Evolution of V1R pheromone receptor genes in vertebrates: diversity and commonality

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The vomeronasal organ (VNO) plays a key role in sensing pheromonal cues, which elicit innate responses and induce social and sexual behaviors. The vomeronasal receptor 1 genes, V1Rs, encode members of a pheromone receptor family that are mainly expressed in the VNO. Previous studies have revealed that the V1R family shows extraordinary variety among mammalian species owing to successive gene gains and losses. Because species-specific pheromonal interaction may facilitate species-specific reproductive behaviors, understanding the evolution of V1Rs in terms of their origin, repertoire and phylogeny should provide insight into the mechanisms of animal diversification. Here I summarize recent studies about the V1R family from its initial discovery in the rat genome to extensive comparative analyses among vertebrates. I further introduce our recent findings for V1Rs in a broad range of vertebrates, which reveal unexpected diversity as well as shared features common among lineages.

Key words: evolution, pheromone receptor, V1R, vertebrate, vomeronasal organ

INTRODUCTION

The olfactory sense is essential to acquire chemical information from the external world, allowing animals to respond with appropriate behavior. Pheromones, which are secreted by an individual and perceived by another individual of the same species, are especially important for eliciting social and sexual behaviors (Karlson and Lüscher, 1959). In terrestrial vertebrates, pheromones are predominantly detected by the vomeronasal organ (VNO). The VNO is anatomically distinct from the main olfactory epithelium (MOE), which detects general odorants (Firestein, 2001; Brennan and Zufall, 2006). Neurons of the VNO (vomeronasal sensory neurons: VSNs) project to the accessory olfactory bulb (AOB), whereas those of the MOE (olfactory sensory neurons: OSNs) project to the main olfactory bulb (MOB) (Chamero et al., 2012, Fig. 1). The VSNs have a specific transduction mechanism, involving a diacylglycerol-activated cation channel and TRPC2 (transient receptor potential channel 2), which is essential for a fully functional VNO (Stowers et al., 2002).

Vomeronasal receptor genes are classified into two multigene families, namely type-1 (V1Rs) and type-2 (V2Rs), which are both seven-transmembrane G protein-coupled receptors (Nei et al., 2008). These two receptor families are distinct in that the V1R-positive VSNs reside within

Fig. 1. Schematic diagram of the mammalian olfactory system highlighting the VNO. Sagittal view of a mouse head showing the main olfactory epithelium (MOE), vomeronasal organ (VNO), main olfactory bulb (MOB) and accessory olfactory bulb (AOB). A coronal view of the VNO is shown in the box. In the VNO, apical VSNs express V1Rs and project their axons to the anterior (red) AOB, and basal VSNs express V2Rs and project to the posterior (yellow) AOB.
the apical layer of the VNO, co-express \textit{Gai}_2, and project to the anterior AOB, whereas V2R-positive VSNs reside within the basal layer, co-express \textit{Gzo}, and project to the posterior AOB (Chamero et al., 2012, Fig. 1). Previous bioinformatic analyses for several mammalian genomes revealed that V1Rs and V2Rs show extraordinary variation in gene number and in the proportion of intact genes compared with pseudogenes (Grus et al., 2005; Yang et al., 2005; Young et al., 2005), which have been generated by species-specific gene duplication and loss (Nei et al., 2008). Consequently, size variation of the V1R repertoire exceeds that of any other multigene family (Grus et al., 2005). Given that the evolution of vomeronasal receptors may reflect the history of speciation and adaptation in a broad range of vertebrates, analyses of such genes may provide important insight into the molecular mechanisms underlying pheromone-mediated animal diversification.

In this review, I focus on the \textit{VIR} family in particular, from its initial discovery to very recent findings. Recent \textit{VIR} data are largely dependent on new advances in genome bioinformatics methods, which are still expanding rapidly. After the initial discovery of \textit{VIR}s in the rat genome (Dulac and Axel, 1995) they were characterized extensively in the genomes of a broad diversity of mammals (Grus et al., 2005; Young et al., 2005), frogs (Date-Ito et al., 2008) and reptiles (Brykczynska et al., 2013). Several groups revealed the extreme variability in \textit{VIR} repertoires across tetrapods (land vertebrates, including mammals, amphibians, reptiles and birds) due to species-specific gene duplication and loss. \textit{VIR}s were also found in multiple species of teleost fish, which do not have VNOs, showing that they were highly conserved in these ancestral vertebrate lineages (Pfister and Rodriguez, 2005; Saraiva and Korsching, 2007). In addition to mammals and teleosts, \textit{VIR}s have been explored in the coelacanth, which was found to possess fish-type as well as tetrapod-type \textit{VIR}s (Nikaido et al., 2013). Recently, our group also found a highly divergent \textit{VIR} in cichlid fishes (Nikaido et al., 2014), and a highly conserved \textit{VIR} shared among most bony vertebrates (Suzuki et al., 2018). Both of these discoveries were quite unexpected and forced a reconsideration of previous ideas about \textit{VIR} evolution. Further investigation of \textit{VIR}s will be essential to elucidate the general mechanism for pheromone detection systems as well as the origin of the VNO in vertebrates.

**DISCOVERY OF \textit{VIR} RECEPTOR GENES**

\textit{VIR} genes were initially identified in the rat genome by Dulac and Axel (1995). Prior to this discovery, Buck and Axel (1991) identified a large multigene family encoding olfactory receptors specifically expressed in the MOE. They concluded that ~1,000 copies of olfactory receptor genes exist in mammalian genomes, which enables us to discriminate diverse odorant chemicals. Dulac and Axel (1995) predicted that the pheromone receptors, which may be specifically expressed in the VNO, would be distinct from the olfactory receptors. They designed an experimental strategy based on two hypotheses: 1) expression of the pheromone receptors is restricted to the VNO; and 2) individual VNO neurons express different receptor genes according to the “one neuron–one receptor” rule. Differential screening of cDNA libraries derived from individual VNO neurons identified some genes that were differentially expressed among different VNO neurons. The punctate expression patterns of the mRNA in the VNO suggested that these genes are likely to encode pheromone receptors. Based on genomic Southern hybridization assays as well as quantitative screening of genomic libraries, the authors estimated the number of putative pheromone receptor genes to be 30 or 40 copies. Since then, great advances in whole-genome sequencing technologies have allowed us to characterize these putative pheromone receptor genes for a broad diversity of mammals. This novel multigene family was named vomeronasal receptor type I (\textit{VIR}) or \textit{Vmn1}. The copy numbers of intact \textit{VIR}s were revealed to be 106 and 187 in rat and mouse, respectively, and are much higher than expected from their initial discovery (Nei et al., 2008).

The biological function of \textit{VIR}s has also been extensively examined. Del Punta et al. (2002) generated mutant mice in which a 600-kilobase genomic region that contains a cluster of 16 intact \textit{VIR}s was deleted. The behavioral impairment and chemo-sensory deficit in the mutant mice suggested a role for \textit{VIR}s as pheromone receptors. Furthermore, mutant mice deficient for \textit{Gai}_2, which facilitates signal transduction by \textit{VIR}s in the apical VNO neurons, showed impairment of aggressive behaviors in males and lactation in females, implicating an essential role for \textit{VIR-\textit{Gai}_2} signal transduction in vomeronasal function (Norlin et al., 2003).

**HIGHLY DIVERSE \textit{VIR} REPERTOIRES IN MAMMALS**

Since the \textit{VIR} multigene family was discovered, it has been extensively characterized in a broad diversity of mammals in order to elucidate how highly diversified social and sexual behaviors have been acquired during evolution. Rodriguez et al. (2002) identified a total of 293 \textit{VIR} sequences (137 intact, 60 partial, 96 pseudo) in the mouse genome, showing rapid evolution of these genes. Next, two research groups conducted a genome-wide comparison, showing the extreme variability in \textit{VIR} repertoires across mammalian lineages due to species-specific gene duplication and loss (Grus et al., 2005; Young et al., 2005, 2010; reviewed in Rodriguez, 2005; Nei
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Fig. 2. Number of intact V1Rs among a broad range of vertebrates. In the graph on the right, bars for species having more than 100 gene copies are truncated and the actual numbers are indicated. The phylogenetic tree of vertebrates with taxonomic classification names is shown on the left side. The copy number was based on the following references (Saraiva and Korsching, 2007; Date-Ito et al., 2008; Grus and Zhang, 2009; Young et al., 2010; Ota et al., 2012; Nikaido et al., 2013; Zapilko and Korsching, 2016; Moriya-Ito et al., 2018). Note that the V1R repertoire is highly variable in mammals, from 283 in platypus to 0 in dolphin and bat, but much less variable in teleost fish (6 copies in most species). The number of V1Rs increases dramatically in species belonging to the Sarcopterygii.

et al., 2008, summarized in Fig. 2). Zhang et al. (2007) compared the gene repertoires between V1Rs and olfactory receptor genes (ORs) in mice and rats, showing that V1Rs evolved more rapidly than ORs in a species-specific manner. Moreover, Kurzweil et al. (2009) and Karn et al. (2010) revealed differences in V1Rs in closely related mouse species and subspecies, respectively. These lines of evidence suggest that V1Rs contribute to the speciation and diversification of mammals.

Grus et al. (2005) also proposed that the number of intact V1Rs correlates with the anatomical complexity of the VNO. This hypothesis is consistent with the absence of intact V1Rs in whales, dolphins and some bats (Young et al., 2010; Kishida et al., 2015), which do not possess a
VNO. The decline of the number of intact V1Rs is also evident in anthropoid primates (hominoids, Old World monkeys and New World monkeys; Yoder and Larsen, 2014; Moriya-Ito et al., 2018, Fig. 2), in which the VNO is anatomically oriented to be reduced or vestigial (Smith et al., 2014). Only one apparent exception was observed in the dog genome (Boxer dog), in which the number of intact V1Rs is unexpectedly small in view of the functional VNO and a complex system of pheromone-based behaviors in wild canine species (Young et al., 2005, Fig. 2). Interestingly, several intact V1Rs have been characterized in humans and were shown to be expressed in the MOE (Rodriguez et al., 2000) even though we lack an anatomically intact VNO.

LESS DIVERSE V1R REPERTOIRES IN TELEOST FISH

Although teleost fish do not have a VNO, they have V1Rs. Pfister and Rodriguez (2005) revealed the existence of a mammalian V1R-like sequence in multiple species of teleost fish. Subsequently, extensive bioinformatic analyses revealed that a total of six highly divergent V1Rs exist in five model fish species: zebrafish, medaka, stickleback, fugu and pufferfish (Saraiva and Korsching, 2007; Pfister et al., 2007). The teleost V1Rs were also called ORAs (olfactory receptor class A) because they are expressed in the olfactory epithelium (OE), which is not differentiated into the MOE and VNO. In addition to the above five fish species, V1Rs were also characterized in lamprey (Grus and Zhang, 2009; Libants et al., 2009), elephant shark (Grus and Zhang, 2009), cichlids (Ota et al., 2012), eel (Churcher et al., 2015), salmon (Johnstone et al., 2012) and anchovy (Zhu et al., 2016). The presence of V1Rs in lamprey and elephant shark indicates that their origin is old in aquatic vertebrate evolution (Grus and Zhang, 2009). Indeed, the phylogenetic positions of three V1Rs of lamprey, the basal lineage of all vertebrates, are close to V1R3 or V1R4 of teleost fish or a much older clade (Fig. 3), implying that vertebrate V1Rs originated in these ancestral clades.

Although the V1Rs of mammals and fish belong to the same gene family, they are distinct in terms of the diversity of their repertoires. Whereas the repertoires of V1Rs are highly diverse in mammals, they are conserved among teleost species. Indeed, most teleosts possess a set of six orthologous V1Rs except for some gene gain or loss in pufferfish, salmon and anchovy (Fig. 2). In addition to this repertoire, each of the V1Rs is highly conserved among teleost fishes at the DNA sequence level (Saraiva and Korsching, 2007; Ota et al., 2012). For example, comparisons of the V1R sequences among distantly related rockfishes (Johansson and Banks, 2010) as well as nine salmonid species (Johnson and Banks, 2011) revealed that they are all highly conserved among species. Although the ligands for fish V1Rs remain largely unexplored, the high level of conservation of teleost V1Rs in repertoires as well as in sequence implies that they recognize a very small number of ligands, which are common across a broad range of fish species. Johansson and Banks (2010) proposed that the fish V1Rs are involved in assessing mate condition, gender, reproductive status and other fitness-related traits, which are important across multiple species regardless of species specificity.

V1R EVOLUTION DURING WATER TO LAND TRANSITION

In addition to mammals and teleosts, V1Rs have been further explored in other vertebrates. Given that terrestrial vertebrates experienced a massive environmental transition from water to land, their V1Rs are also expected to have changed their ligands during that period. Indeed, Shi and Zhang (2007) have characterized and compared the V1Rs as well as other chemoreceptor genes among 11 vertebrates (mammals, chicken, frog and teleosts), proposing that the V1Rs increased in the frog (Fig. 2), in which a morphologically identifiable VNO first appeared during evolution. Saraiva and Korsching (2007) also revealed that frog V1Rs, which are phylogenetically close to mammalian V1Rs, increased in a species-specific manner. They suggested that the expansions of these V1Rs is related to the origin of the VNO in the common ancestor of tetrapods (Shi and Zhang, 2007). Hereafter, I refer to less diverse “fish-type” V1Rs as t-V1Rs and highly diverse “tetrapod-type” V1Rs as t-V1Rs. Phylogenetic analyses revealed that t-V1Rs are derived from the common ancestor of t-V1R1 and t-V1R2 (Fig. 3).

Nikaido et al. (2013) explored the V1Rs in the genome of the coelacanth, which is believed to be one of the closest extant relatives of tetrapods. Interestingly, the number of intact V1Rs (20 intact genes) of coelacanth was comparable to that of frog (Fig. 2). Indeed, the coelacanth has highly diverse t-V1Rs, suggesting that the increase of t-V1Rs occurred earlier than the emergence of tetrapods (Fig. 3, Nikaido et al., 2013). Furthermore, Zapilko and Korsching (2016) found a few t-V1Rs in the spotted gar, an early-diverging ray-finned fish, and proposed that t-V1Rs originated far earlier than expected. This earlier origin of t-V1Rs in vertebrate evolution may provide important insight into the ligands of V1Rs. Thus, the above results imply that the t-V1Rs already existed in the common ancestor of bony fishes and allowed them to detect pheromones underwater, and that subsequently these ancestral t-V1Rs were co-opted to detect pheromones in the terrestrial environment.

It is noteworthy that few or no V1Rs are found in the genomes of lizards, snakes, birds, alligators and turtles even though they are terrestrial vertebrates (Silva and
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Fig. 3. Evolutionary relationships of t-V1Rs, f-V1Rs and ancV1R. The maximum likelihood tree of the amino acid sequences of V1Rs of 10 representative vertebrates under the JTT+G+F model constructed by RAxML 8.2.4 (Guindon and Gascuel, 2005). The scale bar indicates amino acid substitutions per site. The species are indicated as follows: cow (gray), frog (green), coelacanth (red), five teleost fishes – zebrafish, stickleback, fugu, pufferfish and medaka – (blue), elephant shark (hash #), sea lamprey (star *). The bitter taste receptor type 2 (bitter taste receptor genes) Outgroups (bitter taste receptor genes)

The lack of V1Rs in birds and alligators is consistent with the anatomical observations that VNOs are degenerate or vestigial in these groups (Døving and Trotier, 1998). The VNO of turtles is also equivo-
UNEXPECTEDLY HIGH DIVERSITY OF \textit{V1R}s IN CICHLID FISH

Whereas each of the \textit{f-V1R} orthologs is highly conserved among distantly related fish species, our group revealed an unexpectedly large diversity of \textit{f-V1Rs} in closely related cichlid species. The cichlids in the African Great Lakes have been intensely studied by evolutionary biologists because they are highly diverse in species (Kocher, 2004). Ota et al. (2012) and Nikaido et al. (2014) characterized and compared six \textit{f-V1Rs} in more than 30 cichlids and found that \textit{f-V1R2 (ORA1)} alleles were highly conserved among distantly related fish species, whereas each ortholog is highly conserved among closely related fish species.

**Fig. 4A** Nikaido

(A) Evolutionary scenario of \textit{ancV1R} during vertebrate evolution. \textit{ancV1R} emerged in the common ancestor of Sarcopterygii and Actinopterygii more than 400 million years ago and was lost in each of the common ancestors (gray circles) of teleost fishes as well as turtles, alligators and birds (gray dashed lines and silhouettes) by pseudogenization. The white silhouette and dashed black lines associated with sharks indicate that \textit{ancV1R} has not yet emerged. Figure adapted from Suzuki et al. (2018).

(B) Distinct pattern of expression between canonical \textit{VRs} and \textit{ancV1R} in the VNO. \textit{In situ} hybridization on coronal sections of the VNO of C57BL/6 mice was performed using probes against canonical \textit{V1R (V1Rh5, left)} and \textit{V2R (V2Ra, middle)}, and \textit{ancV1R} (right). Note that canonical \textit{V1R} and \textit{V2R} show punctate expression in the apical and basal regions of the vomeronasal epithelium, respectively, according to the one neuron–one receptor rule. However, \textit{ancV1R} shows broad expression in most vomeronasal sensory neurons, suggesting a new “one neuron–two receptors” (canonical \textit{V1R} or \textit{V2R} + \textit{ancV1R}) rule. Scale bar indicates 200 μm.
dimorphic between closely related species pairs. Evolutionary analyses revealed that these two \( f-VIR2 \) alleles emerged through a process of positive Darwinian selection, implying a functional distinctiveness of these two alleles in detecting pheromones. We thus suggested that highly dimorphic \( f-VIR2 \) led to the incipient step of population divergence and speciation. Signatures for the operation of positive Darwinian selection were further detected in \( f-VIR1, f-VIR3 \) and \( f-VIR6 \). The occurrence of such events and the unexpected gene diversity in one \( f-VIR \) family is suggestive of the contribution of \( f-VIRs \) to species diversification in cichlids. Specification of the ligands of each \( f-VIR \) and evaluation of the relevant physiological effects may provide crucial insight into the mechanism of speciation in cichlids.

A SINGLE \( VIR \) CONSERVED ACROSS 400 MILLION YEARS OF VERTEBRATE EVOLUTION

Recently, our group found a novel clade of \( VIR \) gene, named ancient \( VIR \) (anc\( VIR \)), which is shared among most bony vertebrates from the basal lineage of ray-finned fishes to mammals (Suzuki et al., 2018, Fig. 4A). Given that no anc\( VIR \) sequences were found in the genomes of elephant shark and lamprey (Fig. 3), anc\( VIR \) emerged after the divergence of cartilaginous fish (Fig. 4A). Importantly, the orthologous relationship of anc\( VIR \) has been conserved for more than 400 million years of vertebrate evolution. Conservation of orthologous relationships is quite rare in mammals, in which \( f-VIRs \) are highly diverse and in which no genes orthologous to \( f-VIRs \) have been found so far. Actually, although \( f-VIR2 \) shares a common ancestry with mammalian \( t-VIRs \), they do not have a one-to-one orthologous relationship. We also found a strong correlation between the pseudogenization of anc\( VIR \) and the loss of an intact VNO (Suzuki et al., 2018). Furthermore, anc\( VIR \) was shown to be expressed in most vomeronasal sensory neurons (VSNs) in contrast with canonical \( VIRs \), which show punctate patterns of expression called the “one neuron—one receptor” rule (Fig. 4B). For the first time, we showed that anc\( VIR \) is co-expressed with canonical \( VIRs \) or \( V2Rs \) in the VNO, and proposed the new hypothesis of the “one neuron–two receptors” rule. The high degree of evolutionary conservation as well as the broad expression pattern imply an essential functional role(s) for anc\( VIR \) in VNO-mediated pheromone detection.

In addition to the function of the VNO, anc\( VIR \) may shed light on its origin. Given that anc\( VIR \) is expressed in most VSNs, it can be used as an ideal molecular marker to explore the VNO in these groups. Traditionally, the VNO was believed to have originated in the common ancestor of tetrapods (Bertmar, 1981). However, a primitordial VNO was later identified in the lungfish, implying an earlier origin (González et al., 2010; Nakamuta et al., 2012). To examine the possibility of a much earlier origin of the VNO, we need to focus on the basal lineages of ray-finned fishes (e.g., Polypterus, gars), which possess anc\( VIR \) even though morphologically apparent VNOs have not been identified in these ancient vertebrates.

In this review, I provide a summary and overview of the evolutionary dynamics of \( VIRs \) in a broad range of vertebrates. Several recent findings allow us to more accurately guide further investigation of the evolution and function of \( VIRs \) and their role in driving the diversification of vertebrates. Specifically, the evolution of \( f-VIRs \) and \( t-VIRs \) can be more precisely elucidated by exploring \( VIR \) sequences in basal lineages of ray-finned fishes, whose genomes are now becoming available. Clarifying the function of anc\( VIR \) is of primary importance in understanding the mechanism of pheromone detection, much of which remains to be elucidated. To test these novel discoveries in a diversity of non-model vertebrates within a hypothesis-driven framework, our group is also currently setting up the experimental system in well-established animal models to compare phenotypic differences (e.g., reproductive behavior, physiology) between wild type and anc\( VIR \)-knockout mice.

The illustration of the mouse vomeronasal organ in Fig. 1 was drawn by Ms. M. Nishimori at Tokyo Institute of Technology. The phylogenetic tree was constructed by Mr. Zicong Zhang at Tokyo Institute of Technology. The in situ hybridizations of mouse VNO sections in Fig. 4 were provided by Dr. J. Hirota at Tokyo Institute of Technology. This work was funded by JSPS KAKENHI (16H04820, 25440189), MEXT KAKENHI (22IS0002), the Asahi Glass Foundation and the Hitachi Global Foundation to M. N.

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