Inactivation of *ancV1R* as a predictive signature for the loss of vomeronasal system in mammals

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Abstract

The vomeronasal organ (VNO) plays a key role in sensing pheromonal cues, which elicit social and reproductive behaviors. Although the VNO is highly conserved across mammals, it has been lost in some species that have evolved alternate sensing systems during diversification. In this study, we investigate a newly identified VNO-specific gene, \textit{ancV1R}, in the extant 261 species of mammals to examine the correlation between genotype (\textit{ancV1R}) and phenotype (VNO). As a result, we found signatures for the relaxation of purifying selection (inactivating mutations and the elevation of $dN/dS$) on \textit{ancV1R}s in VNO-lacking mammals such as catarrhine primates, cetaceans, the manatees, and several bat lineages, showing the distinct correlation between genotype and phenotype. Interestingly, we further revealed signatures for the relaxation of purifying selection on \textit{ancV1R} in true seals, otters, the fossa, the owl monkey, and alcelaphine antelopes in which the existence of a functional VNO is still under debate. Our additional analyses on \textit{TRPC2}, another predictive marker gene for the functional VNO, showed a relaxation of purifying selection, supporting the possibility of VNO-loss in these species. The results of our present study invite more in-depth neuro-anatomical investigation in mammals for which VNO function remains equivocal.

Key words: vomeronasal organ, \textit{ancV1R}, pheromone, mammal, pseudogene
1. Introduction

The vomeronasal organ (VNO) is a chemosensory structure found in terrestrial vertebrates that is anatomically separated from the main olfactory epithelium (MOE). VNO detects pheromones, whereas MOE mainly detects odorants (Firestein 2001; Brennan and Zufall 2006). Although some overlaps were found between VNO and the MOE in pheromone detection (Mandiyan et al. 2005; Ohara et al. 2009), the VNO plays a major role in eliciting the pheromone-induced sexual and social behaviors. Actually, the surgical ablation of the VNO in mice led to a number of behavioral and physiological deficits in their response to pheromonal cues (Meredith 1986; Wysocki and Lepri 1991). The VNO structure can be found across a broad diversity of mammals because of its importance to pheromone mediated communication, essential for fitness and survival (Doving and Trotier 1998). However, morphological studies have shown that some mammals do not possess anatomically intact VNOs. For example, cetaceans and sirenians lost the VNO in parallel accompanied by their independent adaptation to fully aquatic lifestyles (Lowell and Flanigan 1980; Switzer et al. 1980; Mackay-Sim et al. 1985; Oelschläger 1989). Catarrhine primates also lost their functional VNO (Bhatnagar and Meisami 1998) which may be due to their adaptation to a diurnal lifestyle and the acquisition of trichromatic color vision (Dixson 1983). In bats, most species have lost their VNO except for two families (Phyllostomidae and Miniopteridae; Wible and Bhatnagar 1996). These independent losses on each bat lineage are still puzzling. The VNO structure in New World monkeys is still intact, but reductions of some microanatomical components have been suggested (Hunter et al. 1984; Smith et al. 2011).
Among semi-aquatic pinnipeds, otariids (fur seals, sea lions) and odobenids (walrus) retain the VNO whereas this organ is lost in phocids (true seals), (Mackay-Sim et al. 1985).

*TRPC2* (transient receptor potential C), which is specifically expressed in most VNO sensory neurons, was shown to be essential for a fully functional VNO (Stowers et al. 2002; Liman and Dulac 2007). Previous studies reported that the inactivation of *TRPC2* is correlated with the loss of anatomically intact VNO in catarrhine primates (Liman and Innan 2003; Zhang and Webb 2003), cetaceans (Yu et al. 2010) and some bats (Zhao et al. 2011; Yohe et al. 2017, 2018), otters and true seals (Yu et al. 2010, Hecker et al. 2019). Thus, *TRPC2* has been used as a genetic marker to examine the existence of a functional VNO (Summarized in Fig. 1).

Recently, we characterized a novel VNO-specific gene, named *ancV1R* (Suzuki et al. 2018). *ancV1R* belongs to the vomeronasal receptor type-1 (*V1R*) gene family, which encode members of a pheromone receptor family and is highly variable in number and repertoire among mammals due to extensive gene gain and loss (Grus et al. 2005; Young et al. 2010, Silva and Antunes 2017; Nikaido 2019). However, *ancV1R* is quite distinct from canonical *V1Rs* of mammals in that just one orthologous gene is shared among most bony vertebrates. In addition, *ancV1R* is expressed in all VNO sensory neurons and co-expressed with canonical *V1Rs*, implying the essential role of *ancV1R* in the function of the VNO (Suzuki et al. 2018).

In this study, we conducted comprehensive analyses on *ancV1R* sequences using all currently available vertebrate genome assemblies in the NCBI database. The *ancV1Rs*...
were investigated based on two criteria: 1. The presence/absence of inactivating mutation(s), which includes frameshift or premature stop codon mutations; and 2. the $\omega$ ($dN/dS$) ratios, which can be an indicator of the selective pressure acting on a protein-coding gene. As a result, the inactivating mutations were observed in cetaceans, sirenians, catarrhine primates, and most bat lineages, supporting our previous study (Suzuki et al. 2018, Fig. 1). The elevation of $\omega$ ratios were also observed in these groups, strongly suggesting the relaxation of purifying selection. The relaxation of purifying selection on \textit{ancV1R} is consistent with the loss of functional VNO in these groups. These results demonstrate that the presence/absence of inactivating mutation(s) as well as the $\omega$ ratios of \textit{ancV1R} can be used as an ideal genetic marker to evaluate the biological status of the VNO. By applying \textit{ancV1R} as a reliable diagnostic indicator of VNO function, we revealed the relaxation of purifying selection in \textit{ancV1Rs} for true seals, the sea otter, the giant otter, the fossa, the owl monkey and alcelaphine antelopes, in which the existence of a functional VNO is still under debate. To evaluate the possibility of VNO-loss in these species, we additionally investigated \textit{TRPC2}s and found the signatures for the relaxation of purifying selection, supporting the results of \textit{ancV1R}. By showing the relaxation of purifying selection in \textit{ancV1R} as well as \textit{TRPC2}, we propose the possibility that the VNOs were degenerated in these species. Thus, \textit{ancV1R}, together with additional molecular markers may provide valuable new insights into the neuro-anatomical status of the VNO in a broad diversity of mammals.
2. Methods

2.1. Sequence collection, alignments and inactivating mutations

Nucleotide tBLASTn searches were performed using intact *ancV1R* sequences identified by a previous study (Suzuki et al. 2018) as queries against all vertebrate species with genomic sequences in the NCBI whole-genome shotgun contig database. A representative *ancV1R* sequence in each order was used as the query for BLAST searching against genomes of all species in the same order. Complete *ancV1R* sequences were identified from 341 species, which include four orders of ancient fishes, two orders of amphibians, four orders of reptiles, and 23 orders of mammals (summarized in Supplementary Table S1). We excluded the *ancV1R* sequences, which contain multiple “N” base call ambiguities due to reference assembly problems. We also excluded the redundant *ancV1R* sequences of closely related species belonging to the same genus and used only one representative species (see Supplementary Table S1). In total, 298 sequences were used for the analyses. All obtained nucleotide sequences were translated into amino acid sequences and the sequences containing premature stop codons were identified as pseudogenes using Biopython (Cock et al. 2009) and visual inspection. The remaining amino acid sequences were identified as intact and aligned in MAFFT using default parameters of mafft-linsi (Katoh et al. 2002). This multiple alignment of amino acid sequences was converted into a codon alignment by PAL2NAL (Suyama et al. 2006). To align nucleotide sequences for pseudogenes into this codon alignment, we used the “--add” option (Katoh et al. 2012) in MAFFT and minor adjustments by eye (Supplementary Alignment File 1). We also used this integrated multiple alignment to inspect inactivating
mutations including frameshifts, altered start codons, and premature stop codons. The mutations of inactivated \textit{ancV1R} sequences were inspected to distinguish between two possibilities (assembly error or true inactivation) by megablast searches against the multiple reads of their corresponding assemblies (NCBI SRA: https://www.ncbi.nlm.nih.gov/sra/). In addition to \textit{ancV1R}, we also mined the longest exon 12 of \textit{TRPC2} (720 bp, starting from phase 0 of the reading frame) for Afrotheria, Primates, Carnivora, Cetartiodactyla, and Chiroptera, using \textit{TRPC2} sequences of cow and mouse (Ensembl ID: ENSBTAE00000396459 and ENSMUSE00001323118) as queries. The resultant sequences were inspected for the presence/absence of inactivating mutations. The entire alignment for the exon 12 of \textit{TRPC2} is shown in Supplementary Alignment File 2.

2.2. Phylogenetic analysis

Phylogenetic trees for \textit{ancV1R} genes were generated using RAxML 8.2.4 (Guindon and Gascuel 2003) with the GTR+I+G nucleotide substitution model. The best fitting nucleotide substitution model was selected by jModelTest2 (Darriba et al. 2012) based on the Akaike Information Criterion (AIC) scores. Rapid bootstrap analyses were performed with 1000 replicates for assessing the reliability of nodes. We also used some sequences of fish \textit{V1R5} and \textit{V1R6} included in Suzuki et al. (2018) as outgroups.

2.3. Selection analyses
Calculations of the nonsynonymous to synonymous ratios ($\omega = dN/dS$) for measuring natural selection on sequences of ancV1R or exon 12 of TRPC2 were performed with the codeml program in PAML 4.8 (Yang 2007). Generally, functional genes are under purifying selection and their $\omega$ values are < 1, but the $\omega$ values for nonfunctional genes are elevated to 1. We used the branch models (Yang 1998; Yang and Nielsen 1998) in codeml for examining changes of $\omega$ values between foreground and background branches. Selection analyses were performed with separate data sets for each lineage that have inactivated ancV1R or TRPC2 and their corresponding backgrounds which contain all intact sequences in the same order. In the present study, we included the pseudogenized sequences in the selection analyses because the inactivation should be validated from several lines of evidence, 1. existence of the frameshift or premature stop codon, 2. existence of the inactivating mutation in short read data (SRA), and 3. elevation of $\omega$ value. We used branch-site model in codeml for examining the possibility of positive selection in ancV1Rs with high $\omega$ values without frameshift or premature stop codon mutations. We also used free ratio model in codeml for exploratory search of the branches with elevated $\omega$ values of ancV1Rs in the mammalian tree. For this analysis, we eliminated the ancV1R sequences for which the relaxation of purifying selection was examined in detail by using branch model analyses and statistical test.

The data sets were composed of subsets of aligned sequences extracted from the comprehensive sequence alignment (Supplementary Alignment File 1 or 2) in accordance with the tree topologies for foreground and background branches (Fig. 3). Frameshift insertions, lineage-specific in-frame insertions, and stop codons were deleted prior to
each selection analysis. For the tree topologies of mammals used in codeml analyses, we referred to the Open Tree of Life database (Hinchliff et al. 2015) (Fig. S2). For the tree topology for antelopes, which was not available in the database, we referred to Bärmann et al. (2013) and Chen et al. (2019).

The most appropriate codon frequency models for each selection analysis were selected from the following list: CF0 (codon frequencies are assumed to be equal); CF1 (codon frequencies are calculated from the average nucleotide frequencies); CF2 (codon frequencies are calculated from the average nucleotide frequencies at the three codon positions); and CF3 (codon frequencies are used as free parameters), based on AIC scores of the analyses which performed with all intact \textit{ancV1R} or \textit{TRPC2} sequences of mammals. CF0 was found to be the most appropriate model for \textit{ancV1R} (Table S4) and CF1 was for \textit{TRPC2} (Table S5). To evaluate the significance of $\omega$ for foreground branches, likelihood ratio tests were used to compare likelihoods of the multiple $\omega$ category models which assumed selective pressures were changed at the foreground branches, against that of their corresponding one $\omega$ category models which assumed selective pressures were not changed.
3. Results

3.1. Characterization of ancV1Rs in vertebrates

In the present study, we extensively analyzed a total of 298 ancV1R sequences of vertebrates (see details for methods section). ancV1Rs were not found in teleosts and birds, and were inactivated by many insertions and deletions in crocodilians and testudines (crocodiles, alligators, and turtles; Supplementary Table S3), which confirms our previous study (Suzuki et al. 2018) and is consistent with the absence of intact VNO in these species (e.g. Silva and Antunes, 2017). We found only one copy of ancV1R in most species except for the African clawed frog, which has both an intact gene and a pseudogene on chromosome 1S and 1L, respectively. Importantly, all ancV1R sequences identified in this study are located in the first intron of SNCAIP (synuclein alpha interacting protein), suggesting that the synteny relationship between ancV1R and SNCAIP is conserved among all bony vertebrates from the polypterus to mammals (Suzuki et al. 2018). Supplementary Table S2 summarizes the variation in length of the intact ancV1Rs of mammals. In the intact sequences, the lengths are 972bp in most cases with some exceptions due to in-frame insertions or deletions. Fig. 2 and Supplementary Fig. S1 shows the maximum-likelihood tree of ancV1R including intact and pseudogene sequences. The teleost V1R5 and V1R6 were used as outgroups of ancV1R because of their phylogenetic closeness (Suzuki et al. 2018). The resultant ancV1R gene tree is mostly consistent with species tree. The two lines of evidence, the conservation of the synteny relationship as well as the consistency in gene tree and species tree, suggest that the ancV1R sequences obtained in the present study are single copy and orthologous.
3.2. Relaxation of purifying selection in ancV1R of the manatee, cetaceans, and catarrhines

Next, we focused on the inactivating mutations of ancV1Rs in mammals. The inactivation of ancV1Rs in the manatee, cetaceans, and catarrhines are consistent with the previous study of ancV1R (Fig. 1; Suzuki et al. 2018). More specifically, the manatee has two frameshift insertions, two nonsense mutations, and one frameshift deletion (Fig. 3A). Cetaceans share one frameshift deletion that has occurred on the stem cetaceans branch (Fig. 3B). In catarrhine primates, each of the three families of catarrhines (Hominidae, Cercopithecidae, Hylobatidae) has independent inactivating mutations, which are not shared among the different families. Hominidae shares one nonsense mutation, Cercopithecidae has one frameshift deletion and one frameshift insertion, and Hylobatidae has one frameshift deletion and one nonsense mutation (Fig. 3C). These data add further evidence that ancV1Rs are not intact in the three groups. In addition to the inactivating mutations, we newly estimated selective pressures on each lineage by calculating and comparing the \( \omega (= dN/dS) \) ratio. The selection analyses for the manatee, cetaceans, and catarrhines were performed with two \( \omega \) categories to examine whether or not selective pressures are relaxed in these lineages: 1) a category for each foreground branch for the manatee, cetaceans or catarrhines; and 2) a category for each background branch of afrotherians, cetartiodactyls and perisodactyls, and Primates, respectively (Fig. 3 A-C). The \( \omega \) values for the manatee (1.99), cetaceans (1.09), and catarrhines (1.08) are higher than their background \( \omega \) values (0.38, 0.46 and 0.44), indicating that purifying
selection on background branches is relaxed in these lineages, that were all supported by likelihood ratio test ($p = 2.7 \times 10^{-4}, 1.4 \times 10^{-7}$ and $1.0 \times 10^{-5}$; Table S6).

In the manatee, cetaceans, and catarrhines, we also investigated the exon 12 of TRPC2 using same strategies with ancV1R. We found inactivating mutations and signatures for the relaxation of purifying selection (Fig. S3A, B, C). The $\omega$ values for the manatee (0.48), cetaceans (0.66) and catarrhine primates (1.24) are higher than their backgrounds (0.08, 0.11 and 0.13), that were all supported by likelihood ratio test ($p = 5.0 \times 10^{-6}, 3.2 \times 10^{-21}$ and $1.4 \times 10^{-21}$; Table S7). Thus, the signatures for the relaxation of purifying selection on ancV1R and TRPC2 are correlated with each other in these groups. It is noteworthy that the $\omega$ values are lower in TRPC2 than in ancV1R for all comparison regardless of the relaxation of purifying selection. The lower $\omega$ values of TRPC2 imply that the selective constraint on amino acid sequence is strict in this protein as compared with ancV1R.

3.3. Additional signatures for the relaxation of purifying selection in ancV1R of semi-aquatic mammals

In addition to the previously described species, we newly discovered the inactivations of ancV1R in semi-aquatic mammals including otters and true seals. We found that ancV1Rs of both giant otter and sea otter are inactivated by a frame-shift mutation at different positions (Fig. 3D). The $\omega$ value of otters (0.88) is higher than background (0.40) ($p = 0.029$; Table S6), and suggests that selective constraints on ancV1R were relaxed by aquatic adaptation. We found the signature for relaxation of purifying selection in true seals but not in other pinnipeds (fur seals, walrus). Among the
four true seals, *ancV1R* of northern elephant seal is inactivated by a frame-shift deletion and the others are intact (Fig. 3E). To assess whether selective constraints are relaxed not only in northern elephant seal but also in the other three true seals, selection analyses were performed for true seals with three ω categories that separated categories for northern elephant seal, the other three true seals, and background. The elevated ω value of 999 in northern elephant seal is due to the absence of synonymous substitutions. The ω value (1.28) in three true seals is higher than background (0.40) even though that they are intact at the sequence level. These results are supported by the likelihood ratio test (*p* = 0.0068; Table S6), suggesting that relaxation of purifying selection on *ancV1R* in all true seals.

We next performed the evolutionary analyses on *TRPC2* of otters and true seals. Although exon 12 of *TRPC2* were intact at the sequence level in giant otter and sea otter, ω value of the otters (0.49) is higher than background (0.12) (Fig 3D; *p* = 4.2 x 10⁻⁴, Table S7). The results are consistent with the previous studies showing that river otter has a nonsense mutation (Yu et al. 2010) and sea otter has a mutation at the splicing site (Hecker et al. 2019). These data suggest that selective constraint on *TRPC2*s were relaxed among otters of the subfamily Lutrinae. In true seals, a nonsense mutation on *TRPC2* is shared among all of the four species (Fig S3D). The ω value in true seals (0.57) is higher than background (0.12) (Fig 3E; *p* = 8.7 x 10⁻⁷, Table S7). Thus, the relaxation of purifying selection on *TRPC2* are consistent with the results of *ancV1R* in otters and true seals.

3.4. Further signatures for the relaxation of purifying selection in *ancV1R* of several
terrestrial mammals

We further discovered the inactivating mutations on ancV1R in the fossa, the owl monkey and alcelaphine antelopes. ancV1R of the fossa has one nonsense mutation and one frameshift deletion (Fig. 3F). ancV1R of the owl monkey is also inactivated by one frameshift deletion (Fig. 3G). Although it is not statistically significant, the $\omega$ values of the fossa (1.52) and the owl monkey (0.83) are higher than their background (0.40, 0.40; $p = 0.055$, 0.26, Table S6). The present findings of the inactivating mutations and elevation of $\omega$ values in the fossa and the owl monkey suggest the relaxation of purifying selection on ancV1Rs in these species.

In the fossa, we revealed that TRPC2 is inactivated by a nonsense mutation and the $\omega$ value (0.45) is higher than background (0.12) (Fig. S3E; $p = 0.0055$, Table S7). In the owl monkey, TRPC2 is intact but the $\omega$ value (0.83) is higher than background (0.13) (Fig 3G; $p = 0.028$, Table S7), which is consistent with the previous study (Liman and Innan 2003). Taken together, selective constraints are relaxed in ancV1R of the fossa and the owl monkey, that are supported by the results of TRPC2.

Among four alcelaphine antelopes (the hartebeest, the hirola, the topi, and the blue wildebeest), ancV1Rs are intact at the sequence level except for the hartebeest, which has a start codon mutation (Fig. 3H; in-frame “ATG” was not found within 100bp upstream or downstream). It is worth noting that flehmen response, which is a typical behavior for catching pheromones, is observed solely in blue wildebeest but not in the hartebeest, the hirola and the topi (Hart et al. 1987). To examine whether or not selective pressure was relaxed in alcelaphine antelopes, we assigned separate $\omega$ categories for branches of the
hartebeest, the blue wildebeest, and the hirola and the topi. The \( \omega \) value of 999 for the hartebeest branch is due to the absence of synonymous substitutions. The \( \omega \) value for hirola and topi (1.77) is higher than background (0.45) (Fig. 3H). The \( \omega \) value for the blue wildebeest (0.44) is similar to background (0.45). Although the result is not significant by likelihood ratio test (\( p = 0.091 \); Table S6), the selective constraint on \( \text{ancV1R} \) is likely to be relaxed in the hartebeest, the hirola and the topi but not in the blue wildebeest.

In alcelaphine antelopes, exon 12 of \( \text{TRPC2} \) are all intact at the sequence level. However, the \( \omega \) values of the hartebeest (999, absence of synonymous substitutions), the hirola and the topi (0.97) are higher than background (0.10). The \( \omega \) value of blue wildebeest (0.00, absence of nonsynonymous substitutions) was similar to background (0.10). This result is significant by likelihood ratio test (Fig 3H; \( p = 0.001 \), Table S7). Thus, the \( \text{TRPC2} \) show same evolutionary trend with \( \text{ancV1R} \) in that the selective constraint was relaxed in the hartebeest, the hilora and the topi, but not in the blue wildebeest. The results of \( \text{ancV1R} \) and \( \text{TRPC2} \) are both consistent with behavioral study in that the flehmen response is observed solely in the blue wildebeest among alcelaphine antelopes.

3.5. Existence of intact and inactivated \( \text{ancV1Rs} \) in bats

Previously, Suzuki et al. (2018) analyzed 8 bat species and identified the inactivation of \( \text{ancV1Rs} \) in 3 (Vespertilionidae, Rhinolophidae, Pteropodidae) of 5 families. In the present study, we newly added a total of 11 families (33 species) for the analyses. Inactivation of the VNO, \( \text{ancV1R} \) and \( \text{TRPC2} \) were correlated in most bats but not in
some bats (Table S8). The precise sequence alignment revealed that \( ancV1R \)s are inactivated in four (Vespertilionidae, Rhinolophidae, Molossidae, Pteropodidae) of 11 families (Fig. 3I). The frameshift insertions independently occurred in Pteropodidae (Long-tongued fruit bat, etc.) and Rhinolophidae (Greater horseshoe bat). Vespertilionidae (Little tube-nosed bat, etc.) possess a frameshift insertion except for the big brown bat, in which a 6 bp insertion at the same site makes \( ancV1R \) apparently in-frame (Fig. 3I). In Molossidae (Brazilian free-tailed bat), start and stop codon positions are both moved to 16bp and 25bp downstream, respectively, which may inactivate \( ancV1R \).

Next, we estimated the \( \omega \) values of \( ancV1R \) and \( TRPC2 \) for each family of bats. Given that there are several bat families which have intact \( ancV1R \) in spite that their VNO and \( TRPC2 \) are inactivated (for example, Mormoopidae, Noctilionidae, Hipposideridae and Megadermatidae; Table S8), we assigned separate \( \omega \) categories to each family to evaluate \( \omega \) values. The \( \omega \) values of \( ancV1R \) for Pteropodidae (0.90), Rhinolophidae (0.73), Molossidae (1.93) and Vespertilionidae (1.06) are higher than background (0.43) (Fig. 3I; \( p = 4.8 \times 10^{-4} \), Table S6), suggesting the relaxation of purifying selection in these families. The \( \omega \) values of \( TRPC2 \) for Pteropodidae (0.86), Rhinolophidae (0.83), Molossidae (1.56) are higher than background (0.11) (Fig. 3I; \( p = 2.3 \times 10^{-13} \), Table S7), except for Vespertilionidae, in which exon 12 of \( TRPC2 \) were not found in the genome. The results of \( ancV1R \) and \( TRPC2 \) are both consistent with the absence of intact VNOs in these four families.

The \( \omega \) values of \( ancV1R \) for Phyllostomidae (Jamaican fruit-eating bat, etc.) (0.54)
and Miniopteridae (Natal long-fingered Bat) (0.48) were similar to the background (0.43). The ω values of TRPC2 for Phyllostomidae (0.15) and Miniopteridae (0.10) were also similar to background (0.11) (Fig. 3I). The results of ancV1R and TRPC2 are both consistent with that they possess intact VNOs (Yohe et al. 2017).

The inactivation of ancV1R and the loss of an anatomically intact VNO as well as the inactivation of TRPC2 are tightly correlated in most bat species analyzed. However, such correlations were somewhat weak in the following bat families, Hipposideridae (Great roundleaf bat), Megadermatidae (Greater false vampire bat) and Noctilionidae (Greater bulldog bat). Although they possess intact ancV1Rs, of which the ω values (Fig. 3I; 0.55-0.63) are slightly higher than bats with intact VNOs (0.48-0.54), their VNOs are vestigial and TRPC2s are inactivated (Table S8). It is difficult to conclude whether or not purifying selection was relaxed on ancV1Rs in these groups. Furthermore, in Mormoopidae, the ω values of intact ancV1R in Parnell’s mustached bat (0.92) and in Antillean ghost-faced (0.68) are not consistent with the presence/absence of intact VNO (Table S8). We performed additional analyses using branch-site model in codeml, and found no signatures for positive selection (Table S9).

3. 6. Examples of ambiguous inactivation.

The southern two-toed sloth has a 4bp deletion in its sequence (Fig. S4), which was confirmed in multiple reads (SRX4501348). However, the ω value for this species (0.30) is lower than backgrounds (0.40). In addition, TRPC2 is intact and the ω value (0.08) is also lower than background (0.15) (Fig. 4S). We also found that the closely related
species of three-toed sloth has intact \textit{ancV1R}. Thus, it is possible that the 4bp deletion exists only in the sequenced individual and/or was very recent evolutionary event. To examine these possibilities, we need a PCR validation of the 4bp deletion for multiple individuals of wild populations in future study.

3. 7. Exploratory analyses for the elevation of \( \omega \) values in \textit{ancV1Rs}

In addition to the hypothesis testing method using branch model, we also comprehensively explored the branches with elevated \( \omega \) values of \textit{ancV1Rs} in mammalian tree by using free ratio model. Fig. S5 showed the summary of \( \omega \) values on each branch. As a result, we found that the \( \omega \) values are around 0.4 in most of the branches, suggesting the operation of purifying selection in these mammals analyzed. However, we also detected apparent elevation of \( \omega \) values in several species (\( \omega > 0.8 \); Table S10A), which were subjected to the additional analyses using branch model and likelihood ratio test. As a result, the elevation of \( \omega \) values were statistically significant in Lesser panda (\( p = 0.043 \)) and Pacarana (\( p = 0.0064 \)). The branch-site analysis for these species showed that the operation of positive selection in these species is unlikely (Table S10B). To argue the possibility of the degeneration of the VNO, we further examined the \textit{TRPC2}, revealing that the \( \omega \) values of Lesser panda (0.074) and Pacarana (0.070) were comparable to the background (0.12 and 0.050, respectively). Thus, \( \omega \) values were elevated in \textit{ancV1R} but not in \textit{TRPC2}. The results imply that the elevation of \( \omega \) values in \textit{ancV1R} could be the noise in the low number of mutations, or the selective constraint was relaxed only in \textit{ancV1R} of these species. Given that the description of the VNO still remains limited in

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Lesser panda and Pacarana, these group could be the next target of the anatomical and molecular investigation.

4. Discussion

Understanding the source of phenotypic diversity at the DNA level is a key challenge in molecular evolutionary biology. The coincidental loss of a particular gene and a specific trait may provide important insight into the molecular mechanisms that underlie phenotypic diversity (Sharma et al. 2018). Recently, such phenotype-genotype relationships have been uncovered in several studies of mammals; the inactivation of C4orf26, MC5R, and OMP showed strong correlation with the loss of teeth (Springer et al. 2016), the sebaceous gland (Springer and Gatesy 2018), and olfactory sense (Springer and Gatesy 2017), respectively. Given that \textit{ancV1R} is expressed in all VNO sensory neurons and co-expressed with canonical \textit{V1Rs} (Suzuki et al. 2018), we suppose that \textit{ancV1R} possesses general and essential function in the VNO. Therefore, it is of primary interest to examine the correlation between phenotype (VNO) and genotype (\textit{ancV1R}). Here, we found clear correlations between the loss of the VNO and the inactivation of \textit{ancV1R}. Furthermore, we revealed unexpected inactivation of \textit{ancV1R} in multiple lineages of mammals for which the existence of a functional VNO is not well described.

4.1. Consistent pattern of inactivation of \textit{ancV1R}, \textit{TRPC2} and the loss of the VNO

In all catarrhine primates, our observations of gene inactivation and the elevation of \( \omega \) values in \textit{ancV1R} and \textit{TRPC2} are consistent with previous studies (Liman and Innan
2003; Zhang and Webb 2003) and anatomical loss of both the VNO and the distinct accessory olfactory bulb (Wysocki 1979; Bhatnagar and Meisami 1998, Smith 2014). The degeneration of the VNO in these groups could be based on an evolutionary trade-off shifting toward more reliance upon visual and auditory signals rather than the chemical signals for reproductive communication.

In cetaceans, the inactivation and the elevation of $\omega$ values in ancV1R occurred in their stem lineage (Fig. 3B). This is consistent with the inactivation of TRPC2 (Fig. S3B; Yu et al. 2010) and the absence of the VNO and the accessory olfactory bulb (Lowell and Flanigan 1980; Mackay-Sim et al. 1985). It is noteworthy that the timing of the inactivation of ancV1R and TRPC2 occurred earlier than that of OMP, which was inactivated in most toothed whales but is still intact in baleen whales (Kishida et al. 2015; Springer and Gatesy 2017). Springer and Gatesy (2017) noted that the olfactory sense was retained in the common ancestor of Cetacea but was significantly reduced in odontocetes by a trade-off with the evolution of echolocation ability. Given that ancV1R was inactivated in both toothed and baleen whales, the vomeronasal system may have been less important than olfaction during the adaptation toward a fully aquatic environment.

In bats, the inactivation and the elevation of $\omega$ values in ancV1R occurred in Pteropodidae, Rhinolophidae, Vespertilionidae and Molossidae, which are consistent with the inactivation of TRPC2 and loss of the VNO (Fig. 3I; Zhao et al. 2011; Yohe et al. 2017). Conversely, the existence of an intact ancV1R and similar $\omega$ values with background in Phyllostomidae and Miniopteridae are also consistent with the presence of
an anatomically intact VNO in representative species belonging to these two families (Bhatnagar and Meisami 1998). Given that the first inactivating mutations are different among each family (Fig. 3I), the timing of inactivation of \textit{ancV1R} does not date back to the common ancestor of all bats but rather to the ancestors of each family. These results imply that the loss of the VNO occurred relatively recently and independently in bat evolution, which is also consistent with the finding of Yohe et al. (2017).

4.2. Complex history of the vomeronasal sensory system in bats

Although we obtained clear correlations between phenotype and genotype, we also obtained a few ambiguous results in several bat species investigated. This may reflect the rapid and complex evolutionary history of the vomeronasal sensory system in this group (e.g. Yohe et al. 2017). First, Hipposideridae, Megadermatidae, Noctilionidae, and one species of Mormoopidae (Antillean ghost-faced bat) possess intact \textit{ancV1R}s, of which the \(\omega\) values were only slightly higher than background (Fig. 3I; 0.62, 0.55, 0.56 and 0.68, respectively). It is difficult to judge whether or not selection pressure on VNO functionality was relaxed by solely focusing on \textit{ancV1R}. However, considering that \textit{TRPC2} was inactivated in these species (Yohe et al. 2017), the slightly higher \(\omega\) values of \textit{ancV1R} may reflect some relaxation of purifying selection, which is consistent with anatomical observations of lacking VNO (Wible and Bhatnagar 1996). The recent loss of the vomeronasal sensory system in bats may not have allowed sufficient time for the accumulation of deleterious mutations in \textit{ancV1R}, making it difficult to detect an obvious signature for the relaxation of purifying selection. Conversely, Parnell’s mustached bat
(Mormoopidae) possesses an intact \textit{ancV1R}, of which the $\omega$ value (0.92) was higher than background. The operation of positive selection in this bat species was shown to be unlikely by branch-site test (Table S9), implying the possibility of the relaxation of purifying selection. However, its \textit{TRPC2} is intact (Yohe et al. 2017) and the VNO is also anatomically intact in this species (Bhatnagar and Meisami 1998). These data imply that only \textit{ancV1R} sequence may not be sufficient to judge the presence/absence of functional VNO in this species. To further broaden our understanding about the evolution of the VNO in this complex bat family, investigation of additional \textit{ancV1R} as well as \textit{TRPC2} of closely related species are indispensable. It is also noteworthy that Hog-nosed bat (Craseonycteridae) possesses intact \textit{ancV1R} and \textit{TRPC2} with mostly similar $\omega$ values with each background (Fig. 3I). This suggests that VNS is still functional in Craseonycteridae, in spite that most of yinpterochiropteran bats (from Rhinolophidae to Pteropodidae, Fig. 3I) lack intact VNOs (Table S8).

4.3. Novel insights into VNO functionality from \textit{ancV1R} evolution

By applying \textit{ancV1R} as a reliable and useful genetic marker, we obtained several novel insights into the vomeronasal sensory systems in mammals that was supported by the additional analyses of \textit{TRPC2}. In Pinnipedia, \textit{ancV1R} was inactivated in only one species of true seals (Northern elephant seal). In addition, the elevation of $\omega$ values was also observed in three other species of true seals (Weddell seal, Hawaiian monk seal, and harbor seal), implying the relaxation of purifying selection (Fig. 3E), that was supported by the inactivation of \textit{TRPC2} (Fig. S3D; Yu et al. 2010). Thus, both of the two
independent molecular markers suggest that the vomeronasal sensory system became nonfunctional in true seals. It is noteworthy that in pinnipeds the existence of the accessory olfactory bulb could not be clearly identified only in true seals investigated (harbor seal) (Meisami and Bhatnagar 1998), implying a reduction or loss of vomeronasal sensory system function in true seals. Molecular, morphological and ecological studies now consistently suggest that true seals are more aquatic than other pinnipeds (Jefferson 2008) and thus are expected to be less dependent on vomero-olfaction.

In the sea otter and the giant otter, \(ancV1R\)s are inactivated by a frameshift insertion of T at different sites (Fig. 3D), suggesting that \(ancV1R\)s were independently inactivated in these species. To our knowledge, the VNO has not been described in otters. Given that the selective constraint on \(TRPC2\) was also relaxed the river otter (Yu et al. 2010), sea otter and the giant otter (Fig. 3D), it is likely that the vomeronasal sensory system became vestigial in the common ancestor of these two groups coinciding with adaptation to aquatic environments. Although a behavioral study described the flehmen response in sea otter, they also pointed out that the response was not stereotypical for this behavior (Island et al. 2017). It is possible that the flehmen response in sea otter could be induced by MOE rather than VNO. It is worth noting that the number of olfactory receptor genes has decreased in both the sea otter and the giant otter (Beichman et al. 2019), implying that aquatic adaptation may have affected not only the vomeronasal but also the olfactory sensory system during the evolution of these species.

We further obtained an unexpected example of inactivation, namely, the frameshift mutation, as well as an elevated \(\omega\) value (Fig. 3G) for \(ancV1R\) in the owl monkey (\(Aotus\)
nancymae). In a previous study, Liman and Innan (2003) revealed an elevated $dN/dS$ ratio for TRPC2 in the owl monkey that was confirmed by our study (Fig. 3C), suggest that the VNO is either already nonfunctional or else on the way to becoming nonfunctional. Although the possibility of a reduction of the VNO in New World monkeys has been discussed in both anatomical (Smith et al. 2011) and molecular (Liman and Innan 2003; Yoder et al. 2014; Moriya-Ito et al. 2018) studies, no precise description was done in the owl monkeys.

We also found an unexpected example of inactivating mutations as well as an elevated $\omega$ value (Fig. 3F) for aneV1R in one species of Malagasy carnivorans; the fossa (Cryptoprocta ferox), that is consistent with the existence of premature stop codon in TRPC2 (Fig. S3E). The fossa has once been classified in Viverridae (most civets and their relatives), but it was revealed to be more closely related to Herpestidae (mongooses) and included in the monophyletic group of Malagasy carnivorans (Yoder et al. 2003). To our knowledge, the vomeronasal systems of the fossa and the other carnivorans in Madagascar have not yet been described. Thus, the anatomical status of the VNO and the reproductive behavior of these species represent an important next target of continuing research. It is important to note that an olfactory receptor gene with premature stop codon was shown to encode functional protein due to the efficient translational read-through (pseudo-pseudo gene; Prieto-Godino et al. 2016). Therefore, we cannot completely exclude the possibility that the TRPC2 are still functional in this species. More comprehensive analyses on VNO-related genes may provide insights into the status of the vomeronasal system in this species.
The relaxation of purifying selection for \textit{ancV1R} in the hartebeest, the hirola and the topi, and the operation of purifying selection in the blue wildebeest (Fig. 3H) are all highly consistent with the presence/absence of flehmen responses in alcelaphine antelopes (Hart et al. 1987), which are also supported by \textit{TRPC2}. Anatomically, however, VNO structures were described in all alcelaphine antelopes (Hart et al. 1987). It is important to note that incisive papilla and incisive ducts, which are involved in transferring pheromones from the oral cavity to the VNO, were degenerated in the hartebeest, the hirola and the topi. On the other hand, very small incisive duct still exists in the wildebeest (\textit{Connochaetes taurinus}), implying that the vomeronasal system is retained to be functional in this species. Our analyses for \textit{ancV1R} of alcelaphine antelopes suggest that purifying selection was relaxed in the common ancestor of the topi, the hirola and the hartebeest, and likely corresponds with the loss of the flehmen response and the complete loss of incisive papilla and ducts in this group. Our study provides the first genetic insight into the evolution of vomeronasal systems in alcelaphine antelopes.

In the present study, our evolutionary analyses of nearly all 23 mammalian orders showed obvious correlations between the phenotype of the VNO and the genotype of \textit{ancV1R}, demonstrating that the presence/absence of inactivating mutation(s) and the $\omega$ values of \textit{ancV1R} can be used as valuable genetic markers to elucidate the biological status of the VNO. Furthermore, five unexpected examples of inactivation events in true seals, otters, the owl monkey, the fossa, and alcelaphine antelopes, as revealed by \textit{ancV1R} and \textit{TRPC2} sequences, provide a timely and important opportunity to re-examine the anatomical condition of the VNO in these ecologically diverse species for which VNO
functionality remains uncertain.

Acknowledgements

This work was funded by JSPS KAKENHI (16H04820, 25440189), MEXT KAKENHI (221S0002), Asahi Glass Foundation, and Hitachi Global Foundation to M.N.

Appendix A. Supplementary material
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Legend to the Figures

Figure 1. Summary of the inactivation of *ancV1R* and *TRPC2* sequences identified in the previous studies and this study. The status of the anatomy of the VNO is also shown for comparison. Note that the inactivation of *ancV1R* is correlated with the inactivation of *TRPC2* and with the loss of functional VNO. Alcelaphine antelopes and the owl monkey are indicated as “putatively nonfunctional” in the *TRPC2* column because they are intact at the sequence level but the $\omega$ values are elevated to almost 1.0 (see results). The anatomical status for alcelaphine antelopes (topi, hartebeest, hirola) is also controversial because they possess intact VNO structures but lack flehmen responses. *ancV1R* of true seal in Suzuki et al. (2018) is indicated as “no data” because the $\omega$ value was not available. See Table S3 for more details and references.

Figure 2. The maximum likelihood tree of *ancV1R* gene tree for 261 mammals. Gray rectangles indicate members of major orders and superorders. Colored circles on each node indicate bootstrap values described in the legend. Scale bar indicates the number of nucleotide substitutions/site. The phylogenetic tree including all bony vertebrates is shown in the Supplementary Fig. S1.

Figure 3. The sequence alignments of *ancV1R* showing the inactivating mutations shared among each family for the manatee (A), cetaceans (B), catarrhines primates (C), otters (D), true seals (E), the fossa (F), the owl monkey (G), alcelaphine antelopes (H), and bats (I), with closely related outgroups. Inactivating mutations are highlighted in grey. Start
codon mutations and stop codon mutations are highlighted in yellow and blue, respectively. Each alignment is extracted from the alignment of all *ancV1R* sequences used in this study (Supplementary Alignment File 1). The numbers above the line indicate the nucleotide positions for each alignment. The colors of each branch indicate different ω categories. The ω values of *ancV1R* and *TRPC2* for each branch are indicated in the *dN/dS* columns.

**Supplementary Fig. S1**

Maximum-likelihood tree of *ancV1R* orthologues of bony vertebrates. Colored circles on each node indicate bootstrap values described in the legend. Scale bar indicates the number of nucleotide substitutions/site.

**Supplementary Fig. S2**

The species trees are downloaded from the Open Tree of Life (https://tree.opentreeoflife.org/; Hinchliff et al. 2015) using all mammalian taxa analyzed in this study.

**Supplementary Fig. S3**

The sequence alignments of exon 12 of *TRPC2* showing the inactivating mutations shared among each family for the manatee (A), cetaceans (B), catarrhines primates (C), otters (D), true seals (E), the fossa (F), the owl monkey (G), alcelaphine antelopes (H), and bats (I), with closely related outgroups. Inactivating mutations are highlighted in grey. Start
codon mutations and stop codon mutations are highlighted in yellow and blue, respectively. Each alignment is extracted from the alignment of all TRPC2 exon 12 sequences used in this study (Supplementary Alignment File 2). The numbers above the line indicate the nucleotide positions for each alignment. The colors of each branch indicate different $\omega$ categories. The $\omega$ values of ancV1R and TRPC2 for each branch are indicated in the $dN/dS$ columns.

**Supplementary Fig. S4**

The sequence alignments of ancV1R showing the inactivating mutation in southern two-toed sloth. No inactivating mutation is found in TRPC2. Note that the $\omega$ value is lower than the background in both of ancV1R and TRPC2, implying that the mutations on ancV1R is an artifact due to the assembly error. However, we cannot examine this possibility because the multiple reads for the assembly are not available for this species.

**Supplementary Fig. S5**

The result of the free ratio model, which assigned separate dN/dS categories for each branch, for (A) Euarchontoglires, (B) Laurasiatheria, and (C) the other mammals (marsupials, afrotherians and xenarthrans) which have intact ancV1R sequences. The branches with low numbers of nonsynonymous substitutions ($< 5$) or synonymous substitutions ($< 5$) were masked by gray.

**Supplementary Table S1**
All species names and assembly names for \textit{ancV1R} sequences used in this study.

**Supplementary Table S2**
Variation in the length of \textit{ancV1R} in mammals with intact sequences.

**Supplementary Table S3**
Classification of vertebrate species used in this study and the status of the VNO, \textit{ancV1R} and \textit{TRPC2}.

**Supplementary Table S4**
Akaike Information Criterion (AIC) comparisons of different codon frequency models in \texttt{codeml} for \textit{ancV1R} sequences. All comparisons were performed with the one category model.

**Supplementary Table S5**
Akaike Information Criterion (AIC) comparisons of different codon frequency models in \texttt{codeml} for \textit{TRPC2} sequences. All comparisons were performed with the one category model.

**Supplementary Table S6**
Results of selection analyses and likelihood ratio tests for \textit{ancV1R} sequences of each group.
Supplementary Table S7

Results of selection analyses and likelihood ratio tests for TRPC2 sequences of each group.

Supplementary Table S8

Classification of bat species and the status of the VNO, ancVIR and TRPC2.

Supplementary Table S9

Results of selection analysis based on the branch-site model in several bat species with high $\omega$ values without frameshift or premature stop codon mutations in Fig. 3I.

Supplementary Table S10

(A) Results of the selection analysis based on the branch model (two categories) for new candidates with elevated $\omega$ values (> 0.8) detected by free ratio model (Fig. S5) and (B) results of the additional selection analysis based on branch-site model for Lesser panda and Pacarana.

Supplementary Alignment File 1

Fasta format alignment of ancVIR sequences.

Supplementary Alignment File 2
Fasta format sequence alignment for *TRPC2* exon 12.
|                      | VNO* | TRPC2                      | ancV1R | previous studies* | This study | Suzuki et al. (2018) | This study |
|----------------------|------|----------------------------|--------|-------------------|------------|----------------------|------------|
| Manatee              | absent |                           |        |                   |            |                      |            |
| Otters               |       |                           |        |                   |            |                      |            |
| True seals           | absent |                           |        |                   |            |                      |            |
| Fossa                |       |                           |        |                   |            |                      |            |
| Bats                 | various | various                   | various | various           | various    | various              | various    |
| Cetaceans            | absent |                           |        |                   |            |                      |            |
| Alcelaphine antelopes (topi, hartebeest, hirola) |       | controversial              |        |                   |            |                      |            |
| Catarrhine primates  | absent |                           |        |                   |            |                      |            |
| Owl monkey           | controversial |                      |        |                   |            |                      |            |

*See Supplementary Table S3 for references and details

- **Nonfunctional**: (inactivating mutation, elevated \(\omega\))
- **Putatively nonfunctional**: (elevated \(\omega\))
- **No data**

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**Fig. 3**

(A) Phylogenetic tree and nucleotide sequences for the Manatee, Elephant, Hyrax, Yellow-spotted-hyrax, and Other Afrotherians (5).

(B) Phylogenetic tree and nucleotide sequences for Bowhead_whale, North_Pacific_right_whale, Gray_whale, Humpback_whale, Northern_minke_whale, Sperm_whale, Pygmy_sperm_whale, Indus_river_dolphin, Sowerbys_beaked_whale, Cuviers_beaked_whale, Yangtze_river_dorphin, Boutu, Harbor_porpoise, Yangtze_finless_porpoise, Narwhal, Beluga, Killer_whale, Pacific_white-sided_dolphin, Indo-pacific_humpbacked_dolphin, Bottlenose_dolphin, Hippopotamus, Other_Cetartiodactylans (50), and Perissodactyla (5).
Fig. 3 (continued)

![Graphical representation of evolutionary relationships]

| Species                          | dN/dS 0.44 | dN/dS 0.13 | dN/dS 1.08 | dN/dS 1.24 |
|----------------------------------|------------|------------|------------|------------|
| Human                            |            |            |            |            |
| Chimpanzee                       |            |            |            |            |
| Western_Lowland_gorilla          |            |            |            |            |
| Orangutan                        |            |            |            |            |
| Gibbon                           |            |            |            |            |
| Red_shanked_douc_langur          |            |            |            |            |
| Long-nosed_monkey                |            |            |            |            |
| Golden_snub-nosed_monkey         |            |            |            |            |
| Hanuman_langur                   |            |            |            |            |
| Angola_colobus                   |            |            |            |            |
| Ugandan_red_colobus              |            |            |            |            |
| De_Brazza's_monkey               |            |            |            |            |
| Vervet_monkey                    |            |            |            |            |
| Red_guenon                       |            |            |            |            |
| Sooty_mangabey                   |            |            |            |            |
| Drill                            |            |            |            |            |
| Olive_baboon                     |            |            |            |            |
| Gleda                            |            |            |            |            |
| Macaque                          |            |            |            |            |
| Other Primates (20)              |            |            |            |            |
| Sunda_flying_lemur               |            |            |            |            |
Fig. 3 (continued)

| dN/dS | ancV1R TRPC2 | ancV1R          | 570 | 580 | 850 | 860 |
|-------|--------------|-----------------|-----|-----|-----|-----|
|       | Giant otter  | AGCTC-TGATT     |     |     |     |     |
| 0.88  | Sea otter    | AGCTC-TGATT     |     |     |     |     |
| 0.49  | American mink| AGCTC-TGATT     |     |     |     |     |
| 0.40  | Ferret       | AGCTC-TGATT     |     |     |     |     |
| 0.12  | Other Carnivorans (24) | AGCTC-TGAGT |     |     |     |     |
|       | Sunda pangolin |                 |     |     |     |     |

| dN/dS | ancV1R TRPC2 | ancV1R          | 500 |
|-------|--------------|-----------------|-----|
|       | Northern elephant seal | TCACGGGATTT |     |
| 999   | Weddell seal | TCACAGGGATT     |     |
|       | Hawaiian monk seal | TCACAGGGATT     |     |
|       | Harbor seal  | TCACAGGGATT     |     |
| 1.28  | California sea lion | TCACAGGGATT     |     |
|       | Steller sea lion | TCACAGGGATT     |     |
|       | Antarctic fur seal | TCACAGGGATT     |     |
|       | Northern fur seal | TCACAGGGATT     |     |
|       | Walrus       | TCACAGGGATT     |     |
| 0.40  | Other Carnivorans (21) | TACAGGGATT     |     |
| 0.12  | Sunda pangolin |                 |     |

| dN/dS | ancV1R TRPC2 | ancV1R          | 570 | 580 | 920 |
|-------|--------------|-----------------|-----|-----|-----|
|       | Fossa        | TTGACACTTCA     |     |     |     |
| 1.52  | Meerkat      | TCTCCACTTCA     |     |     |     |
|       | Banded mongoose | TTTCCACTTCA |     |     |     |
|       | Dwarf mongoose | TCTCCACTTCA     |     |     |     |
| 0.40  | Other Carnivorans (23) | TCTCCACTTCA |     |     |     |
| 0.12  | Sunda pangolin |                 |     |     |     |
### (G)

| dN/dS | ancV1R TRPC2 | AncV1R |
|-------|---------------|--------|
|       | 0.83 0.83     | 50 60  |
| 0.45  | 0.13          |        |

| Species          | Sequence   |
|------------------|------------|
| Owl_monkey       | CCTTTTGAGC |
| Tamarin          | TCTTTGTGAGC|
| Marmoset         | TCTTTGTGAGC|
| Squirrel_monkey  | TCTTTGTGAGC|
| White-headed_capuchin | TCTTTGTGAGC|
| Other Primates   |            |
| (16)             |            |
| Sunda_flying_lemur|           |

### (H)

| dN/dS | ancV1R TRPC2 | AncV1R |
|-------|---------------|--------|
|       | 999 999       | 1 10   |
| 0.44  | 0.00          |        |
| 0.45  | 0.10          |        |

| Species                  | Sequence       |
|--------------------------|----------------|
| Hartebeest               | GTGAAGTCCT     |
| Hirola                   | ATGAAGTCCT     |
| Topi                      | ATGAAGTCCT     |
| Blue_wildebeest          | ATGAAGTCCT     |
| Gemsbok                  | ATGAAGTCCT     |
| Other Artiodactyla (46)  |                |
| Perissodactyla (5)       |                |
