frugivory across the entire OR subgenome. Particular shifts in diversity within functional genes and pseudogenes, rather than the simple pseudogenization of a large number of ORs, suggests adaptive changes linked to ecological specialization in the highly derived frugivorous phyllostomids.

The timing and morphology of adaptation to frugivory within Yinpterochiroptera has not been investigated in as much depth. We found that significant differences within Yinpterochiroptera *OR*s were concentrated in pseudogene OR diversity, and power analyses also support this, as detecting differences in functional gene diversity would require an impossibly large sample size. The patterns of differentiation in OR repertoires between frugivores and nonfrugivores in both Yinpterochiroptera and Yangochiroptera suggest *OR* genomic adaptations to frugivory in pteropodids were mainly achieved through pseudogenization, rather than functional variation alone. Recent genetic studies suggest that there are detectable genetic adaptations to frugivory

in pteropodids. For example, a study of the glucose transporter gene, *Slc2a4*, found a change in selection pressure within Yinpterochiroptera but no positive selection in Yangochiroptera, including frugivorous phyllostomids (Shen et al. 2012). This potential "differential" adaptation to frugivory should be further explored.

Recent analyses have revealed that the transition from antagonistic and less specialized to mutualistic, specialized trophic interactions has increased taxonomic diversification in mammals generally (Price et al. 2012) and phyllostomid bats in particular (Rojas et al. 2012). In phyllostomids, this transition involved both a reorganization of skull architecture to enable high bite force despite small body size (Dumont et al. 2012), changes in selective pressures in one gene responsible for ATP synthesis (Dávalos et al. 2012), and adaptation in the mitochondrial-targeting sequence in a dietary enzyme (Liu et al. 2012). In an aerial nocturnal niche, animal prey can be located using sound and echolocation, but locating fruit requires an alternative sensory strategy. The changes in OR repertoires detected here suggest shifts in key gene families are linked to this new sensory strategy. These sensory adaptations are at least as important as morphological and physiological changes in providing access to the frugivorous adaptive zone and may prove crucial to explaining the evolutionary success of frugivorous bats on two continents.

Ecological Opportunity and Morphological Innovation Open New Adaptive Zones

A growing body of evidence shows that evolutionary innovations that provide access to ecological opportunities open new adaptive zones across the mammalian tree (Losos 2010; Dumont et al. 2012). The unhinged jaw of baleen whales combined with the newly discovered sensory organ coordinating lunge feeding in rorqual whales enabled these mammals to become the largest known vertebrates (Pyenson et al. 2012). Within the phyllostomid bats, one of the most diverse dietary radiations within mammals, the evolution of skull morphology allowed certain species to feed on harder foods opening a whole new adaptive zone (Dumont et al. 2012; Santana et al. 2012). Our study provides more evidence for sensory innovation opening new adaptive zones. Molding of the *OR* gene repertoire results in access to a new ecological niche, frugivory, for both phyllostomid bats and members of Yinpterochiroptera.

Behavioral studies on the ability of *Cynopterus sphinx* (greater short-nosed fruit bat), an Old World, fruit feeding species included in this study, to discriminate between different food odors, showed that *C. sphinx* preferred to visit odor containing sample tubes to odorless ones and had a preference for ethyl acetate odors (Elangovan et al. 2006). Interestingly, gas chromatography-mass spectrometry analysis has highlighted ethyl acetate as a compound that occurs at elevated levels in overripe fruit of the New World tree, *Annona muricata* (Marquez et al. 2011), a plant commonly visited by fruit feeding phyllostomid bats, including *Artibeus jamaicensis* (Jamaican fruit-eating bat), also included in this study (Goodwin and Greenhall 1961). Wherever it has been introduced in the Old World, frugivorous bats have acquired a taste for the fruit of *A. muricata* (Spencer and Fleming 1989; Hall and Richards 2000). We speculate that given the link between *OR* genes families OR 1/3/7 and OR 2/13 and frugivorous phyllostomids, these OR gene families may be directly involved in the detection of ethyl acetate; however, this will need to be confirmed and explored with future functional assays.

Future Directions

Although our sampling technique provided a true representation of the *OR* gene repertoire, our conclusion is based on a representative sample and not all *OR* genes. To obtain a better sample of the *OR* gene repertoire of species whose genomes have not yet been sequenced, next-generation sequencing techniques should be used. These approaches would enable analyses of all *OR* genes amplified by degenerate primers (Hughes et al. 2013). More thorough sampling, though still restricted by the range of genes amplifiable by our primers, could unveil signals of ecological niche specialization that are currently masked by inadequate sampling. Gene-capture methods aimed at isolating the entire OR subgenome followed by next-generation sequencing should be developed for nonmodel, nonhuman samples to augment taxonomic and genic representation in future studies. Increasing species sampling within Yinpterochiroptera will also unveil the extent to which the *OR* gene repertoire has been molded by pseudogenization with adaptation to frugivory. Further sampling of specialized nectar-feeding bats would elucidate OR gene families involved in the detection of flowering plants. Increasing taxonomic representation to include other frugivorous nonchiropteran mammals will allow a further exploration into the link between *OR* gene families OR 1/3/7 and OR 2/13 and adaptation to a frugivorous niche.

Conclusion

We have demonstrated a connection between environmental niche specialization and the *OR* gene repertoire. Frugivorous bats in the family Phyllostomidae have a significantly different *OR* gene repertoire when compared with their relatives that do not feed exclusively on fruit. This *OR* gene repertoire appears to be linked to an increase in *OR* gene families OR 1/3/7 and OR 2/13. Within pteropodids, the link between the same *OR* gene families and frugivory can be seen as a trend in combined genes and is associated with pseudogenization in other *OR* gene subfamilies. This study respresents a further step in uncovering the function of *OR* gene families, associating OR 2/13 and OR 1/3/7 gene family diversity with a dietary shift to frugivory forms basis for further studies in nonchiropteran frugivorous mammals.

Materials and Methods

Taxonomic Sampling

The species sampled comprised variation in echolocation abilities, vomeronasal function, and feeding ecology with a particular emphasis on frugivorous lineages (fig. 1). The variation in sensory modality and feeding ecology sampled

included the independent evolution of echolocation and frugivory in two clades, along with the possible loss of function of the VNO in two clades.

OR Gene Sampling

Numbers of *OR* genes, proportion of *OR* pseudogenes, and distribution of genes into *OR* gene families were all examined to elucidate what factors can be associated with the diverse OR subgenomes exhibited within bats. *OR* genes (~700 bp) from genomic DNA of 16 bat species were obtained by polymerase chain reaction (PCR) amplification using the degenerate primers (GPC1 and GPC2), conditions, and protocols as detailed in Hayden et al. (2010). Briefly, the PCR products were cloned into *Escherichia coli* via a Topo-TA cloning kit (Invitrogen Corporation, USA) to isolate the individual genes. Initially, 192 colonies were picked but if this did not yield enough *OR* genes, more colonies were sampled. These clones were screened using M13 vector primers in PCR. Clones containing an insert of correct length were then subjected to another round of PCR using M13 primers and Platinum *Taq* (Invitrogen Corporation, USA). PCR products were purified and Sanger sequenced in forward and reverse directions. These data were combined with raw sequence data from the 11 bat species previously published in Hayden et al. (2010), totaling 27 bat species (fig. 1).

Sequence Analysis

The nucleotide sequences of the *OR* genes were examined using Aligner (CodonCode Corporation, USA). Forward and reverse sequences were aligned and checked for ambiguities. Assembly of the consensus sequences was carried out at 99% similarity level to allow for 1% *Taq*-generated mutations. Each consensus sequence was counted as one gene. Genes were confirmed as *OR* genes and assigned into *OR* gene families using the OR family Assigner, ORA v1.9 (Hayden et al. 2010). To account for the variance in numbers of *OR* genes sampled per species, when comparing the number of genes in each *OR* gene family between species, all values were normalized. For example, within *M. lucifugus,* 89 genes were amplified for family OR 1/3/7 out of a total of 399 genes, this gave *M. lucifugus* a value of 0.301 for gene family OR 1/3/7. When normalizing functional or pseudogenes, the number of genes in a particular family was divided by the total number of functional or pseudogenes. As published previously, any *OR* gene was considered a pseudogene if it did not have an open reading frame of 650 bp or more, which corresponds to the seven transmembrane domains required to bind odors (Hayden et al. 2010; Hughes et al. 2013). Between 152 and 448, colonies were picked and between 100 and 390, *OR* genes were sequenced from each study species (see supplementary table S1, Supplementary Material online, for numbers of sequences and genes).

PCR Screening

It was estimated that at least 25% of potentially *OR* genes amplifiable with these primers needed to be amplified to represent an unbiased sampling of the OR genome. The number of potentially amplifiable genes was assessed using the Gazey and Staley (1986) mark-recapture algorithm (described in detail in Hayden et al. 2010). The 25% threshold value was obtained by comparing the distribution of *OR* genes into gene families from laboratory-generated data and from whole-genome sequence data (see Hayden et al. 2010 for full details). At this threshold, no significant difference was found between experimental and whole-genome sequence samples.

Supplementary table S1, Supplementary Material online, shows three species below the 25% sampling effort *Lophostoma silvicola* (white-throated round-eared bat; 23%), *Carollia perspicillata* (Seba's short-tailed bat; 20%), and *Leptonycteris curasoae* (Southern long-nosed bat; 12%). As these species are below the 25% sampling threshold, it is not clear whether their sequences provided an accurate *OR* gene distribution. To test whether these "below-threshold" species affected the principal component analysis (PCA), PCAs were performed on the normalized distribution of functional genes into OR gene families with, and without, these species in the data set (supplementary fig. S1, Supplementary Material online). No difference was found in the distribution of species across the PCA, therefore the species were retained in all further analyses.

Separation of Data into Eco-Groups and Phylogeny

Bat species were assigned into groups to test the following influences on the OR gene repertoire: echolocation, presence of VNO, and feeding ecology (fig. 1). Phylogenetic trees were obtained from Teeling et al. (2005) and Dumont et al. (2012). Echolocation data were collected from Nowak and Walker (1994). VNO data were collected from Wible and Bhatnagar (1996) and Bhatnagar and Meisami (1998). Feeding data were collected from Nowak and Walker (1994) for bats in general and Voigt et al. (2011) for Phyllostomidae. Bats were classified as echolocators if they were capable of laryngeal echolocation, frugivores if their diet consisted solely of fruit, or in the case of Phyllostomidae, if stable isotope data classified them as frugivorous.

Statistical Analyses

We used phylogenetic PCA to quantify variation within our data sets. Phylogenetic PCA were performed using normalized frequencies on "all genes," "functional," and "pseudogene" OR data sets (supplementary table S2, Supplementary Material online). Separate phylogenetic PCA for all species, phyllostomid species only, and yinpterochiropteran species only were conducted using the *phyl.pca* routine in the *phytools* R package v0.3-72 (Revell 2012). To account for the phylogenetic correlation structure between observations, we grafted the 50% majority rule consensus of dated phylogenies of Dumont et al. (2012) onto the chiropteran phylogeny of Teeling et al. (2005). The resulting tree (supplementary data S1, Supplementary Material online) was used together with normalized *OR* gene frequencies as input in phylogenetic PCA (Revell and Collar 2009). The PCA algorithm was the covariance matrix of the data.

The first two PCs of *OR* genes and the proportion of pseudogenes in ecologically and phylogenetically distinctive sets of species were compared across the entire taxon sample while accounting for phylogeny. The phylogeny described earlier was used to fit phylogenetic generalized least-squares (PGLS) models with correlation structures that applied Brownian motion (BM) or lambda models of evolution (Grafen 1989; Pagel 1997). These analyses were conducted using the *pgls* routine of the *caper* v0.5 R package (Orme et al. 2012). The best-fit model of either BM or lambda was selected using the Akaike information criterion (AIC) with a correction for small sampling sizes (AICc). The criteria of Burnham and Anderson (2002) were applied: Models within 2 units of the lowest AICc (Δ_i) were considered substantially supported by the data, whereas differences of more than 4 units were considered unsupported.

Frugivory

We conducted more detailed analyses of the first PCs of *OR* genes and the proportion of pseudogenes in phyllostomid frugivores (subfamily Stenodermatinae) and nonfrugivores. To account for phylogenetic correlations among observations, we used the summary of the posterior distribution of dated phylogenies of Dumont et al. (2012). This tree were used in comparative analyses fitting linear regression models of OR evolution with frugivory as a factor in BM and lambda (λ) models implemented in *caper*. The AICc of each model was used in model selection and to calculate Akaike weights (w_i) that can be interpreted as the probability that a given model is the best model given the data.

We examined the general trend of distinction along PCs among Yinpterochiropteran frugivores and nonfrugivores (fig. 3). The number of species available for comparison was very small, fewer than six for each diet class. We asked: given the differences found using PGLS, what sampling size would yield statistically significant results ($\alpha = 0.05$)? These power analyses yield the minimum number of independent observations needed to detect differences between group means. Because species are not independent observations, these analyses provide a minimum sampling size needed to statistically differentiate groups. First, we calculated effect sizes from the best-fit models of PCs as a function of diet using the formula:

$$f^2 = \frac{R^2}{1 - R^2}$$

where R^2 values were obtained from phylogenetic generalized squares. Second, the numerator degrees of freedom (number of groups = 2) were obtained based on the models, and the desired power was set at 1-β of 0.80. Finally, we used the *pwr.f2.test* function of the *pwr* v.1.1.1 package (Champely 2012) to estimate the sampling size needed to obtain a statistically significant result ($\alpha = 0.05$).

Heat Maps

Heat maps were generated in R to visualize the normalized levels of *OR* genes in each *OR* gene family across all species. The heat map gradient spanned from blue to red, with blue representing a low level or no genes to red representing a high level of *OR* genes.

Supplementary Material

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

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