Dramatic variation of the vomeronasal pheromone receptor gene repertoire among five orders of placental and marsupial mammals

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Pheromones are chemicals emitted and sensed by conspecifics to elicit social and sexual responses and are perceived in terrestrial vertebrates primarily by the vomeronasal organ (VNO). Pheromone receptors in the mammalian VNO are encoded by the V1R and V2R gene superfamilies. The V1R superfamily contains 187 and 102 putatively functional genes in the mouse and rat, respectively. To investigate whether this large repertoire size is typical among mammals with functional VNOs, we here describe the V1R repertoires of dog, cow, and opossum based on their draft genome sequences. The dog and cow have only 8 and 32 intact V1R genes, respectively. Thus, the intact V1R repertoire size varies by at least 23-fold among placental mammals with functional VNOs. To our knowledge, this size ratio represents the greatest among-species variation in gene family size of all mammalian gene families. Phylogenetic analysis of placental V1R genes suggests multiple losses of ancestral genes in carnivores and artiodactyls and gains of many new genes by gene duplication in rodents, manifesting massive gene births and deaths. We also identify 49 intact opossum V1R genes and discover independent expansions of the repertoire in placental and marsupials. We further show a concordance between the V1R repertoire size and the complexity of VNO morphology, suggesting that the latter could indicate the sophistication of pheromone communications within species. In sum, our results demonstrate tremendous diversity and rapid evolution of mammalian V1R gene inventories and caution the generalization of VNO biology from rodents to all mammals.

Abbreviations: MY, million years; VNO, vomeronasal organ.

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Materials and Methods

Database Searches. TBLASTN searches for V1R genes were conducted on the dog (Canis familiaris), cow (Bos taurus), and opossum (Monodelphis domestica) genome sequences. The 7.6× coverage dog genome sequence (www.ncbi.nlm.nih.gov/genome/guide/dog) and 3.3× coverage cow genome sequence (www.ncbi.nlm.nih.gov/genome/guide/cow) are available in the National Center for Biotechnology Information. The 7.2× coverage opossum genome sequence is available at ENSEMBL (http://pre.ensembl.org/Monodelphis_domestica). Mouse and rat V1R genes from refs. 8, 10, and 12 were used as query sequences. Putative V1Rs were identified with an E value cutoff of 10−5. They were then used as queries to BLAST the NR database of GenBank. A putative V1R gene was considered to be real if its best hit was a previously known V1R. Use of human V1Rs from ref. 9 as query sequences did not yield additional V1Rs.
V1Rs were considered pseudogenes if they contained premature stop codons or were incomplete across the 13 internal domains (7 transmembrane, 3 extracellular, and 3 intracellular). The database searches were independently conducted, yielding identical results. The sequences of newly identified intact V1Rs from the dog, cow, and opossum are listed in Data Set 1, which is published as supporting information on the PNAS web site.

**Sequence Alignment and Phylogenetic Analysis.** Gene sequences were aligned per protein sequence alignment by CLUSTALX (18) with manual adjustment. Phylogenetic trees were reconstructed by using the neighbor-joining method (19) with protein-Poisson distances (20) and were evaluated by 1,000 bootstrap replications (21). We also used protein p-distance (20) and found that the branching patterns with high bootstrap support in the Poisson-distance tree remained unchanged in the p-distance tree. After convention (10), we defined gene families by a minimum of 40% amino acid identity among all family members and confirmed the monophyly of gene families by phylogenetic analysis. We conceptually translated each pseudogene sequence according to its alignment with all functional V1R genes (mouse, rat, and dog or cow), and determined the phylogenetic position of each pseudogene by making a neighbor-joining tree with the protein-sequence of the pseudogene and those of all functional genes. The distribution of LINE repetitive elements was determined by the REPEATMASKER program (www.repeatmasker.org). For easy comparison, we used the same criterion as used in ref. 22 to calculate the L1 density. More specifically, we estimated the L1 density in the intergenic regions for tandem-linked V1R genes and that in 10,000 nucleotides upstream and downstream of the coding regions for non­tandem-linked V1R genes.

**Results**

**Dramatic Variation in V1R Repertoire Size in Placental Mammals.** Placental mammals can be classified into four superorders (23). Rodents and primates belong to the superorder Euarchontoglires, to which all previous V1R evolutionary analyses have been restricted. Here, we investigate the V1R repertoire in the dog and cow, which are members of the superorder Laurasiatheria, the sister clade to Euarchontoglires. From the dog genome sequence, we identified eight complete V1R ORFs, which are presumably functional genes. In addition, 22 V1R pseudogenes were detected. Because the dog genome sequence has a high (7.6×) coverage, it is likely that the majority of, if not all, dog V1R genes have been found. Similarly, we detected 32 functional V1R genes and 41 pseudogenes from the cow genome sequence. However, because the cow sequence has a relatively low coverage (3.3×), it is possible that a few additional genes and pseudogenes may be discovered when a more complete genome sequence becomes available. Even with this limitation, our observations clearly show that the V1R repertoires in cow and dog are substantially smaller than those in mouse and rat, which contain 187 and 102 putatively functional genes, respectively (12). Even when humans are disregarded, there is still a 23-fold variation in the size of functional V1R repertoire among rodents, artiodactyls, and carnivores (Fig. 1). The proportion of putatively functional V1R genes is lower in dogs (27%) than in rodents (49%, refs. 10 and 11), but the proportion in cows (44%) is similar to that in rodents.

**Phylogenetic Relationships of Placental V1R Genes.** To understand the evolutionary relationships of the V1R genes from the four placental orders (rodents, primates, artiodactyls, and carnivores), we reconstructed a protein neighbor-joining tree with all putatively functional V1Rs from the mouse, rat, human, dog, and cow (Fig. 2). We also included four human V1Rs with complete ORFs in the phylogenetic analysis, although these ORFs are likely relics of an ongoing pseudogenization process (13). A fifth human V1R gene, hV1RL3, was not included because a previous study found this gene to be nearly fixed with a nonfunctional allele in human populations (allele frequency >98%) (13). Putatively functional nonhuman primate V1R sequences from ref. 24 were not included because only partial sequences were available.

The dog and cow V1R genes cluster within the previously described rodent V1R superfamily (8). The mouse and rat V1R genes were previously classified into 14 families (VIRA to V1RN) in ref. 8. A new family (V1RO) is found when the seven recently described rat V1R genes (12) are added (see Fig. 2). Because the families were originally described in rodents, some of the families could be rodent-specific, meaning that their origins postdated the origin of rodents (8, 23). To classify other mammalian V1Rs, we clustered the families into family groups. The family groups contain families, described in rodents, that split around the time of the most recent common ancestor of rodents, cow, and dog. The 15 rodent families may be grouped into nine family groups based on phylogeny (Fig. 2 and Table 1). Based on the rodent synonymous substitution rate, we previously estimated that the V1R family groups appeared before 95 MY ago (8). This finding suggests that the most recent common ancestor of primates, rodents, artiodactyls, and carnivores should have the V1R family groups that are currently observed in rodents, because this ancestor lived ≈95 MY ago (23, 25). Note that this estimate for the age of the V1R family groups is conservative because it is likely that the synonymous substitution rate has been enhanced in rodents compared with that in other mammals (26, 27). We found that all nine family groups contain putatively functional mouse genes and eight of nine (except V1RH/I) contain functional rat genes (Fig. 2 and Table 1). However, only five of the nine family groups include functional dog genes and five include functional cow genes (Fig. 2), suggesting that cows and dogs lost many ancestral V1R genes. For instance, the family groups V1RC and V1RG each contain >10 mouse genes and 10 rat genes, but neither contains functional dog or cow V1Rs. Additionally, there are no functional dog V1RD or V1RE genes, and there are no functional cow V1RH/I genes. Surprisingly, the second-largest V1R gene family
in cows (V1RD) contains no functional dog genes. We also identified two cow-specific families, V1RP and V1RQ, each containing a single gene.

Interestingly, several family groups that do not contain functional cow or dog genes possess their pseudogenes. We were able to classify 17 of the 22 dog V1R pseudogenes and 35 of 41 cow V1R pseudogenes by family group (Table 1; see also Fig. 5, which is published as supporting information on the PNAS web site). The remaining pseudogenes have degenerated too much to be included. Family groups V1RC and V1RD each contain two dog pseudogenes, but no functional dog genes. When both functional genes and pseudogenes are considered, only two family groups in dog (V1RE and V1RQ) and two in cow (V1RC and V1RG) have been completely lost if the unclassified pseudogenes do not belong to these family groups. These observations provide further evidence that the small sizes of the dog and the cow V1R repertoires can be partially explained by loss of ancestral genes.

The second factor causing the repertoire-size variation among species is the lineage-specific expansion of families as occurred most prominently in rodents. The phylogenetic analysis shows that most of the dog and cow V1Rs diverged from their closest rodent homologs before the expansions of rodent V1R families (Fig. 2). This divergence pattern is consistent with our previous estimate that the earliest duplication events within rodent V1R families took place ~88 MY ago (8), postdating the separation of rodents, carnivores, and artiodactyls ~95 MY ago (23, 25). Family expansions were virtually absent in the dog but were evident in three V1R families of cow. Only two dog families, V1RF and V1RJ/K, contain more than one V1R gene. The other three dog V1Rs are single genes in V1RL/M/N, V1RH, and V1RA/B/O, respectively. Six of the 22 dog pseudogenes were part of the two larger dog V1R families. In contrast, three cow V1R families, V1RF, V1RD, and V1RQ, exhibited the duplications characteristic of the rodent V1R gene families. The remaining cow V1Rs are in V1RA/B/O, V1RF/K, V1RL/M/N, V1RP, and V1RQ. Although some of these families contain more than one cow gene (Table 1), the multiple genes are not the product of species-specific duplication events. Twenty-eight of the 41 cow pseudogenes were part of the three largest cow families. The dog-cow V1RF clade was similar to the rodent gene families with species-specific duplications (Fig. 2).

Based solely on comparison with the mouse, Rodriguez and Mombaerts (9) reported that human V1R genes do not belong to the reported V1R families. Our analysis showed that three human V1Rs can be classified into V1RF and V1RL/M/N, and the fourth (hV1RL5) forms a new human-specific family (V1RJ). Giorgi and Rouquier (15) identified several V1R sequences from the chimpanzee, gorilla, and orangutan. We found that these sequences cluster closely with the human sequences, although we did not present them in the phylogeny of Fig. 2 because they are not from complete V1R repertoires. Thus, when all four placental orders are considered, there are 12 V1R family groups, 10 of which have moderate to high bootstrap support (Fig. 2). In the case of V1RF, we maintained the family by at least 40% amino acid identity among all genes. Fig. 2 also shows V1RF as paraphyletic; however, the bootstrap values for the deep branches defining V1RF are low, indicating that it could be monophyletic.

**Opossum V1R Repertoire.** We also identified 49 putatively functional V1R genes and 53 pseudogenes from the opossum genome sequence. Because the opossum genome sequence has a high coverage (7.2x), we expect that almost all opossum V1R genes have been detected. We reconstructed a phylogenetic tree of the 49 opossum V1Rs with all functional V1Rs of the mouse, rat, dog, and cow (Fig. 3). The phylogeny shows that the opossum genes can be classified into eight opossum-specific families (oV1RA to oV1RH). The families range in size from a single gene (oV1RH) to 15 genes (oV1RA and oV1RC), with variable levels of bootstrap support. The tree shows that the placental and marsupial genes do not form two separate monophyletic groups, suggesting that more than one V1R gene was present in the
common ancestor of placentals and marsupials. However, it is difficult to estimate the number of V1R genes in the common ancestor because of the low resolution of deep nodes in the tree. Nevertheless, the presence of many well supported opossum-specific and placental-specific gene clusters in the tree provides strong phylogenetic evidence that V1R families expanded independently in marsupials and placentals.

Table 2. Size variation of some gene families among mammalian species

<table>
<thead>
<tr>
<th>Gene family</th>
<th>Function</th>
<th>Size variation, ratio</th>
<th>Smallest size (organism)</th>
<th>Biggest size (organism)</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1R</td>
<td>Pheromone receptor</td>
<td>23.4</td>
<td>8 (dog)*</td>
<td>187 (mouse)</td>
<td>12, this study</td>
</tr>
<tr>
<td>Morpheus</td>
<td>Nuclear pore complex interacting protein</td>
<td>21</td>
<td>1 (Old World monkeys)</td>
<td>21 (chimpanzee)</td>
<td>28</td>
</tr>
<tr>
<td>EAR</td>
<td>Antiparasitic RNases</td>
<td>17</td>
<td>1 (New World monkeys)</td>
<td>17 (ricefield mouse)</td>
<td>29, 30</td>
</tr>
<tr>
<td>Ly49</td>
<td>Immunity</td>
<td>17</td>
<td>1 (baboon)</td>
<td>17 (rat)</td>
<td>31</td>
</tr>
<tr>
<td>OTEX/PEPP2</td>
<td>Reproduction-related</td>
<td>7.5</td>
<td>2 (human)</td>
<td>15 (mouse)</td>
<td>32</td>
</tr>
<tr>
<td>Granzyme</td>
<td>Mast cell chymases</td>
<td>7</td>
<td>4 (human)</td>
<td>28 (rat)</td>
<td>27</td>
</tr>
<tr>
<td>KIR</td>
<td>Immunity</td>
<td>7</td>
<td>2 (mouse)</td>
<td>14 (human, macaque)</td>
<td>33, 34</td>
</tr>
<tr>
<td>OR</td>
<td>Olfactory receptor</td>
<td>3.7</td>
<td>388 (human)</td>
<td>1,430 (rat)</td>
<td>27, 35</td>
</tr>
<tr>
<td>Keratin-associated protein</td>
<td>Epithelial cell function</td>
<td>3.3</td>
<td>3 (human)</td>
<td>10 (mouse)</td>
<td>27</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>Polymerase</td>
<td>2.6</td>
<td>25 (mouse)</td>
<td>65 (human)</td>
<td>36</td>
</tr>
</tbody>
</table>

Only functional genes are considered. Species with at least one functional gene in the gene family are compared. Gene families with a size ratio >2 are presented.

*Human V1Rs are likely relics of an ongoing pseudogenization process (see ref. 13). Therefore, the dog has the smallest functional repertoire.

Discussion

In this study, we described the vomeronasal pheromone receptor V1R gene superfamily from the dog, cow, and opossum, extended the study of the superfamily outside of rodents and primates, and revealed extremely high variation in the sizes of functional V1R repertoires among mammals. The sizes of the dog and cow V1R repertoires are vastly smaller than those of rodents and primates. We found only 8 putatively functional V1Rs in dogs and 32 in cows compared with 102 in rat and 187 in mouse (8, 10–12). In humans, the functional V1R repertoire is also small with only four ORFs in most individuals (9, 13). However, the entire human V1R repertoire, including both functional genes and pseudogenes, is quite large, with ~200 members. In opossum, we identify 49 putatively functional V1Rs, intermediate among what we identify here for dog and cow and what had been previously identified for rodents.

Even when humans who have lost functional VNOs are disregarded, the size of the functional V1R repertoire varies by >23-fold among all mammals or among placental mammals (Table 2). Several gene families, particularly those involved in sensory, immune, and reproductive functions, are known to vary substantially in size among mammalian species (27). For instance, the number of functional olfactory receptor genes in rat is approximately four times that in humans (27, 33). The putatively anti-parasitic eosinophil-associated RNase gene family is 6–17 times larger in rodents than in New World monkeys (29, 30, 37). The human X-linked testis-expressed homeobox genes OTEX and PEPP2 have a total of 15 orthologous genes in the mouse genome, because of multiple gene duplications that postdated the primate-rodent separation (32). The human genome contains >200 Ig heavy chain variable region (VH) genes and ~80 of them are functional (38). Rabbit also contains >100 VH genes, but only one of them is predominantly used, resulting in very few functional genes (39). The exact number of functional rabbit VH genes is yet to be determined, although at least five have been identified (39). Thus, the number of functional VH genes may vary up to 16-fold among different mammals. Table 2 lists additional gene families known to have wide variations of family size among mammalian species. However, to our knowledge, the size variation in V1R repertoire among mammals exceeds that in any other mammalian gene family. This high variation might be in part because V1Rs are involved in both sensory and reproductive functions. Our phylogenetic analysis indicates that the dramatic size difference in the V1R repertoires of placental mammals is due to two molecular evolutionary mechanisms. First, some ancestral gene families that are still present in rodents have been lost in dogs and cows. Second,
species-specific duplication events characteristic of rodent V1R families were less frequent in cows and dogs. Thus, massive gene deaths and births (40, 41) in different lineages explain the observed size variation.

Is it possible that the smaller V1R repertoire in dogs and cows indicates that the VNO is not functional in these organisms? Pseudogenization of vomeronasal genes and loss of VNO function happened in catarrhine primates (i.e., humans, apes, and Old World monkeys), presumably after the acquisition of full trichromatic vision (13, 42, 43). It is possible that stereoscopic vision in both primates and carnivores (44) compensates for reduced pheromone communication. Complete loss of VNO function, however, is unlikely to be responsible for the small V1R repertoires of dogs and cows, because TRP2, the ion channel necessary for VNO pheromone signal transduction (45, 46), is apparently functional in cows (47), and we were able to identify a complete ORF for dog TRP2 from the genome sequence. Furthermore, there have been reports of bovine pheromones that induce estrus, which is likely mediated by the VNO (48, 49). Is it possible that the V1R repertoires have shrunken during the domestications of dog and cow because of either artificial selection or genetic drift? We think it is unlikely because the domestication started no earlier than 15,000 years ago for dogs and 10,000 years ago for cows (50, 51). Even if a small number of functional genes have become nonfunctional during domestication, their relics should remain readily detectable as pseudogenes. We thus believe that the sizes of V1R repertoires in dogs and cows should be very close to those in their wild ancestors. Another possibility is that the small V1R repertoire could be compensated by a large V2R repertoire. This explanation also seems unlikely, because all V2Rs we identified from dogs and cows were pseudogenes (data not shown). Furthermore, all V2R genes identified from the human and goat genomes are pseudogenes (17). In fact, no functional V2Rs have been reported in nonrodent mammals. If the estimated size of ~100 genes in the rodent V2R repertoire (6) is accurate, the phylogenetic surveys suggest an even more dramatic variation in the V2R repertoire among placental mammals. In this respect, it is interesting to note that a recent study found two types of vomeronasal systems in mammals, with rodents and opossums having both Gαz- and Gαo-expressing vomeronasal sensory neurons and all other species examined (goats, dogs, horses, musk shrews, and marmosets) having only Gαz-expressing vomeronasal sensory neurons (ref. 52, but also see ref. 53). Because V1Rs are expressed in Gαz-positive neurons and V2Rs are expressed in Gαo-positive neurons (16), it is possible that functional V2Rs exist only in rodents and opossums among mammals. Indeed, our preliminary search confirms the presence of V2R ORFs in the opossum genome (P.S. and J.Z., unpublished results).

It should be noted that not all VNO-mediated functions in rodents are VNO-mediated in other mammals (53), and it is possible that during the evolution of rodents some olfactory cues became detectable by the VNO. In fact, a goat V1R gene is known to be expressed in the olfactory epithelium (17). Before we can determine the effects of gene loss in V1R superfamly evolution, we must understand the functional difference between family groups. Two studies have investigated the functions of V1Rs, one in a strain of mice mutant for V1Rb2 (54) and one in a strain of mice that had 16 genes of the families V1RA and V1RB knocked out (2). Both studies showed that the mutations resulted in physiological or behavioral changes in the mice. But, no study has compared the phenotypic effects of eliminating different V1R families, and it remains unknown whether each family is necessary for a specific function. Such information would allow us to reason why certain families are lost in some species.

In addition to gene loss, our analysis clearly demonstrated V1R family expansions in some placental mammals. However, it is unknown what factors have promoted the dramatic expansions in rodents but have only allowed limited expansion in cows and virtually no expansion in dogs. Lane and colleagues (22, 55) suggested that the V1R gene duplications in rodents were mediated by L1 repetitive elements. These elements, which are known to have been active in rodents around the time of the mouse-rat divergence, densely populate the regions of the mouse and rat genomes where V1Rs are located. L1 density differs between species, so it would be interesting to see how V1R repertoire size correlates with L1 density. After the procedure of Lane et al. (22), we found that 21% of the DNA sequences in the genomic regions harboring dog V1R genes are L1 elements, lower than the corresponding density (40%) in the mouse V1R loci (22) or the average density (25%) in the mouse genome (56). The L1 density of the genomic regions containing cow V1R genes is also 21%. Thus, the low duplicability of both dog and cow V1R genes might be in part due to the low density of L1 elements in the genomic regions. L1 density in cow V1R genomic regions is similar to that in dogs, but cows have four times as many V1Rs as dogs have. Thus, L1 elements might not play as great a role in V1R duplications as originally thought, or the role of L1 elements in V1R duplication might be limited to rodents.

The phylogenetic reconstruction of placental V1Rs with the opossum V1Rs (Fig. 3) suggests that there was more than one V1R gene in the common ancestor of marsupials and placental, and the mammalian V1R families are then at least 170 MY old (23). Many V1R families expanded in placental and marsupials independently. Sequencing another marsupial genome will significantly broaden our understanding of V1R evolution in marsupials. Because both primates and rodents have >100 V1R genes (or pseudogenes in the case of humans) and because primates and rodents are more closely related to each other than either of them is to carnivores or artiodactyls (23, 25), one might infer that after the separation of the common ancestor of cows and dogs from the common ancestor of rodents and primates, there was a dramatic expansion of the V1R repertoires. Thus, the large size of the V1R superfamly as observed in rodents might be restricted to organisms derived from the common ancestor of primates and rodents, including the five orders of Rodentia, Lagomorpha (e.g., rabbit), Dermoptera (e.g., flying lemur), Scandentia (e.g., tree shrew), and Primates (25). However, our previous molecular dating indicates that rodent V1R families expanded after the primate-rodent split (8), suggesting independent expansions in rodents and primates. [In the future, the independent expansions in primates and rodents could be tested by examining the phylogenetic positions of human pseudogenes when their sequences (ref. 9) become available.] This independence would further imply that some of the aforementioned mammalian orders might not contain expanded V1R families. Even if expansion is characteristic of these five orders, it is also possible that a functional V1R repertoire subsequently shrank after expansion, as in catarrhine primates (13). These considerations suggest that mouse and rat may be atypical mammals in terms of their pheromone receptor genes and pheromone sensitivities. Of course, independent expansions would also imply great differences in V1R receptors and pheromone sensitivities. Thus, one should be cautious in applying to other mammals the V1R and pheromone-related knowledge learned from the model organisms of mouse and rat. Furthermore, although a large variation in the V1R repertoire is described here, the species we have examined (human, mouse, rat, dog, cow, and opossum) represent only five of ~24 orders of placental and marsupial mammals. A more thorough investigation of the V1R repertoires in other orders will give a better picture of the variation and evolution of V1Rs in mammals.

Interestingly, the small repertoire of vomeronasal pheromone receptor genes that we report in the dog may not be entirely unexpected and may be common to carnivores in general.
Although rodents have a complex VNO with a thick layer of vomeronasal sensory epithelium (VNSE), where vomeronasal receptors are found, carnivores have a different type of VNO with a much thinner VNSE (14, 52). Furthermore, the VNO of the ferret Mustela putorius, another carnivore, is rudimentary in size and development and does not change with respect to season, which is in contrast to the seasonal variation observed in other ferret organs involved in sexual reproduction and behavior and in rodent VNOs (57). Additionally, Woodley and Baum (58) found that the main olfactory system, but not the VNO, is necessary for mate identification in the ferret. The findings in dogs and ferrets suggest a limited role of the VNO, which may be necessary for mate identification in the ferret. The findings in carnivores and in rodent VNOs (57). Additionally, Woodley and Baum (58) found that the main olfactory system, but not the VNO, is necessary for mate identification in the ferret. The findings in dogs and ferrets suggest a limited role of the VNO, which may be necessary for mate identification in the ferret. The findings in carnivores and in rodent VNOs (57).

Note Added in Proof. Recently, Young et al. (59) reported the same eight dog V1R genes.

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