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

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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, V2R7 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

Many tetraps 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 Gaolf, Gi2 and Gao, 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 Gaolf, while microvillous ORNs express V2Rs and Gao [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...
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 Goαl and Goα have been demonstrated both in the UCE and LCE [41, 42, 55]. Moreover, expression of Gaα1-3 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 CNAG2, 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 acetate 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 ml 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.
The pellets were washed with 1 ml 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 µg 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-005303611]. A list of primers is shown in Table 2. PCR was performed using Takara Ex Taq (Takara). The PCR products were divided by the area of each epithelium to obtain the density of cells expressing V2Rs and TRPC2.

### Table 2. Primers for PCR amplification

| Genes               | Forward primers | Reverse primers          |
|---------------------|------------------|--------------------------|
| Pelodiscus sinensis | gtagtctggeatcaaggtt | cctcaactgtaacctagggtt    |
| P. sinensis V2R1    | ttctaaccacagctagcgg | egaaccacagagacggggtt     |
| P. sinensis V2R26   | ggeaacctgcttgtcttt   | tttcaggccacacacgcttt     |
| P. sinensis V1RA14  | tggagagggctctcagcga | agattggaagacacagtggc      |
| P. sinensis CNGA2   | ctccgactgctgtgctgt  | cgattgagatctcggc         |
| Chrysemys picta V2R1| tggctcactctgtgccagc | agattgctgtaaaccgagg      |
| C. picta V2R26      | tcccaactcegacgcatat | caaccacagctcgagaaa       |
| C. picta V1R3       | gaaaagtaaagcacgtgta | ecatgctcggtgtgataa        |
| C. picta V1RA14     | acacactacaacagctagg | gcagacacatggagcctg       |

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/ml 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 stained with 4% PFA and immersed in 0.1% acetic anhydride in the acetylation buffer for 15 min followed by washing in 4×saline-sodium citrate (SSC) for 20 min. Post-hybridization was carried out using hybridization buffer ISSR7 (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 MgCl2, 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 V2Rs 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 V2Rs 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 V2Rs and TRPC2 were divided by the area of each epithelium to obtain the density of cells expressing V2Rs and TRPC2 (cells per mm²). Data was represented as mean ± standard error (SE). The difference in the density of cells expressing V2Rs 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.

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 VIRA14, in western painted turtle, while the V1R is encoded by a single gene VIRA14 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 VIRA14 (Fig. 6A). Also, the expression of VIRA14 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$^2$).
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/mm² in the LCE and 2.394 ± 0.106 cells/mm² in the UCE).

**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/mm² in the LCE and 2.394 ± 0.106 cells/mm² in the UCE).
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)† | V2R26-cells (cells/mm²)† |
|---------------|--------------------------|--------------------------|
| 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            |
| 12            | 0                        | 1.083 ± 0.123            |
| 13            | 0                        | 2.542 ± 0.178            |
| 14            | 4.075 ± 0.368            | 2.542 ± 0.178            |

† Data are represented as mean ± standard error.

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.

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.
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/mm² in the UCE and 8.661 cells/mm² in the LCE, while those expressing VRs were 1.72 cells/mm² in the UCE and 6.64 cells/mm² 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/mm² in the rostral, 6.081 ± 1.085 cells/mm² in the intermediate and 6.317 ± 0.79 cells/mm² 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 soft-shelled turtle. In situ hybridization analysis unveiled an extensive expression of CNGA2 both in the UCE and LCE (Fig. 12), in support of previous reports suggesting the expression of ORs by the majority of ORNs in the olfactory organ of turtles [41, 42, 55].
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, \( V1R \) 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
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 $VIR$ 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 $V1R$s in terrestrial vertebrates, suggesting that the expansion in the number of genes encoding $V1R$s 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 $V2R$s 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 $V2R$s 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 $V2R$s 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 $V1R$s 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 $V1R$s in terrestrial animals over aquatic animals [43, 48, 49]. These pieces of evidence suggest that, although the ligand for $V1R$s 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 $V1R$ 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 $V2R$s 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 $VIR$ 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 began 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 Goαlf express ORs and those expressing Goα express V2Rs [10, 12, 19]. Therefore, by the extensive expression of both Goαlf and Goα [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 Goα 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 Goα. The involvement of Goα in the synaptic function, such as cell-to-cell contact, has been suggested in the nervous system [26]. Thus, the Goα 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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