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Evolutionary transition from water to land in vertebrates illuminated by basal ray-finned fish vomeronasal type 2 receptor (OlfC) genes

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Abstract

The vomeronasal type 2 receptor (V2R, also called OlfC) multigene family is found in a broad range of jawed vertebrates from cartilaginous fish to tetrapods. V2Rs encode receptors for food-related amino acids in teleost fish, whereas for peptide pheromones in mammals. In addition, V2Rs of teleost fish are phylogenetically distinct from those of tetrapods, implying a drastic change in the V2R repertoire during terrestrial adaptation. To understand the process of diversification of V2Rs in vertebrates from “fish-type” to “tetrapod-type”, we conducted an exhaustive search for V2Rs in cartilaginous fish (chimeras, sharks, and skates) and basal ray-finned fish (reedfish, sterlet, and spotted gar), and compared them with those of teleost, coelacanth, and tetrapods. Phylogenetic and synteny analyses on 1897 V2Rs revealed that basal ray-finned fish possess unexpectedly higher number of V2Rs compared with cartilaginous fish, implying that V2R gene repertoires expanded in the common ancestor of Osteichthyes. Furthermore, reedfish and sterlet possessed various V2Rs that belonged to both “fish-type” and “tetrapod-type”, suggesting that the common ancestor of Osteichthyes possess “tetrapod-type” V2Rs although they inhabited underwater environments. Thus, the unexpected diversity of V2Rs in basal ray-finned fish illuminates the process of how the osteichthyan ancestors adapt from water to land.
Introduction

Olfaction is a critical chemosensory system for eliciting social behaviors in vertebrates, including reproduction, kin recognition, aggression, and feeding. The vertebrate olfaction system has experienced drastic changes in anatomy and neurophysiology during adaptation from water to land, one of the most important events is vertebrate evolution. Specifically, aquatic vertebrates detect water-soluble chemicals using their olfactory epithelium (OE), whereas terrestrial vertebrates detect both volatile and nonvolatile chemicals by differentiating their OE into main olfactory epithelium (MOE) and vomeronasal organ (VNO). Accompanied by the terrestrial adaptation of olfaction, the chemosensory receptors are proposed to have undergone major innovative diversification, although the detailed evolutionary process of diversification remains to be poorly understood at the DNA level.

Olfaction of vertebrates is mainly composed of four types of G protein-coupled receptors (GPCRs), namely, the olfactory receptor (OR), vomeronasal receptor type I (V1R), vomeronasal receptor type II (V2R), and trace amine-associated receptor (TAAR), all forming multigene families. In teleost, V2Rs, also referred to as OlfC's (olfactory receptors classified as type C GPCRs), are expressed in OE of the nasal cavity. Several independent studies have shown that teleost V2Rs detect amino acids that elicit certain feeding behaviors. For example, V2Rs are expressed in microvillous sensory neurons of zebrafish and respond to amino acids, but not bile acids or sex pheromones. In addition, the genetic blockage of neural transmission in the V2R-expressing neurons abolishes the attractive response to a mixture of amino acids. However, given that Yang et al. have proposed a possible contribution of some V2Rs to elicit fright reactions, it is premature to rule out the possibility that V2Rs detect some chemicals other than amino acids. In tetrapods, two anatomically distinct organs, that is, MOE and VNO, each mainly detecting odorants and pheromones. V2Rs are specifically expressed in VNO of mice, frogs, and reptiles. Hence, they are believed to encode pheromone receptors. Indeed, it has been shown that V2Rs detect peptide pheromones and peptides for the major histocompatibility complex.

Until now, phylogenetic analyses of V2Rs have revealed that teleost possesses 20–60 V2Rs. These V2Rs are divided into 16 subfamilies, whereas mammalian V2Rs are genetically closely related to each other. V2Rs of teleost are revealed to be distinct from those of tetrapods in the phylogenetic tree, implying a drastic change in the V2R repertoire during terrestrial adaptation. Therefore, for simplicity, we used the term “fish-type” V2Rs to refer to putative amino acid receptors of teleosts, while “tetrapod-type” to refer to putative peptide pheromone receptors of tetrapods. The nomenclature with “fish-type” and “tetrapod-type” was also adopted for V1Rs, based on the initial discoveries from genomes of model organisms (mouse and zebrafish). The “fish-type” and “tetrapod-type” V2Rs are also found to be distinct in their synteny relationships in that the former V2Rs form single large cluster in particular chromosomes and the latter V2Rs are scattered on several chromosomes. It is notable that the coelacanths, which are the close relatives of tetrapods, have both “fish-type” and “tetrapod-
type” $V2R$s\textsuperscript{16}, showing that the coelacanth is an important organism as it serves as a missing link to fill an evolutionary gap between vertebrates under water and land\textsuperscript{20}. Recent studies on several shark genomes have shown that olfaction in cartilaginous fish is dominated by $V2R$s rather than conventional $OR$s\textsuperscript{21,22,23}, which is consistent with the ultrastructural observation that the presence of only microvillous sensory neurons in OE\textsuperscript{24} and with the immunohistochemical observations that most neurons in OE are positive for Go antibodies\textsuperscript{25}. In addition, a previous study has shown that $V2R$s were absent in lamprey genomes\textsuperscript{26}. Based on the above findings, $V2R$s are considered to have originated in the jawed vertebrate ancestor before the split between the two extant descendant lineages, that is, cartilaginous fish and Osteichthyes (ray-finned and lobed-finned fish including tetrapods).

In this study, we have performed a comprehensive exploration and phylogenetic analyses for $V2R$s in nine cartilaginous fish species (one elephant shark, one rabbit fish, four sharks, two skates, and sawfish), three basal ray-finned fish (reedfish, sterlet, and spotted gar), two teleost fish (eel, zebrafish), a coelacanth, two amphibians (western clawed frog and caecilian), an anole lizard, and a mouse to elucidate the process of diversification of “fish-type” and “tetrapod-type” $V2R$s in vertebrates. As a result, we have characterized 9 to 42 $V2R$s in cartilaginous fish, and a large amount (47 to 189) of $V2R$s in basal ray-finned fish. Phylogenetic analyses of $V2R$s of 19 various vertebrate species have revealed the existence of “tetrapod-type” $V2R$s in the genomes of reedfish and sterlet, implying the presence of an evolutionary seed for mammalian peptide pheromone receptors in the basal ray-finned fish. The result of this fine-scale phylogenetic study would provide important insights into understanding the process of olfactory adaptations in the $V2R$s of vertebrates from cartilaginous fish to tetrapods.
Results

Characterization of V2Rs from the genomes of a broad range of vertebrates

We explored V2R gene repertoires from the genomes of nine cartilaginous fish (one elephant shark, one rabbit fish, four sharks, and three rays), three basal ray-finned fish (reelfish, sterlet, and spotted gar), two teleost fish (zebrafish, Japanese eel), a coelacanth, two amphibians (western clawed frog, caecilian), an anole lizard, and a mouse using an original software “fate”\textsuperscript{27}. The copy numbers of V2Rs in individual species are summarized and provided in Table 1. The number of V2Rs in teleost fish was mostly comparable to previous studies with some update. For example, zebrafish that possessed 72 V2Rs, which was larger than in previous studies\textsuperscript{13,20}, comprising 19 genes from unplaced scaffolds and 53 genes from chromosome 18. The numbers of V2Rs in cartilaginous fish ranged from 8 (rays) to 41 (chimera), which were found to be smaller than those of the teleost fish. In contrast, basal ray-finned fish possessed an unexpectedly large number of V2Rs. In reedfish, we identified 188 intact V2Rs, including 68 genes from unplaced scaffolds, which was the largest in all ray-finned fish studied until now. The numbers of V2Rs in sterlet and spotted gars were also high (46 and 49, respectively). The copy number of V2Rs in the western clawed frog (691), which far exceeded that previous studies\textsuperscript{15}, was the largest among vertebrates studied so far; it is much higher than those of other tetrapods, such as caecilian (275), anole lizard (64), and mouse (154). It is noteworthy that we found no V2Rs in the genomes of agnathans and amphioxus. According to a recent study by Bi et al.\textsuperscript{28}, reports on more than 50 V2R-like sequences in hagfish and a few in amphioxus were observed. However, our phylogenetic analyses revealed that they did not form a cluster with known V2Rs. Therefore, we need to be still cautious to designate these sequences as V2Rs, reaching a conclusion that no typical V2Rs exist in agnathans and amphioxus.

Phylogeny and classification of the identified V2Rs

To classify and infer the evolutionary history of V2Rs in vertebrates from cartilaginous fish to mammals, we constructed a phylogenetic tree of 1897 V2Rs identified in this study. The whole vertebrate V2R tree (Supplementary File 1) shows the dichotomy between two clades, including “fish-type” and “tetrapod-type” V2Rs supported by high BPs (89, 92). Therefore, the phylogenetic trees of “fish-type” (Fig. 1A) and “tetrapod-type” V2Rs (Fig. 1B) were shown separately for better visibility in detail classifications. A simplified and overall phylogenetic tree, including all V2Rs of “fish-type” and “tetrapod-type” V2Rs, and other two additional clades are shown in Fig. 1C.

Previous studies have shown that “fish-type” V2Rs were subdivided into 16 subfamilies\textsuperscript{13}. In this study, the subfamily memberships of cartilaginous and basal ray-finned fish were consistent with those of previous studies; however, we identified additional subfamilies. Notably, a large number of V2Rs from the basal ray-finned fish were expanded in a species-specific manner at the stem of subfamily 4–9 (Fig. 1A). Although these V2Rs do not form an exclusive cluster in the tree, we named it subfamily...
“a1” for convenience in classification. Over half of the V2Rs of the basal ray-finned fish belonged to this subfamily “a1” (Table 1, reedfish 101; sterlet 21; spotted gar 29). Also, we have found a cartilaginous fish-specific subfamily “a2” and “a3” at the stem of subfamily 4–10 and at the stem of subfamily 15, 16 (Table 1, Fig. 1A, Fig. S1). In particular, the large number of V2Rs of the cartilaginous fish belonged to subfamily “a2,” in which the genes were expanded in a species-specific manner (Table 1, Supplementary Fig. S1). Taken together, a large fraction of V2Rs in cartilaginous and basal ray-finned fish were classified into these new subfamilies “a1”–“a3, which were the ancestral clades for some known 16 subfamilies. In addition to the subfamily “a1”, species-specific expansion of V2Rs in basal ray-finned fish was observed in subfamily two of sterlet (11) and in subfamily 16 of reedfish (67) and spotted gar (9). In contrast to ray-finned fish in which V2R subfamilies were highly diverse, cartilaginous fishes were relatively less diverse in that they lacked some subfamilies (Table 1). One exceptional finding was that only one sequence of caecilian V2R was classified as being a member of the “fish-type” subfamily 14 (Table 1, Fig. 1A, marked with an asterisk).

The “tetrapod-type” V2Rs were shown to be dominated by closely related V2Rs that have duplicated more recently than those of cartilaginous and ray-finned fish. Notably, V2Rs of amphibians (western clawed frog and caecilian) are highly diverse in that they are subdivided into more than 10 and 3 species-specific clusters, while all of the 147 V2Rs of mouse belonged to a single cluster. The 63 V2Rs of anole lizard were grouped into 2 clusters, which were close to those of mammals (Fig. 1B, Table 1). The 75 V2Rs of coelacanth were classified into the “tetrapod-type,” while 13 V2Rs were classified into the “fish-type” (Fig. 1A, Table 1). The existence of both the “fish-type” and “tetrapod-type” V2Rs in coelacanth was consistent with a previous study. One of the most striking results of this study was that some V2Rs of ray-finned fishes (three genes from each of reedfish and sterlet) were classified into “tetrapod-type”. Indeed, in the phylogenetic tree (as shown in Fig. 1B and Supplementary File S1), these V2Rs are nested within the “tetrapod-type” V2Rs. These “tetrapod-type” V2Rs of basal ray-finned fish were close to those of coelacanth.

Newly identified V2R orthologs conserved from cartilaginous fish to amphibians

Previous studies have shown that V2Rs were divided into three well-supported clades, namely, the V2R2s, “fish-type” V2Rs, and “tetrapod-type” V2Rs. The orthologous V2R2s were shared in all vertebrates from cartilaginous fish to mammals with a few species-specific duplications in mice. In this study, only one V2R2 ortholog of all species investigated was located at the basal position of the V2R tree (Table 1, Fig. 1C, Supplementary Fig. 1). In addition to the three clades, we identified a novel clade, in which V2Rs of cartilaginous fish, basal ray-finned fish, and amphibians were included (Fig. 1C). Since this clade contained one V2R from each species, and the tree topology was identical to the species tree, these V2Rs were considered orthologous. Considering that the origin of these V2R orthologs was comparably as old as V2R2, they would be evolutionarily distinct from the “fish-type”
and “tetrapod-type” V2Rs, which amplify in a species-specific manner. Therefore, we designated them as **anc** (ancient) V2R. In this analysis, highly conserved **anc**V2R sequences were found in the genomes of cartilaginous fish to amphibians, but not in teleost fish, coelacanth, and mammals (Fig. 1C).

**Conserved gene clusters of the “fish-type” V2Rs**

In addition to phylogenetic analysis, the synteny relationships have provided important insight into the classification of V2Rs. Previous studies have shown that the V2Rs in teleost were clustered in one particular chromosomal region, which was flanked by two landmark genes, **phospholipase C (PLC) eta1** and **neprilysin**. In contrast, no V2Rs were found between these two genes in tetrapods, and “tetrapod-type” V2Rs were scattered into several chromosomes. Therefore, to ascertain if the V2Rs are “fish-type” or “tetrapod-type”, we examined the synteny relationships in vertebrates. Figure 2 shows the gene arrangements in the genomic region of two landmark genes in various vertebrates from cartilaginous fish to mammals. Notably, V2R2 and **anc**V2R was found in tandem of the neighboring regions of the PLC eta1, implying the evolutionary conservation of these two genes and evolutionary distinction from known “fish-type” V2Rs. Consistent with the previous study, no V2R was found in tetrapods in this region, except for only one caecilian V2R, which was classified as “fish-type” in the phylogenetic tree (Fig. 1A). In basal ray-finned fish (reedfish, sterlet spotted gar), it was obvious that the V2Rs were clustered between PLC eta1 and neprilysin. Importantly, we revealed that all “fish-type” V2Rs were located in this cluster region, while three “tetrapod-type” V2Rs identified in reedfish and sterlet were located on different chromosomes (chr.3 in reedfish, chr.52, 53 and VTUV01000346.1 in sterlet, Supplementary Table S1). In sterlet, we identified two distinct chromosomal regions of the “fish-type” V2R clusters, which were due to polyploidization specific to this group. The synteny of coelacanth also showed a conservation of the “fish-type” V2R cluster. In the elephant shark and bamboo shark, some “fish-type” V2Rs were located outside the cluster, but considering that they were all found in short scaffolds, it is likely that the cluster region was not properly assembled. Overall, the phylogenetic and synteny analyses both supported the conservation of the cluster for “fish-type” V2Rs as well as the existence of the “tetrapod-type” V2Rs in basal ray-finned fish.

**Expression of V2Rs in the olfactory epithelium of basal ray-finned fish**

To evaluate the functional role of V2Rs found in the genomes of basal ray-finoned fish, we examined the cellular expression patterns for these receptors in the OE. Figure 3 shows the location of the transcripts detected by **in situ** hybridization on frozen sections of the OE of *Polypterus senegalus* (bichir), which is a basal ray-finoned fish closely related to reedfish. The probes of the four V2Rs – “fish-type” V2R, “tetrapod-type” V2R, V2R2, and **anc**V2R – each of which has been identified as a distinct clade in the phylogenetic tree (Fig. 1C), were used in the experiments. The expression of a
member of the “fish-type” V2R showed a sparse pattern in the sensory cells of the OE, typical of canonical V2Rs (Fig. 3A). The expression of a member of “tetrapod-type” V2R, which was newly identified in basal ray-finned fish, also showed similar sparse pattern in the OE of P. senegalus (Fig. 3B). The expression of V2R2, of which the ortholog was highly conserved among jawed vertebrates, has showed widespread patterns in their OE (Fig. 3C). The expression pattern of V2R2 in P. senegalus was consistent with the ubiquitous expression in zebrafish and mouse. The expression of ancV2R, of which the orthologs were also highly conserved from cartilaginous fish to amphibians, showed a sparse pattern in their OE (Fig. 3D) distinct from that of V2R2. Overall, the V2Rs belonging to four clades were all expressed in the OE, suggesting their functions as olfactory receptors. However, the patterns of expressions were ubiquitous in V2R2, while they were sparse in V2Rs of other clades.
Discussion

In this study, we have conducted a comprehensive exploration of V2R sequences from the genomes of 19 vertebrate species. Phylogenetic analyses of a large number of V2Rs allowed us to gain a panoramic view of the diversity in terms of copy number and repertoire of V2Rs across vertebrates. Here, we discuss the tempo and mode of evolution of V2Rs and how these factors drive the adaptive evolution of the olfactory system in vertebrates.

It is obvious that V2Rs were abundant in ray-finned fish compared to cartilaginous fish, both in terms of copy number and repertoire, which is achieved by a species-specific expansion of “fish-type” V2Rs. The syntenic analyses revealed that “fish-type” V2Rs constituted a large gene cluster between PLC eta1 and neprilysin in ray-finned fish (Fig. 2). Although the V2R clusters were ambiguous in the elephant shark, bamboo shark, and sawfish, which may be due to the complex chromosomal rearrangements in cartilaginous fish, the “fish-type” V2Rs exist near the two marker genes. Notably, species-specific expansions of V2Rs did not occur uniformly in all subfamilies, but were rather concentrated in certain ones. For example, the expansion of V2Rs was mainly observed in subfamilies 4–9, 16, ‘a1’, and ‘a2’, while the copy numbers of other subfamilies remained one or two. Therefore, distinction in copy numbers between subfamilies reflects the difference in ligand recognition and biological functions of V2Rs among each subfamily. At present, V2Rs are expected to detect amino acids and their derivatives, eliciting feeding behaviors in teleost fish. It is reasonable to assume that a limited number of amino acids in diets were received by evolutionarily conserved V2R subfamilies. However, it is plausible to assume that the V2R subfamily with frequent lineage-specific gene duplications is responsible for receiving some species-specific variable chemicals for social communication. For example, Yambe et al.33 showed that an amino acid derivative, L-kynurenine, secreted in the female urine, acts as the male-attracting pheromone in masu salmon. In addition, a previous study showed a possible correlation between expansions of V2Rs in subfamily 9 and the evolution of fright reactions in teleost fish. Thus, to elucidate the function of V2Rs in ray-finned fish in addition to amino acid reception, it is necessary to further examine the V2Rs from a multidisciplinary framework, including the ligand binding, and behavioral experiments using candidate chemicals.

We showed that orthologous sequences of V2R2 and newly identified ancV2R have long been conserved during the evolution of vertebrates. The conservation pattern of orthologs in V2R2 and ancV2R is distinct from the canonical V2Rs, such as those in the “fish-type” and “tetrapod-type” V2Rs, which were diversified via species-specific gene duplications. The existence of V2R2 and ancV2R in close genomic proximity was also conserved in many vertebrates (Fig. 2). Studies by Silvotti et al. in the mouse29 and DeMaria et al. in zebrafish31 have revealed that highly conserved V2R2 was expressed in a broad area of the OE and was co-expressed with one of the many canonical V2Rs. Consistent with previous studies, V2R2 of Polypterus senegalus showed widespread expression patterns in the OE (Fig.
3C), while the “fish-type” and “tetrapod-type” V2Rs showed sparse patterns (Figs. 3A, B). In contrast, although ancV2R was similar to V2R2 in terms of evolutionary conservation and genomic proximity, the pattern of expression in the OE was sparse rather than widespread. Thus, it is implicative to note that ancV2R has characteristics between V2R2 and canonical “fish-type” and/or “tetrapod-type” V2Rs. Thus, taking the evolutionary conservation as well as the sparse pattern of expression into account, ancV2R may have retained ancestral nature inherited from a protogene before the split between “fish-type” and “tetrapod-type” V2Rs, which are now highly diversified in jawed vertebrate genomes. This study showed that all four clades of V2Rs, including the “tetrapod-type” V2Rs, were expressed in the olfactory organs of basal ray-finned fish, which propose that they have functional roles in olfaction. At the same time, the degree of conservation and expression patterns was distinct among those clades. In particular, it was of interest that the expression patterns of V2R2, ancV2R, and “fish-type” V2Rs were distinct despite their location on the same genomic cluster. A detailed investigation into this genomic region would lead to the elucidation of a cis-regulatory mechanism that controls the expression of canonical V2Rs, as to say “one neuron one receptor” rule.34 Figure 4 illustrates the evolutionary scenario of the four clades of V2R in vertebrates from agnathans (lamprey and hagfish) to mammals by showing the distribution of these four V2R clades in extant species and two ancestral nodes, as estimated by the parsimonious principle. No V2R was found in the agnathans, implying the acquisition of a V2R-mediated olfaction as a common ancestor of jawed vertebrates (cartilaginous fish and Osteichthyes). Given that agnathans have three other types of chemoreceptor genes, namely, ORs, TAARs, and V1Rs, it is likely that V2Rs were originated later than these three receptors during vertebrate evolution.26,35 V2R2 was conserved in all extant vertebrates with no exceptions, which suggests the highly important and fundamental role of V2R2 in the detection and subsequent signaling pathway for olfactory substances in both underwater and terrestrial environments. Importantly, except for evolutionarily conserved V2R2, mammal and teleost fish possessed only specific clades of V2Rs, namely, “tetrapod-type” and “fish-type” V2Rs, respectively. In contrast, basal ray-finned fish and amphibians possessed both “tetrapod-type” V2Rs and “fish-type” V2Rs. This finding suggests that all four clades of V2Rs were present at least in the common ancestor of Osteichthyes, or even earlier in the common ancestor of jawed vertebrates. However, subsequently, the repertoires of V2Rs were lost in teleost fish and mammals during adaptation to their specific environments. Because the previous studies of V2Rs have been limited to teleost fish and mammalians, the diversity of V2Rs that should exist in the other variety of species has not been recognized. Thus, the genome sequences of non-model animals such as cartilaginous fish, basal ray-finned fish, coelacanth, which become available due to the recent advancement of sequence technique, allowed us to uncover the hidden and unexpectedly large diversity in the genomes of vertebrates. One of the worthwhile discussions is the possible great diversity of V2Rs in the Osteichthyes ancestor. The repertoires of the subfamily of the “fish-type” V2Rs are abundant in ray-finned fish and
coelacanth compared with those in cartilaginous fish (Table 1). In addition, all four clades of V2Rs were shown to be present in basal ray-finned fish and amphibians. The abundance of copy numbers and repertoires in these groups show that V2Rs were highly diversified in the common ancestor of Osteichthyes. Given that some of the olfaction-related genes also emerge in the common ancestor of the Osteichthyes (e.g., ancV1R\textsuperscript{36}; OMP\textsuperscript{37}), it might be possible that an innovative evolution of the olfactory system occurred in this timing. It is worth mentioning here that the polypterids (bichir and reedfish) possess large paired openings (spiracles) on top of their head, in which they use for breathing air\textsuperscript{38}. Similar spiracle-like structures were observed in the fossil records of stem tetrapods\textsuperscript{39}. Thus, breathing air using spiracles may have been an important respiratory strategy in the stem Osteichthyes, which inhabit shallow freshwater environments and use lungs in addition to gills for respiration\textsuperscript{40}. Specifically, the evolution of air-breathing by spiracles may increase the opportunity to raise their head above water, which led to the acquisition of the primitive capabilities of detecting airborne chemicals before terrestrial adaptation. Thus, such dual functional roles of the olfactory system in stem Osteichthyes were related to the diversification of V2Rs, including “tetrapod-type” V2Rs. Deorphanization of V2Rs of various vertebrates in the near future is necessary to evaluate the above possibility.
Materials and methods

Sequence retrieval

To estimate the evolutionary history of V2Rs, we conducted a comprehensive exploration of V2R sequences in the genome assemblies of a broad range of vertebrates, including nine cartilaginous fishes and three basal ray-finned fishes. In addition to two teleost fishes (Japanese eel and zebrafish), coelacanth, two amphibians (caecilian and western clawed frog), anole lizard, and mouse were explored (Table 1). To identify the V2R sequences from the genome assemblies, we performed tBLASTn searches using the transmembrane (TM) domain of V2Rs as queries against all of the genomic sequences. The query sequences were generated by aligning V2R sequences of mouse, anole lizard, tropical clawed frog, coelacanth, spotted gar, and zebrafish deposited in the Ensembl database and of elephant shark and cloudy catshark\textsuperscript{22} using the MA-T --dash option\textsuperscript{41}. Then, the TM domains, from 50-aa sequences upstream of the 1st TM region to 50-aa downstream of the 7th TM region, were extracted using a protein structure of the glutamate receptor (PDB: 4OR2)\textsuperscript{42} as reference.

Next, the V2R-coding sequences were predicted for tBLASTn hit regions using GeneWise v.2.4.1\textsuperscript{43}. Sequences shorter than 600 nucleotides were discarded. The homology of intact outputs was then determined by the next phylogenetic analysis. The queries used in the BLAST search were critical to the estimated number of V2Rs. Indeed, the number of V2Rs identified in this study was consistent with previous studies, whereas a few differences were observed in some cases. For example, the numbers of V2Rs in elephant shark and catshark identified in this study (29 and 24 copies) were slightly smaller than those reported by Sharma \textit{et al.}\textsuperscript{22}. The difference in query sequences used for the BLAST search reflects results. Specifically, we used the seven-transmembrane regions of the V2R, whereas Sharma \textit{et al.}\textsuperscript{22} used the entire region of V2R, including the extracellular Venus flytrap module region (VFTM)\textsuperscript{44}. The VFTM region of the V2R protein has been determined to be highly diverse and difficult in predicting the exon-intron structure of the gene. This result affects the judgment of intact or pseudogenes. Therefore, we compared the number of V2Rs estimated under a unified condition using the seven-transmembrane region as a query.

Phylogenetic and synteny analyses

Sequences were translated into amino acid sequences and aligned using MAFFT with the -ginsi option\textsuperscript{45} and default parameters. The sites with <50 % coverage among all sequences were removed. Maximum likelihood trees for V2R genes were then inferred using RAxML-NG v.1.0.1\textsuperscript{46} with the JTT+G+F amino acid substitution model. This result was the best fitting model selected by the ModelTest-NG\textsuperscript{47,48} based on AIC scores. Rapid bootstrap analyses were performed using 100 replicates to assess the reliability of nodes. The V2R sequences for coelacanth, western clawed frog, and mouse were then clustered based on the criteria of 80 % similarity at the amino acid level to save computational costs. To avoid false positives in identification of the V2R genes and prevent long
branch attractions, we obtained other GPCR family C sequences from GenBank or Ensembl for use as outgroups, including CaSR (NM_013803.3), Tas1r1 to Tas1r3 (ENSMUSG00000028738, ENSMUSG00000029072), GPCR6 (NM_153071.1), GRM1a to GRM8 (NM_016976.1, NM_001160353.1, NM_181850.2, NM_001081414.2, NM_001081414.2, NM_173372.2, NM_177328.3, NM_001361125.1), and GABA B1 to GABA B2 (NM_019439.3 and NM_001081141.2), for constructing an initial gene tree. All genes in the sister clade to CaSR were named homologs of V2R (including V2R2). Using these genes, we constructed the V2R gene tree again (CaSR was used as an outgroup). We also included sequences of all 16 teleost fish subfamilies classified in previous studies, as markers to indicate V2R subfamilies.

The synteny relationships of “fish-type” V2Rs of vertebrates were then illustrated based on the genomic location of the identified V2Rs (Supplementary Table S1). The numeric data for the genomic position of V2Rs, which were identified as “fish-type” V2Rs, V2R, and ancV2R in the phylogenetic tree, were then compiled, with those of PLC eta1 and nephrilysin.

**In situ hybridization**

Bichir (*Polypterus senegalus*) individuals of 11–25 cm long, which were used for the preparation of frozen sections and the extraction of total RNA from the olfactory organs, were purchased from a commercial supplier; they were kept under standard conditions suitable for tropical fish breeding until experimental manipulations. All experimental studies using the animals were approved by the Institutional Animal Experiment Committee of the Tokyo Institute of Technology were performed in accordance with the institutional, governmental ARRIVE guidelines. TRIzol (Invitrogen) was then used for total RNA extraction from the olfactory organs of the bichir. Using the total RNA extracted from the olfactory organs of the bichir, cDNA was synthesized by reverse transcription reaction using SuperScript III RTase (Invitrogen). Coding regions of V2R were amplified by PCR using the primer sets, which were designed on the basis of V2R sequences of reedfish, as has been summarized in Supplementary Table S2. The PCR products were cloned using the pGEM-T vector (Promega) and the DH5α strain of *E. coli*. Digoxigenin-labeled RNA probes were synthesized using the plasmid vector as a template using T7 or SP6 RNA Polymerase (Roche) and DIG RNA labeling mix (Roche) as well. The olfactory organs of the bichir were then fixed with 4 % PFA, replaced with sucrose, and embedded in an O.C.T compound (Sakura Finetek). *In situ* hybridization was performed according to the method as previously described. Briefly, hybridization was performed using DIG-labeled RNA probes. The antibody reaction was conducted using anti-digoxigenin-POD, Fab fragments (Sigma-Aldrich), or anti-fluorescein-POD, Fab fragments (PerkinElmer). The signal was then amplified with Tyramide Signal Amplification Plus Biotin kit (Kiko Tech), and detected by streptavidin, Alexa Fluor 488 conjugate (Thermo Fisher). Finally, the sections were sealed using a VECTASHIELD Mounting Medium with DAPI (VECTOR). The sealed sections were observed using a fluorescence microscope.
Axioplan (Carl Zeiss). All fluorescence photographs were taken using an Axiocam 503 color (Carl Zeiss) and optimized for brightness and contrast in Adobe Photoshop.
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Author contribution
Project design, coordination: M.N.; bioinformatics and evolutionary analyses: Z.Z.; molecular experiments: A.S.; manuscript writing: Z.Z., S. K., M.N.

Competing interests
The authors declare no competing interests

Additional information
The online version contains supplementary materials available at XXXX.

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Figure legends

Figure 1. Phylogenetic relationships of V2Rs of a broad range of vertebrates from cartilaginous fish to tetrapods. (A) Phylogenetic tree of “fish-type” V2Rs. Note that “fish-type” V2Rs were subdivided into 16 known and 3 novel subfamilies, as indicated by gray thick bars. Triangles in red, orange, blue, white, and brown indicate the expanded V2R clusters specific to reedfish, sterlet, spotted gar, cartilaginous fish, and teleost fish, respectively. Only one “fish-type” V2R found in the caecilian species was marked using an asterisk. (B) Phylogenetic tree of “tetrapod-type” V2Rs. Triangles in violet, pink, yellow, green, and gray indicate expanded V2R clusters specific to coelacanth, caecilian, western clawed frog, anole lizard, and mouse, respectively. Asterisks were used to mark the “tetrapod-type” V2Rs identified in reedfish and sterlet. Note that the “tetrapod-type” V2Rs, in contrast to the “fish-type” V2Rs, are composed of many clusters that are expanded in a species-specific manner. (C) Overview of the phylogenetic tree of all V2Rs showing novel orthologous clade ancV2R. The calcium-sensing receptor (CaSR) gene was used for outgrouping all V2Rs. The OTU names consist of the common name and locus as summarized in Supplementary Table S1. The “fish-type” V2Rs, “tetrapod-type” V2Rs, and V2R2 clades were compressed into black triangles. The number on the branches indicates the bootstrap support values for particular nodes. Note that the grouping of the orthologous ancV2Rs of cartilaginous fish, basal ray-finned fish, and western clawed frog was suggested by maximum bootstrap support (100%). The numbers next to triangles indicate the copy number of V2Rs included in the clusters. The filled circles on each node indicate bootstrap supports (black > 75, 75 ≥ gray ≥ 50, 50 > white). Scale bar indicates the number of amino acid substitutions per site.

Figure 2. Synteny relationships for the “fish-type” V2R clusters among basal ray-finned fish (reedfish, sterlet, spotted gar), teleost fish (zebrafish), lobe-finned fish (coelacanth), tetrapods (caecilian, western clawed frog, mouse), and cartilaginous fish (elephant shark, bamboo shark, and smalltooth sawfish), respectively. Triangles in yellow, blue, red, and green indicate two landmark genes (PLC eta1, neprilysin), V2R2, and ancV2R, respectively. Black squares indicate “fish-type” V2Rs. Indicated at the upper-left of each line were several chromosomes or scaffolds and its directions. Indicated below the ends of the line are the start and end of the cluster regions. Unplaced scaffolds are not shown in this figure. Note that “fish-type” V2Rs were flanked by two landmark genes and that V2R2s and ancV2Rs were located in tandem close to the clusters. No “tetrapod-type” V2Rs were observed in these cluster regions. In the elephant shark, some “fish-type” V2Rs are located outside the cluster because the cluster regions were not properly assembled.

Figure 3. The expression patterns of V2Rs in the olfactory epithelium of the basal ray-finned fish, Polypterus senegalus. Expression of four V2R genes were confirmed by FISH on horizontal sections of the olfactory organ of the bichir using DIG-labeled RNA antisense probes for “fish-type” V2R (A)
and “tetrapod-type” V2R (B), V2R2 (C), and ancV2R (D). Green indicated the expression signals. The blue area indicates the cell nucleus stained with DAPI. V2R2 was globally expressed in the deep layers of olfactory folds (C). In contrast, “fish-type” V2R, “tetrapod-type” V2R, and ancV2R were sparsely expressed in a small number of neurons in the deeper layers of the olfactory folds (A, B, and D). (A’-D’) High magnification view of the dotted squares in A–D. Scale bars show 100 µM (A–D) and 20 µM (A’–D’).

**Figure 4.** Evolutionary scenario of V2Rs during vertebrate evolution. The presence/absence of the four major V2R clades was plotted on the phylogenetic tree of vertebrates from agnathans to mammals. The red circle with “2,” yellow with “a,” gray with “t,” and blue with “f” indicate V2R2, ancV2R, “tetrapod-type” V2R, and “fish-type” V2R, respectively. In contrast to basal ray-finned fish with all four clades of V2Rs, teleost fish, mammals, and lizards were determined to possess only two of them. The reduction of specific V2R clades in these lineages would be due to adaptation to specific oceanic and terrestrial environments. Note that the origin of “tetrapod-type” V2Rs dates back to the era of the common ancestor of extant Osteichthyes, but its antiquity in the jawed vertebrate ancestor remains to be examined with complete genome sequences of more cartilaginous fish (dotted circle with “t” inside).

**Table 1.** Number of intact V2R genes identified in the genomes of a broad range of vertebrates.

**Supplementary Table S1.** Summary of the intact V2Rs in the genomes of vertebrates. Gene name, locus (position in the chromosomes, scaffolds), and directions were summarized in a bed file format.

**Supplementary Table S2.** PCR primers used to amplify four V2R genes of Polypterus senegalus.

**Supplementary Figure S1.** A phylogenetic tree of subfamily “a2” and the cartilaginous fish-specific V2Rs. Note that this subfamily consists of 132 V2Rs, most of which were expanded in species-specific birth and death processes. Only one orthologous V2R was shared among cartilaginous fish (indicated by asterisk).

**Supplementary File 1.** The Newick format phylogenetic tree of all 1897 intact V2Rs identified by a broad range of vertebrates. Note that grouping of “fish-type” V2Rs, “tetrapod-type” V2Rs, V2R2, and ancV2R was supported in sufficient bootstrap values.
Supplementary File 2. The aligned V2R sequences of all 1897 intact V2Rs identified from various vertebrates.
| Scientific name | Common name            | Assembly name       | V2R sum | ancV2R sum | tetrapod-type V2R sum | fish-type V2R sum |
|-----------------|------------------------|---------------------|---------|------------|-----------------------|-------------------|
| Callorhinchus milii | Elephant shark       | Callorhinchus_milii-6.1.3 | 1       | 1          | 0                     | 26                |
| Hydrologus affinis | Small-eyed rabbitfish | UP_Haf               | 1       | 1          | 0                     | 39                |
| Scyllorhinus torazame | Cloudy catshark       | Storazame_v1.0       | 1       | 1          | 0                     | 21                |
| Carcharodon carcharias | Great white shark | ASM360424v1          | 1       | 1          | 0                     | 12                |
| Rhinodon typus | Whale shark           | ASM164234v2          | 1       | 0          | 0                     | 21                |
| Chilomycterus punctatum | Brownbanded bambooshark | Cpmctatum_v2.1      | 1       | 1          | 0                     | 24                |
| Leucoraja erinacea | Little skate          | LER_WGS_1            | 1       | 1          | 0                     | 6                 |
| Amblyraja radiata | Thomy skate           | sAmbRad1.pri         | 1       | 1          | 0                     | 6                 |
| Chiloscyllium punctatum | Brownbanded bambooshark | Cpmctatum_v2.1      | 1       | 1          | 0                     | 24                |
| Pristis pectinata | Smalltooth sawfish   | sPset02.pri          | 1       | 1          | 0                     | 22                |
| Erpetoichthys calabaricus | Reedfish             | fErpCal1.1           | 1       | 1          | 3                     | 183               |
| Acipenser ruthenus | Sterlet               | ASM1064508v1         | 1       | 1          | 3                     | 41                |
| Leptosomus oculatus | Spotted gar          | LepOcu1              | 1       | 1          | 0                     | 47                |
| Anguilla japonica | Japanese eel          | Ajp_01               | 1       | 0          | 0                     | 63                |
| Danio rerio | Zebrafish             | GRCz11               | 1       | 0          | 0                     | 71                |
| Latimeria chalumnae | Coelacanth            | LatCha               | 1       | 0          | 0                     | 71                |
| Xenopus tropicalis | Western clawed frog   | UCB_Xtro_10.0        | 1       | 1          | 0                     | 41                |
| Gonioplectrus sarrahini | Caecilian            | aGeoSer1.1           | 1       | 0          | 0                     | 41                |
| Anolis carolinensis | Anole lizard          | AnoCar2.0            | 1       | 0          | 0                     | 41                |
| Mus musculus | Mouse                 | GRCm38.p6            | 7       | 0          | 147                   | 0                 |

Note: The gray highlighted the cells in which no genes were found in the corresponding subfamilies.
Supplementary Files

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