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Ancient and Nonuniform Loss of Olfactory Receptor Expression Renders the Shark Nose a De Facto Vomeronasal Organ

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Abstract

Cartilaginous fishes are renowned for a keen sense of smell, a reputation based on behavioral observations and supported by the presence of large and morphologically complex olfactory organs. At the molecular level, genes belonging to the four families coding for most olfactory chemosensory receptors in other vertebrates have been identified in a chimera and a shark, but it was unknown whether they actually code for olfactory receptors in these species. Here, we describe the evolutionary dynamics of these gene families in cartilaginous fishes using genomes of a chimera, a skate, a sawfish, and eight sharks. The number of putative OR, TAAR, and V1R/ORa receptors is very low and stable, whereas the number of putative V2R/OlfC receptors is higher and much more dynamic. In the catshark *Scyliorhinus canicula*, we show that many V2R/OlfC receptors are expressed in the olfactory epithelium in the sparsely distributed pattern characteristic for olfactory receptors. In contrast, the other three vertebrate olfactory receptor families are either not expressed (OR) or only represented with a single receptor (V1R/ORa and TAAR). The complete overlap of markers of microvillous olfactory sensory neurons with pan-neuronal marker HuC in the olfactory organ suggests the same cell-type specificity of V2R/OlfC expression as for bony fishes, that is, in microvillous neurons. The relatively low number of olfactory receptors in cartilaginous fishes compared with bony fishes could be the result of an ancient and constant selection in favor of a high olfactory sensitivity at the expense of a high discrimination capability.

Key words: gene family dynamics, gene expression, Chondrichthyes, odorant receptors, trace amine-associated receptors, vomeronasal receptors.

Introduction

The sense of smell is involved in many essential tasks of vertebrates, including Chondrichthyes (cartilaginous fishes), from food and prey location over reproductive functions and social interactions to danger avoidance (DeMaria et al. 2013; Gardiner et al. 2014; Gardiner et al. 2015). The study of Osteichthyes (bony fishes, such as mouse and zebrafish), but also lampreys (jawless fishes), has shown that four large olfactory receptor families (OR, TAAR, V1R/ORa, and V2R/OlfC) are expressed in olfactory sensory neurons (OSNs) and constitute the molecular basis of odor detection (Mombaerts 2004). These families of olfactory receptors were first identified in mammals (Buck and Axel 1991; Dulac and Axel 1995;

Matsunami and Buck 1997; Liberles and Buck 2006), but subsequent studies have shown their presence in other tetrapods and Actinopterygii (ray-finned fishes) (Niimura 2009), in cartilaginous fishes (Grus and Zhang 2009; Hussain et al. 2009; Niimura 2009; Sharma et al. 2019), and in jawless fishes (Grus and Zhang 2009; Libants et al. 2009; Dieris et al. 2021; Kowatschew and Korsching 2022), suggesting that they were present in the last common ancestor of all extant vertebrates.

We have recently described the olfactory repertoire of the small-spotted catshark *Scyliorhinus canicula* to be dominated by the V2R/OlfC family, whereas the V1R/ORa, OR, and TAAR families are only represented by a handful of members each (Sharma et al. 2019). This

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repertoire is similar to that of four other sharks—the cloudy catshark *Scyliorhinus torazame*, the brownbanded bamboo shark *Chiloscyllium punctatum*, the whale shark *Rhincodon typus*, and the white shark *Carcharodon carcharias* (Hara et al. 2018; Marra et al. 2019)—and a more distantly related species, the chimaera *Callorhinchus milii* (Grus and Zhang 2009; Hussain et al. 2009; Niimura 2009). These olfactory gene repertoires are distinctly different from those of both jawless and bony fishes, which are dominated by the OR family (Niimura 2009).

The expression of olfactory receptor genes has been overwhelmingly studied in mammals, in particular mouse and rat, and to some extent in teleosts, in particular zebrafish. The expression for all four families is very similar in mammals and teleosts: A monogenic expression pattern and a characteristically sparse expression of individual receptor genes are features common to both (Mombaerts 2004; Korsching 2020a). In both mammals and teleosts, olfactory receptor expression is segregated between two main types of OSNs, ciliated and microvillous neurons. ORs and TAARs are expressed in ciliated neurons, whereas V2Rs (in mammals also V1R) are expressed in microvillous neurons (Korsching 2020b). Much less is known about the expression of olfactory receptors in jawless fishes (Berghard and Dryer 1998; Freitag et al. 1999; Libants et al. 2009; Kowatschew and Korsching 2022). To the best of our knowledge, no in situ expression studies have been performed for any olfactory receptor of any cartilaginous fish (sharks, rays, and chimaeras).

The olfactory organ of cartilaginous fishes looks similar to that of many ray-finned fishes: a rosette with bilaterally symmetric rows of lamellae (fig. 1). However, all cartilaginous fishes examined exhibit the so-called secondary lamellae, emanating from the primary lamella, which also contain olfactory epithelium (OE) (fig. 1) (Holl 1973; Theisen et al. 1986; Ferrando et al. 2019). Furthermore, and in contrast to tetrapods and ray-finned fishes, cartilaginous fishes seem not to possess ciliated OSNs (Holl 1973; Theisen et al. 1986; Takami et al. 1994) and their OSN repertoire is dominated by microvillous neurons, with rare crypt neurons (Holl 1973; Theisen et al. 1986; Takami et al. 1994; Ferrando et al. 2006). Thus, it is an open question, if and how ORs and TAARs—which are expressed in ciliated OSNs in both ray-finned fishes and tetrapods—are expressed in cartilaginous fish OSNs.

Here, we made use of the recent availability of genomes for species from the 3 main groups of cartilaginous fishes—chimaeras, rays/skates/sawfishes, and sharks, in total 11 species—to obtain a comprehensive picture of the evolutionary dynamics of the 4 olfactory gene families in chondrichthyans. We report that consistently, OR, TAAR, and V1R/ORa repertoires are very small and stable, whereas the V2R/OlfC repertoire is larger and more dynamic.

Furthermore, we examined the expression of olfactory genes in the catshark *S. canicula*. None of the few *or* genes present in its genome are expressed in the olfactory organ. For TAAR and V1R/ORa families, we observed expression in the olfactory organ for a single gene each. In contrast,

V2R/OlfCs showed robust expression with several different probes. Individual olfactory receptor genes are expressed in sparsely distributed cells, and their spatial expression patterns are characteristically different between different receptors, both features as observed in other vertebrates. Globally, these results suggest that olfaction in cartilaginous fishes essentially relies on a relatively small set of *v2r/olfC* genes and that in several aspects the olfactory system of this vertebrate class could be considered a vomeronasal system (cf. Ferrando and Gallus 2013).

Results

Diversity and Evolutionary Dynamics of the Olfactory Gene Repertoire in Cartilaginous Fishes

We counted the number of complete coding sequences and 3 different types of incomplete sequences (pseudogenes, truncated, and edge; for definition, see Materials and Methods) belonging to the OR, TAAR, V1R/ORa, and V2R/OlfC families in 11 genomes of cartilaginous fishes. Five genomes have been previously examined, but often, only the number of complete genes has been reported and not always for all gene families. For *S. canicula*, we refined the identification of complete genes in each family (cf. Sharma et al. 2019). For *C. milii*, the number of complete *or* and *v2r/olfC* genes was underestimated. For *S. torazame*, *C. punctatum*, and *R. typus*, the number of complete genes was much underestimated or not examined at all. Once corrected, the numbers of genes in these species were similar and similar to those found in the six species for which the olfactory gene repertoire has never been studied before (fig. 2; supplementary fig. S1 and table S1, Supplementary Material online).

In the cartilaginous fishes examined, the number of *or* genes varies between 6 and 13, and only 2 pseudogenes, 7 truncated, and 1 edge sequences were found. This pattern implies 12 gene duplications and 18 gene losses and the presence of 7 *or* genes in the last common ancestor. The number of *taar* genes varies between 3 and 5, and in total, 25 pseudogenes and 15 truncated sequences were found. This pattern implies 1 gene duplication and 14 gene losses and the presence of 6 *taar* genes in the last common ancestor. The number of *v1r/ora* genes varies between 2 and 4, and only 3 pseudogenes were found. This pattern implies no gene duplication and six gene losses and the presence of four *ora* genes in the last common ancestor. The number of *v2r/olfC* genes varies between 8 and 43, and 189 pseudogenes, 16 truncated, and 15 edge sequences were found. This pattern implies 94 gene duplications and 121 gene losses and the presence of 21 *v2r/olfC* genes in the last common ancestor (fig. 2).

Overall, it appears that the number of *or*, *taar*, and *v1r/ora* genes is low and stable and very few pseudogenes and truncated genes are present in the genome of cartilaginous fishes, in deep contrast with large and highly dynamic numbers of genes belonging to these gene families in bony fishes, often associated with the presence of many

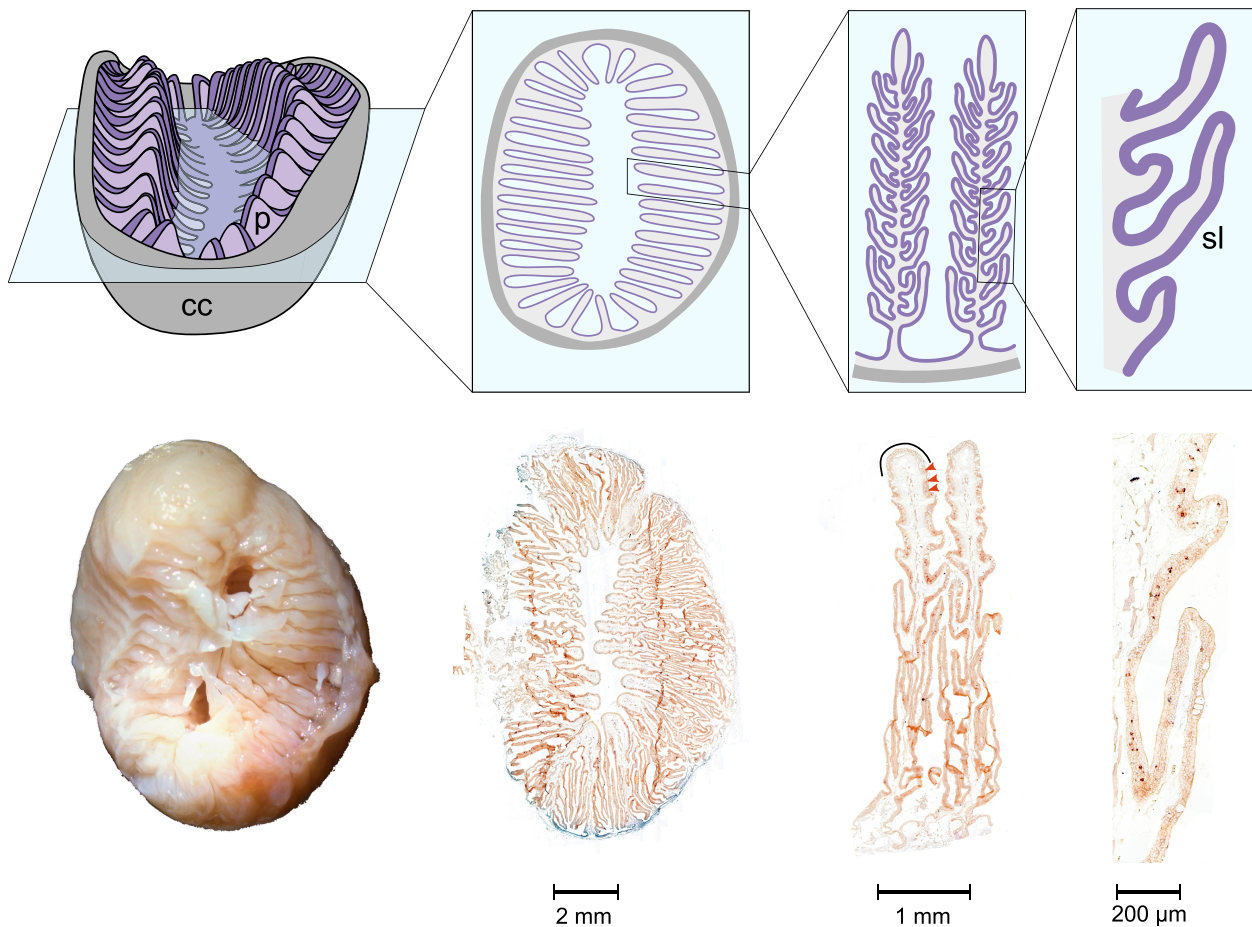


FIG. 1. Morphology of the catshark olfactory organ. The catshark olfactory organ is shown at increasing resolution from left to right, starting with the whole organ enclosed by a connective capsule (cc), down to a segment of a single lamella. Top row, schematic representation; bottom row, micrographs of the same features, scale bars as indicated. Micrograph sections are from an in situ hybridization (ISH) experiment. Note the presence of primary (*p*) and secondary (*sl*) lamellae. The sensory surface covers both primary and secondary lamellae, excluding lamellar tips (solid black line), where mucous cells (large ovals) are enriched. Some labeled OSNs are pointed out by arrowheads.

pseudogenes and truncated genes. On the contrary, and as in bony fishes, the number of *v2r/olfC* is often high and much more variable. Several species-specific expansions exist (supplementary fig. S1 and table S1, Supplementary Material online), and large proportion of pseudogenes are often present, a hallmark of a multigene family coding for olfactory receptors.

Microvillous Neuronal Markers TRPC2 and Go Label the Entire Neuronal Population in the Catshark Olfactory Epithelium

We employed the pan-neuronal marker HuC to visualize the entire neuronal population in the catshark olfactory epithelium (fig. 3). The lamellae of the olfactory organ are covered almost entirely by the sensory olfactory epithelium; only the tip region of the lamellae is covered by nonsensory epithelium (fig. 1). The HuC-immunoreactive neurons form an almost continuous irregular monolayer of perikarya (fig. 3b), which are situated in the middle layer of the epithelium, below the apical layer of supporting cells recognizable by their palisade-like arrangement.

Proliferating cells form the basal layer of the olfactory epithelium and were visualized by proliferating cell nuclear antigen (PCNA) antibody (fig. 3). As expected, no overlap between HuC and PCNA immunoreactivity was observed (fig. 3a and d).

We then examined the expression of two established microvillous markers (transient receptor potential channel TRPC2 and G alpha protein Go) (Hansen et al. 2004; Sato et al. 2005) within the entire neuronal population as defined by HuC immunoreactivity. Notwithstanding different subcellular compartments for HuC (perikarya) and Go (dendrites and axons), all HuC-positive cells appear to be Go-positive (fig. 4d and g). This was confirmed in double-labeling experiments using HuC antibody and Go in situ hybridization (fig. 4h–j). Moreover, TRPC2, which labels all microvillous neurons in bony fishes (Sato et al. 2005; Omura and Mombaerts 2014), colocalizes completely with HuC immunoreactivity (fig. 4k–m). We did not detect any HuC-positive but TRPC2-negative or Go-negative cells. However, we cannot exclude the potential presence of a minor population of Go-negative ciliated neurons, on the scale of crypt neuron frequency (i.e., very minor),

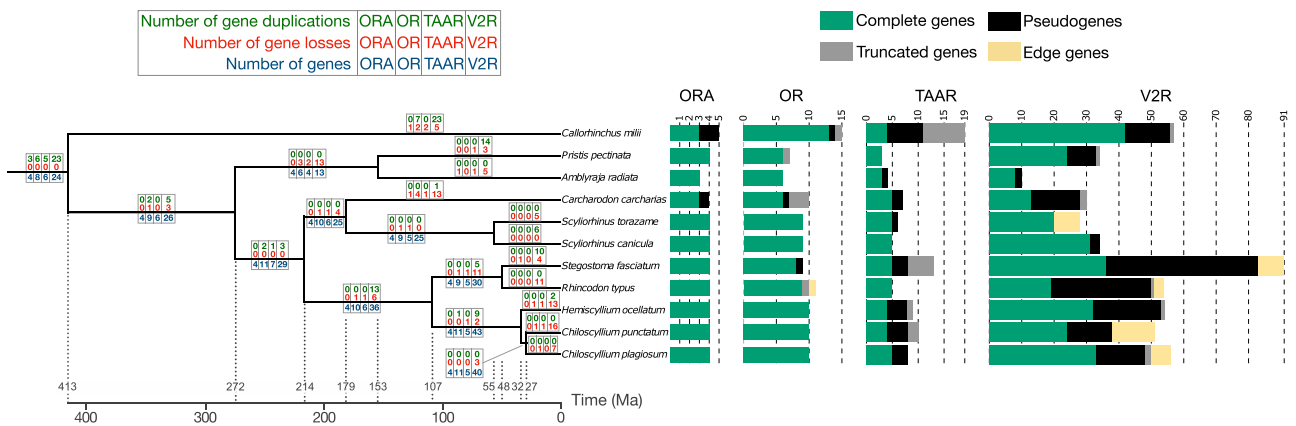


Fig. 2. Time-calibrated chondrichthyan tree. The species tree topology was inferred by maximum likelihood using 1,068 BUSCO genes, and node ages were inferred using the least square dating method. The numbers of genes in the four olfactory receptor families are represented by multiple values bar plots. Complete genes, pseudogenes, truncated genes, and edge genes are shown separately. The numbers of gene losses (red numbers, above branches) and gene gains (green, top rows) in each branch of the tree and for the four gene families were inferred using the gene tree–species tree reconciliation method. Total numbers of genes given below branches (blue numbers) refer to the subsequent node. The complete species tree with confidence intervals of node dates and with *H. affinis* is available in [supplementary figure S5, Supplementary Material](#) online.

since this population is known to be negative for Go (Ferrando et al. 2009) and was not detected in our analysis. Nevertheless, the present data are consistent with the absence of ciliated OSNs in catshark olfactory epithelium.

Taken together, (nearly) all OSNs within the sensory surface of the catshark olfactory epithelium appear to express Go and TRPC2, suggesting that the entire OSN population of catshark consists of microvillous neurons. This fits well with the predominance of V2R/OlfC in the olfactory receptor repertoire of cartilaginous fishes, since in both tetrapods and teleosts, V2R/OlfC receptors are characteristically expressed in microvillous neurons and absent from ciliated neurons (Hansen et al. 2004; Mombaerts 2004; Syed et al. 2017).

A Comprehensive Approach to Study the Expression of the Entire Olfactory Repertoire

The catshark olfactory receptor repertoire is dominated by V2Rs/OlfCs, with 34 *v2r/olfc* genes in contrast to 4–9 receptors for the other 3 families (OR, TAAR, and V1R/ORA) (fig. 2; [supplementary table S1, Supplementary Material](#) online). Here, we have examined the expression patterns for all four olfactory receptor families in catshark.

We performed reverse transcription polymerase chain reaction (RT-PCR) for all *or*, *taar*, *taar-like*, and *v1r/ora* genes identified in Sharma et al. (2019) and for one *v2r/olfc-like* and five *v2r/olfc* genes (*v2r14*, *v2r1*, *v2r6*, *v2r14*, *v2r19*, and *v2r29*). With the exception of *v2r14*, expression was observed for all genes examined ([supplementary fig. S2, Supplementary Material](#) online).

To examine expression at the cellular level, we performed in situ hybridization (ISH) with cRNA probes on horizontal cryostat sections from adult catshark olfactory epithelia (fig. 1). For the V2R/OlfC gene family, we used both specific and cross-reacting probes. The expression of *v2r1* and two *v2r*-like genes (*v2r11* and *v2r13*) was analyzed with specific probes. In addition, we employed four

probes from different *v2r* subclades which are expected to cross-react with several other genes (table 1), resulting in coverage of a considerable proportion of that family. For three families, ORs, TAARs, and V1R/ORAs, we examined the expression for each gene with a single, specific probe. In all cases where the characteristic pattern of sparsely distributed labeled cells was observed ([supplementary fig. S3, Supplementary Material](#) online), we quantified the expression frequency as well as the spatial distribution of receptor-expressing cells.

The Earliest-Diverging V2R/OlfC Gene Exhibits the Highest Frequency of Expression

A common feature of the tetrapod and teleost V2R/OlfC repertoires is the presence of genes coding for V2R/OlfC coreceptors and belonging to a monophyletic sister group of the main group of V2R/OlfC receptors. This receptor is a single gene in zebrafish, *olfCc1* (DeMaria et al. 2013), but has expanded to a small family in rodents, *vmn2r*, also known as *v2r2* (Martini et al. 2001). Because these genes serve as coreceptor for many individual *v2r/olfc* genes (Ishii and Mombaerts 2011; Akiyoshi et al. 2018), their expression frequency is characteristically high compared with the other *v2r/olfc* genes (DeMaria et al. 2013). The V2R1 receptor of catshark is the ortholog of OlfCc1 and *Vmnr2r1-7*. We were therefore interested in determining its expression frequency in relation to that of other *v2r/olfc* genes.

We performed in situ hybridization for *v2r1*, two of the five *v2r/olfc-like* (*v2r11,3*) and four *v2r* from the main clade *v2r6*, *v2r14*, *v2r19*, and *v2r29*, which are expected to cross-react with one to six other *v2r/olfc* (see table 1 for details). No expression in the OE was seen for the *v2r/olfc-like* genes, but all other probes resulted in labeling of sparse cells within the OE as expected for olfactory receptor genes (figs. 5 and 6). The gene *v2r1* was expressed in a considerable population of neurons (fig. 5 a–c), which

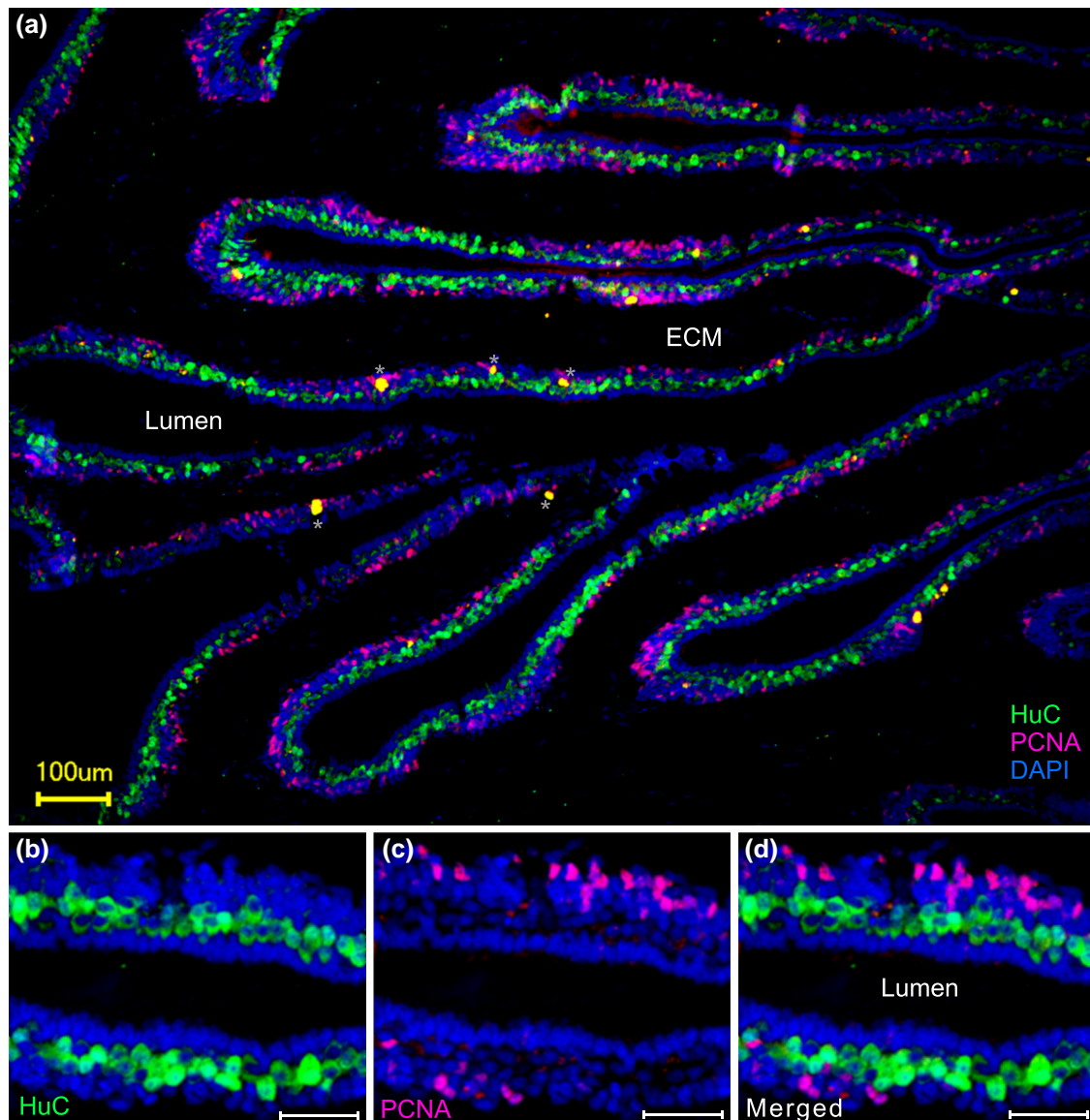


FIG. 3. Catshark OSNs form a monolayer above the proliferative zone of the olfactory epithelium. Double immunofluorescence for a pan-neuronal marker (HuC, green) and a marker for mitotic cells (PCNA, purple) was performed on cryostat sections of the olfactory organ of the catshark. Nuclei are stained by DAPI (blue). Neurons (green) form an irregular monolayer below the supporting cells (dense palisade facing the lumen, blue) and above the basal layer (purple cells). (a) Overview, merged fluorescence, (b–d) higher magnification, fluorescent label as indicated. Scale bars, 100 μm for panel a and 40 μm for panels b–d. Asterisks, no nuclei are associated with these structures. ECM, extracellular matrix.

appeared to be clearly larger than the populations labeled by each of the four cross-reacting probes (fig. 6; table 1). (For a quantitative evaluation of position, see below.)

Two of the Three Minor Olfactory Receptor Families Are Expressed in Catshark OSNs

We analyzed the expression of all *or*, *taar*, and *v1r/ora* genes in the catshark olfactory receptor repertoire (Sharma et al. 2019) by in situ hybridization. All probes were generated from olfactory organ RNA, allowing a first glimpse at expression. Indeed, we identified the expression of all *or*, *taar*, and *v1r/ora* genes in the RT-PCR of olfactory organ of catshark (supplementary fig. S2, Supplementary Material online).

In cartilaginous fishes, the OR family is very small and the OSN normally expressing ORs are absent (Holl 1973; Theisen et al. 1986; Takami et al. 1994; Hara et al. 2018; Marra et al. 2019; Sharma et al. 2019). Here, we examined the expression of all catshark *ors* identified by Sharma et al. (2019) with individually specific probes using in situ hybridization. In no case expression could be seen, suggesting that the levels of mRNA for *ors* are sufficient for the more sensitive RT-PCR but below the detection threshold of in situ hybridization.

The V1R/ORa family of cartilaginous fish is small, similar to that of many teleosts (Saraiva and Korsching 2007; Zapilko and Korsching 2016), but see Policarpo et al. (2022). All genes were examined for expression individually using in situ hybridization. Expression was observed only

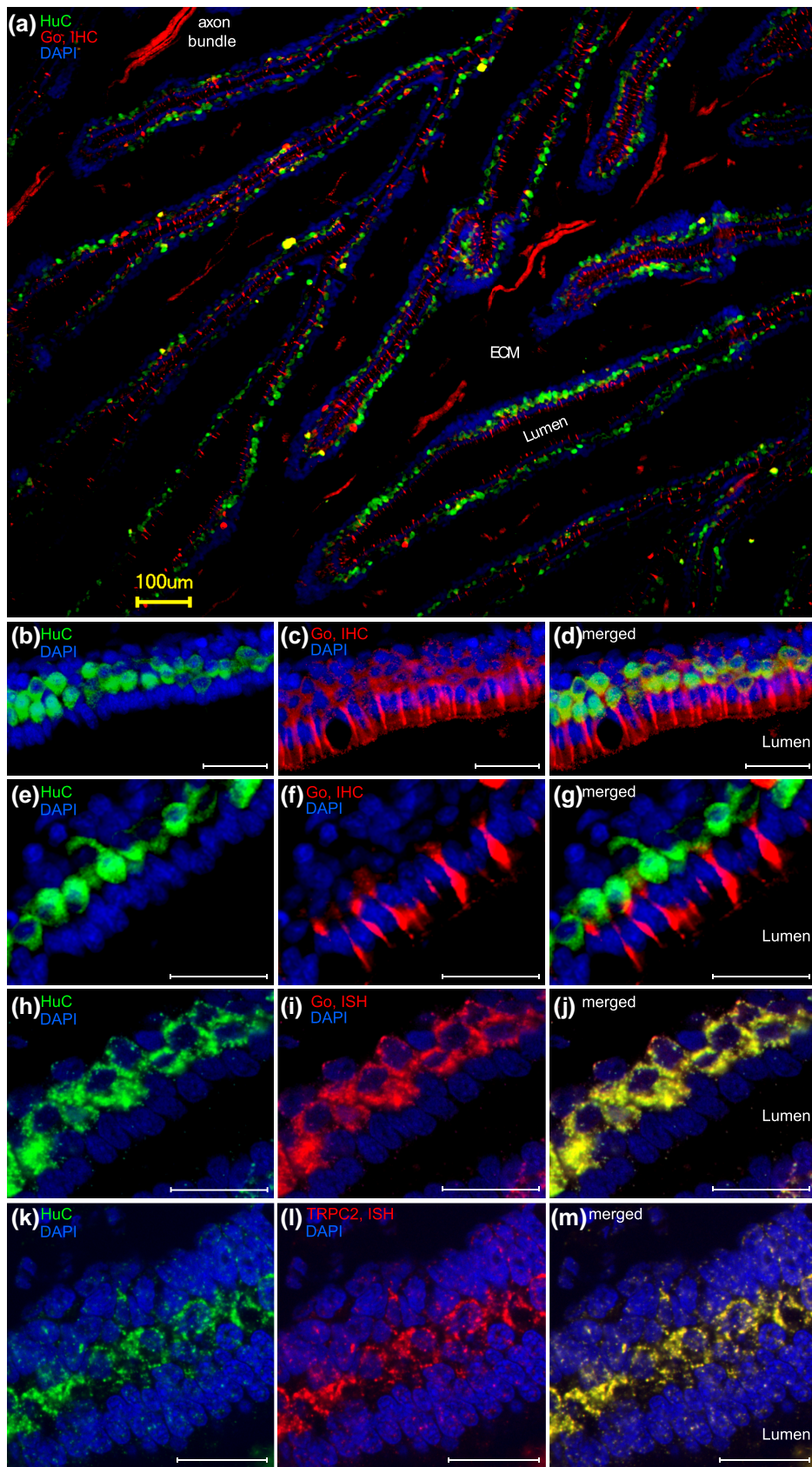


FIG. 4. Go and TRPC2 label the entire OSN population. Cryostat sections of the olfactory organ of the catshark. ECM, extracellular matrix, IHC, immunohistochemistry. (a–g) Double immunofluorescence for Go (red) and HuC (green); nuclei are stained by DAPI (blue). (a, c, and f) Go immunoreactivity is seen in dendrites and axon bundles in the lamina propria of the lamellae, labeled here as ECM (extracellular matrix). Single axons are below threshold. (d) Note that due to different subcellular localizations, the overlap between HuC and Go immunoreactivity, albeit clearly visible, is limited to the base of the dendrites. (h–j) HuC immunofluorescence (green) and in situ hybridization for Go (red). All HuC-immunoreactive cells express Go. (k–m) HuC immunofluorescence (green) and in situ hybridization for TRPC2 (red). All HuC-immunoreactive cells are labeled with the probe for TRPC2. Scale bars, 100 µm for panel a and 40 µm for panels b–m.

for *ora2*. Very sparse cells situated in the neuronal layer (midlayer) of the olfactory lamellae are labeled (supplementary fig. S4a–d, Supplementary Material on-line; table 1).

The TAAR family of catshark consists of three *taar* genes proper and two *taar-like* (*tarl*) genes. In situ hybridization with individually specific probes showed expression of *taar1a* in sparse neurons within the sensory surface of

Table 1. Quantitative Evaluation of Expression for Olfactory Receptor Genes From Three Different Families.

1A						
Olfactory Receptor	Density (# OSN/mm Lamellar Length)	% Neurons in Primary/Secondary Lamellae	# Cross-Reacting Genes			
<i>ora2</i>	0.45 ± 0.05	47.3/52.7	0			
<i>taar1a</i>	2.76 ± 0.24	27.5/72.5	0			
<i>v2r1</i>	5.94 ± 0.81	81/19	0			
<i>v2r6</i>	0.89 ± 0.05	62.9/37.1	6			
<i>v2r14</i>	1.075 ± 0.06	47.3/52.7	1			
<i>v2r19</i>	0.71 ± 0.04	78.9/1.1	3			
<i>v2r29</i>	0.78 ± 0.07	85.8/14.2	1			

1B						
P values	<i>ora2</i>	<i>taar1a</i>	<i>v2r1</i>	<i>v2r6</i>	<i>v2r14</i>	<i>v2r19</i>
<i>ora2</i>		<0.01	<0.001	n.s	<0.001	<0.001
<i>taar1a</i>	<0.001		<0.001	<0.001	<0.001	<0.001
<i>v2r1</i>	<0.001	<0.001		<0.05	n.s	n.s
<i>v2r6</i>	<0.001	<0.001	<0.001		<0.05	<0.01
<i>v2r14</i>	<0.001	<0.001	<0.001	<0.001		n.s
<i>v2r19</i>	<0.01	<0.001	<0.001	<0.05	<0.001	
<i>v2r29</i>	<0.001	<0.001	<0.001	n.s	<0.01	n.s

NOTE.—**1A**, Density of OSN expressing a particular olfactory receptor gene is given as number of labeled OSNs per mm lamellar length. Distribution of labeled OSN between primary and secondary lamellae is given as percentage of total cells. Values are given as mean ± SEM (110 ≤ *n* < 410). **1B**, *P* values are estimated by *t*-test (two-sided, nonpaired) and shown as matrix. Top triangle, *P* values for primary/secondary lamellae distribution; bottom triangle, *P* values for density of OSN comparison.

the olfactory organ (supplementary fig. S4e–h, Supplementary Material online), with clearly higher expression frequency than that observed for *ora2* (table 1). No expression was seen for the other two *taar* genes and the two *tarl* genes. The latter parallels the nonolfactory expression of *tarl* genes in bony fishes (Dieris et al. 2021).

Distinctly Different Spatial Distributions of Neurons Expressing Different Olfactory Receptor Genes

A characteristic feature of olfactory receptor expression in vertebrates is the restriction of expression of individual receptor genes to the so-called expression zones or domains. Here, we wished to investigate whether similar patterns are present in a shark olfactory epithelium. Moreover, we examined whether there are differences between primary and secondary lamellae in terms of receptor expression.

We report that the ratio of expression (primary to secondary lamellae) is significantly different between receptor families (fig. 7b; table 1). *v2r1*-expressing cells are predominantly located on primary lamellae, whereas *ora2/v1r2*-expressing neurons have an equal probability to be present in primary and secondary lamellae. *taar1a*-expressing neurons show a third type of distribution and are strongly enriched on the secondary lamellae (fig. 7). Moreover, within the V2R/OlfC family, individual genes show different distributions, with *v2r6*-expressing cells showing the smallest preference for primary lamellae (fig. 7b; table 1).

In bony vertebrates, the average distance of OSNs from the center of the olfactory epithelium (radial distance) is

characteristically different for different olfactory receptors (Weth et al. 1996; Mombaerts 2006). A recent study showed similar differences in radial distribution in a jawless fish (Kowatschew and Korsching 2022). Here, we have evaluated a similar parameter, the distance of labeled neurons from the base of the lamella, close to the connective capsule, which can also be considered a radial parameter. The distance was normalized to total length of the primary lamella (for details of the measurements, see Materials and Methods). We observed very similar distributions for *v2r1*- and *ora2*-expressing neurons, but a highly significant difference to the distribution of *taar1a*-expressing cells (fig. 7). Over three quarters of the *v2r1*- and *ora2*-expressing neurons are localized in the apical half of the lamellae, whereas the *taar1a*-expressing neurons are rather homogeneously distributed along the baso-apical axis (fig. 7c).

Overall, considering the distribution between primary and secondary lamellae together with the radial distribution (along the lamellar axis), we found several distinctly and significantly different spatial patterns among the genes investigated. Although the differences in basic morphology between cartilaginous, jawless, and ray-finned fishes do not lend themselves to a direct comparison between spatial patterns, we wish to emphasize that the underlying principle of nonrandom spatial organization is shared among all three groups and thus may have emerged in the common ancestor of extant vertebrates.

Discussion

Evolutionary Dynamics of the Olfactory Gene Repertoire in Cartilaginous Fishes

We present a comprehensive analysis of the evolutionary dynamics of gene families coding for putative olfactory receptors in cartilaginous fishes, using 11 genomes of species belonging to the 3 main clades, that is, chimaeras, rays/skates/sawfishes, and sharks. The patterns of family size variation are strikingly different to those observed in bony fishes, the other clade of jawed vertebrates. First of all, the number of olfactory coding genes is on average much smaller and more stable in cartilaginous fishes (fig. 8). In most bony fishes, there are hundreds to thousands of olfactory receptor coding genes, but the genome of cartilaginous fishes codes only for ~10–50 olfactory receptors. Such a low number of olfactory receptors is otherwise only known for species having a highly degenerated olfactory system, for example, toothed whales (Kishida et al. 2015) and ocean sunfishes (Policarpo et al. 2021, 2022). However, in contrast to these species, cartilaginous fishes have well-developed olfactory organs with a large sensory surface. The maintenance of a small repertoire of olfactory receptors may be necessary to support a high sensitivity for a small number of molecules. Indeed, it is likely that for a given number of olfactory neurons, there is a trade-off, that is, the higher the size of the olfactory repertoire, the lower the sensitivity for different molecules.

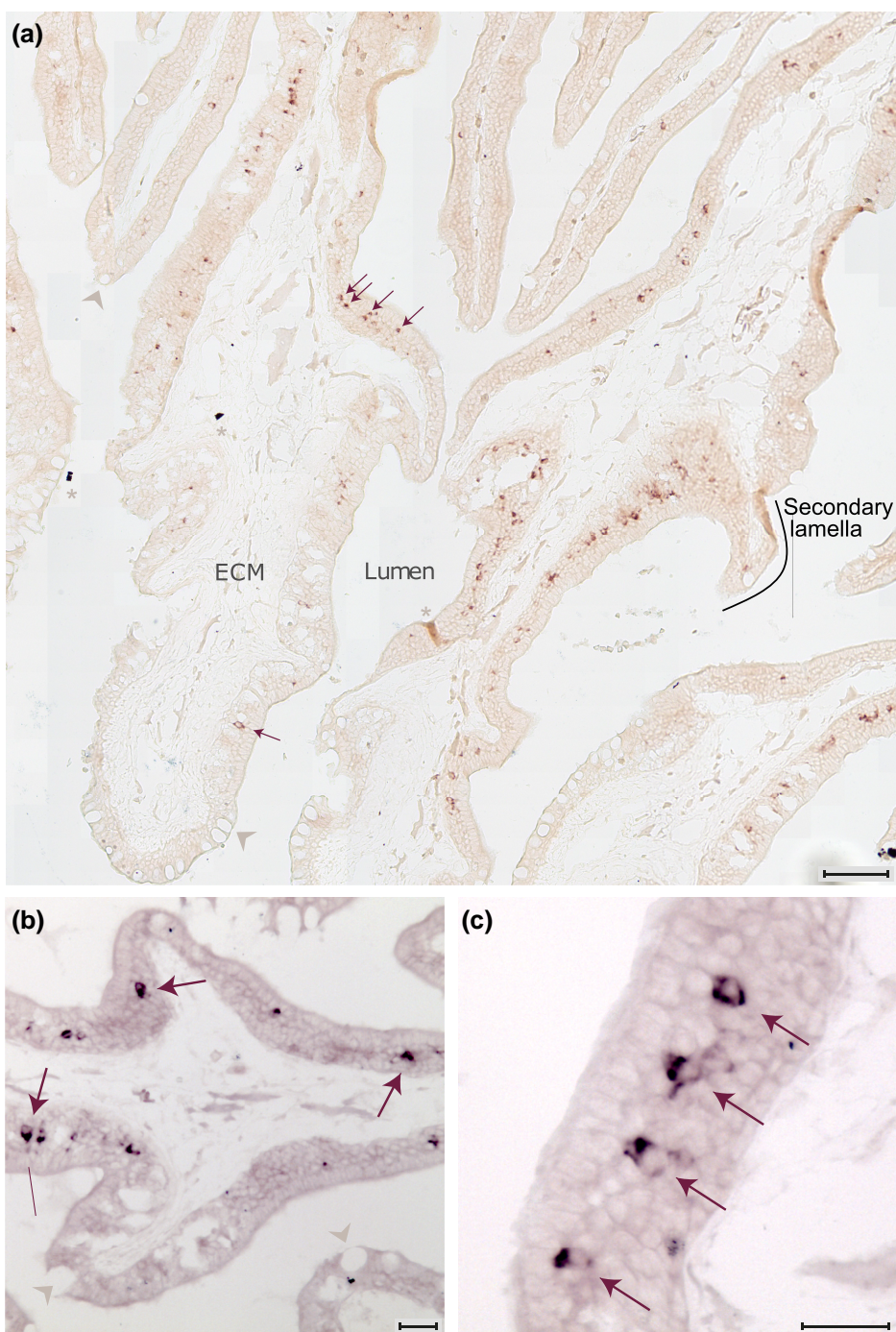


Fig. 5. The basal gene of the main *v2r* clade, *v2r1*, is expressed at moderate frequency in the OE. Horizontal cryostat sections of the olfactory organ of the catshark were hybridized with a probe for *v2r1*. (a) *v2r1*-expressing OSNs are localized in the middle layer of the sensory epithelium, along both the primary and the secondary lamellae. A secondary lamella is indicated (black line). ECM, extracellular matrix; asterisks, artifacts; gray arrowheads, mucous cells. Scale bar, 100 μm . (b and c) Higher magnifications from different sections; some labeled neurons are pointed out by arrows. Scale bar, 40 μm .

Secondly, the relative importance of the four olfactory receptor gene families is drastically different between cartilaginous and bony fishes. The VR2/OlfC family is by far the largest family in all cartilaginous fish examined, whereas in bony fish, the OR family is far larger than the V2R/OlfC family (fig. 8). The ligands of cartilaginous fish V2R/OlfC receptors are unknown, but teleost V2R/OlfCs are activated by amino acids, which serve as food odors. Thus, a large sensory surface together with a relatively small repertoire of V2R/OlfC receptors may enable extremely sensitive localization of prey.

Olfactory Gene Expression Patterns in Catshark

Few vertebrate olfactory receptor repertoires have been analyzed by comprehensive in situ hybridization. However, the available information points to the expression of many if not most of olfactory receptor genes in mammalian and teleost fishes OSNs (Young et al. 2003; Alioto and Ngai 2006; Churcher et al. 2015; Yoon et al. 2015). This is in contrast to the situation we report here for the three minor shark receptor families. We could detect expression in OSNs only for one member per family for TAARs and V1R/ORAs and for none of the 2 *tarl* genes

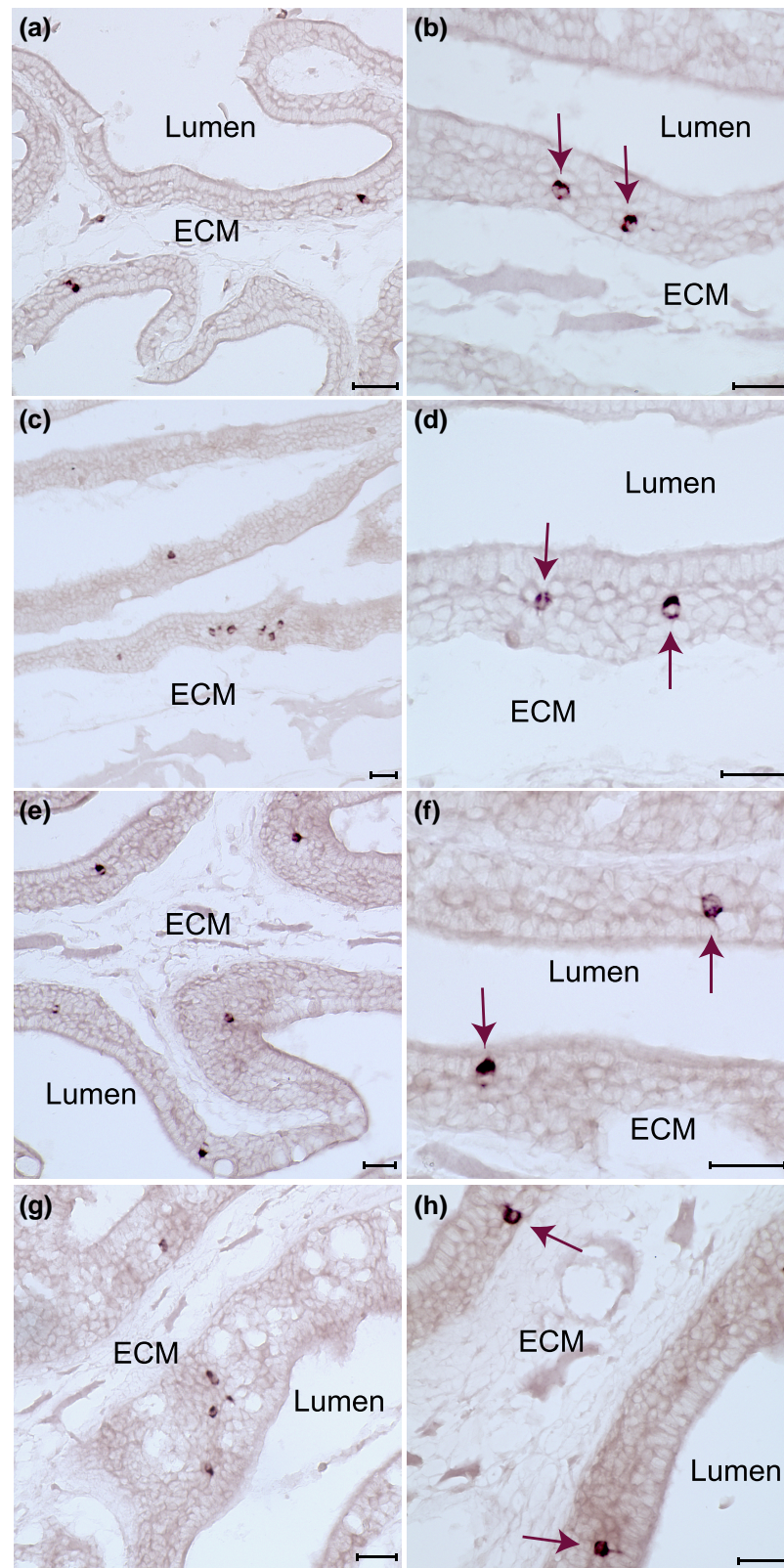


FIG. 6. Sparse expression of *v2r* genes belonging to the main clade. Horizontal cryostat sections of catshark olfactory epithelium were hybridized with probes for *v2r29* (a and b), *v2r19* (c and d), *v2r6* (e and f), and *v2r14* (g and h). Right column, higher magnifications; all scale bars represent 40 μm . All probes show expression in small subsets of scattered OSNs, which are situated on primary and secondary lamellae. ECM, extracellular matrix. Some labeled neurons are indicated by arrows.

and 8 or genes examined (fig. 8). Although we cannot rule out technical reasons or developmental differences (all tissues analyzed stem from a similar stage, nearly adult juveniles), the rarity of expression for the TAAR and OR families could be related to the absence of ciliated OSNs

in the shark olfactory organ (Theisen et al. 1986). Ciliated OSNs are the neuronal subpopulation which expresses ORs and TAARs in bony vertebrates (Hansen et al. 2004; Mombaerts 2004). Both families are very small and stable in all cartilaginous fishes examined, which

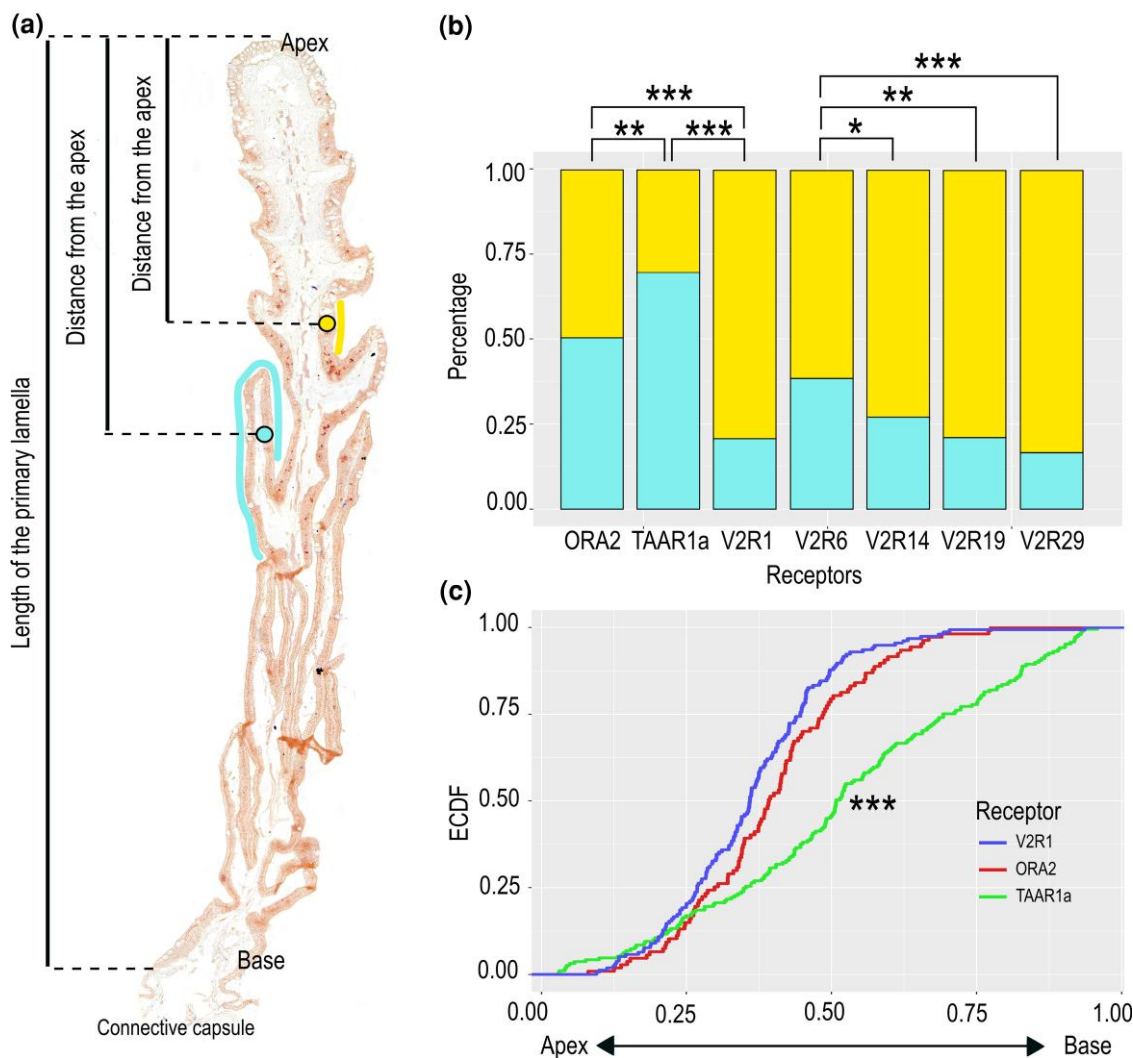


Fig. 7. *Taar*, *ora*, and *v2r* genes show distinctly different, if overlapping spatial patterns of expression. (a) Micrograph of a primary lamella with secondary lamellae from the olfactory organ of a catshark. To analyze the distribution of neurons expressing particular genes, the distance from the apex was measured and normalized to the total lamellar length from the apex to base, both for neurons situated on primary and secondary lamellae (yellow and cyan circles, respectively). (b) Percentage of neurons located on primary or secondary lamellae is shown as bar graph for seven genes from three different olfactory receptor families as indicated. Asterisks denote significance; see table 1 for numerical values. (c) For three genes, the position of labeled neurons along the lamellar length (cf. panel a) is shown as ECDF; blue, *v2r1*; red, *ora2*; green, *taar1a*. Significance of observed differences in distributions was estimated by the Kolmogorov–Smirnov test (Marsaglia et al. 2003). *** $P < 0.001$ for *taar1a* versus each of the other two genes. Distributions for *v2r1* and *ora2* are not significantly different from each other (see table 1 for exact values).

would be consistent with a nonolfactory function in this taxon.

Expression profiling for several organs of two shark species showed broad expression for three ORs, with high expression levels observed only in nonolfactory organs (Hara et al. 2018). A comparison with lamprey suggests the absence of OR expression in OSNs to be a derived feature, since lampreys exhibit a moderately sized OR family (Libants et al. 2009), show olfactory expression of ORs (Freitag et al. 1999), and do possess ciliated OSN expressing Golf, which is the G-protein alpha subunit typically coupled to the OR and TAAR family (Frontini et al. 2003; Laframboise et al. 2007; Spehr and Munger 2009).

The TAAR family is absent in lamprey, which only possesses *taar-like* (*tarl*) genes (Grus and Zhang 2009; Hussain et al. 2009; Dieris et al. 2021). The absence of expression in

OSNs for the two catshark *tarl* genes parallels the absence of OSN expression in teleost fish *tarl* and is in stark contrast to the expression of *tarl* genes in lamprey OSNs (Berghard and Dyer 1998; Dieris et al. 2021). This is consistent with the hypothesis that an olfactory function for *tarl* genes has been acquired independently in the jawless lineage but not in cartilaginous or bony fishes (Dieris et al. 2021). Interestingly, the *taar* gene expressed in the catshark olfactory epithelium, *taar1a*, is the ortholog of a highly conserved *taar1* gene of bony vertebrates, which is nonolfactory in both tetrapods and teleosts (Liberles and Buck 2006; Hussain et al. 2009). Thus, catshark *taar1a* may have acquired olfactory function independently, possibly in microvillous receptor neurons, in contrast to *taar2-n* of bony fishes, which are expressed in ciliated neurons (Mombaerts 2004). We also found expression of only

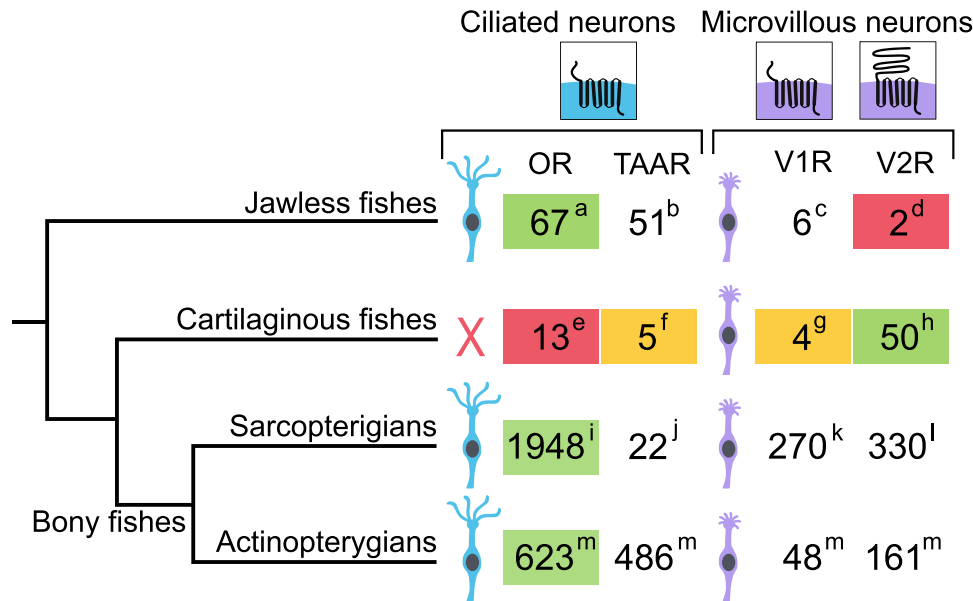


Fig. 8. Schematic representation of evolution of vertebrate olfactory chemosensory receptor repertoires and their expression. Major phylogenetic clades are shown as indicated. The maximal repertoire size for olfactory receptors observed in a species of the respective clade is shown. The dominant repertoires are marked by green overlay; all are majorly expressed in OSNs. Olfactory receptor families not expressed in the olfactory organ (i.e., with nonolfactory function) are marked by red overlay; families with minor expression in OSNs are marked by yellow overlay. Gene numbers are taken from these studies: (a) *Petromyzon marinus* (Niimura 2009); (b) *Lethenteron camtschaticum* (Dieris et al. 2021) (number refers to TARR, the sister group of TAAR, which are absent in lamprey); (c and d) *L. camtschaticum* (Kowatschew and Korsching 2022); (e and h) *C. milii* (this study); (f and g) *S. canicula* (this study); (i) *Loxodonta africana* (Niimura et al. 2014); (j) *Monodelphis domestica* (Grus et al. 2007); (k) *Ornithorhynchus anatinus* (Grus et al. 2007); (l) *Xenopus tropicalis* (Ji et al. 2009); and (m) *Erpetoichthys calabaricus* (Policarpo et al. 2022).

one *v1r/ora* gene in catshark OE, *ora2*. This is again different from the situation in zebrafish, where all *ora* genes show olfactory expression (Saraiva and Korsching 2007; Kowatschew et al. 2022). The cell type of the *ora2*-expressing neurons is unknown, but they could be microvillous neurons, which do express the related family of *v1r* genes in mammals (Mombaerts 2004).

In contrast to the three minor olfactory receptor families OR, V1R/OR, and TAAR/TARL, each V2R/OlfC probe examined resulted in robust expression. Considering the cross-reactivity of several probes, we showed olfactory expression of up to 16 different V2R/OlfCs, a sizable proportion of the entire family. These results extend the olfactory function of the V2R family to the common ancestor of cartilaginous and bony fishes—the family is present with 1–2 genes in lamprey, but these are not expressed in the olfactory epithelium (fig. 8) (Kowatschew and Korsching 2022). Notably, the *v2r1* probe showed a much higher density of labeled cells compared with the four probes cross-reacting with small subsets of genes. The *v2r1* gene is the ortholog of zebrafish *olfCc1* and mouse *vmn2r1-7*, which both have been shown to be coexpressed with many different individual *v2r/olfc* genes (Alioto and Ngai 2006; Silvotti et al. 2007; Ishii and Mombaerts 2011; DeMaria et al. 2013) and accordingly show a much higher density of expression compared with the individual genes. This suggests that the catshark *v2r1* could also serve as coreceptor (cf. Ishii and Mombaerts 2011). The presence of a coreceptor is a characteristic property of the V2R/OlfC family, only paralleled

by insect OR receptors (evolutionarily unrelated to any vertebrate olfactory receptor; Yan et al. 2020) and possibly by a highly conserved V1R/OR, ancV1R (Suzuki et al. 2018).

Since the first discovery of olfactory receptor genes three decades ago, hundreds of expression studies have shown a common theme: Individual receptor genes are expressed in sparsely distributed OSNs. Whenever these distributions have been examined more closely, they were observed to be different for different receptor genes, albeit often broadly overlapping. This “half-random” feature has been described for mouse, rat, frog, and zebrafish (Weth et al. 1996; Miyamichi et al. 2005; Syed et al. 2013; Zapiec and Mombaerts 2020) and recently also for lamprey (Kowatschew and Korsching 2022).

Here, we endeavored to examine whether this characteristic property of bony fishes and lamprey would also be present in cartilaginous fish. We do report that the basic principle of distinctly different spatial distributions for different olfactory receptor genes is present in the cartilaginous fish. This extends previous estimates derived from the comparison of tetrapods, teleost fishes, and lamprey (Strotmann et al. 1996; Horowitz et al. 2014; Ahuja et al. 2018; Kowatschew and Korsching 2022).

It is unclear whether such differences in spatial expression patterns might have functional meaning. The presence of secondary lamellae in the catshark olfactory organ might serve just to increase the surface area of the sensory surface (Ferrando et al. 2019), but differences in

access of odorants to primary versus secondary lamella areas cannot be excluded since existing studies of hydrodynamic properties focus solely on primary lamellae (see, e.g., Cox 2008). Alternatively, differences in radial and primary/secondary lamella distribution could result as consequence of the developmental mechanisms guiding the olfactory receptor expression (cf. Bayramli et al. 2017).

Taken together, we have shown for the first time the cellular expression of olfactory receptors in a cartilaginous fish. The expression is dominated by *v2r/olfC* genes, with minor contributions from a *v1r/ora* and a *taar* gene. The spatial expression patterns of different receptor genes are characteristically different, both for a topological parameter shared with bony fishes (radial parameter) and for a peculiar property of all cartilaginous fishes (secondary lamellae). Thus, the principle of nonrandom spatial organization is shared between jawless, cartilaginous, and bony vertebrates, suggesting similarly broad presence of the underlying molecular mechanisms.

Conclusion

Comparative studies showed that olfactory receptors belonging to the OR, TAAR, and V1R/ORa families were coopted early during the evolution of vertebrates and were present in the last common ancestor of extant vertebrates. Although V2R/OlfC receptors were also present, they were coopted as olfactory receptors later, after the separation of jawless fishes and jawed vertebrates. In bony fishes, V2R/OlfCs are less abundant than ORs and TAARs, but in cartilaginous fishes, V2R/OlfCs constitute the essential component of the entire olfactory receptor repertoire (fig. 8). Why such a difference? Conceivably, this could be an indirect effect of the loss of the ciliated subtype of OSNs which are expected to express ORs and TAARs. Whether the dearth of ORs and TAARs amounts to a restriction in the odor space accessible to cartilaginous fish remains to be seen. The correlation between the number of OR, TAAR, and V2R/OlfC receptors in ray-finned fishes might suggest an initial functional overlap between these receptor families which in tetrapods have divergently evolved to detect volatile substances in the main olfactory epithelium and nonvolatile substances in the vomeronasal organ. Overall, cartilaginous and jawless fish have a small olfactory receptor gene repertoire compared to bony fishes (fig. 8), despite having very well-developed olfactory organs. These divergent evolutionary trajectories could result from different trade-offs between sensitivity and odor discrimination, with cartilaginous fish maximizing sensitivity of odor detection.

Within cartilaginous fishes, there are notable differences in the number of olfactory receptors, which remain to be understood. For example, the thorny skate *Amblyraja radiata* may have less than 10 OlfC receptors, whereas the chimaera *C. milii* has more than 40 OlfC receptors, although they consume relatively similar diet (Froese and Pauly 2022). Further behavioral and functional studies will be necessary to better understand this issue.

Materials and Methods

Genome Data Set and Species Phylogeny

Seventeen chondrichthyan genome assemblies, corresponding to 13 species, were downloaded from NCBI: *A. radiata* (GCF_010909765.2), *C. milii* (GCF_018977255.1; GCA_000165045.2), *C. carcharias* (GCF_017639515.1; GCA_003604245.1), *Chiloscyllium plagiosum* (GCF_004010195.1), *C. punctatum* (GCA_003427335.1), *Hemiscyllium ocellatum* (GCA_020745735.1), *Hydrolagus affinis* (GCA_012026655.1), *Leucoraja erinacea* (GCA_000238235.1), *Pristis pectinata* (GCA_009764475.2), *R. typus* (GCA_001642345.3; GCA_013626285.1; GCA_013626285.1), *S. canicula* (GCA_902713615.2), *S. torazame* (GCA_003427355.1), and *Stegostoma fasciatum* (GCA_022316705.1).

The completeness of these genomes was assessed with BUSCO v5.1.2 using the vertebrata_odb10 database (Manni et al. 2021). For species with multiple genome assemblies, we retained only the one for which BUSCO retrieved the highest number of complete genes (supplementary table S1, Supplementary Material online). *Leucoraja erinacea* was removed from the phylogenetic analysis described below, as only 7% of BUSCO genes could be retrieved complete from its genome assembly (supplementary fig. S6, Supplementary Material online). We then extracted protein sequences of 1,068 BUSCO genes that were retrieved in common in single copy in the best assemblies for each species and align these sequences individually using MAFFT (auto, v7.407) (Katoh and Standley 2013). These alignments were trimmed using trimAl v1.4.1 (with the option -automated1) (Capella-Gutiérrez et al. 2009) and concatenated using AMAS (Borowiec 2016). A maximum likelihood phylogeny was then computed with IQ-TREE v2.2.0 (Minh et al. 2020), and the best model for each partition was assessed with ModelFinder (option -m MFP + MERGE) (Kalyaanamoorthy et al. 2017). The least square dating method implemented in IQ-TREE was used to build a time-calibrated phylogeny from the inferred tree topology (with the options --date-tip 0 --date-ci 100). Five calibration dates retrieved on TimeTree.org were used (supplementary fig. S5, Supplementary Material online) (Kumar et al. 2022).

Olfactory Receptor Gene Mining

or, *taar*, *v1r/ora*, and *v2r/olfC* genes were mined in the most complete genome assemblies of each species, except for *L. erinacea* and *H. affinis* for which only 7% and 54% of BUSCO genes could be retrieved complete, respectively. Thus, an accurate estimation of the number of olfactory genes was not possible in these two species. The naming for *S. canicula* olfactory receptor genes was based on Sharma et al. (2019) (for changes/additions, see supplementary table S2, Supplementary Material online).

Gene mining was performed following methods described by Policarpo et al. (2022). Briefly, TBLASTN searches (e -value $< 1e-10$) were performed against genome assemblies using known olfactory receptors belonging

to the four families in other vertebrate species as queries. Nonoverlapping hit regions were extracted and extended using SAMTools, and genes were predicted on those regions using EXONERATE (options: --model protein2genome --minintron 50 --maxintron 20,000). Genes used for TBLASTN searches and as queries in Exonerate can be found in [supplementary materials S3–6, Supplementary Material](#) online. We verified that predicted genes were true olfactory receptors with a BlastX against a custom database of olfactory, taste, and other G-protein-coupled receptors and with phylogenetic trees, retaining only sequences that clustered with known olfactory receptors. Retrieved sequences were then classified into four mutually exclusive categories: 1) “complete” if a complete coding sequence was retrieved; 2) “pseudogene” if the coding sequence was incomplete due to at least one loss-of-function mutation (a premature stop codon or a frameshift); 3) “truncated” if the gene was found incomplete but without loss-of-function mutation and located well inside a contig or scaffold; and 4) “edge” if the gene was found incomplete and near a contig or scaffold border, that is, most likely abridged by an assembly artifact. For nucleotide sequences for all validated olfactory receptor genes, see [supplementary file S2, Supplementary Material](#) online.

Protein sequences of complete genes obtained in the previous step, as well as outgroup protein sequences (see [supplementary file S2, Supplementary Material](#) online), were aligned using MAFFT v7.467, and maximum likelihood phylogenies were computed with IQ-TREE 2.0 with the best model found by ModelFinder. Branch supports were obtained with 1,000 ultrafast bootstraps.

Patterns of Gene Birth and Death

A gene tree–species tree reconciliation method was used to infer the number of gene duplications and gene losses in every branch of the species tree. We first collapsed nodes with low bootstrap values (<90%) in gene phylogenies for the four olfactory receptor families using the R package ape v5.0 ([Paradis and Schliep 2019](#)). Treerecs was then used to find the best root and reconcile gene trees with the species tree, with default parameters ([Comte et al 2020](#)).

Tissue Preparation

Paraformaldehyde-fixed whole olfactory organs from two nearly adult juvenile catsharks were kindly provided by Sylvie Mazan and Ronan Lagadec. Organs were stored in methanol at -80°C . Before using, tissues were rehydrated in decreasing concentrations (75%, 50%, and 25%) of methanol and rinsed thrice in phosphate-buffered saline (PBS), followed by equilibration in 15% saccharose in PBS at 4°C until they sank. They were then equilibrated in 30% saccharose in PBS and embedded in Tissue Tek.

mRNA Isolation, cDNA Synthesis, and RT-PCR

Total mRNA was extracted using the easy-spin Total RNA Extraction Kit (iNtRON Biotechnology) resulting in highly

concentrated mRNA ($\text{OD}_{260} > 0.9$). Complementary DNA (cDNA) was synthesized from olfactory organ using SuperScript II Reverse Transcriptase (Invitrogen, No. 18064022). cDNA concentration was determined with a NanoDrop photometer and samples stored at -20°C . Forward (Fwd) and reverse (Rev) primers for each gene were chosen to result in fragment lengths between 350 and 560 bp ([supplementary table S3, Supplementary Material](#) online). For RT-PCR, annealing temperatures between 55°C and 58°C were used. PCR products were validated by gel electrophoresis and sequencing. RNA probes for in situ hybridization were generated from PCR product by adding T3 promoter sequence (ATTAACCCTCACTAAAGG) 5' to the Rev primer ([supplementary table S3, Supplementary Material](#) online).

In Situ Hybridization, Stand-alone and Combined with Immunohistochemistry

Transverse cryostat sections of $10\ \mu\text{m}$ were obtained (Leica CM1900) and dried, according to the cutting plane shown in [figure 1a](#). In situ hybridization was done as described ([Ahuja et al. 2018](#)). In short, hybridization was performed overnight at 60°C in 50% formamide, 5× Denhardt's reagent, 2× standard saline citrate (SSC; 20× SSC is 3 M NaCl and 0.3 M sodium citrate, pH 7.0), 0.4 mg of proteinase K-treated torula yeast RNA per ml (Type VI, Sigma), and 0.1 mg of tRNA from bakers' yeast per ml. Washes were as follows: 30 min with 50% formamide/2× SSC at 60°C , 1 h with 0.2× SSC at 60°C , and 15 min with 0.2× SSC at room temperature. These conditions amount to moderately stringent hybridization conditions, allowing for 12–24% mismatch relative to the probes used. This is stringent enough to ensure probe specificity for the respective gene for all OR, TAAR, and V1R/ORa probes but allows considerable cross-reactivity within the more closely related V2R/OlfCs ([table 1](#)). Immunofluorescence was done as described ([Ahuja et al. 2014](#)). Primary antibodies used in the immunofluorescence are mouse anti-PCNA (1:200, Merck) and mouse anti-HuC (1:200, Invitrogen) anti-Go (K-20) antibody (rabbit IgG; 1:50; sc-387, Santa Cruz Biotechnology). Secondary antibodies used were goat anti-rabbit IgG conjugated to Alexa Fluor 488 (A21206, Invitrogen) or Alexa Fluor 594 (A11012, Invitrogen). 4',6-Diamidino-2-phenylindole (DAPI) was used as counterstain for fluorescent detection. Micrographs were taken using a Keyence BZ-9000 fluorescence microscope, and the absence of crosstalk between channels was confirmed. Double labeling was done by combining ISH and IHC as described ([von Twickel et al. 2019](#)).

Quantification and Statistical Evaluation

Image analysis—length and distance measurements and cell counts—was performed using ImageJ ([Schneider et al. 2012](#)). The normalized position of labeled cells along the length of a lamella was determined by dividing the distance from the apex (toward the center of the olfactory rosette) by the total length of the lamella (from the base, which is attached to the peripheral connective

capsule to the apex). For neurons situated in secondary lamellae, a projection onto the corresponding primary lamella was used.

To determine the density, labeled neurons were counted in up to nearly 400 mm of lamellar length to achieve counts of at least 100 cells per gene. For the most frequently expressed gene, *v2r1*, nearly 100 mm of lamellar length was evaluated, which contained over 400 labeled neurons.

Radial distributions are shown as empirical cumulative distribution function (ECDF) (Feller 1966; Wilk and Gnanadesikan 1968). To estimate whether two distributions were significantly different, we performed the Kolmogorov–Smirnov tests as implemented in R version 4.1.0 (Marsaglia et al. 2003).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

S.K., A.S., and K.S. conceived the study; A.S. and K.S. designed the experiments; A.S., K.S., S.F., and M.P. performed the experiments and conducted the analysis. S.K., K.S., S.F., M.P., and D.C. prepared the figures and wrote the manuscript. All authors read and approved the manuscript.

Data Availability

Concatenated gene alignment of the 1,068 BUSCO genes as well as the calibrations points used and the species tree in nexus format can be found in Figshare: <https://doi.org/10.6084/m9.figshare.21507615.v1>.

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