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Localized expression of the olfactory receptor genes in the olfactory organ of common minke whales

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Abstract

Baleen whales (Mysticeti) possess the necessary anatomical structures and genetic elements for olfaction. Nevertheless, the olfactory receptor gene (OR) repertoire has undergone substantial degeneration in the cetacean lineage following the divergence of Artiodactyla and Cetacea. The functionality of the highly degenerated mysticete ORs within their olfactory epithelium remains unknown. In this study, we extracted total RNA from the nasal mucosa of common minke whales (*Balaenoptera acutorostrata*) to investigate the localized expression of ORs. All three sections of the mucosae examined in the nasal chamber displayed comparable histological structure, whereas the posterior portion of the frontoturbinal region exhibit notably high expression of ORs and another gene specific to the olfactory mucosa. Neither the olfactory bulb nor the external skin exhibited expression of these genes. Although this species possesses four intact class-1 ORs, all the ORs expressed in the nasal mucosa belong to class-2, implying the loss of aversion to specific odorants. These anatomical and genomic analyses suggest that ORs are still responsible for olfaction within the nasal region of baleen whales, enabling them to detect desirable scents such as prey and potential mating partners.

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KEYWORDS

Mysticeti / olfaction / RNA-seq / histology / epithelium / secondary-aquatic

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ABSTRACT

Baleen whales (Mysticeti) possess the necessary anatomical structures and genetic elements for olfaction. Nevertheless, the olfactory receptor gene (OR) repertoire has undergone substantial degeneration in the cetacean lineage following the divergence of Artiodactyla and Cetacea. The functionality of the highly degenerated mysticete ORs within their olfactory epithelium remains unknown. In this study, we extracted total RNA from the nasal mucosa of common minke whales (*Balaenoptera acutorostrata*) to investigate the localized expression of ORs. All three sections of the mucosae examined in the nasal chamber displayed comparable histological structure, whereas the posterior portion of the frontoturbinal region exhibit notably high expression of ORs and another gene specific to the olfactory mucosa. Neither the olfactory bulb nor the external skin exhibited expression of these genes. Although this species possesses four intact class-1 ORs, all the ORs expressed in the nasal mucosa belong to class-2, implying the loss of aversion to specific odorants. These anatomical and genomic analyses suggest that ORs are still responsible for olfaction within the nasal region of baleen whales, enabling them to detect desirable scents such as prey and potential mating partners.

1. INTRODUCTION

Olfaction, the sense of smell, represents one of the sensory modalities encompassing biologically important behaviors such as foraging, predator avoidance, mother-calf relationships, mating and territorial display (Doty, 1986; Nei et al., 2008; Corona and Lévy, 2015). The sense of smell arises when olfactory receptor proteins within the nasal cavity capture volatile chemical substances. These proteins are localized on the membranes of olfactory cells. Stimulation is subsequently transmitted from the receptor protein through the cribriform plate into the main olfactory bulb by olfactory nerves, extending from the base of olfactory cells. Olfactory receptor genes (ORs) are responsible for encoding these olfactory receptor proteins (Buck and Axel, 1991). ORs comprise the largest gene family in mammals and are broadly classified into two categories, class-1 and class-2, based on their nucleotide sequences (Niimura and Nei, 2005; Niimura, 2009b). Each individual OR encodes a specific olfactory receptor protein that interacts with particular ligands, thereby enabling the discrimination of different odors (Malnic et al., 1999; Saito et al., 2009).

The observed variation in mammalian olfaction is recognized as a result of anatomical and genomic factors, as expounded upon by the aforementioned mechanism. Anatomical features, such as the dimensions of the cribriform plate (Pihlström et al., 2005), generally align with olfactory abilities, exhibiting interspecies variations. Furthermore, an augmented number of OR copies within a species denotes an elevated discriminatory capacity and enhanced olfactory significance (Niimura, 2009a; Zhou et al., 2021). The comparatively diminished size of the main olfactory organ in primates, incontrast to other mammals, indicates a reduced olfactory capacity within the human species (*Homo sapience*) (Moran et al., 1982; Bird et al., 2018). Consistently, humans possess approximately 400 copies of ORs (Niimura and Nei, 2003), which is significantly fewer than the approximate 1000 copies found in mice (*Mus musculus*) or rats (*Rattus norvegicus*) (Niimura and Nei, 2007). Both morphological investigations and genomic studies provide evidence supporting the diminished importance of olfaction in humans.

The main olfactory organ in mammals is positioned within the respiratory passage, enabling the detection of odors with each inhalation. At this juncture, a query arises: can this sensory system suffice for fully aquatic mammals? Cetaceans, having transitioned aquatic environment over 50 million years ago (Roe et al., 1998; Clementz et al., 2006; Gatesy et al., 2013), encompass two distinct monophyletic lineages known as baleen whales (Mysticeti) and toothed whales (Odontoceti) (Nikaido et al., 2001). They breathe air sorely when they ascend to the water's surface. While the frequency of breaths may vary based on activity levels, consistent breathing patterns have been observed across numerous species. For instance, small toothed whales generally take breaths every 1-2 minutes, killer whales (*Orcinus rca*) breathe no more frequently than every 8 minutes, and deep-diving species such as sperm whales (*Physeter macrocephalus*) and beaked whales (Ziphiidae) can remain submerged for approximately 1 hour (Miller and Roos, 2018). In the case of baleen whales, blue whales (*Balaenoptera musculus*) typically exhibit breathing intervals of approximately 4 minutes (Miller

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and Roos, 2018). Humpback whales (*Megaptera novaeangliae*) during the breeding season demonstrate an average interdive breathing interval of 6 minutes and 45 seconds, and in some instances, particularly among singers, this interval can extend to approximately 13 minutes (Chu, 1988; Hedley et al., 2011). Consequently, cetaceans experience periods of interrupted respiration during dives, leading to intermittent reception of sensory information through the olfactory modality.

Recently, the olfactory capabilities in baleen whales have been investigated through morphological and genomic studies. The skeletal components of the main olfactory organ, such as cribriform plate and turbinals, have been observed in common minke whales (*Blaenoptera acutorostrata*) (Godfrey et al., 2013; Ichishima, 2016). In this species, the nasal mucosa covering the cribriform plate demonstrates similarities to the olfactory mucosa found in terrestrial mammals, as it is lined with pseudostratified columnar epithelium and glandular organs like Bowman's gland within the lamina propria (Hirose et al., 2018). Gross and microscopic examinations have provided evidence for the presence of the main olfactory organ in bowhead whales (*Balaena mysticetus*) (Thewissen et al., 2011; Kishida et al., 2015b), and subsequent immunohistochemical staining has identified olfactory nerves in the nasal mucosa of this species (Farnkopf et al., 2022). Han et al. (2022) conducted a search for ORs in seven baleen whale species using publicly available whole genomes, annotating between 54 and 95 intact copies of ORs. The number of ORs identified in baleen whales is lower compared to other mammals, which corresponding to the reduced anatomical complexity of their main olfactory organ.

Both morphological and genomic investigations postulate the hyposmia of cetaceans, and this diminished olfactory capability is discernible subsequent to the divergence of Artiodactyla and Cetacea (Kishida, 2021). However, baleen and toothed whales exhibits this reduction in distinct manners. While the aforementioned genomic studies suggest a relatively less efficient sense of smell in baleen whales compared to terrestrial mammals, they possess a larger repertoire of ORs compared to toothed whales (Kishida et al., 2015a; Kishida, 2021; Han et al., 2022; Christmas et al., 2023). Analysis of the olfactory marker protein gene (OMP), which exhibits high expression in the olfactory epithelium and plays a crucial role in olfaction (Danciger et al., 1989; Buiakova et al., 1996), indicates that the sense of smell in baleen whales is subjected to purifying selection pressures, whereas toothed whales experience more relaxed selective pressures (Kishida and Thewissen, 2012; Springer and Gatesy, 2017). Furthermore, baleen whales exhibit anatomical features essential for olfaction that are reminiscent of those found in terrestrial mammals. In contrast, the nasal cavity morphology of extant toothed whales has undergone significant modifications for biosonar signal generation, and it is widely accepted that olfactory structures are absent in this lineage (Cranford et al., 1996; Berta et al., 2014; Hirose et al., 2022). Airborne odorants have been proposed to serve as a locating cue for krill, attracting baleen whales through olfactory modality rather than toothed whales (Thewissen et al., 2011; Kishida, 2021). Behavioral experiments targeting humpback whales, long-finned pilot whales (*Globicephala melas*) and bottlenose dolphins (*Tursiops truncatus*) have provided support for this hypothesis (Bouchard et al., 2019; Bouchard et al., 2022). The sense of olfaction provides a captivating illustration of how cetaceans interact with their aquatic environment, primarily due to the accelerated evolutionary rate observed in ORs in placental mammals (Christmas et al., 2023). The remarkable diversification of ORs highlights their significant role in shaping species diversity through the influence of olfactory perception.

While exploring ORs provides a powerful methodology for evaluating olfactory capabilities, it has certain limitations in comprehending the sense of smell (Go and Niimura, 2008). It is important that not all ORs are exclusively expressed within the olfactory mucosa, which is intricately linked to olfactory reception (Kishida et al., 2019). The existence of ectopic ORs, which are expressed in various non-olfactory tissues, have been documented (Chen et al., 2018). Notably, specific ORs in humans and mice exhibit expression in the testis and are involved in sperm chemotaxis (Parmentier et al., 1992; Spehr et al., 2003; Fukuda et al., 2004; Rouquier and Giorgi, 2007). Furthermore, a gene known as OR51E2, classified as a class-1 OR, is present in nearly all mammalian species including both baleen and toothed whales (Han et al., 2022) and has been identified in the prostate (Neuhaus et al., 2009). Hence, the mere presence of ORs does not unequivocally signify the capability of odor detection.

Prior investigations have established that baleen whales possess the essential anatomical structures and

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genetic elements for olfaction; however, these findings alone do not guarantee the existence of a sense of smell based on the same mechanism as observed in other mammals. Therefore, the objective of the present study is to determine whether intact ORs are exclusively expressed in the mucosa of the putative main olfactory organ in baleen whales or not. To address this objective, we utilized the common minke whale, Balaenopteridae, as our research subjects and extracted total RNA from nasal mucosa to examine the localized expression of ORs.

2. MATERIALS

The present study examined common minke whales obtained from the coastal regions of Japan. The specimens were acquired from seven animals (Table 1). 16NPCK-M009 and 16NPCK-M012 were procured in 2016 during the second phase of the Japanese Whale Research Program under the special permit in the Western North Pacific (JARPNII). 18NPCK-M001, 18NPCK-M006 and 18NPCK-M008 were obtained in 2018 during the New Scientific Whale Research Program in the western North Pacific (NEWREP-NP). 19SK214 and 19SK215 were acquired in 2019 during Japanese commercial whaling operations. The research programs adhered to the regulations outlined in Article VIII of the International Convention for the Regulation of Whaling (ICRW). All samples were collected in accordance with legal procedures.

Table 1 List of specimens.

Location	year	ID	Sex	Body length (m)	PI* (h)	Techniques used	Description of specimen	Side	Sa
Kushiro, Hokkaido	2016	16NPCK-M009	M	7.01	4.5	Histology	Nasal mucosa	L	H-
Kushiro, Hokkaido	2018	18NPCK-M001	M	5.50	6	RNA-seq Histology	Nasal mucosa (Ethmo-turbinal II)	L	R- H-
		18NPCK-M006	M	7.09	6	RNA-seq	Nasal mucosa (Frontoturbinal)	L	R-
		18NPCK-M008	F	4.62	6	RNA-seq	Olfactory bulb	R	R-
Abashiri, Hokkaido	2018	18NPCO-M046	F	7.30	5	RNA-seq Histology	Nasal mucosa (Anterior portion)	R	R- H-
Kushiro, Hokkaido	2019	19SK214	M	7.33	8.5	RNA-seq	External skin	-	R-
		19SK215	M	7.56	5.5	RNA-seq	External skin	-	R-

* PI means the post-mortem interval.

3. METHODS

Sampling

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The whales were harvested in the offshore waters of Hokkaido, Japan, and transported to fishing facilities for processing. In order to obtain the nasal mucosa, we meticulously dissected the occipital bone of common minke whales and carefully extracted their brain, subsequently identifying the entrances of the left and right olfactory tract tunnels. The head was then trimmed using a chain saw to create a bony block that encompassed the olfactory bulb tract and the nasal chamber. Upon observing the medial view of the sections, we noted three prominent nasal turbinals, namely the lamina semicircularis and the ethmoturbinals I and II, arranged dorsal to ventral position (Klima, 1999), providing important indications for orientation.

The bony block, measuring 12 cm anteroposterior direction, 7 cm dorsoventrally, and 5 cm transversally, was obtained from the left side of 16NPCK-M009. Subsequently, it was fixed in 10% formalin at the collection site and utilized for gross examination of the nasal chamber. A 5 mm square piece of mucosa was extracted from the posterior end of the frontoturbinal from the block, which was then labeled as H-009 for histological analysis.

We obtained two mucosal samples from 18NPCK-M001. Both samples were collected from the left side nasal mucosa on the ethmoturbinal II, situated in front of the cribriform plate (Fig. 1a and b). One sample was designated as R-001 and was preserved by freezing in RNA-later (Thermo Fisher Scientific Inc., Waltham, MA, USA) for subsequent RNA-seq analysis. The other sample, labeled as H-001, was fixed in 10% formalin for microscopic examination.

Another mucosal sample was acquired for RNA-seq analysis from 18NPCK-M006. The nasal chamber on the left side was carefully trimmed off the head (Fig. 1c and d), promptly frozen, and transported to the laboratory. Subsequently, a 5 mm square mucosal sample was excised from the posterior end of the frontoturbinal region and designated as R-006.

The bony block derived from specimen 18NPCO-M046 was sectioned in a transverse manner, yielding two mucosal pieces extracted from the anterior portion of the right nasal chamber. The mucosal piece intended for RNA-seq analysis was labeled as R-046 and enclosed in a vinyl bag containing RNA-later, ensuring preservation in a freezer. Additionally, an adjacent mucosal piece, designated as H-046, was collected and preserved in 10% formalin for subsequent microscopic examination.

To facilitate comparisons of gene expressions across different organs, the samples were also obtained from the olfactory bulb and the external skin. 18NPCK-M008 was dissected, and the anterior 5 mm tip of the right olfactory bulb was excised and stored in a freezer with RNA-later. This particular piece was labeled as R-008. Furthermore, external skin samples were obtained from specimens 19SK214 and 19SK215, identified as R-214 and R-215 respectively, and preserved in a freezer.

The sampling procedures were recorded through both handwritten and digital macrophotography (Tough TG-5; Olympus Corporation, Tokyo, Japan). We quantified the post-mortem interval as the duration between the animal's capture into the boats above the sea and the preservation of the collected sample in either a freezer or formalin. The maximum recorded post-mortem interval was 8.5 hours.

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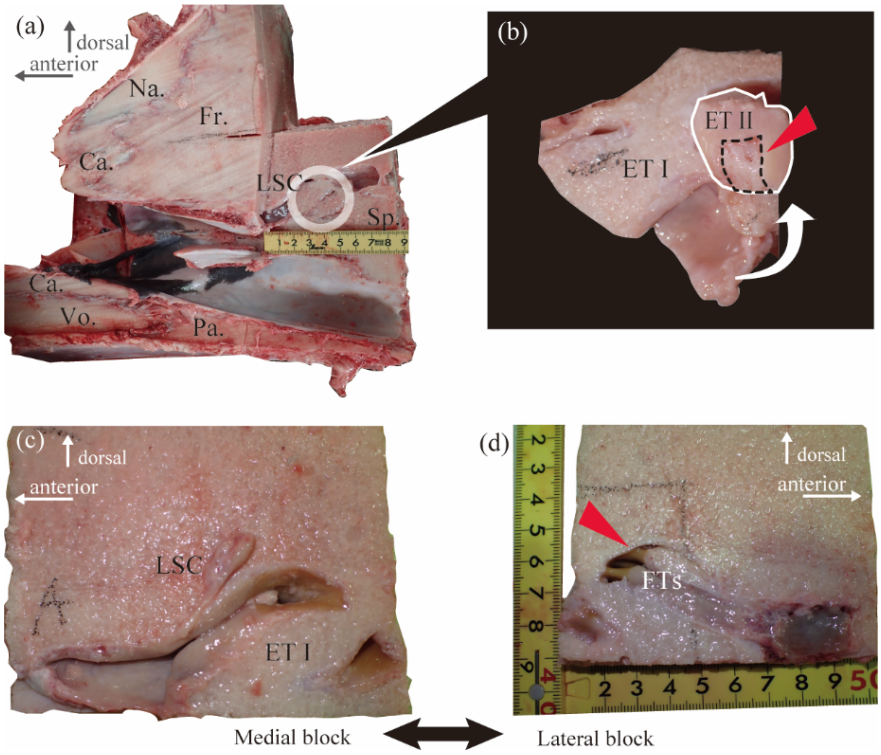

Figure 1 Portions where samples R-001, H-001 and R-006 were harvested. (a) A lateral view of a parasagittal section of the cranium from 18NPCK-M001, illustrating the localization of the nasal turbinals (marked by a white circle) within left nasal chamber. (b) An enlarged view of the ethmoturbinals I and II, positioned anterior to the cribriform plate. The highlighted region, delineated by a white line, represents the posterior end of the ethmoturbinal II. To expose its medial side, this segment is folded over. The red arrowhead denotes the site of mucosal sample R-001 extraction (enclosed by a dashed line). Additionally, in proximity to this area, histological sample H-001 was obtained. (c) A parasagittal section of the left nasal chamber from 18NPCK-M006. This section, depicted in a medial block, provides a lateral view. (d) The corresponding lateral side (in medial view) of the section shown in (c). This photograph displays two frontoturbinals. Mucosal sample R-006 was collected from the posteriormost region of the dorsal frontoturbinal (indicated by the red arrowhead). Abbr: Ca., cartilage; ET I and II, ethmoturbinal I and II, respectively; Fr., frontal bone; FT, frontoturbinal; LSC, lamina semicircularis; Na., nasal bone; Pa., palatine bone; Sp., presphenoid bone; Vo., vomer bone.

RNA expression

The RNA expression analysis in the present study employed the same methods as described in Kishida et al. (2019). The OR genes were queried against the common minke whale genome assembly (GenBank accession GCA_000493695.1) (Yim et al., 2014) using the TBLASTN program in the BLAST+ v. 2.6.0 package (Camacho et al., 2009) with a cut-off E-value of 1×10^{-5}. Deduced amino acid sequences of all intact ORs of green anole (*Anolis carolinensis*) and western clawed frog (*Xenopus tropicalis*), cow (*Bos tauros*), and mouse identified by Niimura (2009b) and Niimura et al. (2014) were used as queries. Each obtained sequence was searched against the GenBank protein database using the BLASTX program and if its best hit did not correspond to an OR, it was discarded. A sequence was deemed a non-functional pseudogene if it contained premature stop codons and/or frame shifts, or it lacked five or more consecutive amino acids, including a transmembrane domain. Sequences interrupted by contig-gaps, though not classified as pseudogenes, were labelled as 'truncated'.

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To search for the OMP gene, FATE (*https://github.com/Hikoyu/FATE/blob/master/fate.pl*) was employed to search the common minke whale genome assembly (GenBank accession GCA_000493695.1) using the annotated query sequence NW_006728793.1, identified as OMP, from GenBank Refseq GCF_000493695.1. The resulting single sequence found in GCA_000493695.1 was used for OMP gene mapping.

Total RNA was extracted from the nasal chamber mucosa (R-001, R-006, R-046), olfactory bulb (R-008) and external skin (R-214, R-215) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines. The olfactory bulb and external skin samples served as negative controls. The extracted RNA was used to construct paired-end sequencing libraries using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina Inc., San Diego, CA, USA). Subsequently, Illumina NovaSeq platform (2x101 bp) was employed for sequencing, generating RNA-seq reads with the following sizes: R-001, 5.89 G bp; R-006, 4.57 G bp; R-046, 5.54 G bp; R-008, 5.37 G bp; R-214, 5.35 G bp; R-215, 6.03 G bp. Low-quality sequences and adapters were removed using Trimmomatic v. 0.38 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa: 2:30:10, LEADING:20, TRAILING: 20, SLIDINGWINDOW: 4:20, and MINLEN: 36. HISAT2 (Kim et al., 2015) v. 2.1.0 with default parameters was used to map trimmed RNA-seq reads to the conspecific genome assembly. The expression levels of genes were quantified using fragments per kilobase of exon per million mapped fragments (FPKM) values with Cufflinks (Trapnell et al., 2010; Roberts et al., 2011) v. 2.2.1 after removing duplicated reads. The expression level of ORs was calculated by dividing it by that of β-actin and multiplying it by 100, giving expression-percentage.

The annotated intact ORs of common minke whales were incorporated into a phylogenetic tree. The nucleotide sequences were aligned using MAFFT (Kuraku et al., 2013; Katoh et al., 2019) v. 7, and a suitable model was determined by Modeltest, IQtree (Trifinopoulos et al., 2016) v. 1.6.12. Subsequently, the TIM3+F+I+G4 model was selected, and the phylogenetic tree was constructed using RAxML-ng (Kozlov et al., 2019) v. 0.9.0 with the root set as Class-1 ORs.

Gross observation

The bony block obtained from 16NPCK-M009 was bisected along the medial wall to reveal the inner side of the nasal chamber. The cross-section of the lamina semicircularis and ethmoturbinals I and II were observed, as shown in Fig. 2. Upon removal of the skeletal tissues of the medial nasal turbinals (ethmoturbinal I and II), the laterally positioned turbinals became visible. The anatomical nomenclature used to identify the nasal turbinals was based on Ito et al. (2022) and supplemented by relevant references (Klima, 1999; Maier and Ruf, 2014).

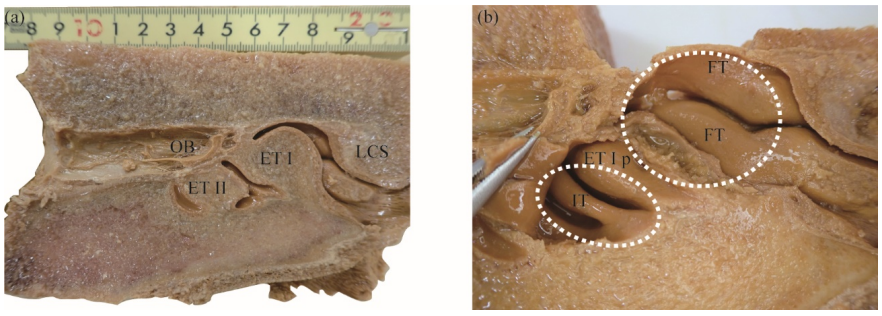

Figure 2 The parasagittal section of the left nasal chamber of 16NPCK-M009. (a) Medial view of the nasal chamber and the posteriorly adjoining olfactory bulb chamber. (b) A closer medial view of the nasal chamber following the removal of ethmoturbinals I and II. The lateral region of the nasal chamber becomes visible. The regions previously occupied by the ethmoturbinals I and II are indicated by circles outlined with white dashed lines. The left and bottom sides correspond to the posterior and ventral directions, respectively. Abbr: ET I and II, ethmoturbinal I and II, respectively; ET I p, ethmoturbinal I posterior part; FT, frontoturbinal; LSC, lamina semicircularis; OB, olfactory bulb.

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Histology staining

The epithelial specimens underwent standard histological techniques. The samples were dehydrated using a series of ethanol concentrations and then cleared with xylene. Following infiltration and embedding in paraffin wax (melting point 56-58), they were sectioned using a rotary microtome (PR-50; Yamato Kohki Industrial Co., Ltd, Saitama, Japan) into slices measuring 4-6 μm. These sections were spread out on warm water, carefully transferred onto glass slides, and subsequently dried in an incubator at 60 for 30 minutes. During the staining process, they were sequentially immersed in deparaffinization solution, hydration medium, and stain solution. Following mounting, the epithelial samples were examined and photographed using a digital microscope (VHX-7000; Keyence, Osaka, Japan). We evaluated whether these epithelial tissues qualified as olfactory epithelium based on the criteria proposed by Farnkopf et al. (2022): epithelium constructed of basal cells, supporting cells, and olfactory sensory neurons; the presence of Bowman's glands; the absence of goblet cells; and the distance between the apical surface and the nuclei of the supporting cells.

4. RESULTS

Expression of the olfactory receptor genes

Transcriptome sequencing using RNA-seq was performed on the nasal mucosa of the putative olfactory organ (R-001, R-006, R-046), the olfactory bulb (R-008), and the external skin (R-214 and R-215) of common minke whales. The FPKM values of β-actin were as follows: R-001, 983; R-006, 285; R-046, 549; R-008, 371; R-214, 161; R-215, 143 (Table 2), indicating successful RNA extraction from all the samples. In this study, 81 intact, 12 pseudo, and 266 truncated genes were annotated. The maximum expression level (as a percentage of FPKM for β-actin) of intact ORs in negative controls (the olfactory bulb, R-008, and external skin, R-214 and R-215) was 0.75 in R-008 (Fig. 3). The average expression of intact ORs with non-zero expression across all samples was 1.014. This expression level was used as the criterion for significant expression in this study.

Among the nasal mucosa samples, R-006 exhibit the presence of 22 significantly expressed intact ORs, while five of them were also expressed in R-001. OMP, on the other hand, was exclusively expressed in R-006, with an expression level greater than 1.014. In contrast, R-046, obtained from the anterior portion of the nasal chamber, did not demonstrate significant expression of ORs or OMP. Out of the 81 annotated intact ORs, all the genes expressed in the nasal mucosa samples belonged to Class-2 OR. Although four copies of intact Class-1 OR were annotated, none of them were expressed in the six samples investigated. The significantly expressed intact ORs did not form a cluster in the phylogenetic tree (Fig. 4). It is worth noting that some pseudogenes were expressed in the nasal mucosa, and one pseudogene was exclusively expressed in the external skin, as per our investigation.

Gross observation

Three distinct nasal turbinals were observed in the medial aspect of the nasal chamber in all the investigated animals (Figs. 1 and 2). These structures were identified as the lamina semicircularis, ethmoturbinals I and II. The configuration of these nasal turbinals closely resembled the 'ethmoturbinates/olfactory recess' described in a common minke whale (Figs. 6 and 7, Godfrey et al., 2013), as well as in bowhead whales (Fig. 7, Farnkopf et al. 2022).

The cranial bony block from 16NPCK-M009 exhibited a complicated morphology of nasal turbinals situated laterally to the ethmoturbinals I and II (Fig. 2). the ethmoturbinal I was positioned just anterior to the olfactory bulb chamber and was accompanied ventrolaterally by the posterior part of ethmoturbinal I (Fig. 2b, ET I p). Laterally to the ethmoturbinal I, the dorsal region of the nasal chamber was occupied by two slender frontoturbinals (Fig. 2b, FT), which corresponded to the same area from which R-006 was obtained (Fig. 1c and d). A delicate turbinal structure, known as the interterbinal, was located lateral to the ethmoturbinal II (Fig. 2b, IT).

Table 2 FPKM and calculated expression percentage.

	R-001	R-006	R-046	R-008	R-214	R-215
β αστιν (ΦΠΚΜ)	983	285	549	371	161	143
OMP						
FPKM	9.510	21.101	0	0	0	0
Expression*	0.966	7.378	0	0	0	0
Average of expressing intact OR genes	**Average of expressing intact OR genes**	**Average of expressing intact OR genes**	**Average of expressing intact OR genes**			
FPKM	3.640 ±4.946	6.537 ±8.735	0.244 ±0.372	0.543 ±0.876	0.171 ±0.098	0.112 ±0.054
Expression*	0.370 ±0.503	2.286 ±3.054	0.045 ±0.068	0.146 ±0.236	0.106 ±0.061	0.078 ±0.038

* Expression (%) was calculated as: FPKM of a gene was divided by that of β actin, then multiplied by 100.

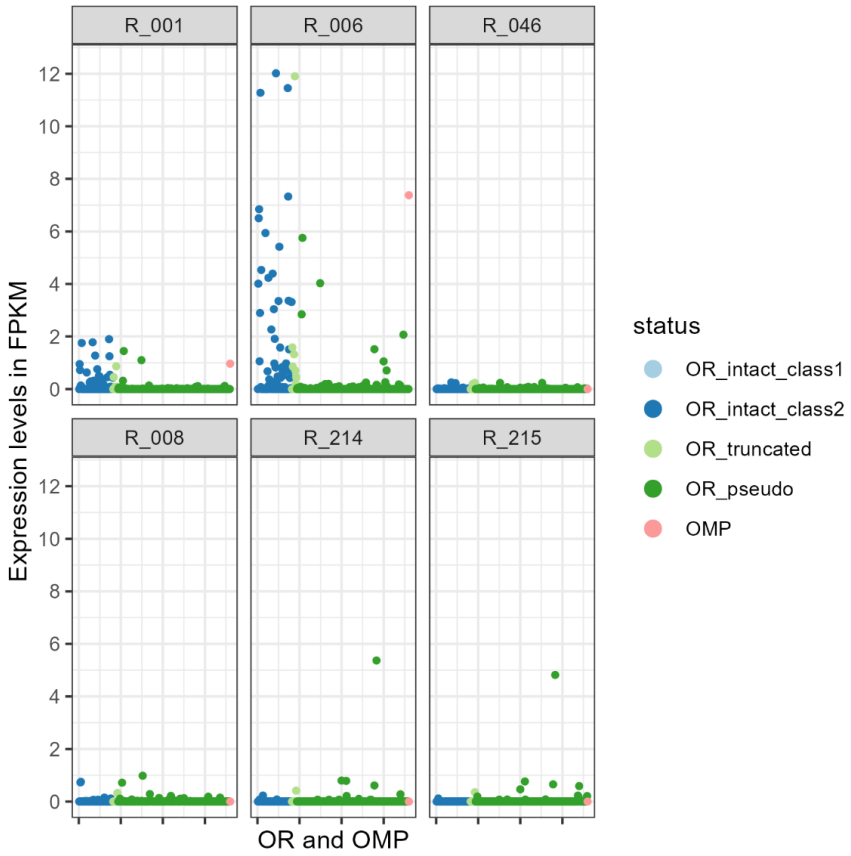

Figure 3 Expression of ORs and OMP. R-001, R-006, and R-046 correspond to the nasal mucosae of the ethmoturbinal II, frontoturbinal, and anterior nasal chamber, respectively. R-008 originated from the olfactory bulb, and both R-214 and R-215 were obtained from the external skin. The average expressions of intact ORs exhibiting non-zero expression were as follows: R-001, 0.370±0.503; R-006, 2.286±3.054; R-046, 0.045±0.068; R-008, 0.146±0.236; R-214, 0.106±0.061; R-215, 0.078±0.038.

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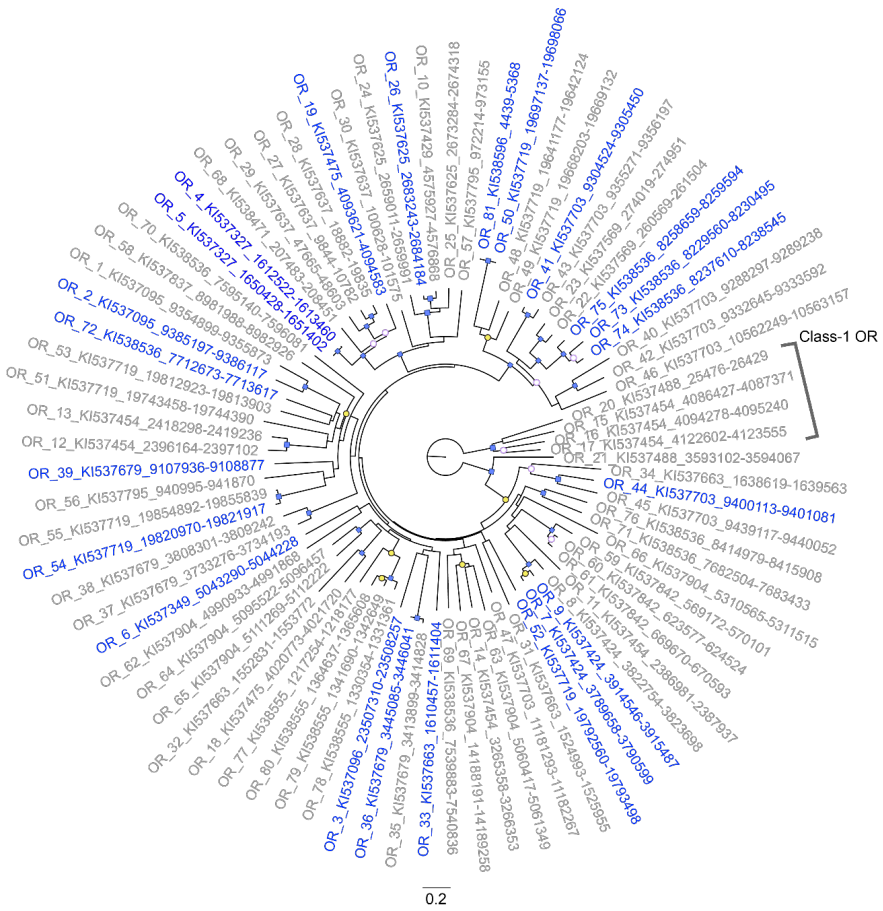

Figure 4 Phylogenetic tree constructed of intact ORs. Genes labeled in blue were determined to have significant expression in R-006. The root of the tree established based on class-1 ORs. Nodes displaying bootstrap values of 100, 90-99, 70-80 are denoted by blue squares, yellow circles, and open circles outlined in purple, respectively, while nodes with bootstrap values below 70 are not marked.

Histological staining

Histological examination of the mucosae (H-001, H-009, H-046) revealed that they were all covered with pseudostratified columnar epithelium and contained glands with visible orifices (Table 3, and Fig. 5). These glands appeared to be serous glands, and the presence of goblet cells was not observed. In all three mucosae, a nucleus-free zone was observed between the apical surface of the epithelium and the nuclei of supporting cells; however, this phenomenon was particularly evident in H-009. Additionally, peripheral nerves were distributed beneath the lamina propria in H-009 (Fig. 5).

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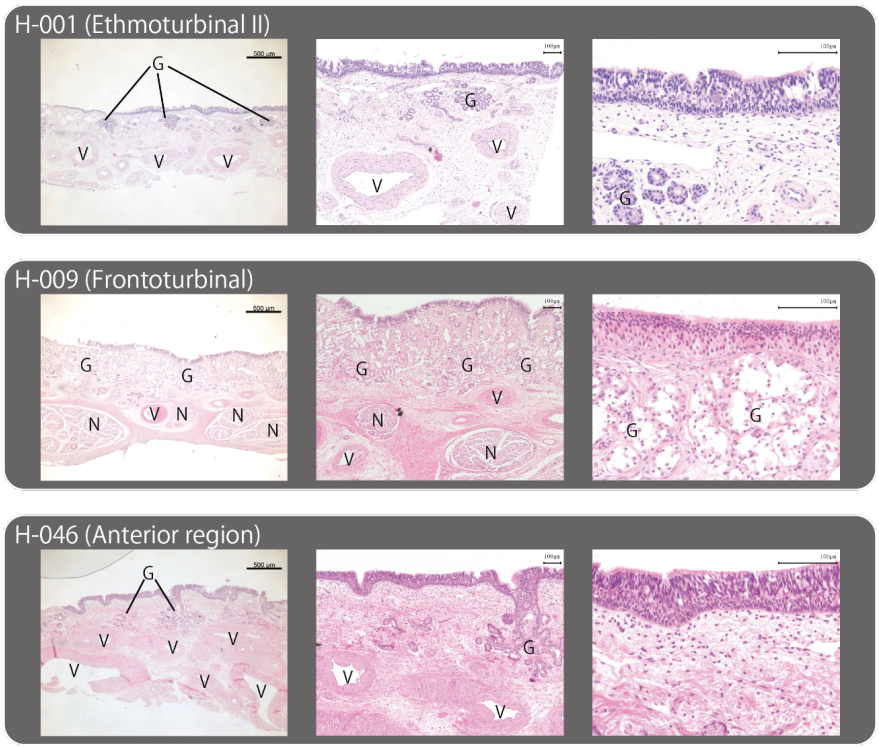

Figure 5 Mucosal samples. The photographs in the far-left, center, and far-right columns were taken at magnifications of ×100 (scale bars indicating 500 μm), ×200 (100 μm), and ×700 (100 μm), respectively. The nasal cavity is located at the top of each image. Abbr: G, glands; N, peripheral nerve; V, blood vessel.

Table 3 Results of RNA-seq and histological observation of mucosal samples.

Description of Specimen	Specimen number	OR gene expression	OMP gene expression	Pseudostratified columnar epithelium	Bowman's grands	Absence of goblet cells	Nucleus free zone	Periphe nerve
Ethmoturbinal II	H-001 R-001	low	low	Y	Y	Y	Y	N
Frontoturbinal	H-009 R-006	Y	Y	Y	Y	Y	Y	Y
Anterior portion	H-046 R-046	No	N	Y	Y	Y	Y	N

5. DISCUSSION

This study has confirmed the specific expression of 22 copies of ORs in the bottom region of the nasal chamber in common minke whales. The ORs were predominantly expressed in the posterior portion of the nasal chamber, facing towards the olfactory bulb, with higher expression levels observed in the frontoturbinal region (R-006) and moderately in ethmoturbinal II (R-001). It has been previously reported that these nasal turbinals in typical mammals are covered with the olfactory epithelium (Van Valkenburgh et al., 2014a). Furthermore, the expression level of OMP was found to be highest in sample R-006, indicating that the

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posterior portion of the frontoturbinal region can be identified as olfactory region. Although the OMP expression in R-001 (0.966) did not meet the criteria of 1.014, it was higher than expression levels observed in R-046, R-008, R-214 and R-215 which showed no expression of OMP. Moreover, the expression of ORs in R-001 was distinctly higher than in R-046, R-008, R-214 and R-215, although lower than R-006 (Table 3). These findings lead us to hypothesize that the sampled region R-001, specifically the posterior medial surface of ethmoturbinal II (Fig. 1b), encompasses both respiratory and olfactory areas. It is commonly reported that respiratory and olfactory mucosae are distributed in a mosaic-like pattern, and this distribution may hold for cetaceans as well.

In this study, we conducted microscopic examination of the ethmoturbinal II (H-001), frontoturbinal (H-009), and anterior region of the nasal chamber (H-046). These samples were assessed based on the histological criteria proposed by Farnkopf et al. (2022), and were likely identified as olfactory mucosa. However, it was the proximal region of the frontoturbinal (R-006) that strongly suggested being the olfactory epithelium based on RNA-seq data. Notably, the same region (H-009) exhibited a rich abundance of peripheral nerves, indicating its high sensitivity. On the other hand, within H-046, which was obtained from a more anterior region of the nasal cavity, dense clusters of vessels with thick walls were observed (Fig. 5). This region may serve as a respiratory area where the vascular epithelium plays a role in thermoregulation.

Although the expressed ORs did not form a distinct cluster in the phylogenetic tree (Fig. 4), it should be noted that the present study does not exclude the possibility of a concealed cluster consisting of ORs expressed in unexplored regions. This is because the distribution of ORs, which mediate odoriferous stimuli to the olfactory bulb, is not uniform across the olfactory epithelium (Ressler et al., 1993; Vassar et al., 1993; Marchand et al., 2004). Furthermore, the distribution pattern of the olfactory mucosa within the nasal chamber varies among lineages; (Smith et al., 2011; Ruf, 2014; Smith et al., 2014)(Smith et al., 2011; Ruf, 2014; Smith et al., 2014; Ito et al., 2021). It is plausible that common minke whales possess additional ORs that contribute to their olfactory modality, and identifying such receptors would enhance our understanding of the molecular mechanisms underlying cetacean olfaction.

To comprehend the expression pattern of ORs, a thorough anatomical examination of the cetacean nasal chamber is indispensable. Our observations unveiled the presence of additional nasal turbinals positioned laterally to ethmoturbinals I and II (Fig. 2b, FT and IT), which have been scarcely documented in cetaceans. However, due to the dearth of comprehensive anatomical data encompassing the entire nasal chamber, precise determination of the exact locations from which H-046 and R-046 were obtained remained elusive. Nasal turbinals can be broadly categorized into olfactory and respiratory turbinals, both of which play a pivotal role in unraveling the aquatic adaptation of these creatures (Van Valkenburgh et al., 2011; Van Valkenburgh et al., 2014a; Martinez et al., 2020). In the nasal chamber of common minke whales, the anterior segment from which H-046 and R-046 were harvested is inferred to represent the respiratory region. It is conceivable that baleen whales also possess respiratory turbinals, and investigating this structure is warranted in future studies. Although the present study primarily focused on the posterior region adjacent to the cribriform plate, it is crucial to obtain a comprehensive understanding of the entire architecture of this intricate labyrinth (Van Valkenburgh et al., 2014b).

Fundamental anatomical data can function as an Atlas during dissection process. As highlighted by Farnkopf et al. (2022), the extraction of the nasal chamber from the cranial bones of large whales presents a formidable challenge due to its remarkable dimensions, thickness, and robust structure. The identification of the nasal turbinals from sectional images can prove challenging, as their appearance exhibits variations with slight deviations in cutting angles. Moreover, the intricate nature of this structure impedes the efficient penetration of fixation solutions into the tissues. To surmount these sampling difficulties, the comprehensive description of the entire nasal chamber using CT imaging becomes imperative. Subsequently, the determination of olfactory epithelium distribution emerges as the next crucial step. The locations of the olfactory epithelium might be predicted based on the surface coloration of the nasal mucosae. While the majority of the nasal mucosa in common minke whales displayed a pale pink hue, R-006, which exhibited distinct olfactory characteristics, was obtained from an epithelium displaying a yellowish appearance. A previous study tentatively proposed

the possibility of yellow pigmentation in the olfactory epithelium of bowhead whales (Farnkopf et al., 2022).

The entirety of the expressed ORs identified in this study exclusively belong to class-2 (Fig.4). While there remained significant room for exploration, the probability of class-1 OR expression in common minke whales is presumed to be minimal even when thoroughly screening the entire lining mucosa of the nasal chamber. This presumption is rooted in the observed morphology, which unveiled the absence of the dorsal domain of the olfactory bulb in common minke whales. Class-1 olfactory receptors typically transmit input to the dorsal domain of the olfactory bulb. Consequently, it is anticipated that class-1 ORs would not be expressed in the nasal mucosa of an animal lacking this specific region of the olfactory bulb. It has been documented that bowhead whales have also lost the dorsal domain of the olfactory bulb (Thewissen et al., 2011; Kishida et al., 2015b), and the investigated common minke whales exhibit a dorsoventrally flattened olfactory bulb, akin to that of bowhead whales.

This study strongly suggests that the class-1 ORs do not partake in olfactory reception in baleen whales. In mice, class-1 ORs receive stimuli that trigger avoidance behaviors and project them into the dorsal domain of the olfactory bulb (Kobayakawa et al., 2007). Hence, our findings imply the loss of the typical avoidance response to specific odorants, such as predators or putrefying substances, in whales.

Considering that Sirenians, another lineage of fully aquatic mammals, still retain their olfactory organ and possess a large repertoire of ORs (Barboza and Larkin, 2020; Han et al., 2022; Christmas et al., 2023), the diminished olfactory ability of baleen whales cannot be sorely attributed to their aquatic lifestyle, which restricts continuous respiration. One possible explanation for this lies in the necessity for discerning ingested foods. Anatomically, the esophagus of cetaceans is separated from the airway (Tyack and Miller, 2002), preventing them from detecting smells emanating from the oral cavity. Genomic research has revealed a degeneration of sense of taste, the other form of chemoreception, in cetaceans (Feng et al., 2014; Zhu et al., 2014; Kishida et al., 2015a; Policalpo et al., 2023). Furthermore, there is no anatomical description of taste buds in the tongues of baleen whales (Sonntag, 1922; Ogawa and Shida, 1950; Tarpley, 1985). Consequently, it follows that baleen whales do not rely on chemosensory modalities to evaluate food within their mouths, which may have contributed to the reduction of their olfactory capabilities.

Our histological and genomic investigations suggest that residual class-2 ORs are responsible for olfaction in baleen whales. Specifically, the present study indicates that baleen whales are able to receive preferred odors, such as those associated with prey and potential mating partners. This research establishes a foundational link between the anatomy and genome of baleen whales, and further investigations hold the potential to illuminate the natural social and behavioral biology of these animals, thereby aiding in their conservation.

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DATA AVAILABILITY STATEMENT

All sequence reads were deposited in the DDBJ Sequence Read Archive under BioProject accession no. PRJDB16252.

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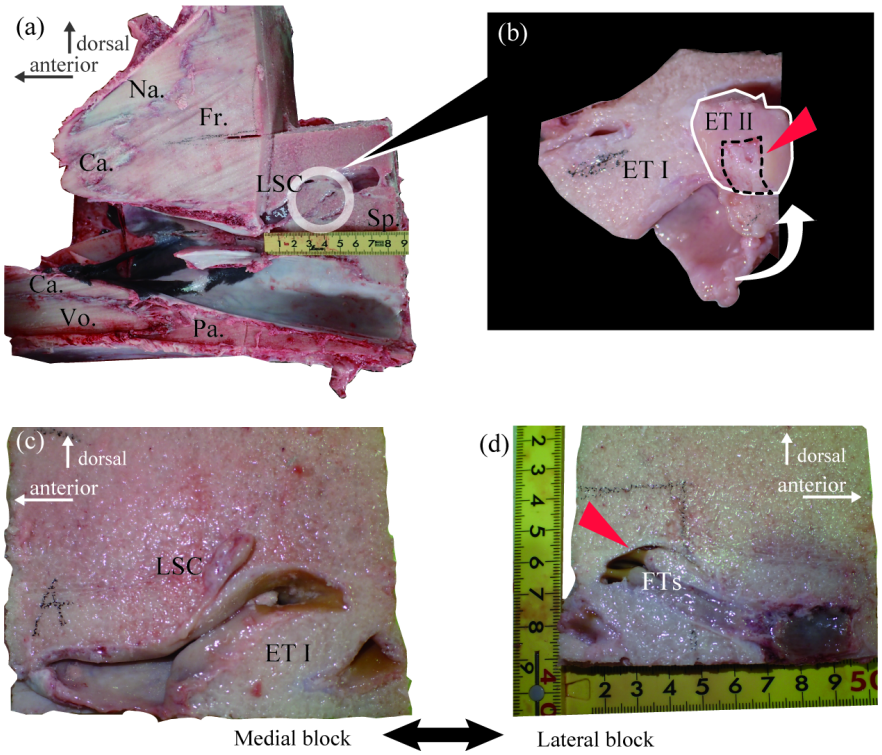

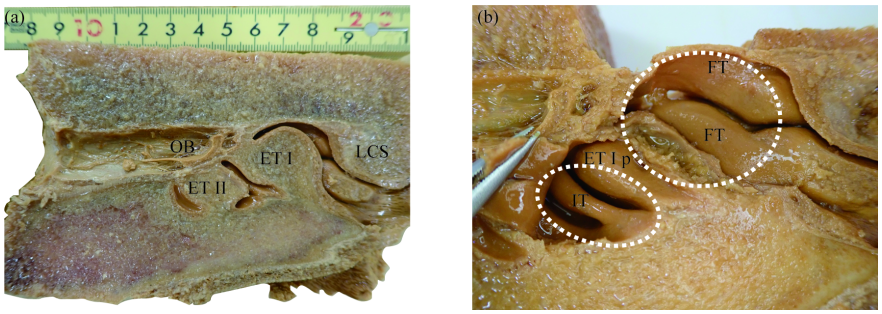

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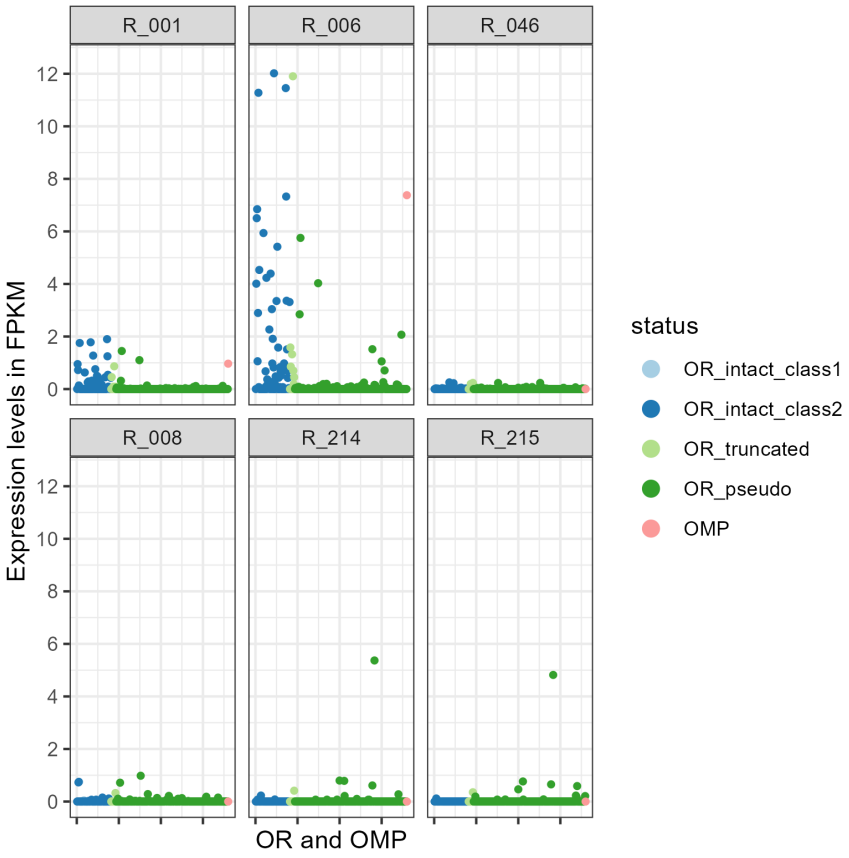

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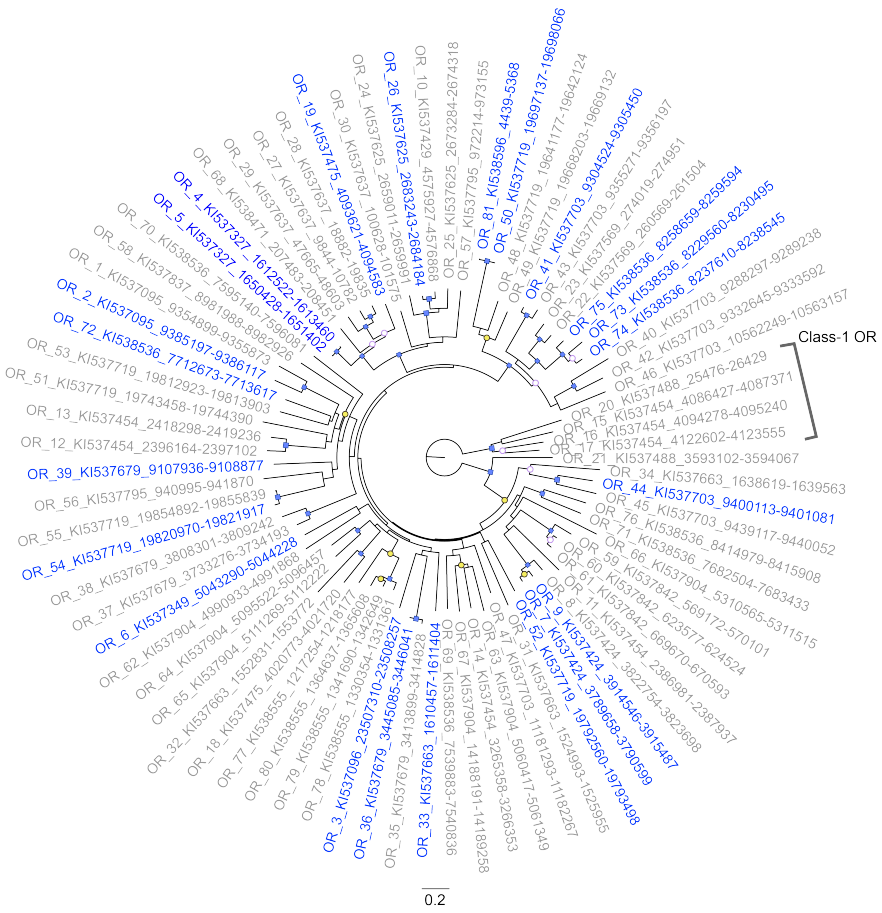

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