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Anatomy

Distribution of cells expressing vomeronasal receptors in the olfactory organ of turtles

Sayed Sharif ABDALI[1,2], Shoko NAKAMUTA[2], Yoshio YAMAMOTO[1,2] and Nobuaki NAKAMUTA[1,2]*

[1]United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan
[2]Laboratory of Veterinary Anatomy, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

ABSTRACT. Generally, the olfactory organ of vertebrates consists of the olfactory epithelium (OE) and the vomeronasal organ (VNO). The OE contains ciliated olfactory receptor neurons (ORNs), while the VNO contains microvillous ORNs. The ORNs in the OE express odorant receptors (*ORs*), while those in the VNO express type 1 and type 2 vomeronasal receptors (*V1Rs* and *V2Rs*). In turtles, the olfactory organ consists of the upper (UCE) and lower chamber epithelia (LCE). The UCE contains ciliated ORNs, while the LCE contains microvillous ORNs. Here we investigated the distribution of cells expressing vomeronasal receptors in the olfactory organ of turtles. The turtle vomeronasal receptors were encoded by two *V1R* genes and two *V2R* genes. Among them, *V2R1* and *V2R26* were mainly expressed in the LCE, while *V1R3* was expressed both in the UCE and LCE. Notably, vomeronasal receptors were expressed by a limited number of ORNs, which was confirmed by the expression of the gene encoding TRPC2, an ion channel involved in the signal transduction of vomeronasal receptors. Furthermore, expression of *ORs* by the majority of ORNs was suggested by the expression of the gene encoding CNGA2, an ion channel involved in the signal transduction of ORs. Thus, olfaction of turtle seems to be mediated mainly by the ORs rather than the vomeronasal receptors. More importantly, the relationship between the fine structure of ORNs and the expression of olfactory receptors are not conserved among turtles and other vertebrates.

KEY WORDS: *in situ* hybridization, olfactory organ, transient receptor potential cation channel subfamily C member 2, turtle, vomeronasal receptors

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Many tertapods have two olfactory organs: the olfactory epithelium (OE) and the vomeronasal organ (VNO) [4, 15, 21, 54]. In mice, the OE lines dorso-caudal portion of the nasal cavity, while the VNO is situated at the base of the nasal septum. Chemosensory cells in the olfactory organs, namely, olfactory receptor neurons (ORNs), are bipolar neurons extending an axon basally and a dendrite apically. Generally, the ORNs in the OE bear cilia at the tip of their dendrites and project their axons to the main olfactory bulb, while those in the VNO bear microvilli at the tip of their dendrites and project their axons to the accessory olfactory bulb [20, 53, 54]. In fish, a discrete VNO does not exist and the olfactory organ is represented solely by the OE. The OE of fish contains both ciliated and microvillous ORNs which project their axons to the distinct parts of olfactory bulb [22, 45, 54].

Olfactory chemoreception is mediated by the olfactory receptors which are members of seven transmembrane, G protein coupled receptors. They are categorized into three families: odorant receptors (ORs), type 1 vomeronasal receptors (V1Rs) and type 2 vomeronasal receptors (V2Rs) coupled to Gαolf, Gαi2 and Gαo, respectively [8, 11, 12, 24, 36, 44]. In mice, each ORN in the OE expresses only one *OR* gene out of a repertoire of over 1,000 *OR* genes [38, 52], whereas that in the VNO expresses one or a few members of vomeronasal receptor (*VR*) genes [25, 34, 50]. Furthermore, the ORNs in the apical layer of the vomeronasal sensory epithelium express *V1Rs*, while those in the basal layer express *V2Rs* [3, 11, 12, 24, 36, 44]. In the OE of fish, as in the olfactory organ of mice, ciliated ORNs express *ORs* and *Gαolf*, while microvillous ORNs express *V2Rs* and *Gαo* [23, 45]. Thus, it is generally believed that the fine structure of ORNs and the gene expression of olfactory receptors are closely related to each other, and that this relationship is conserved among vertebrates [14, 23].

The olfactory organ of turtles is comprised of two types of sensory epithelia: the upper chamber epithelium (UCE), lining the dorsal portion of the nasal cavity, and the lower chamber epithelium (LCE), lining the ventral portion of the nasal cavity. The ORNs in the UCE and LCE project their axons to the ventral and dorsal parts of the olfactory bulb, respectively [1, 37, 46].

As described above, the OE of mammals contains ciliated ORNs while the VNO contains microvillous ORNs. Meanwhile, the OE of reptiles and birds contains ORNs bearing both cilia and microvilli at the tip of their dendrites [13, 29]. In general, the ORNs

*Correspondence to: Nakamuta, N.: nakamuta@iwate-u.ac.jp

in the UCE of turtles bear cilia and microvilli at the tip of their dendrites and thus the UCE is regarded as the OE. On the other hand, the ORNs in the LCE bear only microvilli and thus the LCE is regarded as the VNO [17, 41, 57].

Based on the fine structure of ORNs, it is speculated that the ORNs of turtles in the UCE express both *OR* and *VR* genes, while those in the LCE express only *VR* genes. Nevertheless, immunohistochemical analyses of the olfactory organ of turtles, including Reeve's turtle, snapping turtle and red-eared slider, expression of the Gαolf and Gαo have been demonstrated both in the UCE and LCE [41, 42, 55]. Moreover, expression of Gαi1-3 has been reported in the LCE of the common musk turtle and red-eared slider [40]. These pieces of evidence suggest that ORNs in the UCE of turtles express both *ORs* and *V2Rs*, whereas those in the LCE express *ORs*, in addition to *VRs*.

The inconsistency between the olfactory receptors deduced by the fine structure of ORNs and the G protein expression in the LCE leads to the need for the elucidation of olfactory receptor genes expressed in the olfactory organ of turtles. Thus, we analyzed the expression of *VRs* in the present study. Also, expression of the gene encoding TRPC2, an ion channel mediating the signal transduction of VRs [12, 33, 59], and the expression of the gene encoding CNGA2, an ion channel mediating the signal-transduction of ORs [6, 12], were examined to proxy the type of olfactory receptors expressed in the olfactory organ of turtles.

MATERIALS AND METHODS

Animal handling and tissue preparation

Totally twelve red-eared sliders *Trachemys scripta* of both sexes weighing 457–1,698 g and two soft-shelled turtles *Pelodiscus sinensis*, one male (1,132 g) and one female (1,164 g), were used in the present study (Table 1). Red-eared sliders captured at Hyogo prefecture, Japan, were generous gift from Suma Aqualife Park (Kobe, Japan). Soft-shelled turtles were purchased from a local turtle farm. Samplings were done during 2017–2019.

Animals were anesthetized by intraperitoneal injection of pentobarbital sodium, 60 mg/kg of body weight. For RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR), one soft-shelled turtle and one red-eared slider were killed by decapitation. The UCE and LCE were immediately dissected out and preserved at −80°C until use.

The remaining animals were sacrificed by exsanguination through transcardial perfusion with Ringer's solution and fixed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). After decapitation, tissues surrounding the nasal cavity were removed. Heads were immersed in the same fixative solution overnight at 4°C and then decalcified in 10% ethylenediamine tetra acetic acid in 0.1 M PB for several days. After decalcification, olfactory organs were cryoprotected in sucrose gradient, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and cryosectioned at 20 μm in thickness.

All procedures of the animal handling were carried out in accordance with Standards for Animal Care and Use at Iwate University (approval No. A201720).

PCR cloning

Total RNA was isolated from the turtle olfactory organ according to the manufacturer's protocol. Briefly, the samples containing both UCE and LCE were homogenized using 1 m*l* ISOGEN reagent (Nippon gene, Tokyo, Japan) with a homogenizer and incubated for 5 min at room temperature. An appropriate amount of chloroform was added and mixed vigorously. The samples were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was transferred to new tubes, mixed with isopropanol and kept at room temperature for 10 min. Subsequently, samples were centrifuged at 15,000 rpm for 20 min at 4°C to obtain RNA pellets.

Table 1. Details of animals used in this study

Animal number	Sex	Sampling date (Day/Month/Year)	Body weight (g)	Carapace length (cm)
1	Female	19/06/2017	1,164	19.0
2	Female	19/06/2017	1,294	21.0
3	Female	03/02/2018	995	20.0
4	Female	31/03/2019	1,698	22.7
5	Female	31/03/2019	1,346	20.1
6	Female	02/06/2018	1,143	19.5
7	Female	26/09/2018	1,200	20.0
8	Female	26/09/2018	1,248	20.0
9	Female	17/12/2018	1,200	20.8
10	Male	31/03/2019	883	18.5
11	Male	02/06/2018	457	14.5
12	Male	02/06/2018	442	15.0
13	Male	20/09/2018	916	18.6
14	Male	17/12/2018	556	15.8
15	Male	26/09/2018	1,132	20.0

Animals #1 and #2 were used for RNA extraction; animals #3–15 were used for *in situ* hybridization. #2–-14, red-eared sliders; #1 and #15, soft-shelled turtles.

Table 2. Primers for PCR amplification

Genes	Forward primers	Reverse primers
Pelodiscus sinensis V2R1	gtatgctggccattcaaggt	cctcacttgcaatccaggtt
P. sinensis V2R26	ttctcaacaccagcttgacg	cagaaccacaaggacggttt
P. sinensis V1RA14	ggcaacctcgttgttgtctt	tttcaggcaccaacaggctt
P. sinensis TRPC2	tgcgagaaggacctctacga	agatggaggacagcacttgc
P. sinensis CNGA2	cttccaggactgtgaggctg	cttgatcaggatctcccggc
Chrysemys picta V2R1	tggtgcacctcttgtagcag	agtgctgatgtaacccaggc
C. picta V2R26	tcccaactcacccggactat	caacccaggtccatcggaaa
C. picta V1R3	ggaaaagtcacgccctctga	cctgcttcccgtgctgataa
C. picta V1RA14	acacttaccacacgtgagg	agacaccattgaggacgctg

The pellets were washed with 1 m*l* of 70% ethanol and dissolved into 30 *µl* diethyl pyrocarbonate (DEPC)-treated water. Total RNA was digested with DNase I (Takara, Kusatsu, Japan) to remove contaminating genome DNA. After evaporation of ethanol, an absorbance of 260 nm was measured for RNA by a spectrophotometer and preserved at −80°C until use.

For synthesizing first-strand cDNA, the total RNAs were subjected to reverse transcription. For each sample, 2 *µ*g of total RNA was mixed with ReverTra Ace (Toyobo, Osaka, Japan), (1*µl*, 100 U/*µl*), oligo-(dT) (1 *µl*, 10 pmol/*µl*), dNTPs (1 *µl*, 10 mM; Takara) in a final volume of 20 *µl* by adding DEPC-treated water. PCR primers were designed based on the nucleotide sequences of soft-shelled turtle *V2R1* [GenBank: XM-006123493], *V2R26* [XM-006111317], *V1RA14* [XM-014581607], *TRPC2* [XM-006111779] and *CNGA2* [XM-014580174]. For red-eared slider, primers were designed based on the nucleotide sequence of western painted turtle *Chrysemys picta V2R1* [XM-005287015], *V2R26* [XM-005284259], *V1RA14* [XM-005291513] and *V1R3* [XM-0053033611]. A list of primers is shown in Table 2. PCR was performed using Takara Ex Taq (Takara). The PCR products were mixed with 1 *µl* loading buffer and loaded into 1% gel electrophoresis. The gel extraction was performed to purify the cDNAs by MinElute Gel Extraction Kit (Qiagen, Hilden, Germany), and cDNAs were cloned using TOPO TA cloning Kit (Thermo Fisher Scientific, Rockford, IL, USA). The sequences of cDNA fragments were analyzed by a genetic analyzer (HITACHI 3500). Finally, the sequence similarity search for obtained fragments of red-eared slider with corresponding genes in the western painted turtle were done by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained nucleotide sequences of *V2Rs* and *V1R3* showed 99 and 98% identity with their counterpart sequences in western painted turtle, respectively.

Probe preparation

Digoxigenin (DIG)-labeled cRNA sense and antisense probes were synthesized for soft-shelled turtle and red-eared slider individually by *in vitro* transcription using DIG RNA labeling kit (SP6 and T7) (Roche Diagnostics GmbH, Mannheim, Germany).

In situ hybridization

Sections were washed in 0.1 M phosphate buffered saline (PBS) for 15 min and fixed in 4% PFA for 10 min. Subsequently, sections were treated with 10 *µl*/m*l* proteinase K for 15 min at 37°C followed by washing in PBS glycine for 10 min plus PBS for 6 min. The sections were stabilized with 4% PFA and immersed in 0.1% acetic anhydrate in the acetylation buffer for 15 min followed by washing in 4×saline-sodium citrate (SSC) for 20 min. Pre-hybridization was carried out using hybridization buffer ISHR7 (Nippon gene, Tokyo, Japan) at 55°C. Hybridization was performed using 0.25 *ng/µl* of cRNA sense and antisense probes in the hybridization buffer overnight at 55°C. Post-hybridization washes were carried out in formamide/2×SSC for 1 hr and 0.1×SSC for 2 hr at same temperature. And then sections were treated with blocking buffer for 30 min. The sections were incubated with anti-DIG antibody (Roche Diagnostics GmbH) for 2 hr and washed with Tris-buffered saline (TBS) for 30 min. Sections were transferred in NTM buffer containing 1 M Tris HCl, 1 M $MgCl_2$, 5 M NaCl and distilled water. Lastly, sections were colorized by 5-bromo-4-chloro-s-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) coloring agent (Roche Diagnostics GmbH) and washed with TE and TBS buffers each for 5 min.

Analyzing the density of cells expressing VRs and TRPC2

Serial sections were prepared along the whole extent of the olfactory organ and three to five sections were picked up from each of the rostral, intermediate and caudal regions, to examine the distribution of cells expressing *VRs* and *TRPC2* in all regions of olfactory organ. The number of cell bodies with a clear signal was counted for UCE and LCE, individually. At the same time, the area of the UCE and LCE were measured with ImageJ software (http://rsb.info.nih.gov/ij/). Subsequently, the number of cells expressing *VRs* and *TRPC2* were divided by the area of each epithelium to obtain the density of cells expressing *VRs* and *TRPC2* (cells per mm^2). Data was represented as mean ± standard error (SE). The difference in the density of cells expressing *VRs* and *TRPC2* along the rostro-caudal axis of the olfactory organ and their relative abundance in the UCE and LCE were statistically analyzed by either student's *t*-test for comparison of two means or a one-way analysis of variance for more than two means. $P<0.05$ was considered to be statistically significant.

Spatial distribution analysis

The distribution of cells expressing *VRs* and *TRPC2* along the apical-to-basal axis of epithelium were analyzed as described by Syed *et al*. (2013) [51] with slight modification. Briefly, the relative depth of cells expressing *VRs* and *TRPC2* was defined as the distance of the cell soma center from the apical surface of epithelium divided by the entire thickness of epithelial layer at the position. Data were arranged in bins with 0.1 intervals. Zero corresponds to most apical and one corresponds to most basal.

RESULTS

Expression of the genes encoding V2Rs

In turtles, V2Rs are encoded by two genes, including *V2R1* and *V2R26*. By the RT-PCR conducted to determine the genes encoding V2Rs expressed in the olfactory organ of red-eared slider, a single band was obtained for both *V2R1* and *V2R26* genes (Fig. 1), indicating the expression of *V2R* genes in the olfactory organ of red-eared slider.

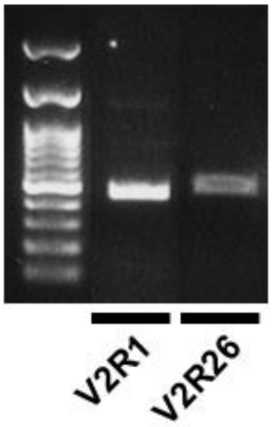

Fig. 1. Reverse transcription-polymerase chain reaction analysis for the *V2R1* and *V2R26* genes showing the expression of both *V2R* genes in the olfactory organ of red-eared slider.

By the *in situ* hybridization performed to clarify the localization of cells expressing *V2Rs* in the olfactory organ of red-eared slider, cells expressing *V2R1* were found only in the LCE (Fig. 2A and 2B), whereas those expressing *V2R26* were found both in the UCE and LCE (Fig. 2D–F). No signals were detected in the sections hybridized with sense probes (Fig. 2C and 2G). As shown in the Fig. 2H, the density of cells expressing *V2R26* was significantly higher in the LCE than in the UCE (1.823 ± 0.132 cells/mm^2 in the LCE, 0.34 ± 0.08 cells/mm^2 in the UCE). Furthermore, statistical analysis indicated no significant difference in the density of cells expressing *V2R26* along the rostro-caudal axis of the olfactory organ (1.303 ± 0.308 cells/mm^2 in the rostral, 1.106 ± 0.269 cells/mm^2 in the intermediate and 0.87 ± 0.24 cells/mm^2 in the caudal regions) (Fig. 3A–C). Similarly, cells expressing *V2R1* distributed almost evenly along the rostro-caudal axis of the olfactory organ (3.342 ± 1.259 cells/mm^2 in the rostral, 3.296 ± 1.531 cells/mm^2 in the intermediate and 3.229 ± 1.172 cells/mm^2 in the caudal regions). Thus, a region containing higher density of cells expressing *V2Rs* was not found.

Moreover, differences in the expression of *V2Rs* were investigated among individuals with different carapace length. The density of cells expressing *V2R1* varied (1.24–9.27 cells/mm^2) among some individuals with different carapace lengths 15.8–22.7 cm (#4, #6, #7, #8 and #14) (Fig. 4A). Furthermore, the cells expressing *V2R1* were not found in other individuals with carapace lengths of 18.5, 14.5 and 15.8 cm (#10, #11 and #13, respectively). Meanwhile, the density of cells expressing *V2R26* was similar (0.2–2.54 cells/mm^2) among individuals with different carapace lengths 14.5–22.6 cm (#5, #6, #8-#14) indicating the absence of individual difference for the expression of *V2R26* (Fig. 4B). Data is summarized in Table 3.

Subsequently, mRNA expression of the genes encoding V2Rs was investigated in the olfactory organ of soft-shelled turtle. RT-PCR analysis revealed the expression of *V2R1* only in the LCE, and *V2R26* both in the UCE and LCE (Fig. 5A). Furthermore, *in situ* hybridization analysis demonstrated the expression of *V2R26* both in the UCE and LCE (Fig. 5B and 5C). No signals were detected in the sections hybridized with sense probes (Fig. 5D). As in the case of red-eared slider, the density of cells expressing *V2R26* was significantly higher in the LCE (1.456 ± 0.094 cells/mm^2) than in the UCE (0.294 ± 0.12 cells/mm^2) (Fig. 5E). Cells expressing *V2R1* were not found either in the UCE or LCE (not shown).

Expression of the genes encoding V1Rs

The V1R is encoded by two genes, *V1R3* and *V1RA14*, in western painted turtle, while the V1R is encoded by a single gene *V1RA14* in soft-shelled turtle. In order to determine the *V1R* genes expressed in the olfactory organ of turtles, the mRNA expression of the *V1R* genes was investigated by RT-PCR. Obtained results indicated the expression of *V1R3* in the olfactory organ of red-eared slider, but not that of *V1RA14* (Fig. 6A). Also, the expression of *V1RA14* was not detected in the olfactory organ of soft-shelled turtle (Fig. 6B).

Subsequently, the localization of cells expressing *V1R3* in the olfactory organ of red-eared slider was analyzed by *in situ* hybridization. *V1R3* gene was sparsely expressed by cells both in the UCE and LCE (Fig. 6C and 6D).

Although the density of cells expressing *V1R3* varied among individuals (0.906 ± 0.153 cells/mm^2 in animal #3, 1.811 ± 0.133 cells/mm^2 in animal #6, 1.845 ± 0.197 cells/mm^2 in animal #11 and 0.254 ± 0.07 cells/mm^2 in animal #12), the cells expressing *V1R3* were almost evenly distributed along the rostro-caudal axis of olfactory organ. A significant difference was not found between the rostral (1.308 ± 0.355 cells/mm^2), intermediate (1.232 ± 0.438 cells/mm^2) and caudal regions (0.951 ± 0.301 cells/mm^2) (Fig. 7A and 7B). Meanwhile, the relative abundance of cells expressing *V1R3* in the UCE and LCE varied among individuals (Fig. 8). In most cases, the density of cells expressing *V1R3* was higher in the UCE (1.492 ± 0.398 cells/mm^2 in animal #3 and 3.366 ± 0.331 cells/mm^2 in animal #11) than in the LCE (0.576 ± 0.118 cells/mm^2 in animal #3 and 0.511 ± 0.151 cells/mm^2 in animal #11). On the other hand, in animal #6, the density of cells expressing *V1R3* was higher in the LCE (2.064 ± 0.207 cells/mm^2) than in the UCE (1.369 ± 0.178 cells/mm^2). Moreover, in animal #12 the density of cells expressing *V1R3* was almost equal between the UCE (0.274 ± 0.081 cells/mm^2) and LCE (0.244 ± 0.078 cells/mm^2).

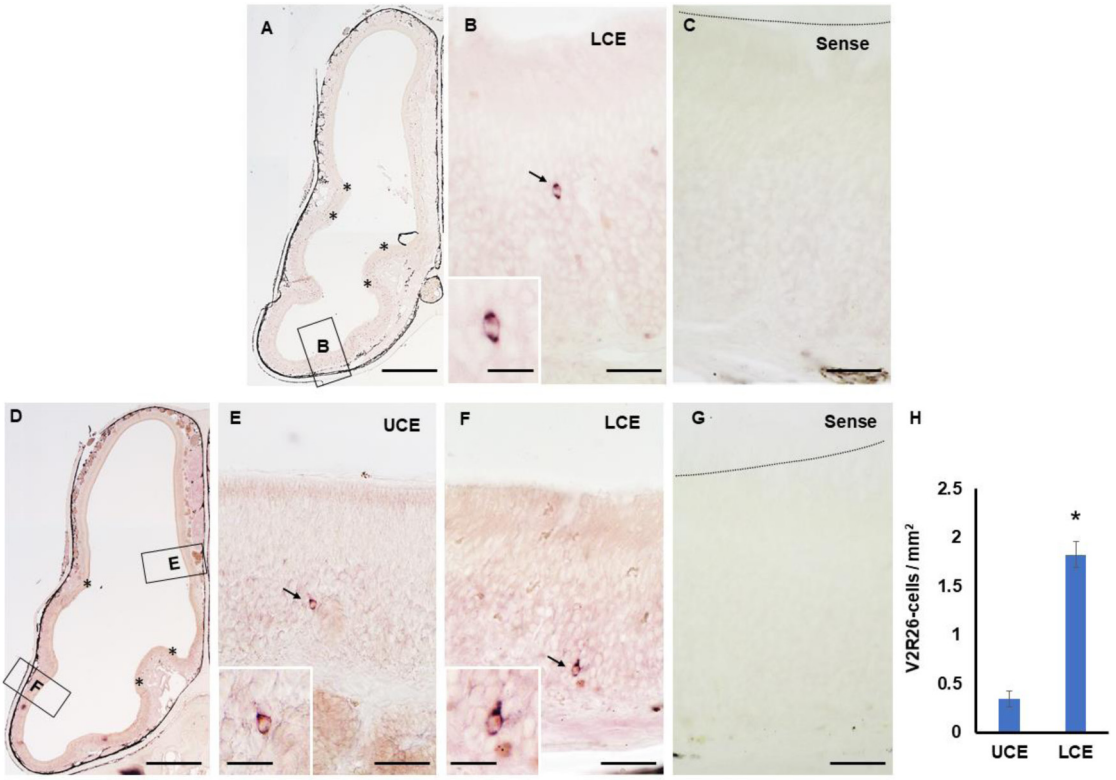

Fig. 2. *In situ* hybridization analysis for the localization of cells expressing *V2Rs* in the olfactory organ of red-eared slider. Cells expressing *V2R1* were localized only in the lower chamber epithelium (LCE) (A, B), while those expressing *V2R26* were localized both in the upper chamber epithelium (UCE) and LCE (D–F). Asterisks in (A, D) indicate the boundary between UCE and LCE. (B, E and F) Higher magnification views for boxed areas in A and D, respectively. No signals were detected in the sections hybridized with sense probes (C, G). Scale bars=1 mm in (A, D), 50 μm in (B, C and E–G), 20 μm in insets. (H) Relative abundance of cells expressing *V2R26* between the UCE and LCE of red-eared slider. The density of cells expressing *V2R26* was significantly higher in the LCE than in the UCE. Each column and vertical bar represent the mean and standard error (n=9). Asterisk indicates a significant difference between the UCE and LCE (P<0.05).

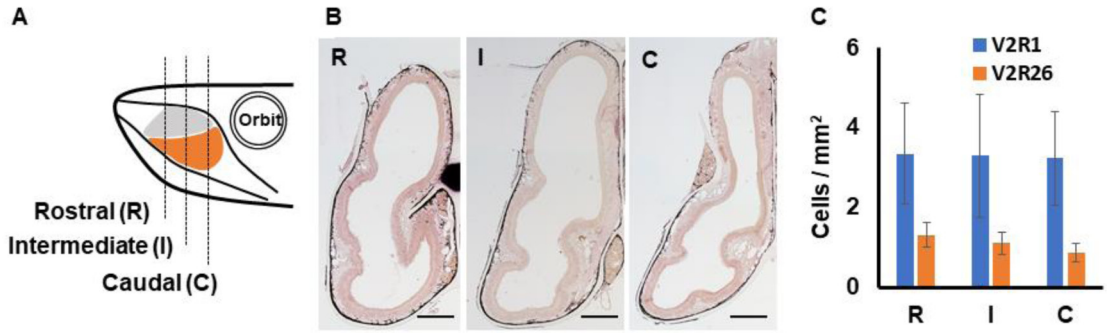

Fig. 3. Distribution of cells expressing *V2R* genes along the rostro-caudal axis of olfactory organ. (A) Schematic drawing of the head of red-eared slider. Dotted lines correspond positions in (B). (B) Transverse sections through the olfactory organ. Scale bars=1 mm. (C) Density of *V2R*-cells along the rostro-caudal axis of olfactory organ. Each column and vertical bar represent the mean and standard error (n=9 for *V2R26* and n=6 for *V2R1*). No significant difference was found in the density of cells expressing *V2Rs* along the rostro-caudal axis of olfactory organ (P>0.05).

Expression of the genes encoding TRPC2 and CNGA2

The expression of *VR* genes by a small number of ORNs described above implies the presence of unknown genes encoding VRs and their expression in the LCE which contains microvillous ORNs. Thus, we further investigated the expression of the gene encoding TRPC2 in the olfactory organ of red-eared slider. *In situ* hybridization analysis demonstrated the expression of *TRPC2* mRNA by sparsely distributed cells both in the UCE and LCE (Fig. 9A–C). The density of cells expressing *TRPC2* was significantly higher in the LCE than in the UCE (9.083 ± 0.813 cells/mm^2 in the LCE and 2.394 ± 0.106 cells/mm^2 in the

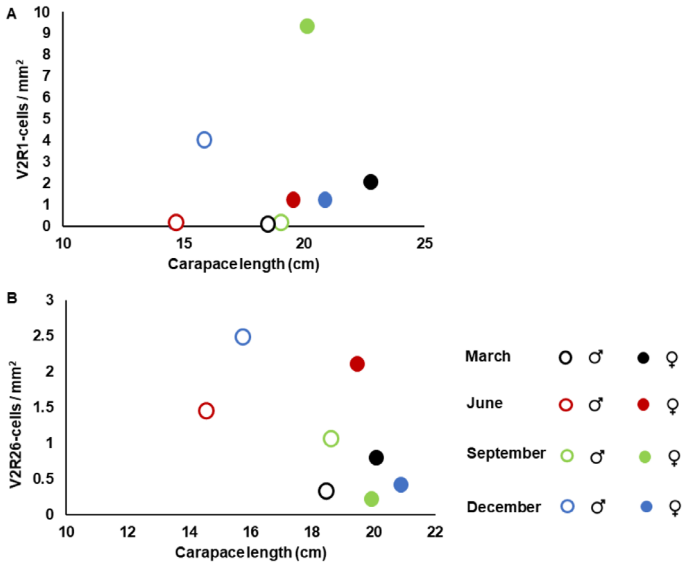

Fig. 4. The density of cells expressing *V2R1* (A) and *V2R26* (B) in the olfactory organ of red-eared sliders with different carapace lengths. Filled circles represent females; open circles represent males.

Table 3. Density of cells expressing type 2 vomeronasal receptors (*V2Rs*) in the olfactory organ of red-eared slider

Animal number	*V2R1*-cells (cells/mm²)[a)]	*V2R26*-cells (cells/mm²)[a)]
4	2.109 ± 0.163	Not available
5	Not available	0.79 ± 0.197
6	1.243 ± 0.143	2.118 ± 0.146
7	9.271 ± 0.522	Not available
8	Not available	0.202 ± 0.05
9	1.253 ± 0.209	0.431 ± 0.059
10	0	0.362 ± 0.079
11	0	1.474 ± 0.197
13	0	1.083 ± 0.123
14	4.075 ± 0.368	2.542 ± 0.178

a) Data are represented as mean ± standard error.

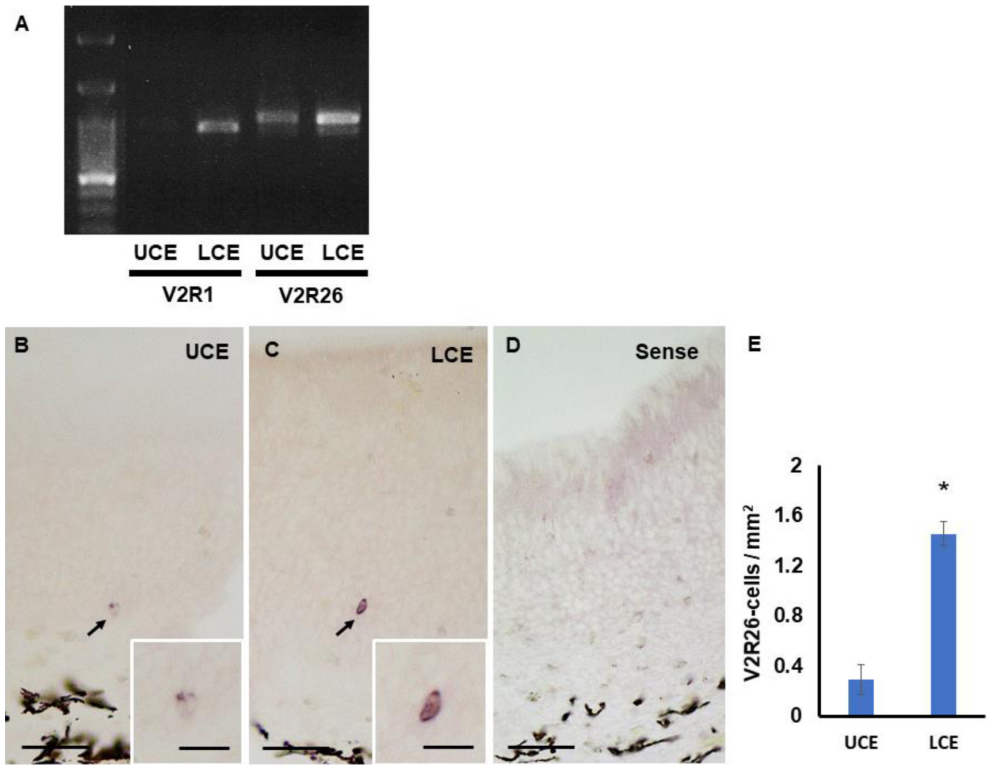

Fig. 5. Reverse transcription-polymerase chain reaction analyses for the expression of genes encoding V2Rs in the olfactory organ of soft-shelled turtle, indicating the expression of *V2R1* only in the lower chamber epithelium (LCE), whereas the *V2R26* was expressed both in the upper chamber epithelium (UCE) and LCE (A). (B, C) *In situ* hybridization analysis for the gene encoding V2R26 in the olfactory organ of soft-shelled turtle. Cells expressing *V2R26* were found both in the UCE and LCE. No signals were detected in the sections incubated with sense probe (D). Scale bars=50 μm in (B–D), 20 μm in insets. (E) The relative abundance of cells expressing *V2R26* in the UCE and LCE of soft-shelled turtle. The LCE contained significantly higher density of cells expressing *V2R26* than the UCE ($P<0.05$). Each column and vertical bar represent the mean and standard error. Asterisk indicates a significant difference between the UCE and LCE.

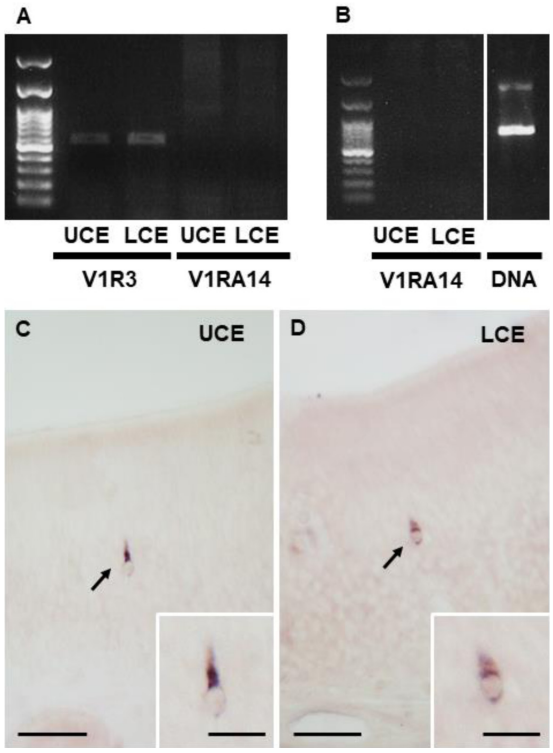

Fig. 6. Reverse transcription-polymerase chain reaction analysis for the expression of *V1Rs* in the olfactory organ of red-eared slider (A) and soft-shelled turtle (B). *V1R3* was expressed both in the upper (UCE) and lower chamber epithelia (LCE), whereas *V1RA14* was not in the olfactory organ of red-eared slider. The expression of *V1RA14* was not detected in the olfactory organ of soft-shelled turtle as well. Genomic DNA was used as a control (B). (C, D) *In situ* hybridization analysis for *V1R3* mRNA in the olfactory organ of red-eared slider. Cells expressing *V1R3* were localized both in the UCE (C) and LCE (D). Scale bars=50 μm in (C, D), 20 μm in insets.

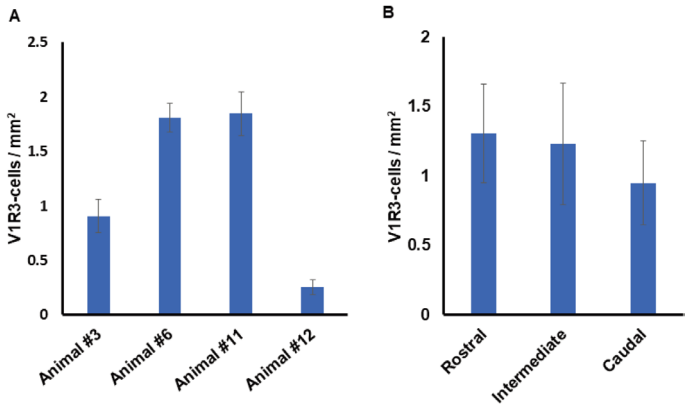

Fig. 7. Density of cells expressing *V1R3* (A) and the distribution of cells expressing *V1R3* along the rostro-caudal axis of olfactory organ (B) in red-eared slider. The density of cells expressing *V1R3* varies among individuals, whereas the density of *V1R3* expressing cells does not show any significant difference along the rostro-caudal axis of olfactory organ (P>0.05). Each column and vertical bar represent the mean and standard error (n=4).

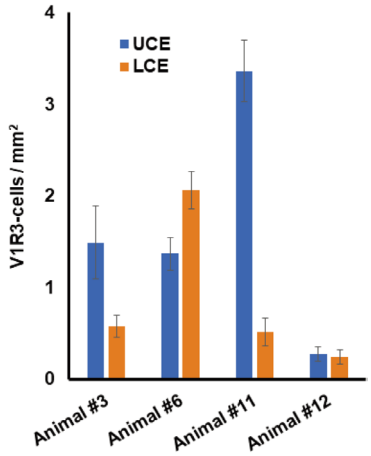

Fig. 8. The relative abundance of cells expressing *V1R3* in the upper and lower chamber epithelia of red-eared slider, showing variations among individuals.

UCE) (Fig. 9D). The density of cells expressing *TRPC2* in each chamber was almost equal to that of the cells expressing *VRs*. For instance, the density of cells expressing *TRPC2* was 2.387 cells/mm^2 in the UCE and 8.661 cells/mm^2 in the LCE, while those expressing *VRs* were 1.72 cells/mm^2 in the UCE and 6.64 cells/mm^2 in the LCE in animal #3 (Fig. 10A). Furthermore, the distribution of cells expressing *TRPC2* along the apical-to-basal axis of the epithelium closely resembled that of the cells expressing *VRs* (Fig. 10B), i.e., cells expressing *TRPC2* and those expressing *VRs* were not found in the most apical part of the epithelium, where the nuclei of supporting cells were situated. Conversely, they were present mainly in the middle to basal parts of the epithelium (bins 0.3–0.9), where the nuclei of ORNs were situated. As in the case of cells expressing *VR* genes, a significant difference was not found in the density of cells expressing *TRPC2* along the rostro-caudal axis of olfactory organ. They were almost evenly distributed along the rostro-caudal axis of olfactory organ: 6.174 ± 0.966 cells/mm^2 in the rostral, 6.081 ± 1.085 cells/mm^2 in the intermediate and 6.317 ± 0.79 cells/mm^2 in the caudal regions (Fig. 11).

Lastly, mRNA expression of the gene encoding CNGA2 was analyzed to proxy the type of olfactory receptors expressed by the majority of ORNs in the olfactory organ of red-eared slider. *In situ* hybridization analysis unveiled an extensive expression of *CNGA2* both in the UCE and LCE (Fig. 12), in support of pervious reports suggesting the expression of *ORs* by the majority of ORNs in the olfactory organ of turtles [41, 42, 55].

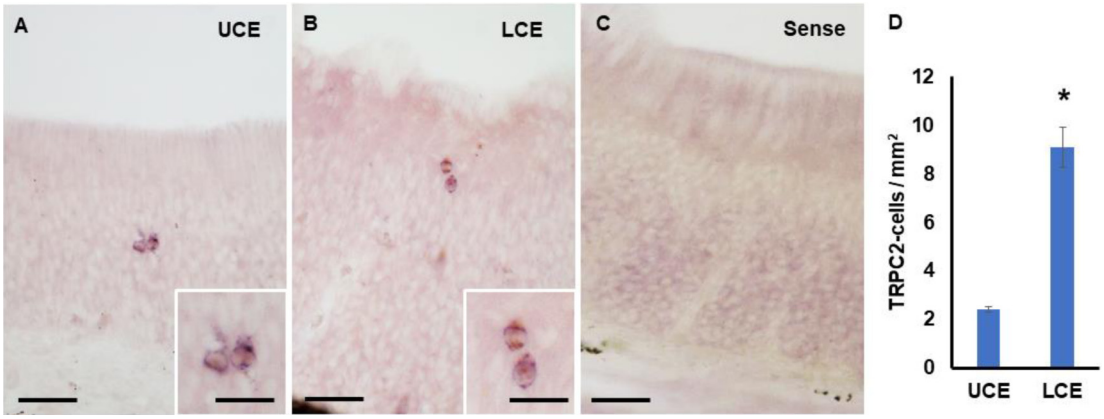

Fig. 9. *In situ* hybridization analysis for the gene encoding transient receptor potential cation channel subfamily C member 2 (TRPC2) in the olfactory organ of red-eared slider showing cells expressing *TRPC2* both in the upper (A) and lower chamber epithelia (B). No signals were detected in the section hybridized with sense probe (C). Scale bars=50 μm in (A–C), 20 μm in insets. (D) Localization of cells expressing *TRPC2* in the olfactory organ of red-eared slider. Each column and vertical bar represent the mean and standard error (n=4). Asterisk indicates a significant difference between the UCE and LCE (P<0.05).

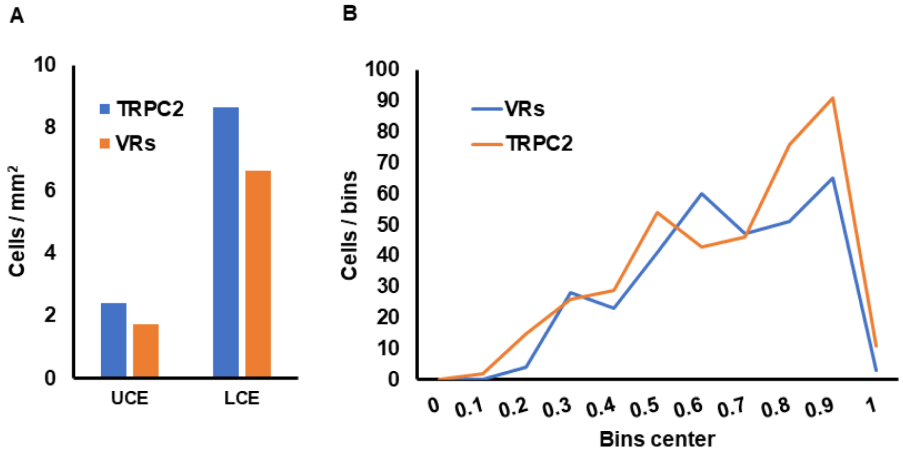

Fig. 10. Density of the cells expressing transient receptor potential cation channel subfamily C member 2 (*TRPC2*) and *VRs* (*V1R3*, *V2R1* and *V2R26*) in the upper and lower chamber epithelia of red-eared slider (A) and the distribution of cells expressing *TRPC2* and *VRs* along the apical-to-basal axis of epithelium (B). 0 is most apical and 1 is most basal.

DISCUSSION

The results in the present study demonstrated the expression of genes encoding VRs in the olfactory organ of turtles. The *V2R* genes were expressed mainly in the LCE, while a single *V1R* gene was expressed both in the UCE and LCE of red-eared slider, but not in that of soft-shelled turtle. Notably, *VR* genes were expressed by a small number of ORNs. In addition, the gene encoding TRPC2, an ion channel mediating the downstream signaling for VRs [12, 33, 59], was expressed by a small number of ORNs in a similar manner to that of *VRs*, suggesting that unknown *VR* genes are less likely to exist in turtles. Meanwhile, the gene encoding CNGA2, an ion channel involved in the signal-transduction of ORs [6, 12], was extensively expressed by the ORNs both in the UCE and LCE, suggesting the expression of *OR* genes by the majority of ORNs in the olfactory organ of turtles. Conceivably, the olfactory chemoreception of turtles might be mediated mainly by the ORs rather than the VRs.

An intimate relationship between the fine structure of ORNs and the gene expression of olfactory receptors, i.e., the expression of *OR* genes by ciliated ORNs and the expression of *VR* genes by microvillous ORNs, has been demonstrated in the olfactory organ of fish and mammals [14, 23]. However, the results in the present study indicated the expression of *VR* genes by a small population of ORNs in the LCE of turtle, despite the fact that the LCE contains microvillous ORNs [17, 41, 57]. In addition, the expression of *OR* genes by the majority of the ORNs in the LCE of turtle was suggested by the extensive expression of the gene

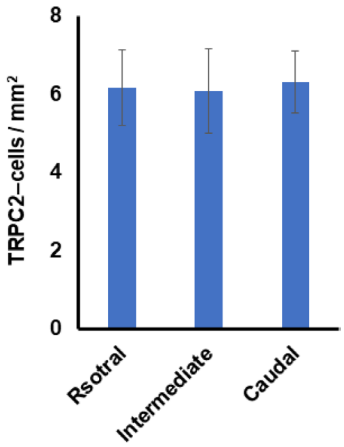

Fig. 11. Distribution of cells expressing transient receptor potential cation channel subfamily C member 2 (*TRPC2*) along the rostro-caudal axis of the olfactory organ of red-eared slider. A significant difference was not found in the density of cells expressing *TRPC2* along the rostro-caudal axis of olfactory organ (P>0.05). Each column and vertical bar represent the mean and standard error (n=4).

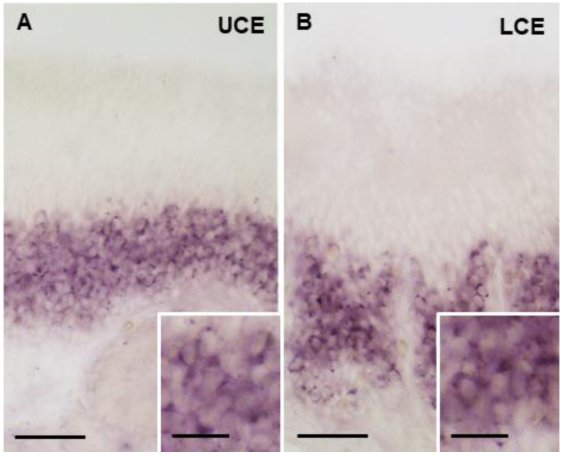

Fig. 12. Localization of cells expressing cyclic nucleotide gated channel alpha 2 (*CNGA2*) in the upper chamber epithelium (A) and lower chamber epithelium (B) of red-eared slider. Scale bars=50 μm in (A, B), 20 μm in insets.

encoding CNGA2. Thus, it is likely that the correlation between the fine structure of ORNs and the gene expression of olfactory receptors is not conserved among turtles and other vertebrates, and that the fine structure of ORNs will not always help us to predict the expression of genes encoding olfactory receptors.

The sparse expression of *V1R* gene in the olfactory organ of turtles demonstrated here is compatible with that of snakes, which is characterized by a punctate expression of *V1R* genes in the VNO [7]. This suggests that the expression of *V1R* genes by a small number of ORNs might be a common feature among reptiles. Meanwhile, comparative genomic analyses of vertebrates indicate an increase in the number of genes encoding V1Rs in terrestrial vertebrates, suggesting that the expansion in the number of genes encoding V1Rs is associated with the terrestrial adaptation [43, 48, 49]. However, such an expansion has not found in reptiles, and they retain a very small number of *V1R* genes [7, 47, 58], implying that reptiles and other vertebrates evolved different disciplinary for olfaction and that the V1R mediated-chemoreception is less important for reptiles.

Furthermore, the small number of *V1R* genes are accompanied by a large repertoire of *V2R* genes in snakes, which are expressed by the majority of ORNs in the VNO [7]. In contrast, there are only two genes encoding V2Rs in the genome of turtles [47, 58], and they are expressed by a small number of ORNs in the olfactory organ as shown here. These pieces of evidence suggest that olfactory chemoreception is much diversified among reptiles and each reptile evolved different strategies for the detection of olfactory chemical cues.

Several lines of evidence suggest that the V2Rs are involved in the detection of non-volatile substances in mammals [9, 18, 20, 28, 31]. Meanwhile, turtles have ability to detect odorants both on land and in water [16, 35, 39]. Moreover, the UCE and LCE of turtles are regarded as the air-nose and water-nose, respectively [46]. Thus, the V2Rs might be involved in the detection of non-volatile substances for turtles as well, since they were expressed mainly in the LCE as shown here.

Expression of a single *V1R* gene by the ORNs both in the UCE and LCE of red-eared slider, but not in that of soft-shelled turtle suggests the importance of V1R-mediated chemoreception in the olfactory organ of semi-aquatic turtles including red-eared slider, but not in that of highly-aquatic turtles including soft-shelled turtle. The involvement of V1Rs in the detection of volatile substances has been reported in the VNO of mice [5, 30, 32, 49]. Moreover, comparative genomic analysis among vertebrates indicates an increase in the number of genes encoding V1Rs in terrestrial animals over aquatic animals [43, 48, 49]. These pieces of evidence suggest that, although the ligand for V1Rs in turtles is not known at present, the V1R might be involved in the detection of volatile substances in the olfactory organ of semi-aquatic turtles, and that the V1R-mediated chemoreception might have been lost secondarily in highly-aquatic turtles.

The *VR* genes have been shown to be expressed in a sexually dimorphic manner in the VNO of several vertebrates [2, 24, 27]. In mice, the density of particular *V2R* genes is biased toward males and is hormone dependent [2]. In addition, the density of cells expressing some *V2Rs* is higher in female salamanders than in males [27]. In the present study, cells expressing *V2R1* were not found in the olfactory organ of male red-eared sliders sampled in March, June and September. In addition, the expression of *V2R1* was not detected by *in situ* hybridization in the olfactory organ of male soft-shelled turtle. Moreover, the density of cells expressing *V1R3* varied among individuals. These pieces of evidence are in accordance with that of Murphey *et al*., (2001) indicating the expression of the components of vomeronasal signaling cascade, including TRPC2 and Gαi1-3, in a sexually dimorphic manner [40]. Collectively, these findings suggest the presence of both sex and seasonal differences for the expression of *VR* genes in the

olfactory organ of turtles. Additional observations will be required to elucidate the sex and seasonal differences for the expression of *VR* genes in the olfactory organ of turtles in detail.

The expression site of *V1Rs* in the olfactory organ varies among vertebrates, e.g., the *V1Rs* of *Xenopus laevis* are expressed in the OE and MCE [10], whereas the *V1Rs* of mammals are principally expressed in the VNO [11, 56, 57]. Therefore, it has been speculated that the *V1R* genes have shifted their expression site from the OE to the VNO during the evolution from amphibians to mammals [10, 20]. Moreover, the results in the present study indicated the expression of a single *V1R* gene both in the OE (UCE) and VNO (LCE) of semi-aquatic turtles. These pieces of evidence suggest that the *V1R* genes begun their expression in the VNO of common ancestor of reptiles and mammals. In support of this, expression of some *V1R* genes in the VNO has been reported in snakes [7].

In the OE of mammals, ORNs bear cilia at the tip of their dendrites and each ORNs express only one *OR* gene [8, 38, 52]. Meanwhile, ORNs in the UCE of turtles bear both cilia and microvilli [17, 41, 55]. Expression of the genes encoding CNGA2 and TRPC2 demonstrated here suggests the expression of *OR* genes by the majority of ORNs and *VR* genes by a small number of ORNs in the UCE. Possibly, ORs might be expressed at the cilia and the VRs at the microvilli of distinct ORNs. Another possibility is that the ORs and VRs are expressed at the cilia and microvilli of a single ORN, respectively. Further analyses are required to ask if multiple receptors are expressed by a single ORN or not.

In the olfactory organ of mammals and amphibians, the ORNs expressing Gαolf express *ORs* and those expressing Gαo express *V2Rs* [10, 12, 19]. Therefore, by the extensive expression of both Gαolf and Gαo [41, 57], expression of both *ORs* and *V2Rs* has been suggested in the olfactory organ of turtles. However, the results in the present study demonstrated the expression of *CNGA2* mRNA by the majority of ORNs and the expression of *TRPC2* mRNA by a small number of ORNs, suggesting the expression of *ORs* by the majority of ORNs and *VRs* by a limited number of ORNs. This implies that the expression of Gαo is not necessarily associated with the expression of genes encoding V2Rs in turtles, i.e., it is not possible to infer the expression of *V2R* genes by the expression of Gαo. The involvement of Gαo in the synaptic function, such as cell-to-cell contact, has been suggested in the nervous system [26]. Thus, the Gαo may play a role in these neural activities in the olfactory organ of turtles rather than the olfactory chemoreception.

Although the fine structure of ORNs in the OE varies among tetrapods, the VNO inevitably contains microvillous ORNs [13]. Therefore, the presence of microvillous ORNs is generally accepted as a definitive characteristic of the VNO. In many turtles, the LCE contains microvillous ORNs and is regarded as the VNO [17, 41, 57]. However, present study suggested the expression of *OR* genes by the majority of ORNs in the LCE. Instead, limited number of ORNs in the LCE were demonstrated to express the genes encoding VRs, not only in the olfactory organ of soft-shelled turtle, where microvillous ORNs are not found [42], but also in the olfactory organ of red-eared slider. This is in marked contrast to the situation in the VNO of many tetrapods, which principally express *VRs* rather than *ORs*. Further studies are required to see if the expression of *VR* genes by a small population of ORNs in the LCE is a common feature among turtles.

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