The nasal cavity in sea turtles: adaptation to olfaction and seawater flow

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Abstract

The nasal cavity of tetrapods has become phylogenetically adapted to the environment in terms of function, respiration and olfaction. In addition, the nasal cavity of sea turtles plays an important role in seawater flow and water olfaction, unlike that of terrestrial species. Here, we describe the functional, morphological, and histological characteristics of the nasal cavity, and the odorant receptors encoded in the genome of sea turtles. The nasal cavity of sea turtles is well-suited to its complicated functions, and it significantly differs from those of other animals, including terrestrial and semi-aquatic turtles.

Keywords

marine lifestyle; olfaction; respiration; sensory epithelium; vomeronasal organ

Introduction

Respiration and olfaction are the main functions of the tetrapod nasal cavity, and nasal morphological features have become phylogenetically adapted to the environment. In term of olfaction, the nasal cavity of tetrapods comprises sensory and non-sensory areas. The sensory area is histologically lined by sensory epithelium, whereas the non-sensory area is mostly covered by pseudostratified respiratory epithelium with goblet cells and associated glands to humidify air and remove foreign objects.

Distribution of the sensory areas in the nasal cavity is phylogenetically diverse. For example, the nasal cavity of urodeles has greater dorsal and lesser ventrolateral sensory areas, and the ventrolateral region in anurans is recessed to form a pair of diverticula called the "vomeronasal organ" (Taniguchi and Taniguchi 2014). In mammals, the vomeronasal organ is separate from the nasal cavity, and absent in some mammalian species. Squamates (snakes and lizards) have sophisticated vomeronasal organs that are independent of the nasal cavity, whereas they are absent in crocodiles and birds, which have only one type of sensory epithelium in the nasal cavity (Taniguchi and Taniguchi 2014).

The nasal cavities of turtles are generally separated into a dorsal olfactory region that mainly holds air, and a ventral intermediate region where water can easily enter (Parsons 1959; Schwenk 2008). Each region has a different type of sensory epithelia (Taniguchi and Taniguchi 2014), and it is generally considered that the epithelium lining the ventral intermediate region corresponds to a "vomeronasal organ (or epithelium)".

Sea turtles in the suborder Testudines are believed to have separated from general turtles about 220 million years ago (Li et al. 2008), and they are presently classified as members of the Cheloniidae (six species) and Dermochelyidae (one species) families. The structure and function of the nasal cavity are more complex in sea turtles than in turtles in general. Here, we reveal details of the sea turtle nasal cavity and its relevance to ecology.

Functions of the sea turtle nasal cavity

The nasal cavity functions not only as an airway, but also as a seawater pathway in sea turtles. They pump seawater swallowed with food through their nostrils to defend against incidental drinking (Lutz 1997). They also use buccal oscillations to move water in and out of the nostrils while swimming (Walker, 1959). Loggerhead and green sea turtles can detect both volatile and water-soluble odorants with high sensitivity, even though their lifestyle is uniquely marine. Loggerhead sea turtles recognize volatile odorants derived from foods (Endres et al. 2009) and coastal mud (Endres and Lohmann 2013), and green sea turtles also detect odorants derived from foods and scent glands of the same species (under revision) in the air as they breathe. Simulations by Endres et al. (2016) suggest that green sea turtles locate remote islands partly by using their olfactory ability to sense volatile and water-soluble odorants. The accidental consumption of marine plastic debris by loggerhead sea turtles, which has recently become an important issue, might also be associated with olfaction (Pfaller et al., 2020).

Thus, the nasal cavity of sea turtles plays critical roles in air and seawater flow and chemoreception, and thus might have quite different morphological and histological nasal characteristics from other animal species.

Morphological features of the sea turtle nasal cavity

The nasal cavity of sea turtles is complex (Parsons 1968; Saito et al. 2000), as recently confirmed by computed tomographic analyses of its detailed structures (Kondoh et al. 2019; Yamaguchi et al. 2020). Each nasal cavity consists of a tube surrounded by cartilage that runs posteroventrally from the external naris (nostril) to the internal naris (choana), which opens into the buccal cavity. The nasal passage is divided into an anterior vestibular region, a middle olfactory region called the cavum and a posterior nasopharyngeal duct. As described below, the cavum is typically complex in form, containing various chambers and fossae, which differ among species and especially between species of the families Cheloniidae and Dermochelydiae (Kondoh et al. 2019).

The cavum is divided into three large diverticula and a small fossa in green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtles belonging to the Cheloniidae family (Kondoh et al., 2019; Yamaguchi et al., 2020). We also confirmed that other Cheloniidae species, namely, hawksbill (*Eretmochelys imbricate*), olive ridley (*Lepidochelys olivacea*) and black (*C. mydas agassizii*) sea turtles, also have these four major structures in the nasal cavity (under revision). These structures are called anterodorsal (AdD), anteroventral (AvD) and

posterodorsal (PdD) diverticula and posteroventral fossa (PvF) according to their location (Fig. 1a and b). Our brief simulation using a three-dimensional model revealed that water hardly enters the PdD among these compartments (Kondoh et al. 2019), as Tucker (1971) hypothesized, suggesting that the PdD corresponds to the olfactory region (Parsons 1959), whereas the AdD, AvD and PvF correspond to the intermediate region (Parsons 1959) of terrestrial and semi-aquatic turtles. Schwenk (2008) proposed that odorants diffuse out of the water and into the air of the PdD, as water moves in and out of the sea turtle nasal cavity, although another mechanism described below apparently also plays an important role in water olfaction.

The nasal cavity of leatherback sea turtles belonging to the Dermochelyidae family is also complicated, but quite different from that belonging to the Cheloniidae family (Yamaguchi et al. 2020). The cavum of leatherback sea turtles does not contain distinct structures in the ventral region, but it has significant AdD, PdD, and two small fossae between the nostril and AdD in the dorsal region (Fig. 1c and d). The nasopharyngeal duct is wider and shorter in the leatherback (Fig. 1c), than in other sea turtle species (Fig. 1a).

Histological features of the sea turtle nasal cavity

Saito et al. (2000) published the first histological description of the nasal cavity of sea turtles. They described two types of sensory epithelium in the nasal cavity of loggerhead sea turtles, with and without associated glands. One type had olfactory glands and lined the PdD region,

whereas the other was apparently located at a region corresponding to the AdD, AvD and PvF.

The nature of the epithelium lining the nasal cavity of green sea turtles has recently been elucidated in detail (Kondoh et al. 2019; 2020) (Fig. 2). The vestibular region and nasopharyngeal duct are lined by keratinized stratified squamous epithelium (Kondoh et al. 2020), like that of whales. This type of epithelium in the main upper airway of sea turtles is more likely to protect against water flow and osmotic pressure during submersion when they forcefully pump seawater through the nostrils (Lutz 1997).

The PdD of the cavum of green sea turtles where water scarcely enters is lined by a thin sensory epithelium containing olfactory glands, like the olfactory epithelium of mammals and terrestrial turtles (Kondoh et al. 2019), suggesting that it receives volatile odorants. On the other hand, the AdD and AvD where water does enter were covered with a single thick sensory epithelium without associated glands (Kondoh et al. 2019). The PvF is lined by a thick type of sensory epithelium separated from that covering the AdD–AvD region, and a single nerve bundle derived from the PvF extends solely to the olfactory nerve tract (Kondoh et al. 2019). Therefore, the AdD–AvD region and the PvF might receive various water-soluble odorants and transfer the information through a different circuit. Because these epithelia have no gland structures in the lamina propria, both or either of these two types of sensory epithelia in the intermediate region (i.e. the AdD–AvD and the PvF) apparently correspond to the vomeronasal epithelium of mammals and squamates in terms of histological features.

Many supporting-like cells are notably negative for anti-neuron marker (HuC/HuD) in the receptor-cell zone of the AdD–AvD and PvF, but the PdD, where water scarcely enters,

contains few such cells (Kondoh et al. 2019). The olfactory or vomeronasal epithelia in mammals are devoid of such supporting-like cells. Their morphology suggests that these cells are immature supporting cells, indicating active turnover of supporting cells in the intermediate region, possibly facilitating recovery from damage caused by the movement of seawater through the nasal passage.

Figure 2 summarizes the histological features of the nasal cavity of green sea turtles described above. In contrast, the histology of the nasal cavity of leatherback sea turtles of the Dermochelyidae family, remains unknown and awaits investigation.

Odorant receptors encoded in the sea turtle genome

Odorant receptors in vertebrates are categorized as Class I and II olfactory (OR), vomeronasal type 1 (V1R) and 2 (V2R), and trace amine-associated (TAAR) types. Class I and II OR are thought to detect non-volatile and volatile odorants, respectively, whereas TAAR recognize water-soluble and volatile substances with amine-like properties. The V1R and V2R mainly bind to volatile and water-soluble substances, respectively. Mammals have many V1R and few V2R receptors, whereas in reptiles it is the converse (Brykczynska et al. 2013; Silva and Antunes 2017).

Vieyra (2011) identified 17, 14, and 20 intact OR genes in leatherback, green, and loggerhead sea turtles, respectively, and concluded that the number of functional ORs is reduced in sea turtles based on comparison of that in terrestrial and freshwater turtles. However, Wang et al.

(2013) developed a draft genome of the green sea turtle, and >100 intact OR genes were identified. More than 10 intact TAAR are also encoded in green sea turtle genome, but only two V1R genes and one V2R gene are encoded (Kondoh et al. 2019). Table 1 shows the numbers of each gene in the genome.

Which types of receptors play a role in water olfaction of sea turtles?

Our phylogenic analysis revealed that V1R in the sea turtle is shared with reptiles (Supplementary Fig. 1), and that V2R in the sea turtle is also found in many vertebrate species (Supplementary Fig. 2), indicating these vomeronasal receptors are not for sea turtle-specific. Interestingly, Green et al. (2014) showed that only one V1R and two V2R receptor genes are encoded in Chinese soft-shelled turtles, and we confirmed that there are few V1R and V2R genes encoded in western painted turtle genome (Shaffer et al. 2013) (three V1R and three V2R, Supplementary Figs. 1 and 2). Recently, *in situ* hybridization analyses by Abdali et al. (2020) revealed that TRPC2, which mediates the signal transduction of V1R and V2R, is expressed in only a few receptor cells of both dorsal (putative olfactory) and ventral (putative vomeronasal) sensory epithelia in soft-shelled and red-eared slider turtles, whereas CNGA2, that mediates the signals from OR, is expressed in most of these receptor cells. These findings suggest that the primary family expressed in the sensory epithelia in the intermediate region of sea turtles is OR, not V1R or V2R, although these epithelia are apparently homologous to the vomeronasal epithelium of mammals and squamates based on anatomical criteria.

Wang et al. (2013) revealed that the number of intact OR genes encoded in soft-shelled and green sea turtle genomes is comparable to or greater than that in mammals. In particular, the number of Class I OR genes, which are known as fish-like OR, binding hydrophilic substances, is increased in sea turtles (Wang et al. 2013), suggesting that turtles can receive a wide variety of water-soluble substances. Considering these findings together, it is speculated that sea turtles detect water-soluble odorants mainly via >150 Class I OR expressed in the sensory epithelium in the intermediate region (usually referred to as "the vomeronasal epithelium" of turtles), although future analyses of receptors in the sea turtle nasal cavity *in situ* should clarify which receptor families play a critical role in water-borne olfaction.

Conclusion

The morphological and histological features of the nasal cavity of sea turtles seem perfectly suited to its functions (air and seawater conduction and air and water olfaction). Thus, the sea turtle nasal cavity significantly differs from those of other tetrapods, including terrestrial turtles. At least in green sea turtles, the nasal cavity is complex with three sensory epithelia, but the genome encodes only a few vomeronasal receptor genes. These findings indicate that sea turtles, and perhaps also other turtles, do not have the same olfactory system as other animal species with main olfactory and vomeronasal systems. A deeper understanding of the sensory organs associated with ecology in sea turtles is needed because they are now endangered.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Table 1. Number of odorant receptor genes in green sea turtle genome.

	Class I OR	Class II OR	V1R	V2R	TAAR
General property of binding odorants	Water-soluble	Volatile	Volatile	Water-soluble	Volatile/Water-soluble
Number of genes	159	95	2	1	12

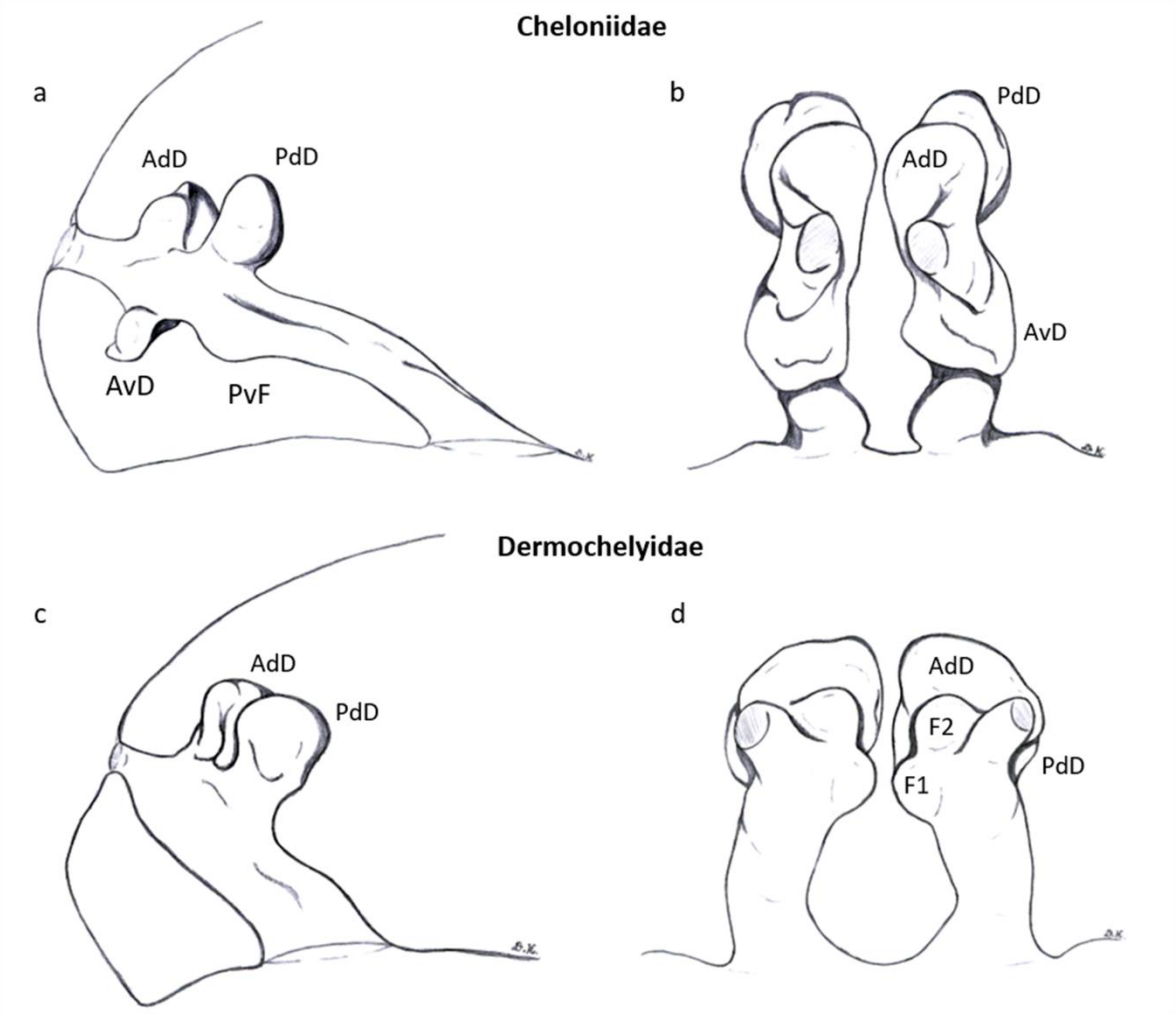

Fig. 1. Internal structure of nasal cavity in green (A and B) and leatherback (C and D) sea turtles respectively belonging to Cheloniidae and Dermochelyidae. Left lateral (A and C) and anterior (B and D) images. Cavum of green sea turtles has anterodorsal (AdD), anteroventral (AvD) and posterodorsal (PdD) diverticula, and posteroventral fossa (PvF). That of leatherback sea turtles has AdD and PdD, and two additional dorsomedial fossae (F1 and F2) near nostril, but no AvD or PvF. These illustrations are based on Yamaguchi et al. (2020).

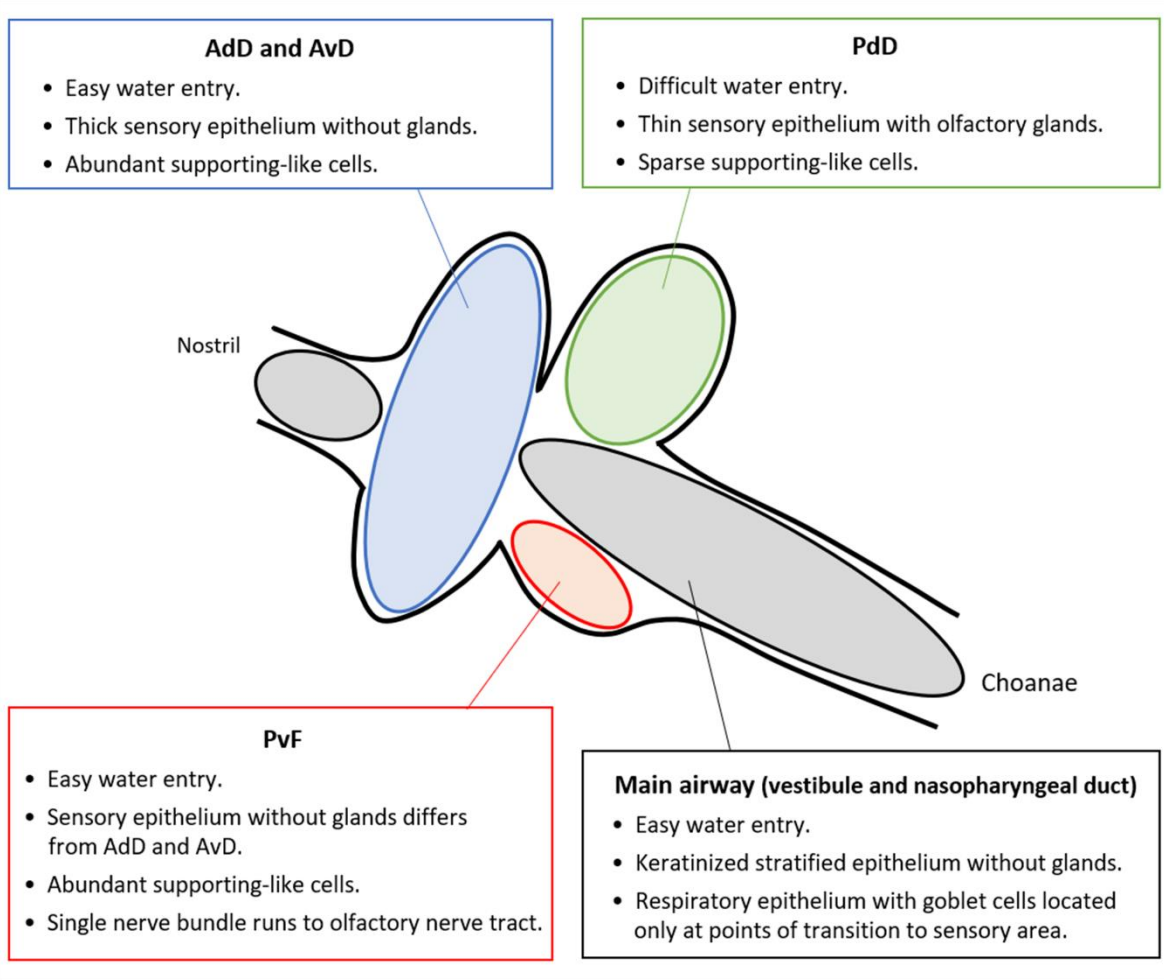

Fig. 2. Distribution of types of epithelial lining in nasal cavity of green sea turtles.

Supplementary Information

(Phylogenetic analysis of V1Rs and V2Rs in green sea turtles)

Materials and Methods

Genomic information shows that two intact V1Rs (XM_007059608.1 and XM_007067150.1) and a single V2R (EMP28964.1 that is not refseq gene) could be expressed in green sea turtles. In green sea turtle one pseudo V2R gene (Gene ID: 102943443) was also found. To investigate evolutionary diversity in green sea turtle V1R genes, TBLASTN search (Camacho et al. 2009) were performed on 11 sequenced reptile RNA sequences. The species are shown in Supplementary Table 1. Each species' RNA information was obtained from the Oct. 2018 GenBank refseq database. Query sequences were 244 individual V1R protein sequences, annotated as "Olfactory Receptor Ancestral (ORA)" or "vomeronasal type 1 receptor" in Ensembl (Zerbino et al. 2018) (release 94) or in NCBI refseq database for zebrafish (*Danio rerio*), western clawed frog (*Xenopus tropicalis*), mouse (*Mus musculus*), green sea turtle, and Chinese soft-shell turtle (*Pelodiscus sinensis*). Genes with obviously different annotation were excluded from query sequences and TBLASTN results. For phylogenetic analysis, V1R sequences in puffer fish were added to the result of TBLASTN and query sequences. Taste receptor type two genes were also added to sequences as outgroup. The amino acid sequences were aligned using multiple sequence comparison by log expectation (MUSCLE) (Edgar 2004) in Molecular Evolutionary Genetics Analysis (MEGA) X (Kumar et al. 2018). The evolutionary history of V1R genes was inferred using the Neighbor-Joining (NJ) method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling

1965) and are in the units of the number of amino acid substitutions per site. This analysis involved 254 amino acid sequences. All positions with less than 95% site coverage were eliminated. There were a total of 238 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

To investigate evolutionary history in green sea turtle V2R genes, TBLASTN search were performed same as V1R genes. Query sequences were 215 individual V2R protein sequences. For phylogenetic analysis, taste receptor type 1 genes were added to sequences as outgroup. The amino acid sequences were aligned same as V1R genes. The V2R sequences in Sauria that were too short or not well aligned were removed from alignment sequences. The sequences were aligned again using MUSCLE and the evolutionary history of V2R genes were inferred using the NJ method (Saitou and Nei 1987). The evolutionary distances were computed using the JTT matrix-based method (Jones et al. 1992) and are in the units of the number of amino acid substitutions per site. This analysis involved 384 amino acid sequences. All positions with less than 90% site coverage were eliminated. There was a total of 421 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Results

Phylogenetic tree of V1Rs

Phylogram of vertebrate V1R genes was constructed of four major clades: “fish ORA1 and other vertebrate V1Rs”, “fish ORA5”, “fish ORA3, ORA4 and turtle V1Rc”, and “reptile V1Rb” (Supplementary Fig. 1). Reptiles V1R genes were divided into three groups that were called here as V1Ra, V1Rb, and V1Rc (Supplementary Fig. 1). V1Ra genes made one clade with ORA1 of zebrafish and V1R genes of frog and mouse. V1Ra

genes were not detected in Chinese soft-shell turtle and three lizard species, and the sequence length of V1Ra in painted turtle was short because of the lack of sequence information. V1Rb genes formed one reptile unique clade and they were possessed by all reptiles that were investigated in this study. The phylogram also revealed V1Rc genes were closely related to fish ORA4 genes, and only common box turtle and painted turtle possess this ORA4-like gene.

Phylogenetic tree of V2Rs

Phylogram of vertebrate V2R genes was constructed of three major clades: "zebrafish frog V2Rs", "vertebrate V2Rs family C", and the other clade that contains "Sauria V2Rs". Reptile V2R genes were divided into two groups that were "vertebrate V2Rs family C" and "Sauria V2Rs" (Supplementary Fig. 2), although in any Crocodilia V2R gene were not detected by TBLASTN. The number of Sauria V2Rs was very small in turtles, and the V2R gene in green sea turtle (EMP28964.1) was contained in family C.

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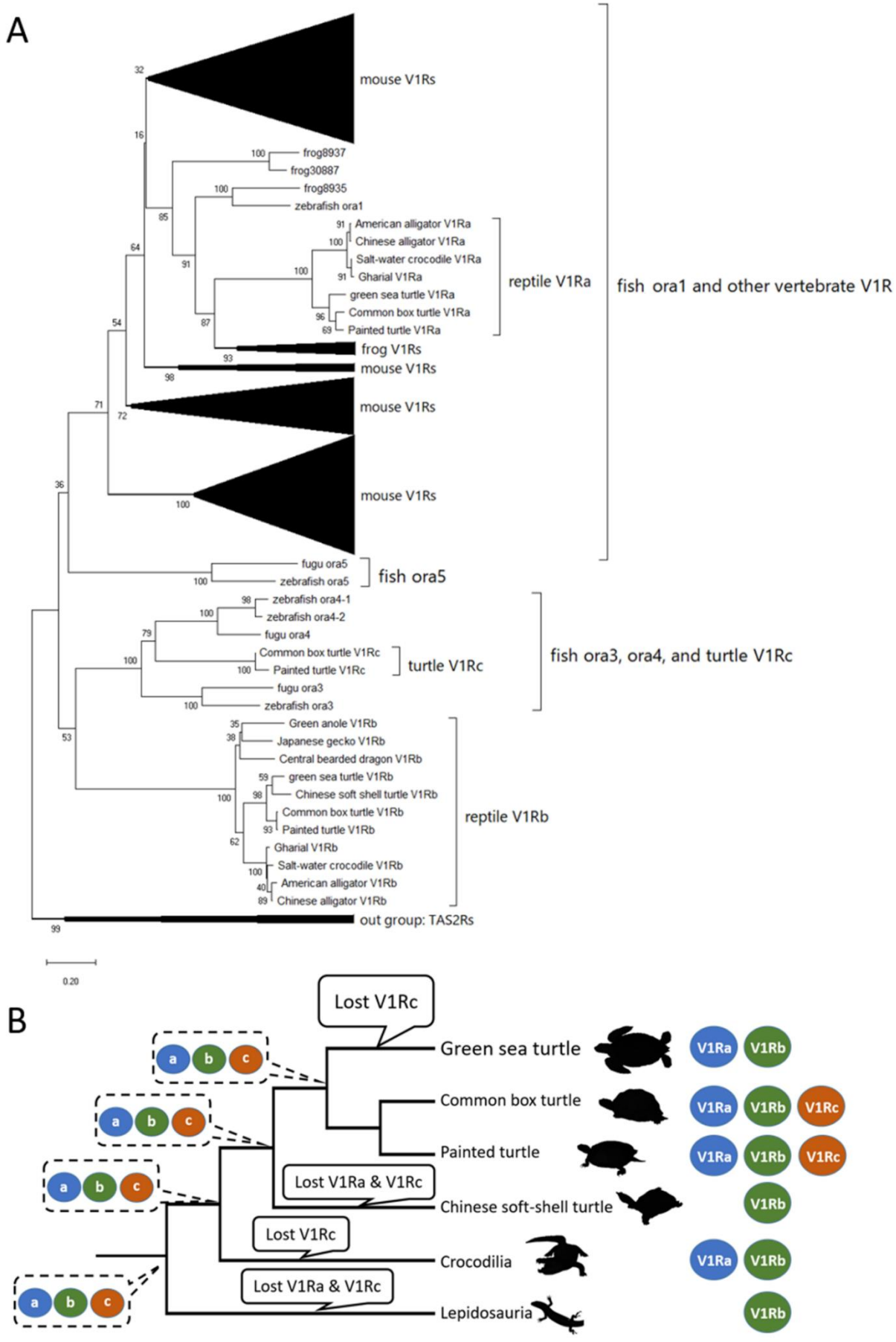
Supplementary Table 1.

The reptile species used in the present molecular phylogenetic analysis.

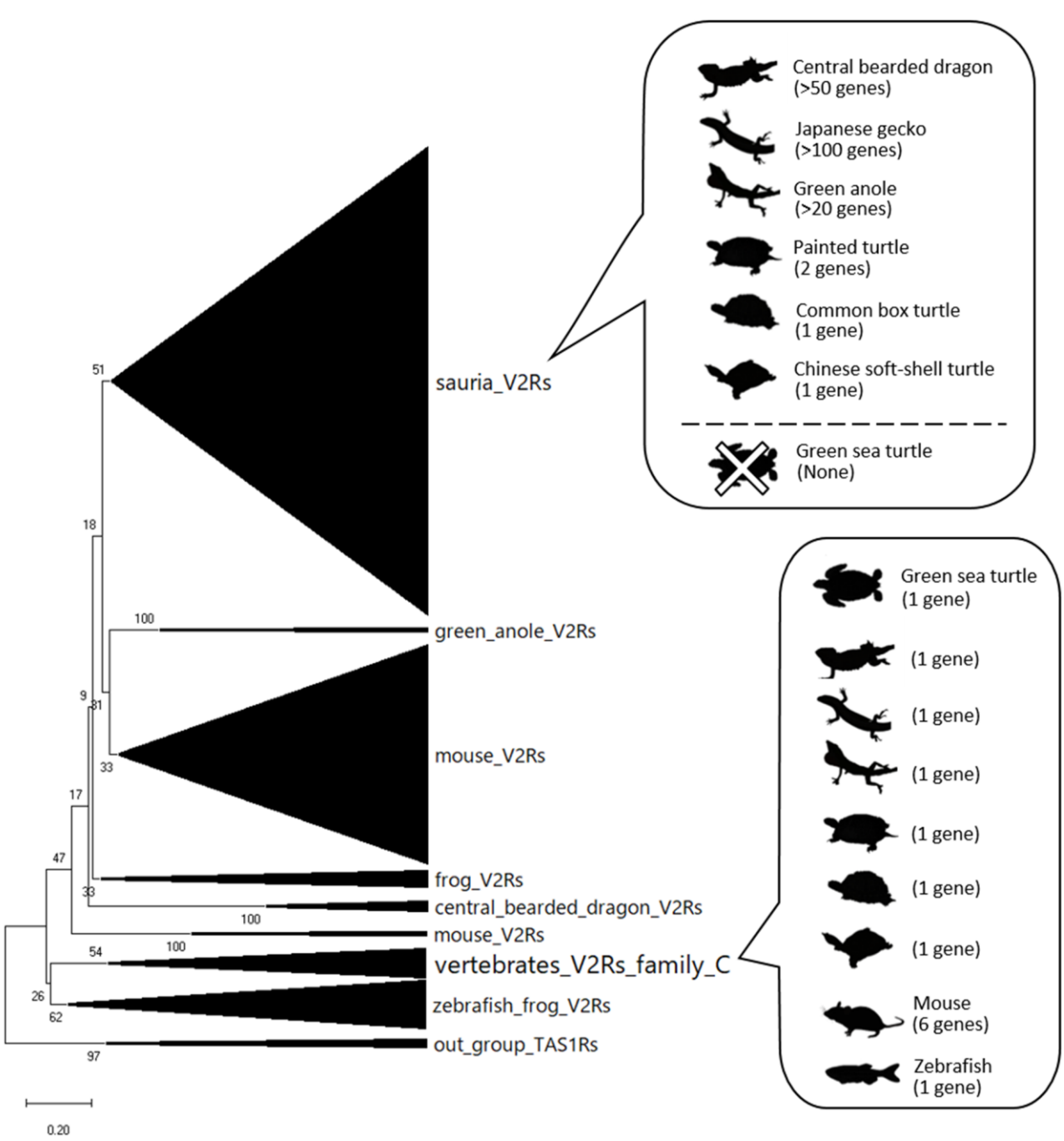
Species	Scientific name
Turtles	
Green sea turtle	*Chelonia mydas*
Chinese soft-shell turtle	*Pelodiscus sinensis*
Common box turtle	*Terrapene mexicana*
Painted turtle	*Chrysemys picta*
Crocodilians	
American alligator	*Alligator mississippiensis*
Chinese alligator	*Alligator sinensis*
Salt-water crocodile	*Crocodylus porosus*
Gharial	*Gavialis gangeticus*
Lizards	
Japanese gecko	*Gekko japonicus*
Central bearded dragon	*Pogona vitticeps*
Green anole	*Anolis carolinensis*



Supplementary Fig. 1.

Supplementary Fig. 1. Phylogram of vertebrate V1R genes.

(A) Phylogenetic tree constructed in MEGA X using 254 amino acid sequences comprises four major clades. Reptilian V1R genes form “V1Ra”, “V1Rb” and “V1Rc” groups. V1Ra genes belong to fish ORA1 and other vertebrate V1R clade. V1Rb genes form one unique reptile clade. V1Rc genes belong to fish ORA3 and ORA4 clade. (B) V1R gene loss in reptiles. Balloons with dotted line, inferred ancestral V1R genes. Pictures of sauria, Crocodilia and Chinese soft-shell turtle are modified from the Togo picture gallery (http://togotv.dbcls.jp/ja/pics.html), licensed under CC-BY 4.0 Togo picture gallery by the Database Center for Life Science (DBCLS), Japan. Other illustrations are modified from resources in public domain.



Supplementary Fig. 2.

Supplementary Fig. 2. Phylogram of vertebrate V2R genes.

Phylogenetic tree constructed in MEGA X using 384 amino acid sequences comprises three major clades. Many vertebrates including zebrafish, mouse, and reptiles except Crocodilia possess “vertebrate V2R family C” genes. Reptilian V2R genes are divided into two groups; family C and a unique reptile clade “Sauria V2Rs”.