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Discoveries

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Genetic and Anatomical Determinants of Olfaction in Dogs and Wild Canids

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Abstract

Understanding the anatomical and genetic basis of complex phenotypic traits has long been a challenge for biological research. Domestic dogs offer a compelling model as they demonstrate more phenotypic variation than any other vertebrate species. Dogs have been intensely selected for specific traits and abilities, directly or indirectly, over the past 15,000 years since their initial domestication from the gray wolf. Because olfaction plays a central role in critical tasks, such as the detection of drugs, diseases, and explosives, as well as human rescue, we compared relative olfactory capacity across dog breeds and assessed changes to the canine olfactory system to their direct ancestors, wolves, and coyotes. We conducted a cross-disciplinary survey of olfactory anatomy, olfactory receptor (OR) gene variation, and OR gene expression in domestic dogs. Through comparisons to their closest wild canid relatives, the gray wolf and coyote, we show that domestic dogs might have lost functional OR genes commensurate with a documented reduction in nasal morphology as an outcome of the domestication process prior to breed formation. Critically, within domestic dogs alone, we found no genetic or morphological profile shared among functional or genealogical breed groupings, such as scent hounds, that might indicate evidence of any human-directed selection for enhanced olfaction. Instead, our results suggest that superior scent detection dogs likely owe their success to advantageous behavioral traits and training rather than an "olfactory edge" provided by morphology or genes.

Keywords: domestication, dog, olfactory repertoire, cribriform plate

Introduction

The olfactory acumen of the domestic dog (*Canis lupus familiaris*), particularly in breeds such as the bloodhound, is well established in popular lore and legend (Pemberton 2013; Worboys et al. 2018). In practice, dogs perform critical scent detection tasks, tracking missing persons, identifying individuals with diseases (e.g. cancer and COVID-19), and locating explosives as well as cryptic and endangered species in the field (Helton 2009; Rooney et al. 2013; Beebe et al. 2016; Wackermannová et al. 2016; Gottwald et al. 2020; Jendrny et al. 2020, 2021; Dargan and Forbes 2021; Grimm-Seyfarth et al. 2021). Canine olfactory detection has even been used as admissible evidence in a court of law (Smith 2021). Aside from genuine feats and murkier legend, comparative studies on mammalian olfactory systems have ranked the dog's olfactory capacity high relative to most other sampled species. This is the case when comparing olfactory anatomy as well as olfactory receptor (OR) gene repertoire size, a metric that has been correlated with scent discrimination performance (Laska and Shepherd 2007; Rizvanovic et al. 2013). Behavioral tests of odorant discrimination and detection thresholds further reinforce the domestic dog's standing as a superior smeller among mammals (Lauruschkus 1942; Marshall et al. 1981; Pihlström et al. 2005; Walker et al. 2006; Niimura et al. 2015; Bird et al. 2018), even if studies comparing detection performance across dog breeds arrive at no clear consensus on exceptional breeds (Jezierski et al. 2014; Hall et al. 2015; Polgár et al. 2016). Because an evolutionary perspective is absent from these studies, two important aspects of dog olfactory systems remain unclear.

First, no comparison of the dog's olfactory system relative to its canid ancestors has yet to be examined. Second, the

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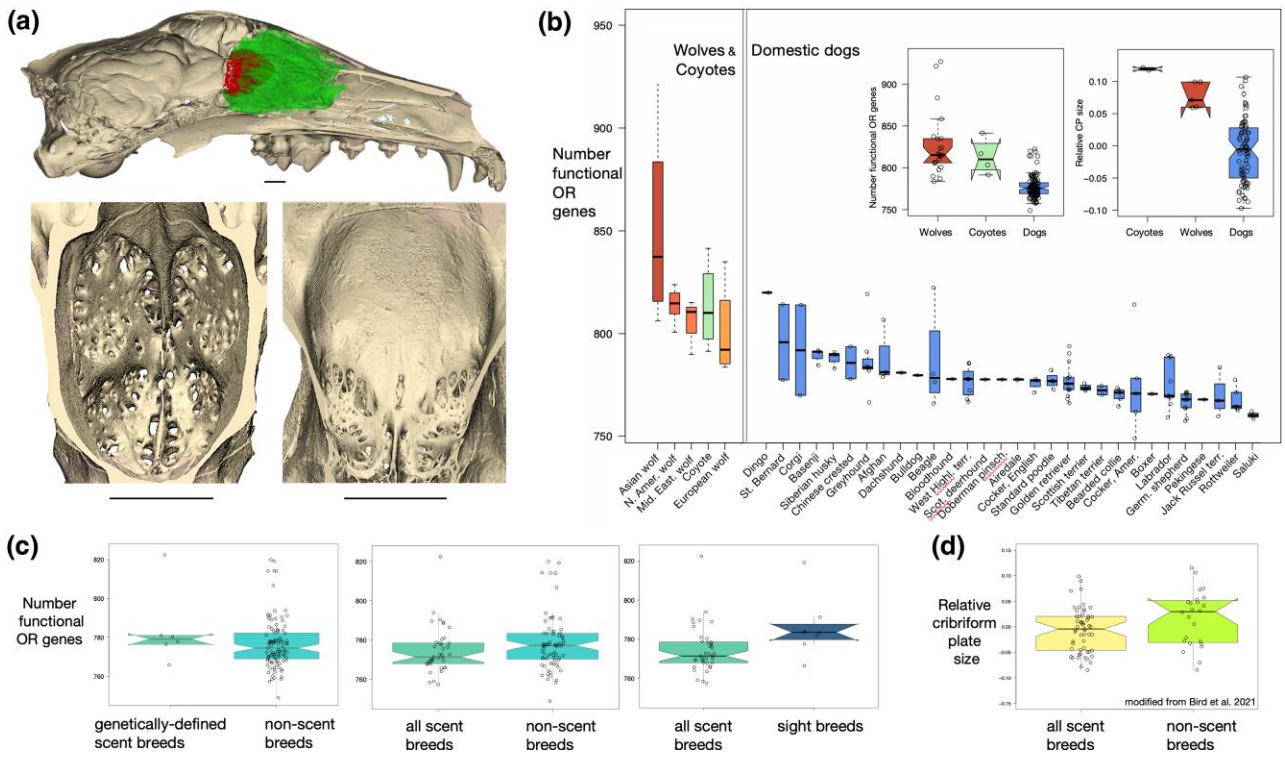

Fig. 1. Comparative olfactory morphology and genomics in wild canids and dog breeds. a) Morphological metric, CP in skull matrix. Top: Borzoi half skull, sagittal view; red, CP; green, olfactory turbinal bones. Bottom, CP, posterior view, showing the relative presence of foramina (holes) for olfactory nerve passage in the gray wolf (left) and Pekingese (right). Scale bars, 10 mm. b) Number of FORGs in wolves (orange), coyotes (green), and domestic dog breeds (blue) in descending order. Left inset: domestic dogs have a smaller FORG repertoire than gray wolves and combined wolves and coyotes ($P < 0.001$). Right inset: RelCP size in dogs is, on average, smaller than in wild canids ($P < 0.001$). Sequencing depth did not significantly affect copy number estimates (supplementary fig. S11, Supplementary Material online). c) Left: FORG count in genetically defined scent breeds is not significantly different from nonscent breeds ($P = 0.41$). Middle: mean FORG count for all scent breeds is not significantly different from that of nonscent breeds ($P = 0.16$) and (right) sight breeds ($P = 0.08$). d) RelCP size is no different between scent and nonscent breeds ($n = 46$, $P = 0.12$). Box plots: midline is median, whiskers are 5% to 95% percentile.

general assumption that concerted artificial selection has resulted in meaningful olfactory differences between dog breeds and breed groupings (e.g. scent breeds) has not been tested. Beginning >15,000 years ago, humans began domesticating individual wolves (*C. lupus*) as commensal or companion animals, a process that would eventually shift to selective breeding for type (Bergström et al. 2020; Morrill et al. 2022). This process has resulted in a new canid lineage with a stunning breadth of variation in form and behavior, exceeding that of their common ancestor, the wolf. Here, we quantify differences between the olfactory morphology, OR gene repertoires, and gene expression of the domestic dog with their closest living ancestors, wolves, and coyotes.

Previous studies have established that mammals rely to varying degrees on olfaction for survival, as evidenced in losses and gains to species' olfactory systems over time (Niimura and Nei 2007; Hayden et al. 2010; Bird et al. 2018). The OR gene superfamily, the largest gene family in terrestrial mammals (Buck and Axel 1991; Hayden et al. 2010), is variably represented in each species' genomes (e.g. humans have a subgenome of 396 functional/or intact, OR genes, whereas African elephants have 1,948). In parallel with OR gene repertoires, olfactory nasal morphology varies markedly across species and reflects selective sensory pressures (Van Valkenburgh et al. 2011; Bird et al. 2018; Bird et al. 2020).

In this light, we predict that the domestic dog, over time, by trading predatory behavior for reliance on human-provided food (Cannon et al. 1999; Arendt et al. 2016; Vonholdt and Driscoll 2017), underwent degeneration of olfactory function relative to its closest living canid relative, the gray wolf. On the other hand, if we accept the widespread assumption that certain dogs, such as scent hounds, have enhanced olfactory traits due to directed artificial selection, we might expect that these dogs have recovered some of this loss through copy number expansion (Gazit and Terkel 2003; Quignon et al. 2012; Pemberton 2013; Greenberg 2017). Moreover, we predict that ancient dogs, a grouping comprised of breeds (e.g. dingo, basenji, Siberian husky, Afghan, and saluki) that show strong evidence of admixture with wolves following domestication, may retain more wolf-like attributes including an enhanced olfactory system (Vonholdt et al. 2010; Freedman et al. 2014; Parker et al. 2017). A recent comparative morphological study of the cribriform plate (CP), the quantifiable bony imprint of olfactory nerves entering the brain from the nose, revealed that domestic dogs had a reduced olfactory skeleton relative to the gray wolf and that, contrary to claims of breeders, scent breeds show no enhanced olfactory phenotypes relative to other breeds (Bird et al. 2021; Fig. 1a; supplementary SI Appendix, supplementary Movies S1 and S2, Supplementary Material online). No comprehensive molecular genetic analysis has been performed comparing OR gene repertoires across dog breeds and closely related wild canids. We hypothesized that OR gene repertoire size and gene expression levels might be more sensitive than morphology alone to directional selection for enhanced scent detection. Here, we report the results of our cross-disciplinary study that combined

morphology and molecular genetics from 56 domestic dog breeds, the gray wolf, and the coyote to better understand the evolutionary dynamics of olfactory systems in domestic dogs and their closest wild canid relatives.

Throughout this paper, we avoid the term olfactory "ability" and instead compare olfactory capacity, that is, chosen genomic and morphological components of olfactory systems. Because olfactory ability is variously described in the literature as detection threshold (Marshall et al. 1981), odorant discrimination (Laska and Shepherd 2007) and identification (Wilson and Richard 2006), and breadth of detectable odorants (Saito et al. 2009), it is too broad a metric difficult to quantify and beyond the scope of this study.

Results

Olfactory Genomics

To test whether losses in olfactory subgenomes accompanied the transition from wolf to dog, we quantified intact functional OR gene (FORG) copy numbers for individual dogs, breeds, and breed groupings (e.g. ancient vs. modern, scent vs. nonscent, American Kennel Club [AKC], etc.) and for gray wolf populations and coyotes (*Canis latrans*; supplementary SI Appendix and Datasets S1 and S2, Supplementary Material online). As the number of intact OR genes is considered a good estimate of the number of functional OR genes (Niimura et al. 2014), we use the terms intact and functional interchangeably here. Overall, FORG repertoire size varies considerably across our sample of domestic dogs and their wild canid relatives (analysis of variance [ANOVA], $P < 0.001$). When comparing our domestic dog and wild canid samples, between-group variation is larger than the within-group variation, and dogs, on average, have significantly fewer FORG than the wolves alone, the wolves and coyotes combined, and the coyotes alone (*t*-tests, $P < 0.001$; Fig. 1b; supplementary SI Appendix and table S1, Supplementary Material online).

Within the entire wild canid sample, the number of FORG does not differ significantly between the gray wolf and coyote ($n = 23$, 4, *t*-test: $P = 0.4$). Within our gray wolf sample alone, the number of FORG ranges from 784 to 927, and the variance between wolf populations is significant (ANOVA, $P = 0.025$; Fig. 1b; supplementary SI Appendix, table S1, and Datasets S1 and S2, Supplementary Material online). Asian (Chinese and Mongolian) wolves ($n = 9$) have, on average, the highest number of functional OR genes (854), and the European wolves ($n = 4$) have the smallest (mean, 801). Mean FORG count in our four European wolf individuals is significantly smaller than that of Asian wolves alone but is not significantly different from that of non-European wolves as a group. Despite having a small repertoire and a sample size of four individuals, the European wolves have, on average, significantly more FORG than domestic dogs if all dog individuals ($n = 111$) are considered (Fig. 1b; Welch *t*-test, $P = 0.007$; supplementary SI Appendix and table S1, Supplementary Material online), but this difference becomes nonsignificant when only dog breed means ($n = 30$) are considered ($P = 0.17$).

Within the domestic dog sample, the number of FORG ranges from 749 to 822 (individual dogs) and 760 to 820 (breed mean). Among breeds, there is a significant variance in gene number (ANOVA, $P = 0.004$). However, this structure is driven by one breed, the dingo, which has more FORG (820) than any other breed mean (Fig. 1b) and significantly more FORG than three other breeds, the German shepherd, saluki, and rottweiler (Tukey honestly significant different (HSD) test; $P = 0.012$, 0.03, and 0.01, respectively). When the dingo is removed from the domestic dog sample, there are no significant differences among breeds in mean FORG count (Tukey HSD test, $P > 0.11$).

We further tested whether concerted artificial selection by breeders has resulted in enhanced FORG repertoires in any breed groupings. First, as regards functional breed groupings, there is no statistically significant difference in FORG repertoire size between nonscent breeds (mean FORG: 778) and scent breeds (mean FORG: 775; Fig. 1c; Welch *t*-tests, $P = 0.37$, supplementary SI Appendix and table S1, Supplementary Material online). The lack of difference was present regardless of how we defined scent breeds, as genetically defined scent breeds alone (3 breeds, 6 individuals; mean FORG: 784), as scent detection breeds (3 breeds, 33 individuals; mean FORG: 773), or as both combined (mean FORG: 775). To test whether years of directed breeding may have favored one sensory specialization over another, we compared scent dogs with sight hounds (Fig. 1c). Scent breeds have, on average, slightly fewer FORG (775) than do sighthounds (786). However, the difference is not significant (Welch *t*-test; using breed mean, $P = 0.47$; using all individuals within each breed, $P = 0.08$; Fig. 1c). When we extended our test for genetic structure across functional groups to include all ten breed groupings assigned by the AKC and dog breeders, we again found no significant differences in OR gene repertoire size (Fig. 2a; ANOVA for breed mean, $P = 0.9$; for individual dogs per breed, $P = 0.09$).

Ancient breeds (dingo, basenji, Siberian husky, Afghan, and saluki; $n = 13$ individuals) distinguish themselves from wild canids with a smaller average number of functional OR genes (785) than wolves alone (826) and wolves and coyotes combined (835) (Welch *t*-test; $P = 0.014$, $P = 0.012$ respectively; Fig. 2b, left). Within domestic dogs, ancient breeds have on average slightly more FORG (785) than do modern breeds (776); however, the difference between these two domestic dog groupings is not significant, regardless of whether we test using breed means alone ($n = 30$; Welch *t*-test, $P = 0.31$) or include all individual dogs ($n = 111$; $P = 0.12$; supplementary SI Appendix and table S1, Supplementary Material online). The dingo is the exception here, with a FORG repertoire (820) closer to the mean FORG count for wolves (826) than to that of other ancient dogs (785) or all other dogs (777) (Fig. 2b).

Finally, as regards morphologically based breed categories, specifically snout length groupings based on cephalic index (Evans and de Lahunta 2012; Stone et al. 2016), we found no difference in FORG number between brachy-, meso-, and dolichocephalic breeds (Fig. 2c; supplementary SI Appendix and table S2, Supplementary Material online; ANOVA, breed mean, $P = 0.62$).

Morphology

To test our hypothesis that differences in olfactory skull morphology across wild and domestic canids would parallel those in olfactory subgenomes, we reexamined and expanded upon CP data documented in Bird et al. (2021). With few exceptions, the morphological results parallel the genomic results. In particular, the relative CP size (i.e. size-adjusted CP: residuals from log–log regression of CP surface area vs. skull length) of the domestic dog is, on average, significantly smaller than that of the wolf and coyote combined as well as the wolf alone ($N = 53$ and 51 respectively; analysis of covariance

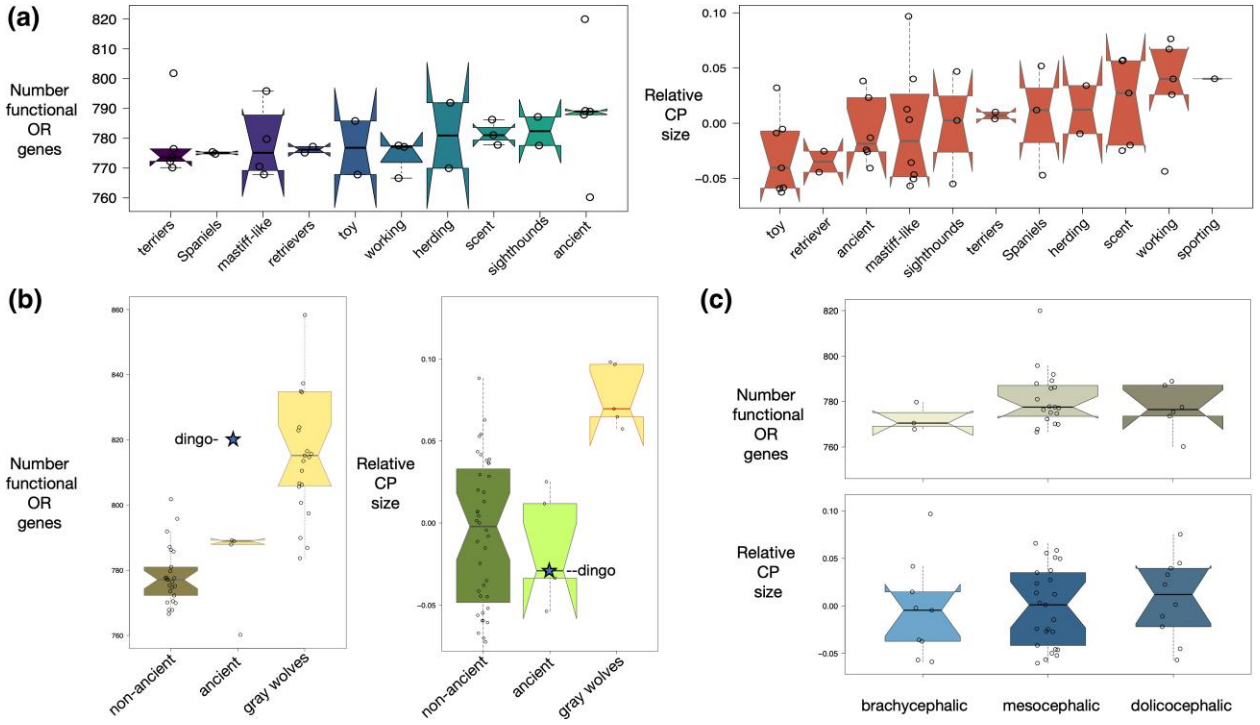

Fig. 2. Differential effects of breed groupings on olfactory subgenomes and morphology. a) No significant difference in FORG count (left) and RelCP size (residuals from log–log regression of CP surface area to skull length; see Materials and Methods) (right) between the ten dog breed groupings defined by the AKC and breeders ($P = 0.9$ and $P = 0.6$ respectively). b) Ancient dog breeds. Left: mean number of FORG in ancient breeds is significantly different from that of wolves ($P = 0.012$) but not from that of nonancient breeds ($P = 0.31$). The dingo FORG count (820) is similar to the mean count in wolves (826) than to that of other dogs (785). Right: the RelCP in the ancient breed grouping is significantly different from that of the wolf but not from that of nonancient breeds. c) Snout length. No difference in FORG count (upper) and RelCP size (lower) between brachy-, meso-, and dolichocephalic dog breeds ($P = 0.62$ and $P = 0.38$, respectively). Box plots: midline is median, and whiskers are 5% to 95% percentile.

[ANCOVA]; $P < 0.0001$). Within the wild canids alone, there is no significant difference between coyote and wolf relative CP size (ANCOVA; $P = 0.25$, Fig. 1b, inset left).

Within the domestic dog sample, there are no significant group differences in olfactory anatomy, specifically, relative CP (RelCP) size. For example, RelCP size does not differ among domestic dog breeds (Tukey HSD pairwise test; all P-values >0.074; Fig. 2a, right, supplementary table S2, Supplementary Material online). Moreover, similar to genomic results, there is no difference in RelCP between nonscent breeds and either genetically grouped scent hounds alone ($n = 6$) or all scent breeds, inclusive of scent detection breeds ($n = 10$; ANCOVA; $P = 0.12$ and 0.19, respectively, Fig. 1d). Similarly, RelCP in scent breeds does not differ from that of sight hounds (ANCOVA; $P = 0.25$).

Among historical breed groupings, morphological and genomic results differ only slightly. Ancient breeds distinguish themselves from the wild canids by having significantly smaller RelCP than wolves alone as well as wolves and coyotes combined (ANCOVA; $P < 0.001$, Fig. 2b, supplementary table S1, Supplementary Material online). Moreover, RelCP in ancient breeds ($n = 6$; dingo, basenji, Siberian husky, chow chow, saluki, and shar-pei) is not statistically different from that of modern breeds (43). A notable difference between our genomic and anatomical results is that whereas the relative CP size of the dingo is near average for dog breeds, its OR gene repertoire size far exceeds that of all other breeds (Figs. 1b and 2b, supplementary SI Appendix and Dataset S1, Supplementary Material online).

Finally, when testing for effects of relative snout length on CP size using direct cephalic index measurements from our skulls, we witnessed no differences in RelCP between brachy-, meso-, and dolicocephalic breeds (ANOVA, breed mean: $P = 0.38$, Fig. 2c, supplementary SI Appendix and table S2, Supplementary Material online).

Evolutionary Relationship Between OR Genes and Olfactory Morphology

To test whether a previously established correlation between FORG and CP morphology across mammals (Bird et al. 2018) persists within the more recent evolutionary history of dog domestication, we regressed the number of functional OR genes ($\log_{10}$) against RelCP within the wolves, coyotes, and all domestic dog breeds for which we have both morphological and genomic data. On the recent time scale of dog breeds alone ($n = 20$), there is no relationship between FORG number and RelCP (Fig. 3a, supplementary SI Appendix and table S1, Supplementary Material online; $r^2 = 0.03$, $P = 0.47$). Widening the evolutionary scale to include wolves, then wolves and coyotes, the correlation is reestablished (Fig. 3a, supplementary Fig. S3, Supplementary Material online, $r^2 = 0.28$, $P = 0.006$; $r^2 = 0.37$, $P < 0.001$, respectively). When the wolf and sampled dog breeds are examined in the context of 26 highly divergent mammal species, the variance among the canids is well within the overall variance, and there remains a strong correlation between OR genes and olfactory morphology (Fig. 3b; $r^2 = 0.69$, $P < 0.001$).

Analysis of OR Genetic Variation

To further investigate whether OR subgenome-wide single-nucleotide polymorphisms (SNPs) reveal genetic structure

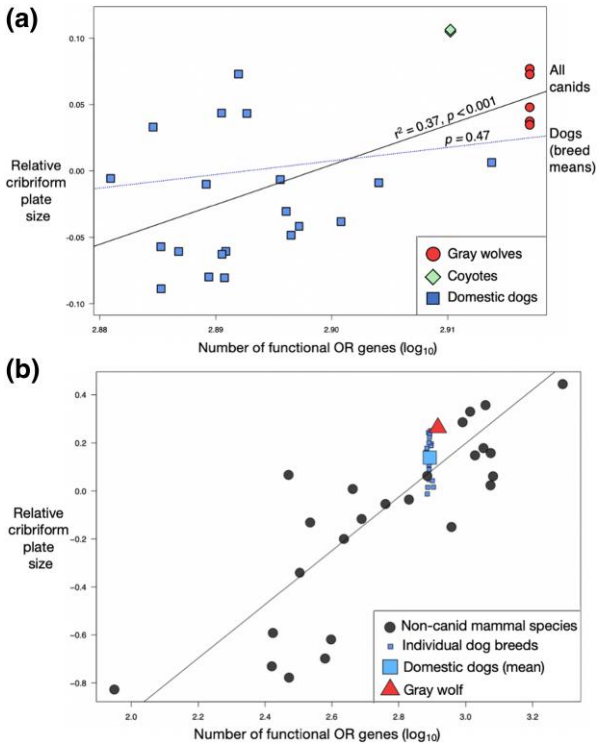

Fig. 3. Relationship between RelCP size and number of FORGs (log_{10}) as a function of evolutionary divergence. a) No significant relationship within the domestic dog breeds (blue) alone. When wolves (red) and coyotes (green) are added, a significant correlation emerges ($r^2 = 0.37$, $P < 0.001$). b) Addition of RelCP size and FORG data from dogs (breed means, $n = 20$, small blue squares; species mean from 39 individuals, large blue squares) and gray wolf (species mean from 5 individuals, red triangle) to 26 highly divergent mammal species (black circles), noncanid species means; plot modified from Bird et al. (2018) (Fig. 2b) reveals a strong correlation between RelCP size and FORG repertoires ($r^2 = 0.69$; $P < 0.0001$). Noncanid mammal species are labeled in supplementary fig. S3, Supplementary Material online.

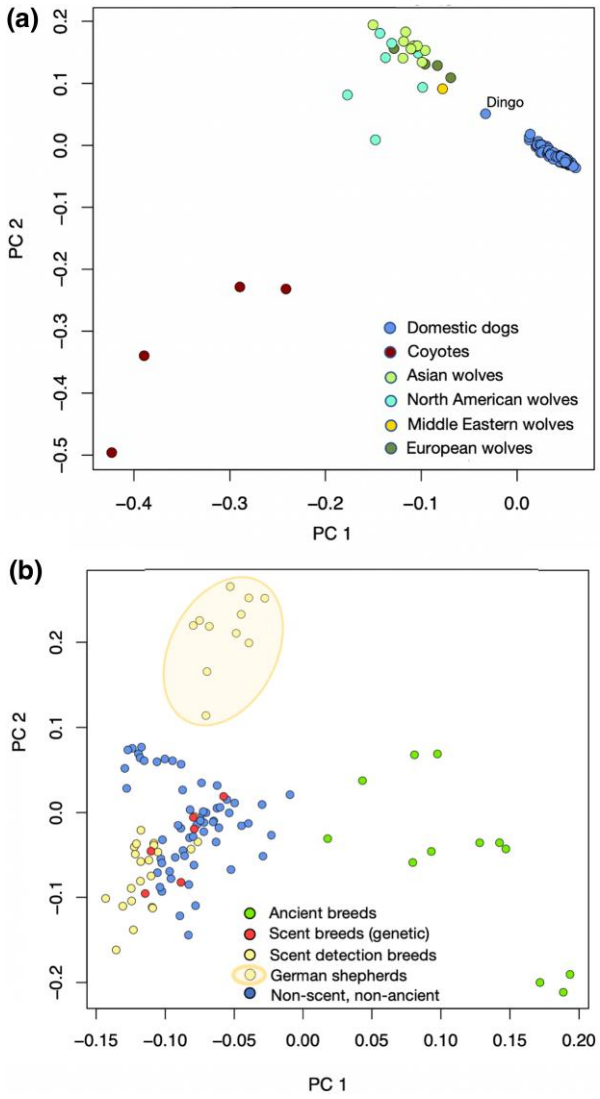

Fig. 4. PCA using the 4,357 OR SNPs with each dot representing an individual animal. a) PCA of 29 wolves, four coyotes, and 111 individual dogs shows a clear division between the domestic dog breeds and the wild canids apart from the dingo, which stands separate from both the wolves and dogs on PC1 and PC2. b) PCA of 111 individual dogs shows a separation between the dogs belonging to ancient breeds (green) and those belonging to modern dog breeds on PC1. Modern dogs cluster together regardless of the functional breed grouping on PC1, but German shepherds form a distinct cluster on PC2.

that reflects canid populations, breeds, and functional breed categories, we performed a principal component analysis (PCA) of 4,357 OR SNPs. Domestic dogs, wolves, and coyotes cluster as three discrete groups. The dingo also separates from wolves and dogs on PC1 and PC2 (variance explained 22% and 9%; Fig. 4a). PCA, including only domestic dogs and omitting the dingo (to enable better resolution of the dogs), shows two distinct clustering patterns (variance 8% to 7%). First, the ancient breeds tend to be separate from the modern breeds on PC1. Second, the German shepherds form a distinct cluster on PC2 (Fig. 4b). However, there is no noticeable structure between functional groups, such as scent and nonscent breeds or AKC breed grouping.

Gene Expression

The RIN scores for the 27 extracts of olfactory epithelium tissues ranged from 5.9 to 9.1 (supplementary SI Appendix and Dataset S3, Supplementary Material online). One sample was removed as an outlier (S15, code 241132). Hierarchical clustering analyses implemented in WGCNA (Langfelder and Horvath 2008) showed that the biological replicates of RNA-seq data from olfactory epithelium clustered well together, and consequently their respective counts were summed for the downstream analyses. After filtering for low counts on the remaining 22 samples (16,893 genes) and normalizing the counts, we used Orth in g:Profiler (Reimand et al. 2016) to retrieve the mouse gene symbols. A gene expression matrix for 15,138 orthologous genes was then submitted into SaVant (Lopez et al. 2017; supplementary SI Appendix, Dataset S4, and fig. S1, Supplementary Material online). We removed five samples based on their expression profiles (supplementary SI text, Supplementary Material online). After controlling for batch effect, no significant correlation was found with scenting abilities in domestic dogs (supplementary SI Appendix, SI text, Dataset S3, and fig. S2, Supplementary Material online).

Discussion

The number and diversity of OR genes within a repertoire vary markedly across species due to gene gains and losses over evolutionary time associated with distinct olfactory niches and

ecological pressures (Niimura and Nei 2007; Hayden et al. 2010; Hughes et al. 2018). Our comparisons of the OR subgenomes in 30 breeds of domestic dogs to that of the gray wolf and the closest outgroup, the coyote, determined that domestication has resulted in a significant loss of FORGs in dogs relative to wild canines (Fig. 1b, supplementary SI Appendix, table S1, and Dataset S1, Supplementary Material online). The loss of FORG number parallels a shift in olfactory skull morphology, specifically to smaller RelCP size (Fig. 1b, supplementary SI Appendix and table S1, Supplementary Material online). Previous studies have established both OR gene repertoire and CP size as informative molecular and morphological metrics of relative olfactory function, respectively. OR repertoire size is linked to ecological niche (Gilad et al. 2004; Niimura and Nei 2006; Hayden et al. 2010, 2014; Niimura 2012; Khan et al. 2015; Niimura et al. 2018), ability to discriminate between structurally similar odorants (Laska and Shepherd 2007; Rizvanovic et al. 2013) and the scope of detectable odorants (Malnic et al. 1999; Saito et al. 2009). Likewise, the CP, a perforated nasal bone that carries a quantifiable imprint of all olfactory nerves on their path from OR cells in the nasal epithelium to the olfactory bulb (Negus 1958; Bird et al. 2014), varies in size across mammals and is linked to habitat and behavioral ecology (Bird et al. 2020). Because RelCP size and FORG repertoire size are strongly correlated across mammalian species (Bird et al. 2018), we investigated this relationship before and after domestication, and in light of artificial selection.

The decline in olfactory metrics in dogs relative to gray wolves suggests that selective pressure for olfactory function has been relaxed in dogs. Although the earliest history of domestication is unclear, dogs likely became increasingly reliant on food sourced from humans as commensal animals, working aides, or pets. In support of this, human and dog diets exhibit parallel shifts in dietary isotopic values over the last 10,000 years (Cannon et al. 1999; Guiry 2012; Sykes et al. 2020). Moreover, coincidental with the expansion of agrarian societies, dogs experienced gene duplications of *AMY2B* (alpha-amylase), an adaptation to improved starch metabolism (Axelsson et al. 2013). Relative to wild canines, dogs generally do not locate and track prey over large home ranges, and losses in olfactory capacity may reflect relaxed selective constraints on maintaining an extensive gene repertoire (David Mech 1966; Gittleman 1991). Alternatively, a reduced olfactory function might be a passive result of drift-related olfactory gene diversity loss due to at least two bottleneck events, first postdomestication and later during breed formation (Wayne and Ostrander 2007; Cruz et al. 2008; Freedman et al. 2014). We detected no significant differences between coyote and wolf FORG repertoires but did recover significant variance among wolf populations. Among the wolves in our sample, those from Europe have, on average, the smallest repertoire, while Asian wolves have the largest. The FORG disparity among wolves may have its roots in ancient population structure. Following the divergence of New and Old-World wolves (ca. 11 to 12 Kya), European wolf lineages, particularly Southwestern European, experienced a marked drop in effective population size relative to other wolf populations, likely due to a severe demographic bottleneck (Lucchini et al. 2004; Sastre et al. 2011; Freedman et al. 2014; Pilot et al. 2014; Fan et al. 2016; Hulva et al. 2018). Long-term isolation and bottleneck events in the European wolves could have led to the contraction of superfamily genes, suggesting that below a certain population size, balancing selection may not be strong enough to maintain genetic diversity (Quignon et al. 2005; Ploshnitsa et al. 2012). However, genetic variability of immunity-related genes seems to have been preserved in European wolves despite these bottlenecks (Arbanasić et al. 2013; Galaverni et al. 2013; Niskanen et al. 2014). Notably, there is a sizable variance within our sample of four European wolves. The three individuals from the Iberian Peninsula and Italy, a population known for long-term isolation, have a particularly low number of functional OR genes (mean = 789). In contrast, the single individual from Croatia has 835 FORG, commensurate with the average FORG count for all the wolves in our sample (826). While all wolf lineages experienced a decline in effective population size, Asian wolves, except Tibetan populations, do not appear to have undergone bottlenecks as severe as those of European wolves. Chinese wolves seem to have experienced both marked population growth and decline during the Late Pleistocene (Fan et al. 2016), which may help explain the high variance in FORG number among our sample of Asian wolves. However, clarification of why Asian wolves exhibit greater variance and a larger mean count of FORG would require a targeted study on the evolution of the OR repertoire in different populations of wolves based on a high-quality wolf reference genome, a task beyond the scope of this study. Because our analyses are based on a dog genome reference assembly (from a boxer), we were unable to detect copy number variants (CNVs) of wolf-specific gene families.

Conditional Relationship Between OR Gene Number and Olfactory Morphology

Previous work established a strong linear correlation between the number of FORGs and RelCP size among 26 species representing all mammalian superorders, ranging in body mass from 0.1 kg to over 2,900 kg, and including the domestic dog (Bird et al. 2018). Here, we asked whether this morphologic-genetic relationship is also supported on a more recent evolutionary timescale between canid lineages that diverged 1 to 3 Mya and, specifically in canine populations that diverged as recently as 15 Kya and in the past few hundred years during breed formation. While some breeds, like the dingo, husky, and basenji, are a genetically distinct cluster relative to most other breeds, a majority have diversified very recently in the Victorian era, beginning about 200 to 300 years ago with the advent of selective breeding (Parker et al. 2017). We found no significant linear correlation between FORG repertoire and RelCP among domestic dog breeds alone. However, adding wolves and coyotes to the analysis restored the relationship (Fig. 3a). Because of the recent development of most dog breeds and extensive crossbreeding, the correlation between FORG and olfactory morphology may only be apparent when more divergent lineages, such as the wolf and other mammal species, are included. Indeed, when other wild canids are added to the divergent group of mammal species included in the previous study (Bird et al. 2018), the relationship between FORG and RelCP size among wolves and dog breeds is well within the overall variance across mammal species, and the overall linear correlation remains strong ($r^2 = 0.69$, $P < 0.001$; Fig. 3b). It is worth noting that across dog breeds alone, variance in RelCP size is larger than variation in FORG number. Because we know that CP shape is informed by extreme skull shape differences in dogs (Jacquemetton et al. 2021), it is conceivable that CP size is

also influenced by the profound variation in dog snout size and shape (Schoenebeck and Ostrander 2013) and that directed artificial selection on snout size and skull phenotype has weakened the relationship between CP size and number of OR genes.

Dog Breeds and Olfaction: Scent Hounds in Name Alone

A central finding in this study was that scent hounds show no expansion in the number of FORG relative to nonscent breeds. Similarly, there is no anatomical signature of olfactory enhancement in scent hounds relative to sight hounds. These findings hold whether the scent grouping is made up solely of those breeds genetically defined as scent dogs (beagle, bloodhound, and dachshund; Vonholdt et al. 2010; Parker et al. 2017) or whether it includes breeds preferentially used as scent detection dogs (German shepherd, Labrador retriever, and golden retriever; Ensminger 2011; Rocznik et al. 2015). Morphological findings parallel that of gene diversity, in that the relative size of the CP in scent dogs is no larger than that of nonscent dogs or even sight hounds (Fig. 1c, supplementary SI Appendix, table S1, and Dataset S1, Supplementary Material online; Bird et al. 2021).

A surprising pattern emerged in the PCA of OR gene SNPs among dogs alone. German shepherds, defined here as scent detection dogs, cluster separately from both the nonscent and scent dogs (Fig. 3b). At this point it is difficult to determine whether this distinct clustering is due to an earlier demographic event, possibly a bottleneck, in the history of the German shepherd breed, and/or whether it represents a functional difference. We note here that all German shepherd dogs in our sample underwent unique gene losses in an OR gene cluster on Chromosome 21 (Chr21:26733324-26734268, Chr21:26751570-26752529); however, it is beyond the purview of this paper to determine whether this loss is tied to the pattern we see among the OR gene SNPs. Future analyses comparing a diversity of haplotype-resolved canine genomes in a pan-genome framework will be enlightening in this regard.

To gain further insight into the specific interaction of genes that might affect olfactory performance, we assessed patterns of OR gene expression between scent and nonscent breeds. Gene regulatory mechanisms allow a range of phenotypes to arise from an otherwise static genome sequence (82). However, we did not find any significant association between gene expression and olfactory function in dog breeds. We acknowledge that this conclusion might be limited by the small number of individuals and unique breeds used in the analysis. Furthermore, olfactory mucosal sampling is challenging given craniofacial variation observed across dog breeds, which raises the possibility that not all OR genes were retrieved during the sampling process.

Overall, we found no morphologic or genetic evidence that breeds categorized as scent hounds are superior smellers or were bred specifically for olfactory ability. Our results challenge claims by breeders that olfactory traits have been selected and managed through strict controls over reproduction among scent breeds (Pemberton 2013). Despite the elevated status given to the best-known scent hound, we found that the bloodhound sits squarely in the middle of domestic dog breeds, both in OR gene repertoire and olfactory anatomy (Figs. 1b, supplementary SI Appendix and fig. S6, Supplementary Material online). To illustrate our struggle to find data that support the acclaimed olfactory ability of scent hounds, we constructed a graphic analysis of a cascade of unsupported references in the primary literature that repeat the misconception that the bloodhound and other scent hounds have an unparalleled olfactory anatomy (supplementary SI Appendix and fig. S7, Supplementary Material online).

The Dingo and Ancient Dog Breeds

"Ancient dogs" comprise a genetically divergent lineage that includes breeds that originated from ancient cultures >500 years ago and have undergone some degree of admixture with wolves postdomestication (Vonholdt et al. 2010; Freedman et al. 2014; Parker et al. 2017). Ancient breeds in our sample include the dingo, basenji, Siberian husky, saluki, and Afghan hound. On average, the ancient dog breeds have a higher number of functional OR genes than modern breeds and a lower number than the wild canids and may reflect the impact of admixture with wolves (Fig. 1b and 2b). However, statistically, the ancient dogs, as a group, align with the modern dogs and differentiate themselves from the wolves and coyotes (supplementary SI Appendix and table S1, Supplementary Material online). The dingo is the exception, with a FORG repertoire that is comparable with that of the wolves and much larger than all other sampled dogs. Dingos belong to a genetically divergent lineage relative to other domestic dog breeds isolated for thousands of years (Freedman et al. 2014; Field et al. 2022). Compared with dogs, dingos are considered feral and hunt prey independently from humans (Savolainen et al. 2004; Zhang et al. 2020). Because they are less reliant on human food sources than dogs consuming starch-rich diets since the Neolithic, dingos likely did not experience relaxed selection on olfactory specialization. Consistent with these observations, the dingo has retained the wolf-like condition of a single copy of AMY2B in contrast to other dog breeds (Arendt et al. 2016; Field et al. 2022). However, it is noteworthy that the dingo's high FORG number is not reflected in a larger CP (Fig. 2b).

Breed Groups and Olfaction

The spectrum of variation in the number of functional OR genes is relatively modest across dog breeds and does not reflect breed grouping. Dog breeds have historically been grouped according to function and genealogical relationships (Wilcox and Walkowicz 1989; American Kennel Club 2007; Judah 2007; Vonholdt et al. 2010; Parker et al. 2017). Breed groupings used today by breeders and the AKC are comprised of the ancient, spitz, toy, spaniels, scent hounds, working dogs, mastiff-like breeds, small terriers, retrievers, herding, and sight hounds (Vonholdt et al. 2010). Although these common classifications have fairly modest genetic support as revealed by haplotype-sharing and allele-sharing analyses (Vonholdt et al. 2010; Parker et al. 2017), they persist throughout the literature.

Our investigations of FORG count show no significant differences among commonly used breed groupings (Fig. 2a). Notably, sight hounds have a slightly higher number of FORG than scent hounds (Fig. 1c), contradicting the notion that sight hounds were selected for their visual abilities, whereas scent hounds were selected for their superior noses. Morphological comparisons of CP size revealed the same lack of significant distinctions across common breed groupings (Fig. 2a). Olfactory gene SNP analysis among dog breeds failed as well to reveal differences between common groupings, except for the ancient breeds, which may be driven by the dingo. We expected that there might be patterns of sensory

specialization that matched the AKC functional groupings, given the evidence of positively selected genes associated with athletic ability in sport-hunting breeds (Kim et al. 2018), as well as a selected trade-off between limb bone strength and stiffness in the American pit bull terrier and greyhound (Kemp et al. 2005). AKC breed groupings undoubtedly have some basis in the history of directed breeding for function; however, this does not appear to apply to olfaction.

Olfaction and Snout Length

Skull shape has been a central focus of artificial selection throughout the domestication of dogs, resulting in a continuum of snout lengths encompassing that exhibited in wolf ontogeny (Wayne 1986; Wilcox and Walkowicz 1989; Drake and Klingenberg 2010; Drake 2011; Schoenebeck and Ostrander 2013; Georgevsky et al. 2014). Most domestic dog breeds have shorter faces (palates) than the wolf, the most pronounced of which are found among the brachycephalic dogs (Pekingese, pug, and collective bulldogs). On the other end of the continuum are the long-faced, dolichocephalic breeds (saluki, collie, and borzoi). Short-snouted breeds are not known for their olfactory performance and are generally avoided by detection dog trainers (Jamieson et al. 2017); however, behavioral studies of detection performance in brachycephalic dogs relative to nonbrachycephalic dogs show contradictory results (Polgár et al. 2016). Here, we found no significant difference in FORG repertoire size among brachycephalic breeds relative to both meso- and dolichocephalic breeds (Fig. 2c), suggesting that individual differences in olfactory performance among brachycephalic dogs might reflect structural constraints imposed on nasal anatomy by positive artificial selection for short-snoutedness. Short-faced dogs are prone to respiratory and upper airway syndromes (Lorinson et al. 1997), which affect airflow and may conceivably influence olfactory function. Notably, in our morphological analysis, there was relatively high variance in RelCP across brachycephalic individuals; however, there was no difference in the RelCP between brachy-, meso-, and dolichocephalic breeds (Fig. 2c). Therefore, relative snout length based on the cephalic index we used here, ratio of maximum skull width to skull length, appears to be a poor predictor of olfactory morphology or gene diversity. However, it is conceivable that a relative snout size metric different from cephalic index may better describe how selection for snout size and shape has regulated the expansion of the olfactory skeleton and innervation.

Conclusion

Our results indicate that relative to their closest living relatives, gray wolves and coyotes, domestic dogs have a reduced FORG subgenome and olfactory skeleton. Within domestic dogs, "ancient" breeds do not appear to have retained markedly wolf-like olfactory attributes relative to modern breeds. One exception is the dingo, which has a larger number of functional OR genes than any dog breed in our sample. We found no genomic or anatomical evidence of direct selection for an elevated sense of smell among scent breeds relative to other breeds. Contrary to popular belief that scent hounds have superior noses, our results reveal that scent breeds are not distinguished from other dog breeds in either functional OR gene repertoire size, OR gene expression or relative CP size. Artificial selection for short faces in brachycephalic dogs has not resulted in any significant reduction of the olfactory variables we measured. Overall, there is considerable variability within breeds in the number of functional olfactory genes; however, no breed grouping had a significantly larger functional repertoire based on genetics or anatomical measures, suggesting that most or all dogs can perform olfactory based functions. The apparent ability of some breeds to perform scent detection tasks better than others likely reflects aspects of behavior, such as motivation and trainability, rather than olfactory gene repertoire size and anatomy.

Materials and Methods

Sampling

Morphometry

We sampled 103 skulls from 45 identified dog breeds, 1 unknown dog breed, and 2 species of wild canid, gray wolf (*C. lupus*) and coyote (*C. latrans*; supplementary SI Appendix and table S1, Supplementary Material online). All specimens were sourced from museum and university collections listed in supplementary SI Appendix and table S1, Supplementary Material online. Sampled wild canid species include only wild-caught adult specimens. Species and breed body masses, as estimated from the literature (Nowak 1991; Crowley and Adelman 1998), ranged from ~2.25 to 68 kg.

Genomic

OR gene copy number variation was estimated by mapping short-read Illumina data 111 domestic dog genomes belonging to 30 different breeds to the well-curated CanFam3.1 genome assembly, including a custom OR annotation (see details below). We compared dog repertoires to 31 wild *Canis* genomes: 27 gray wolves (19 Old World wolves and 8 New World wolves) and 4 coyotes (supplementary SI Appendix and Dataset S1, Supplementary Material online).

Gene Expression

Dogs that were admitted to the Texas A&M University Veterinary Medical Teaching Hospital and euthanized by owner request were included in this study. Animals with a history and physical assessment indicative of nasal or upper respiratory disease or infectious disease were excluded. All samples were acquired within 2 h of euthanasia. The temporal horns of the frontal sinuses were identified by surface palpation and a region on midline 1 to 2 cm rostral to the temporal horns was selected for trephine. A 1 to 2 cm elliptical skin incision was made on midline, and soft tissues dorsal to the nasal and frontal bones were dissected (supplementary SI Appendix and fig. S8, Supplementary Material online). A 4 mm diameter sterile trephine was used to remove a round section of bone, overlying the CP. Sterile forceps were used to grasp multiple pieces of mucosa immediately underlying the punch as well as 5 mm rostral, caudal, and abaxial to the trephine.

Breed Identification and Sample Size

Breed type was assigned by original dog owners or museum collectors. Where possible we sampled two or more individuals per breed, preferably from each sex. We recognize that sample size per breed is relatively low, however, given the large number of breeds and species in our study, a deeper sampling

was prohibitive (limited number of specimens and quality dog genomes available).

Breed Groupings

We used four criteria to classify domestic dogs into breed groupings. First, we grouped the breeds into (i) scent breeds (Vonholdt et al. 2010; Ensminger 2011; Rocznik et al. 2015; Parker et al. 2017) and (ii) nonscent breeds. Within this classification, the scent breeds are divided into two subgroups: genetically defined scent breeds (beagle, bloodhound, and dachshund; see Vonholdt et al. 2010; Parker et al. 2017) and detection dogs that are commonly chosen for scent detection work (German shepherd, golden retriever, and Labrador; Ensminger 2011; Rocznik et al. 2015). This latter classification was used for the gene expression analyses as well. Second, in classifying dogs as ancient and modern breeds, we used criteria used by Vonholdt et al. (2010), Freedman et al. (2014), and Parker et al. (2017) to identify the ancient breeds in our sample (dingo, basenji, Siberian husky, saluki, and Afghan hound). Third, we classified dogs into ten functional breed groupings traditionally used by breeders and the AKC (Wilcox and Walkowicz 1989; American Kennel Club 2007; Vonholdt et al. 2010). Finally, we used relative snout length, specifically the cephalic index (CI; ratio of maximum skull width to skull length × 100) (Roberts et al. 2010; Evans and de Lahunta 2012) to group most dogs into brachy-, meso-, and dolichocephalic breeds. Because we had no access to the skulls of the individual dogs represented in our genome data, we used mean breed CI values already established in an extensive study by Stone et al. (2016).

Data Collection

Morphology

All skulls were scanned on high-resolution industrial computed tomography (CT) scanners (Phoenix v|tome|x S; North Star Imaging ACTIS; XRadia MicroXCT; Nikon Metrology XT H 225 ST). The targeted region of interest was constrained to the CP and the area directly surrounding it in order to increase scan resolution. Scan voxel size ranged from 0.04 to 0.085 mm. All scan data are available through MorphoSource (https://www.morphosource.org/) or Digimorph (http://www.digimorph.org). To visualize and quantify CP morphology, we imported CT scan data into the 3D imaging software Mimics (v. 20.0-21.0, Materialise Leuven, Belgium), segmented the CP into masks that delineate bone and nonbone, and finally reconstructed 3D volumetric models (Fig. 1a, supplementary SI Appendix and Movies S1 and S2, Supplementary Material online). CP surface area is defined here to include only the area of bone perforated by foramina that surround olfactory nerves, a proxy for the amount of olfactory innervation found in an animal's snout. Previous work established a strong linear relationship between the cumulative surface area of the CP foramina and the surface area of the perforated portion of the CP (Bird et al. 2014). This excludes the lateral flanks of the CP perforated by the ethmoid foramen, a distinctly large passageway for the nasociliary branch of the trigeminal nerve that has no olfactory function. We quantified CP surface area first by rendering the perforated area into a continuous surface area in the imaging program 3-matic (v. 11.0-13.0, Materialise) with a wrapping function that fills all foramina in the CP model and then second, by digitally incising the CP surface along the perimeter of the perforated region (supplementary SI Appendix and fig. S9, Supplementary Material online). We digitally calculated the surface area in 3-matic.

RNA Extraction

Total RNA was extracted from 24 epithelium tissues using the Invitrogen TRIzol Plus RNA Purification Kit. Four samples were extracted in two separate batches (supplementary SI Appendix and Dataset S3, Supplementary Material online). The integrity of 28 RNA extracts was then quantified using the Agilent bioanalyzer (Agilent Technologies, USA). One sample (Code 225627) was removed from the library preparation due to low RNA integrity number (RIN) score (2.4). The RIN scores for the remaining extracts ranged from 5.9 to 9.1 (supplementary SI Appendix and Dataset S3, Supplementary Material online). cDNA libraries were constructed using the KAPA mRNA HyperPrep Kit with dual indices (Kapa Biosystems, Ltd). Individual libraries were then pooled in equimolar ratios and sequenced on two lanes of an Illumina Hiseq4000 (150 bp paired-end). Sequencing was performed at Fulgent genetic (https://www.fulgentgenetics.com).

Data Analyses

Morphology

Because CP area increases with body size, we calculated a metric of size-adjusted RelCP size. This size-corrected metric was estimated following (Bird et al. 2018) using residuals from an ordinary least squares regression of $\log_{10}$ values of absolute CP surface area against a body size proxy for all breeds and the two wild canid species. As a body size proxy, we used the distance between the occipital condyles and the anterior extent of the orbit (OOL, occiput to orbit length), a cranial metric shown to correlate well with body mass in carnivorans ($r^2 = 0.9$) (99) (supplementary SI Appendix and fig. S10, Supplementary Material online). In our overall analyses, we chose OOL over total skull length or body mass as a size proxy for two reasons. First, OOL excludes snout length and avoids the confounding effects of large variation in snout length (i.e. brachycephaly and dolichocephaly) present in our sample of dog breeds (Schoenebeck et al. 2012). Second, weight was not available in collectors' notes for most specimens, and weights reported by the AKC are based on breed averages and display large ranges (Crowley and Adelman 1998). A log–log generalized least squares regression of mean absolute CP surface area against OOL was used to derive RelCP from resulting residuals. In a single case, when analyzing the relationship between OR genes and CP plate morphology within a wider context of highly diversified mammals with variable skull morphologies (supplementary SI Appendix and fig. S6, Supplementary Material online), we used body mass as a size correction for CP size in order to match the original study of (Bird et al. 2018). Phylogenetic comparative methods were not used here to account for the effects of phylogeny on CP morphology, as existing cladograms for wolves and between domestic dog breeds are neither resolved nor time-calibrated due to extensive admixture between breed lineages (Parker et al. 2017). To test for significant differences in RelCP between wild canids and domestic dogs and between dog breed groupings, we performed pairwise *t*-tests and one-way ANOVA. Additionally, while testing for differences in RelCP between groupings in various subsets of the data, we performed an ANCOVA, as it is robust to violations of normality. We carried out all analyses in R (v. 3.5.3) (R Core Team, 2014).

Genome Mapping and SNP Calling

We applied Trim galore (Trim Galore, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to filter paired-end Illumina reads. The trimmed paired-end reads were mapped to the domestic dog genome assembly (Version CanFam3.1) using BWA-mem (Li and Durbin 2010) with default settings. SAMtools (Li et al. 2009) was used to remove PCR-induced duplicates. The standard Genome Analysis Toolkit (GATK; Van der Auwera et al. 2013) pipeline was used for base quality recalibration and indel realignment.

OR Gene Annotation of Dog Genome Assembly

To improve the accuracy of OR gene annotation of the dog genome assembly CanFam3.1, we applied a modified Perl script pipeline (https://github.com/GanglabSnnu/OR_identify) to identify all intact (functional) and pseudogene OR genes (Montague et al. 2014). We define functional OR genes as those meeting the following criteria: (i) no premature stop codon, (ii) no frameshift mutations, (iii) no in-frame deletions within a single transmembrane region nor deletions of conserved amino acid sites (Niimura 2013), and (iv) no truncated genes with fewer than 250 amino acids or lacking any of the seven transmembrane domains (Hayden et al. 2010). The updated OR gene annotation was used to estimate OR gene CNV from read-mapping data for all 142 canid genomes.

SNP and CNV Analyses

The software snpEff (Cingolani et al. 2012) was applied to annotate different categories of SNP and INDEL variants (e.g. premature stop codon, frameshift, synonymous substitution, and nonsynonymous substitution). We use a consensus from two structural variant callers, CNVnator (Abyzov et al. 2011), and Delly2 (Rausch et al. 2012) to estimate the CNV of all intact and pseudogene of OR among all analyzed dogs and wolves' individuals. We performed PCA with the R package ggfortify to visualize the relationships of OR gene copy number and substitution variants among all canid species.

OR Gene Expression Analyses

Raw sequences were processed using Trim Galore 0.3.1 (Krueger) to remove Illumina adapters and sequences that did not meet the following quality thresholds: $Q > 20$, length > 25). The alignment of the trimmed reads was performed on STAR 2.5.3 (Dobin et al. 2013) using the dog genome (*C. lupus* familiaris: Ensembl release 95_31). We used HtSeq for read counts on a custom GTF file including all intact olfactory genes. We first checked for biological replicates and outliers. We filtered reads with low counts in the 27 samples and remaining genes were normalized using trimmed mean of *M*-values in the edgeR package (Robinson and Oshlack 2010) in R. Reads were then converted into $\log_2$ counts per million (logCPM) with voom in LIMMA (Law et al. 2014; Ritchie et al. 2015). We performed principal components analyses to identify technical factors from the dataset (Blighe and Lun 2020). We removed the batch effects using the removebatch command in LIMMA for visualization purposes. We explored the data to check for outliers and clustering of biological replicates using hierarchical clustering of the gene expression adjacency matrix with the R package WGCNA (Langfelder and Horvath 2008).

After controlling for biological replicates and outliers, we checked for tissue-type heterogeneity using a web-based tool named SaVant (http://newpathways.mcdb.ucla.edu/savant-dev/; Lopez et al. 2017). SaVant accepts a matrix of gene expression from RNA-seq or microarray and allows a comparison between our own expression data with a repository of more than 10,895 signature profiles. Exploration of expression profiles for the remaining samples was investigated for known olfaction signatures such as "Olfactory bulb," "Kegg olfactory transduction" (389 genes), and "Reactome olfactory signaling pathway" (328 genes). We performed an orthology search of the dog genes with the mouse symbol genes using: Orth in g: Profiler (Reimand et al. 2007, 2016). We suggest that RNA samples with a weak to nonexistent OR signature were likely due to sampling bias (lacking olfactory epithelium) and were removed from the analyses.

Differential Expression and Gene Enrichment Analyses

RNA-seq reads were filtered and processed as explained above. LIMMA (Law et al. 2018) and Deseq2 (Love et al. 2014) were used for our differential gene expressions analyses. Genes falling below FDR < 0.05 in both methods were kept for Gene Ontology analyses in g:Profiler (Reimand et al. 2016). To ensure that the distribution of the false discovery rate satisfied the expectation for FDR under a null model, we ran 100 permutations of the original model where the significant variable was assigned randomly and compared the distribution of *P*-values under the null to the distribution of *P*-values under the true model.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Author Contributions

R.K.W., W.J.M., B.V.V., and J.M.L. designed the study; A.M., D.B., and G.L. performed the data analyses. The manuscript was written by A.M., D.B., R.K.W., and W.J.M. with subsequent contributions and final approval by all authors. M.M. generated the RNA-seq libraries (UCLA QCBio).

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Data Availability

The raw sequencing data, normalized counts, regressed normalized counts, and all associated metadata have been deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO Series accession numbers (GSE291131). Data from CT scanning will be available on Morpho Source and DOI are listed in supplementary table S3, Supplementary Material online (https://www.morphosource.org/).

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