

1 Loss of olfaction in sea snakes provides new perspectives on the aquatic adaptation of amniotes

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20 **Key Words:** OR, V1R, V2R, TAAR, amphibious, fully aquatic

21 **Abstract**

22 Marine amniotes, a polyphyletic group, provide an excellent opportunity for studying  
23 convergent evolution. Their sense of smell tends to degenerate, but this process has not been  
24 explored by comparing fully-aquatic species with their amphibious relatives in an evolutionary  
25 context. Here, we sequenced the genomes of fully-aquatic and amphibious sea snakes, and  
26 identified repertoires of chemosensory receptor genes involved in olfaction. Snakes possess  
27 large numbers of the *olfactory receptor (OR)* genes and the *type-2 vomeronasal receptor (V2R)*  
28 genes, and expression profiling in the olfactory tissues suggests that snakes use the ORs in the  
29 main olfactory system (MOS) and the V2Rs in the vomeronasal system (VNS). The number of  
30 *OR* genes has decreased in sea snakes, and fully-aquatic species lost the MOS which is  
31 responsible for detecting airborne odors. In contrast, sea snakes including fully-aquatic species  
32 retain a number of *V2R* genes and a well-developed VNS for smelling underwater. This study  
33 suggests that the sense of smell also degenerated in sea snakes, particularly in fully-aquatic  
34 species, but their residual olfactory capability is distinct from that of other fully-aquatic  
35 amniotes. Amphibious species show an intermediate status between terrestrial and fully-aquatic  
36 snakes, implying their importance in understanding the process of aquatic adaptation.

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## 44 BACKGROUND

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46 Shifts between terrestrial and aquatic lifestyles are among the most striking types of  
47 evolutionary transitions in the history of life. Vertebrates invaded land during the Devonian to  
48 the Carboniferous in two steps: first, they became amphibious (*i.e.*, both aquatic and terrestrial  
49 habitats are required) with the emergence of tetrapods, and then they adapted for terrestriality  
50 with the emergence of amniotes [1]. Among groups containing mostly terrestrial amniotes, there  
51 are several groups which re-adapted to the aquatic habitat independently from each other.  
52 Amniotes are also suggested to have re-invaded water from land with two major steps: they  
53 become amphibious prior to the completion of aquatic invasion. For example, all extant  
54 cetaceans are fully-aquatic but their intermediate ancestors from the Early Eocene were  
55 amphibious [2] (Fig. 1). Marine elapids (Suborder Serpentes, Order Squamata, Class Reptilia),  
56 collectively called sea snakes, consist of two monophyletic clades, Laticaudini and Hydrophiini.  
57 Laticaudins are oviparous and lay eggs on land, whereas hydrophiins are viviparous and spend  
58 all their life in water. Both groups have a paddle-shaped tail adapted to aquatic locomotion, but  
59 laticaudins retain enlarged ventrals required for terrestrial locomotion which hydrophiins lost  
60 [3]. Although recent studies suggested that laticaudins and hydrophiins adapted to the marine  
61 habitat independently, these two clades are phylogenetically close to each other with a  
62 divergence time of approx. 12–20 million years ago [4-7]. Thus, sea snakes provide an excellent  
63 study system of aquatic adaptation because phylogenetically closely related fully-aquatic and  
64 amphibious species can be compared directly.

65 Aquatic amniotes offer a valuable opportunity for studying convergent evolution because  
66 evolutionary hypotheses of specific adaptation can be tested for multiple aquatic groups that  
67 migrated from land to water independently to each other [8, 9]. One of the most remarkable  
68 differences between terrestrial and aquatic vertebrates involves the sense of smell. Broad taxa of

69 vertebrates detect odorants mainly using four major groups of G-protein coupled receptors  
70 (GPCRs) encoded by different multigene families: olfactory receptors (ORs), trace amine-  
71 associated receptors (TAARs) and two types of vomeronasal receptors (V1Rs and V2Rs) [10]. It  
72 has been hypothesized that olfactory GPCRs are functionally divided into two groups, receptors  
73 for airborne molecules and those for water-soluble molecules [10, 11]. The *OR* gene repertoire  
74 changed drastically in our ancestors during their transition from water to land, and amphibians  
75 show an intermediate form. Modern anurans retain mostly ancestral *OR* gene subfamilies for  
76 detecting water-soluble molecules which amniotes lost, but they also share newly diverged *OR*  
77 gene subfamilies with amniotes which are considered to detect airborne odorants [12-14].  
78 Aquatic tadpoles (larvae of amphibians) possess olfactory organs for smelling underwater, but  
79 extreme remodeling occurs during metamorphosis to meet the requirement of the adult lifestyle,  
80 and adult anurans develop a so-called “air nose” for smelling in the air [15], in which the newly-  
81 diverged *OR* genes are expressed [16]. The *OR* genes possessed by terrestrial amniotes are  
82 prone to secondary loss from the genomes of aquatic amniotes [17-21], and extant toothed  
83 whales possess no olfactory nervous systems [22]. Baleen whales, the other group of extant  
84 cetaceans, still possess a functional olfactory system, but anatomical, histological and genomic  
85 studies suggest that they cannot smell underwater and their olfactory capability is highly limited  
86 [23-26]. However, olfactory capabilities of non-cetacean aquatic amniotes remain largely  
87 elusive. Furthermore, no extensive studies on genomes of amphibious amniotes have ever been  
88 compared and contrasted with those of fully-aquatic relatives, in spite of their importance upon  
89 aquatic adaptation. Here, we sequenced and assembled the genomes of fully-aquatic and  
90 amphibious sea snakes. Olfactory GPCR genes were identified in each genome assembly, and  
91 the expression profiling of these receptors was performed. Our present study explores the  
92 genomic traces of evolution of olfaction in sea snakes and provides new perspectives on the  
93 aquatic adaptation of amniotes.

95

## 96 **RESULTS**

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### 98 **Sea snake genome assemblies**

99 We sequenced and assembled the genomes of two hydrophiins (*Hydrophis melanocephalus* and  
100 *Emydocephalus ijimae*) and two laticaudins (*Laticauda laticaudata* and *L. colubrina*). These  
101 genome assemblies are estimated to contain at least 90% of all protein-coding genes (including  
102 those recognized as ‘Fragmented’ by BUSCO [27, 28] or ‘Partial’ by CEGMA [29]) based on  
103 completeness assessments using an one-to-one reference ortholog set (Table S1).

104 Different methodologies were employed for performing *de novo* assembly of the genomes of  
105 four snakes (see Materials and Methods for detail). We assembled the genome of *L. colubrina*  
106 based on the linked-read sequencing technology [30, 31]. It is known that this method allows us  
107 to generate relatively long genome sequences of diploid species with a single library for short  
108 read sequencing [32]. Among the four species sequenced in this study, the *L. colubrina*  
109 assembly shows the largest scaffold N50 length (3.1 Mbp) and completeness score of one-to-  
110 one ortholog coverage (Table S1). However, the proportion of truncated genes in the *L.*  
111 *colubrina* genome assembly do not differ greatly in comparison with other assemblies (Table  
112 S2). Consistently, the contig N50 lengths do not largely vary between the assemblies of the four  
113 species (Table S1).

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### 115 **Olfactory GPCR gene repertoires in snake genomes**

116 We identified the olfactory GPCR genes in the genome assemblies of sea snakes and their  
117 terrestrial relatives. Snakes possess large numbers of *ORs* and *V2Rs*, which vary between

118 species (mean numbers of intact *ORs* and *V2Rs* are  $194 \pm 136$  and  $240 \pm 136$  [mean  $\pm$  standard  
119 deviation, calculated using all snake species shown in Fig. 2], respectively), whereas the  
120 numbers of *TAARs* and *VIRs* are small and comparable across species (snakes possess only two  
121 or three intact *TAARs* and two intact *VIRs*, as described below) (Fig. 2).

122 Sea snakes possess a smaller number of intact *OR* genes with higher proportions of pseudogenes  
123 (Table S2) compared with terrestrial snakes (mean number of intact *ORs* of hydrophiins:  $63.5 \pm$   
124  $14.9$ , laticaudins:  $114 \pm 12.7$ , terrestrial snakes:  $335 \pm 40.8$  [mean  $\pm$  standard deviation]) mainly  
125 due to massive loss of the *OR* genes in the sea snake lineages (Fig. 3A). They also possess a  
126 relatively small number of intact *V2Rs*, but the numbers of intact *V2R* genes vary greatly  
127 between species (mean number of intact *V2Rs* of hydrophiins:  $137 \pm 95$ , laticaudins:  $177.5 \pm$   
128  $121$ , terrestrial snakes:  $351 \pm 104$  [mean  $\pm$  standard deviation]), and massive gain of the *V2R*  
129 genes is observed in two species of sea snakes, *E. ijimae* and *L. colubrina* (Fig. 3B). Snakes  
130 possess two intact *TAARs* (*TAAR1* and *TAAR5*). In addition, terrestrial snakes and laticaudins  
131 possess one more intact *TAAR* gene (*TAAR2*, which is pseudogenized in the hydrophiin  
132 genomes). All snakes including sea snakes (except for the common viper) possess two intact  
133 *VIRs*, the *ancVIR* [33] and a *VIR* gene which is not orthologous to the mammalian *VIRs*  
134 (*Squamata-VIR*, Fig. S1).

135

### 136 **Expression of the olfactory GPCR genes**

137 There are two anatomically distinctive olfactory systems in terrestrial snakes, the main olfactory  
138 system (MOS) and the vomeronasal system (VNS). The olfactory epithelium of the MOS (the  
139 main olfactory epithelium) is located in the nasal cavity (NC), and that of the VNS (the  
140 vomeronasal epithelium) is located in the vomeronasal organ (VNO) [34]. The snake tongue  
141 also plays a role in the VNS by delivering chemicals to the VNO [34]. We performed

142 transcriptome sequencing with RNA-seq on these potential olfactory organs of *L. laticaudata*  
143 and *H. melanocephalus*. The expression pattern of these genes in *L. laticaudata* suggests that  
144 most of *ORs* are used in the MOS, while *V2Rs* function in the VNS (Fig. 4). It is noted that  
145 some pseudogenes are also expressed in the olfactory organs, coinciding with previous reports  
146 (e.g. Zhang et al. [35]). Expression levels of *VIRs* and *TAARs* suggest that the *ancVIR* is  
147 expressed in the VNO of both species, and the *TAAR2*, which is pseudogenized in the  
148 hydrophiin genomes, is expressed in the NC of *L. laticaudata* (Table S3).

149 An intact *OR* gene is expressed in the tongue in each snake species (Fig. 4, indicated by arrows).  
150 The arrowed gene of *Hydrophis* and that of *Laticauda* are orthologous to each other. All  
151 squamates investigated in this study (except for *Emydocephalus*) possess one-to-one  
152 orthologues of this *OR* gene (Fig. S2).

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154

## 155 **DISCUSSION**

156

### 157 **Evolution of the main olfactory system**

158 The repertoires and expression pattern of the olfactory GPCR genes shown in this study suggest  
159 that snakes mainly use *ORs* in the MOS and *V2Rs* in the VNS. It has been widely considered  
160 that the VNS is the predominant chemosensory system in snakes, being more responsible than  
161 the MOS for their sense of smell [34, 36]. However, in our results, the numbers of intact *ORs*  
162 and *V2Rs* are almost comparable among terrestrial snakes (Fig. 2), implying that terrestrial  
163 snakes potentially detect and discriminate as many chemicals using the MOS as using the VNS.

164 Sea snakes possess an apparently smaller number of intact *OR* genes compared with terrestrial  
165 snakes, and our phylogenetic analysis suggests that it is mainly because of massive loss of the  
166 *OR* genes in the sea snake lineages after the king cobra–sea snakes split (Fig. 3A). Although the  
167 most recent common ancestor of hydrophiins and laticaudins, which lived on land, was also  
168 estimated to possess a smaller number (205) of intact *OR* genes than other terrestrial snakes (Fig.  
169 3A), it is possible that hydrophiins and laticaudins lost *OR* orthologs in their unique lineages  
170 independently. In any case, massive loss of *OR* genes was also confirmed in both hydrophiin  
171 and laticaudin lineages after the Hydrophiini–Laticaudini split, coinciding with their transition  
172 from land to water. Amphibious carnivorans (pinnipeds and otters) and fully-aquatic cetaceans  
173 were also estimated to have lost a large number of intact *OR* genes when they migrated from  
174 land to water [21, 24], showing a remarkable case of convergent evolution on becoming aquatic.  
175 Although both hydrophiins and laticaudins possess fewer intact *OR* genes, their expression  
176 patterns are different from each other. Most of the *OR* genes possessed by *L. laticaudata* are  
177 expressed in the NC, while those possessed by *H. melanocephalus* are not (Fig. 4). This contrast  
178 indicates that most of the *OR* genes possessed by hydrophiins do not have olfactory function,  
179 and that hydrophiins lost a functional MOS. Loss of the MOS in hydrophiins is also supported  
180 by the evolution of the *TAAR* genes — the *TAAR2* gene, which is expressed in the NC of  
181 laticaudins (Table S3), is pseudogenized in the hydrophiin genomes. Histological studies  
182 showed that a relative size of the olfactory region in the NC is highly reduced in sea snakes, and  
183 particularly, that of hydrophiins lacks an external nasal gland which lubricates the olfactory  
184 epithelium [37, 38]. The role of the MOS is poorly understood in snakes, but these findings  
185 suggest that the MOS became less useful for snakes to sense surrounding environment on  
186 becoming aquatic, and it was completely lost in fully-aquatic hydrophiins, probably because the  
187 snake MOS functions only in the air.

188

189 **Evolution of the vomeronasal system**

190 The evolutionary pattern of the gain and loss of *V2R* genes differs from that of *ORs* in snakes.  
191 Although sea snakes possess a relatively small number of intact *V2Rs*, the numbers of intact  
192 *V2R* genes vary largely even among hydrophiins and laticaudins (Fig. 2). Snakes are known to  
193 have a pair of well-developed vomeronasal organs [34, 37], and fully-aquatic hydrophiins are no  
194 exception [37-39]. Most of the intact *V2Rs* are expressed in the VNO even in the case of  
195 hydrophiins (Fig. 4). The snake VNO is linked to the oral cavity, and snakes deliver odor  
196 molecules to their VNOs through tongue-flicking [36]. Underwater tongue-flicking is widely  
197 observed among squamates [37] including hydrophiins [37, 40], and *Hydrophis* can distinguish  
198 fish species solely by tongue-flicking [41]. All these pieces of evidence strongly suggest that the  
199 hydrophiin VNS is functional, and that sea snakes can smell underwater through tongue-flicking.  
200 A recent study reported that presence/absence of a *VIR* gene named *ancVIR* corresponds to  
201 presence/absence of the functional VNO among tetrapods [33]. We found an intact *VIR* gene  
202 orthologous to the *ancVIR* in each snake genome (Fig. S1), and expression of this *VIR* in the  
203 VNO is confirmed in hydrophiins (Table S3), supporting the suggestion that the hydrophiin  
204 VNS is functional. Gene duplication and loss of the *V2R* genes are more frequent than that of  
205 *OR* genes, and massive gain of the *V2Rs* is observed even in two species of sea snakes, *E. ijimae*  
206 and *L. colubrina* (Fig. 3B). Previous studies showed that *Emydocephalus* relies heavily on the  
207 VNS for foraging [40], while not only olfaction but vision plays an important role for  
208 *Hydrophis* to find prey [41]. This implies that *Emydocephalus* relies more on olfaction than  
209 *Hydrophis*, which is consistent with our results that *Emydocephalus* possesses a larger number  
210 of intact *V2Rs* than *Hydrophis*. Olfactory capabilities through the VNS may differ largely  
211 between snake species including sea snakes.

212 We found an *OR* gene expressed in the tongue. This gene is conserved among snake species,  
213 implying that the function of this *OR* is evolutionarily maintained and important for snakes.

214 Unlike most of other *OR* genes, the expression of this gene is confirmed even in hydrophiins  
215 (Fig. 4). Snakes do not use the tongue as a gustatory organ because it lacks taste buds [36]. Still,  
216 the tongue may be used as a chemosensory organ which enables efficient tongue-flicking of  
217 snakes. Further studies are required for testing this hypothesis.

218

### 219 **Olfactory capabilities of sea snakes**

220 In this study, we investigated the molecular basis of snake olfaction and showed the presence of  
221 the VNS but absence of the MOS in fully-aquatic hydrophiins. Although hydrophiins cannot  
222 smell in the air using the MOS, they smell underwater using the VNS. To our knowledge,  
223 hydrophiins are the only vertebrates which possess a functional VNS without presence of a  
224 MOS. The functional VNS is absent from all extant fully-aquatic mammals (cetaceans and  
225 sirenians) though their terrestrial relatives (artiodactyls and terrestrial afrotherians) have it [42],  
226 indicating that the VNS is required only on land in most mammals. However, squamates are  
227 suggested to smell underwater using the VNS. This may be because *V2Rs*, which are abundant  
228 in diverse aquatic vertebrates and putatively detect water-soluble molecules, are predominant in  
229 squamate genomes over *V1Rs*, which have diversified in mammals after their terrestrial invasion  
230 to detect odorants on land [43-47]. Modern anurans, particularly in the larval form, also use the  
231 VNS for smelling underwater [15] with the VNOs in which *V2Rs* are predominantly expressed  
232 [47-49].

233 The olfactory capability of amphibious laticaudins is speculated to be an intermediate between  
234 terrestrial snakes and hydrophiins: they still possess a functional MOS, but their *OR* gene  
235 repertoire has largely degenerated. This is consistent with our assumption that amphibious  
236 species are intermediates between fully-terrestrial and fully-aquatic. Amphibious mammals also  
237 tend to show intermediate status between fully-terrestrial and fully-aquatic mammals. For

238 example, the majority of pinnipeds retain putatively functional VNS, while some species such  
239 as harbor seals lack it [50]. Careful interpretation is required for amphibious species when  
240 studying convergent evolution among marine amniotes.

241

#### 242 **Comparison among fully-aquatic amniotes**

243 Our results show that hydrophiins, which adapted to water independently from aquatic  
244 mammals, also reduced olfaction profoundly. However, the residual olfactory abilities are very  
245 different between fully-aquatic mammals and hydrophiins. Baleen whales smell in the air using  
246 a highly degenerated but functional MOS [23, 26]. Little is known about olfactory capacities of  
247 sirenians, but they also possess a putatively functional though degenerated MOS [42, 51], and  
248 their olfactory anatomy suggests that they smell in the air, not underwater [51]. On the other  
249 hand, hydrophiins possess well-developed VNOs, and behavioral studies suggest that they smell  
250 underwater using the VNS [40, 41] (Table 1). The well-developed underwater-functional VNS  
251 of sea snakes is derived from the V2R-predominant well-developed snake VNS, and the  
252 difference of the olfactory capabilities between hydrophiins and fully-aquatic mammals is  
253 explained by the difference of the olfactory capabilities between their terrestrial ancestors.  
254 Underwater olfaction might have been favored by natural selection if it was adaptive for whales  
255 and sirenians to survive in water, but they have never acquired underwater-functional olfactory  
256 systems. This shows a striking contrast with the terrestrial adaptation of vertebrates. Tetrapods  
257 modified their olfactory organs and generated novel *OR* gene subfamilies for smelling in the air  
258 when they migrated from water to land [13, 15]. In addition, adults of secondarily aquatic pipid  
259 frogs acquired an additional olfactory epithelium called “water nose” for smelling underwater  
260 [47, 52-54]. But no amniotes are known to have modified their olfactory organs for sensing the  
261 newly invaded environment upon aquatic adaptation. Histological and genomic studies imply  
262 that whales lack innate avoidance behavior against predator odors probably because their

263 predators cannot be detected by smelling in the air [24, 25]. Amniotes belonging to various taxa  
264 have to adapt themselves to handle similar problems inflicted by their new environment upon  
265 aquatic adaptation. However, not only the ecological demands but phylogenetic backgrounds  
266 play important roles in the formation of sensory modalities in this process.

267

268

## 269 **MATERIALS and METHODS**

270

### 271 **Specimens**

272 The following specimens were used for DNA/RNA extraction. Specimens used for genome  
273 sequencing were not used for RNA extraction in order to save all internal/external organs for  
274 future studies. All specimens used in the study are deposited in the Zoological Collection of the  
275 Kyoto University Museum (KUZ) with the specimen vouchers shown below.

#### 276 *Laticauda laticaudata* Linnaeus 1758 (blue-lipped sea krait, Laticaudini)

277 1. specimen voucher: KUZ R72402, sex: male, locality: Okinawa Island, Japan (genome  
278 sequencing, Fig. S3A).

279 2. specimen voucher: KUZ R68692, sex: female, locality: Okinawa Island, Japan (RNA  
280 sequencing).

#### 281 *Hydrophis melanocephalus* Gray 1849 (slender-necked sea snake, Hydrophiini)

282 1. specimen voucher: KUZ R72403, sex: male, locality: Okinawa Island, Japan (genome  
283 sequencing, Fig. S3B).

284 2. specimen voucher: KUZ R73056, sex: female, locality: Okinawa Island, Japan (RNA  
285 sequencing).

286 *Laticauda colubrina* Schnieder 1799 (yellow-lipped sea krait, Laticaudini)

287 Specimen voucher: KUZ R77260, sex: male, locality: Ishigaki Island, Japan (genome  
288 sequencing).

289 *Emydocephalus ijimae* Stejneger 1898 (turtlehead sea snake, Hydrophiini)

290 Specimen voucher: KUZ R72604, sex: male, locality: Okinawa Island, Japan (genome  
291 sequencing).

292

### 293 **DNA extraction, sequencing and performing *de novo* assembly**

294 DNA was extracted manually, following the methods of Blin and Stafford [55] with  
295 modifications, from muscle tissues of specimens KUZR72402, KUZR72403 and KUZR72604.  
296 Whole-genome shotgun (WGS) sequences were generated using Illumina platforms. Paired-end  
297 libraries were prepared using the TruSeq DNA PCR-free Sample Prep kit (Illumina) (specimen  
298 KUZR72604) and the TruSeq Nano DNA Sample Prep kit (Illumina) (KUZR72402 and  
299 KUZR72043). Mate-pair libraries were prepared following Tatsumi *et al.* [56] having a size  
300 range of 6-10 kb with a peak of around 7 kb. A PacBio RS II sequencer was also employed for  
301 sequencing the *L. laticaudata* genome using the PacBio DNA Template Prep Kit 1.0 (Pacific  
302 Biosciences). The details of sequencing results are provided in Table S4, and k-mer frequency  
303 spectrum of the WGS reads of each specimen is shown in Fig. S4. `Platanus_trim` and  
304 `Platanus_internal_trim` [57] were employed to trim low-quality regions and adapters of paired-  
305 end and mate-pair sequences respectively with default parameters, except for reads of *L.*  
306 *colubrina*. Regarding PacBio long-reads, following filtering criteria were used to obtain

307 subreads from the polymerase reads: minimum subread length 50, minimum polymerase read  
308 quality 0.75. For the *L. colubrina* (specimen KUZR77260), high-molecular weight (HMW)  
309 DNA was extracted from the liver using Nuclei PURE Prep Kit (Sigma). Using the Chromium  
310 System (10xGenomics), a linked-read library was constructed from 1.25 ng of HMW DNA of  
311 50 kb or longer. The library was sequenced on an Illumina HiSeq platform, and then processed  
312 using Supernova v1.2.2[30, 31] with default settings. Assembling the *L. laticaudata* genome:  
313 Trimmed paired-end reads were used to construct contig assembly using the PLATANUS v1.2.4  
314 [57] with a step size of k-mer extension set at 1. Scaffolds were constructed based on this contig  
315 assembly using the Redundans v0.12c [58]. Finally, the PacBio subreads were merged, and gap-  
316 closing was performed using the PBJelly software in the PBSuite package v15.8.24 [59, 60].  
317 The *H. melanocephalus* genome: PLATANUS v1.2.4 [57] was employed for contig assembling,  
318 scaffolding and gap-closing with step size of k-mer extension set to be 1. Only paired-end reads  
319 were used for contig assembling, and then mate-pair reads were added for scaffolding and gap-  
320 closing. The *E. ijimae* genome: SOAPdenovo2 [61] was employed for contig assembling,  
321 scaffolding and gap-closing with a k-mer set to be 81. Completeness of these genome  
322 assemblies was evaluated using CEGMA v2.5 [29] and BUSCO v3 [27, 28] referring to the  
323 ortholog set CVG [62].

324 In addition to these sea snake genome assemblies, genome assemblies of terrestrial snakes  
325 closely related to sea snakes [king cobra *Ophiophagus hannah* (Elapidae, GenBank Accession  
326 GCA\_000516915.1), garter snake *Thamnophis sirtalis* (Colubridae, GCA\_001077635.2) and  
327 common viper *Vipera berus* (Viperidae, GCA\_000800605.1)] and a green anole *Anolis*  
328 *carolinensis* (Iguanidae, GCA\_000090745.2) were obtained from the GenBank FTP server.

329

### 330 **Identification of the olfactory GPCR genes**

331 **OR:** The *OR* genes were searched against the genome assemblies of seven snake species (four  
332 sea snake genomes assembled in this study, and three terrestrial snake genomes retrieved from  
333 the GenBank) using the TBLASTN program in the BLAST+ v2.6.0 package [63] with the  
334 cutoff *E*-value of  $1 \times 10^{-5}$ . Deduced amino acid sequences of all intact *OR* genes of the green  
335 anole and the western clawed frog identified by Niimura [64] were used as queries. Each  
336 sequence thus obtained was searched against the NCBI protein database using the BLASTX  
337 program and was discarded if its best hit was not an OR. A sequence was judged to be a non-  
338 functional pseudogene if the sequence was interrupted by premature stop codon(s) and/or frame  
339 shift(s), or it lacked five or more consecutive amino acids including a trans-membrane domain.  
340 If a sequence was interrupted by contig-gap(s) although it was not judged to be a pseudogene, it  
341 was labeled as 'truncated'. The *OR* gene repertoires of the anole and the python were retrieved  
342 from the dataset provided by Vandewege *et al.* [65]

343 **TAAR and VIR:** Essentially a uniform method employed to find the *OR* genes was used to  
344 identify intronless *TAAR* and *VIR* genes from the genome assemblies of seven snakes and a  
345 green anole with following amino acid sequences as queries: *TAAR*, all intact mammal TAARs  
346 identified by Kishida *et al.* [24]; *VIR*, all intact vertebrate VIRs identified by Zapilko and  
347 Korsching [46].

348 **V2R:** Only the longest exon (3<sup>rd</sup> exon, approx. 800 bp) of *V2R* genes was analyzed in this study  
349 because *V2Rs* are multi-exon genes and it is difficult to identify all exons derived from a *V2R*  
350 gene if exons are scattered in two or more scaffolds. Using deduced amino acid sequences of the  
351 3<sup>rd</sup> exon of all intact vertebrate *V2R* genes identified by Shi and Zhang [43] as queries, *V2R*  
352 sequences were searched and identified based on the same method employed to identify *OR*  
353 genes.

354

355 **RNA extraction, sequencing and expression analyses**

356 Total RNA was extracted from three potential olfactory organs (the VNO, the NC and the  
357 tongue) and the liver (as a negative control) of specimens KUZR68692 (*L. laticaudata*) and  
358 KUZR73056 (*H. melanocephalus*) using the RNeasy Mini kit (Qiagen) and following the  
359 manufacturer's guidelines. Regarding the NC, we sampled entire tissues around the NC broadly  
360 from both specimens for RNA extraction because the olfactory epithelia of both specimens were  
361 hard to be located exactly. It is noted that this might cause reduction of the estimated expression  
362 level of chemosensory receptors due to contamination of non-olfactory tissues. The extracted  
363 RNA was used to construct paired-end sequencing libraries using the TruSeq RNA Sample Prep  
364 Kit v2 (Illumina), and these libraries were sequenced using an Illumina HiSeq platform  
365 (2×101bp). As a result, the following sizes of RNA-seq reads were obtained: [KUZR68692]  
366 VNO 5.19 Gbp, NC 5.27 Gbp, tongue 5.91 Gbp, liver 5.24 Gbp; [KUZR73056] VNO 4.73 Gbp,  
367 NC 5.74 Gbp, tongue 5.38 Gbp, liver 8.46 Gbp. Low-quality sequences and adapters were  
368 removed using the Trimmomatic v0.36 [66] with the following parameters:  
369 ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10, LEADING:20, TRAILING:20,  
370 SLIDINGWINDOW:5:25 and MINLEN:36. Trimmed RNA-seq reads were mapped to the  
371 conspecific genome assembly using HISAT2 [67] v2.1.0 with default parameters. Gene  
372 expression levels were quantified with FPKM values using Cufflinks [68, 69] v2.2.1 after  
373 removal of duplicated reads.

374

375 **Phylogenetic analyses**

376 Deduced amino acid sequences were aligned using the L-INS-i program in the MAFFT package  
377 v7.266 [70], and gap sites were excluded from further analyses. Trees were inferred using the  
378 neighbor-joining method [71] based on the Poisson-corrected distance matrices. Evolutionary

379 changes in the number of *OR/V2R* genes were inferred using the reconciled tree method [72].  
380 Amniote *ORs* are clearly classified into two classes, class I and class II [73]. All intact *OR* genes  
381 were classified into 35 clades identified by Niimura and Nei (a class I clade and 34 class II  
382 clades) [72] based on sequence similarities, and a calculation was performed for each clade  
383 separately. Eight human class I *ORs* (OR51Q1, OR51G1, OR51L1, OR51I1, OR52K1,  
384 OR52H1, OR52B4, OR56A1) retrieved from the HORDE database [74] build #44 were used as  
385 outgroups for class II *OR* trees; 16 human class II *ORs* (OR1C1, OR1Q1, OR2C1, OR5F1,  
386 OR5J2, OR5P3, OR6B2, OR6N1, OR7D4, OR8D2, OR8U1, OR9Q2, OR10A3, OR10K1,  
387 OR11H4, OR13D1) for a class I *OR* tree. Vandewege et al. [65] reported the *OR* gene repertoire  
388 of a python, a phylogenetically distant snake species which possesses much larger number of  
389 intact *OR* genes (481) than that of any snakes investigated in this study. We included the python  
390 intact *ORs* identified by them for this analysis to estimate the ancestral *OR* gene repertoires  
391 thoroughly. *V2R* genes were classified into two clades (families C and non-C [44]) based on a  
392 phylogenetic tree using green anole *Tas1Rs* (*Tas1R1*; GenBank accession no. XM\_016998922,  
393 *Tas1R2*; XM\_008124605, *Tas1R3*; XM\_003228934) as outgroups, and the evolutionary gains  
394 and losses of non-C *V2Rs* were calculated using the family-C *V2Rs* as outgroups. Because all  
395 snakes possess exactly one family-C *V2R*, we concluded that the number of family-C *V2R* did  
396 not change through the evolution of snakes. Bootstrap values were obtained by 500 resamplings,  
397 and a bootstrap value of 70% was used as a threshold for reconciliation. Truncated genes and  
398 pseudogenes were excluded from this calculation. Phylogenetic trees of class I and class II *OR*  
399 genes are shown in Figs. S5 and S2 respectively, and changes in the number of class I and class  
400 II *OR* genes are shown in Fig. S6.

401

402

403

404 **Acknowledgements**

405 We are grateful to Prof. Tsutomu Hikida for helping dissection of snakes; Takahide Sasai, Koji  
406 Mochida and members of Tropical Biosphere Research Center, University of the Ryukyus for  
407 supporting sampling and fixation of specimens; Dr. Rob Ogden for checking the English of the  
408 text; reviewers of this manuscript for helpful comments. Computations were partially performed  
409 on the NIG supercomputer at ROIS National Institute of Genetics.

410

411 **Data accessibility**

412 Specimens: Zoological Collection of the Kyoto University Museum (KUZ) with specimen  
413 vouchers KUZ R68692, R72402, R72403, R72604, R73056 and R77260. Sequencing data and  
414 assembled genome sequences: GenBank BioProject accessions PRJDB7221 (*E. ijimae* genome  
415 sequencing), PRJDB7226 (*L. laticaudata* genome sequencing), PRJDB7271 (*H.*  
416 *melanocephalus* genome sequencing), PRJDB7284 (*L. colubrina* genome sequencing),  
417 PRJDB7257 (*L. laticaudata* RNA-seq) and PRJDB7258 (*H. melanocephalus* RNA-seq). The  
418 locus of each gene (Supplemental Tables S6-S13) and amino acid sequences of intact olfactory  
419 GPCRs (Supplemental Data S1) identified in this study: Dryad doi:10.5061/dryad.t8sm4m6 [78].

420

421 **Funding**

422 This work was supported by JSPS KAKENHI [grant numbers 15K07184 and 18K06378 to TK,  
423 16H06531 to YG]; and the Collaborative Research of Tropical Biosphere Research Center,  
424 University of the Ryukyus.

425

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#### 659    **Figure captions**

660    **Figure 1.** A schematic view of the evolution of terrestrial adaptation of vertebrates and three  
661    major groups of extant fully-aquatic amniotes. Branch color indicates representative lifestyle in  
662    each branch (brown: terrestrial, purple: amphibious, blue: fully-aquatic), and circles in ancestral  
663    nodes represent lifestyles at these points in evolution. Extinct amphibious species are also  
664    shown for cetaceans (*Amburocetus* [75]) and sirenians (*Pezosiren* [76]).

665    **Figure 2.** Phylogenetic relationship of squamates analyzed in this study, and the numbers of  
666    olfactory GPCR genes identified in the genome assemblies of these species. Red, pink and grey  
667    bars indicate the numbers of intact genes, truncated genes and pseudogenes, respectively.  
668    Approximate divergence time follows Sanders *et al.* [4-6] and Kim *et al.* [77]. Notes: \*Only the  
669    third exon of the *V2R* genes was identified and analyzed. \*\*The *OR* gene repertoire of a green  
670    anole is taken from Vandewege *et al.* [65].

671 **Figure 3.** Evolution of the gain and loss of *OR* and *V2R* genes in snakes. Evolutionary changes  
 672 in the number of intact *OR* (A) and *V2R* (B) genes are estimated using the reconciled-tree  
 673 method [72]. Python intact *OR*s identified by Vandewege *et al.* [65] were included in the dataset  
 674 for this calculation.

675 **Figure 4.** Expression levels of the *OR* and *V2R* genes in the three potential olfactory organs and  
 676 the liver. Each dot represents a single *OR/V2R* gene identified in this study, and the y-axis  
 677 shows normalized gene expression levels in FPKM (fragments per kilobase of exon per million  
 678 mapped fragments) values. Red, pink and black dots represent intact genes, truncated genes and  
 679 pseudogenes, respectively. Mean FPKM values of intact, truncated and pseudogenes in each  
 680 organ are shown as bars in the background. Difference of mean FPKM values of intact *OR/V2R*  
 681 genes between each chemosensory organ and a control (liver) is calculated, and chemosensory  
 682 organs with obviously ( $>1$ ) and significantly ( $p<0.01$ , paired t-test) larger FPKM values  
 683 compared to the control are shown with asterisks (see Table S5 for detail). Arrows indicate an  
 684 intact *OR* gene expressed in the tongue. Approximate position of each organ in a fully-aquatic  
 685 hydrophiin (*H. melanocephalus*) is also shown.

686

687

**Table 1.** Olfactory capabilities of extant fully-aquatic amniotes.

		main olfactory system (MOS)	vomeronasal system (VNS)	references
Cetacea	Odontoceti	absent	absent	[22, 42]
	Mysticeti	present, smelling in the air	absent	[23-26]
Sirenia		present, smelling in the air	absent	[42, 51]
Hydrophiini		absent	present, smelling underwater*	[37-41], this study

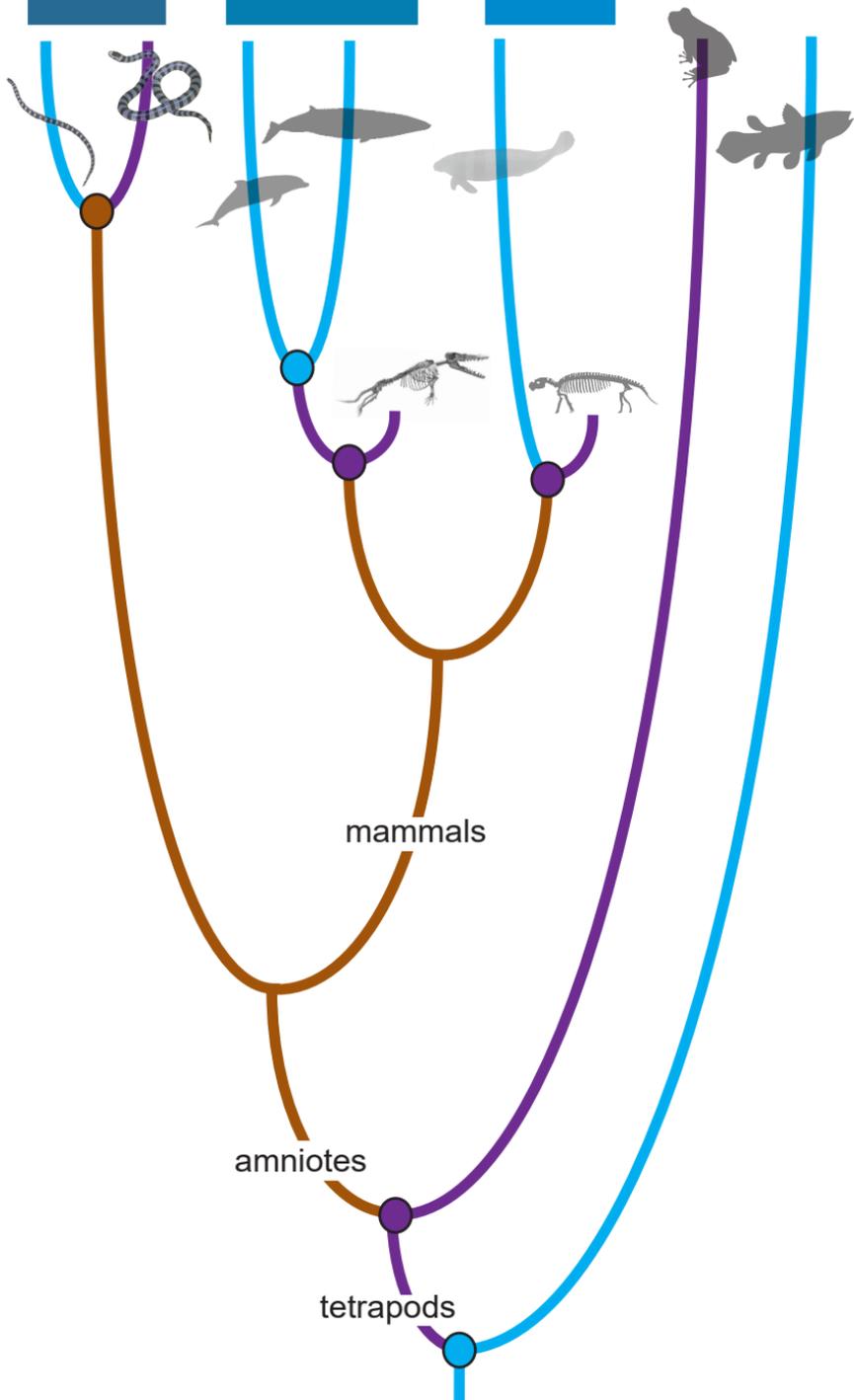
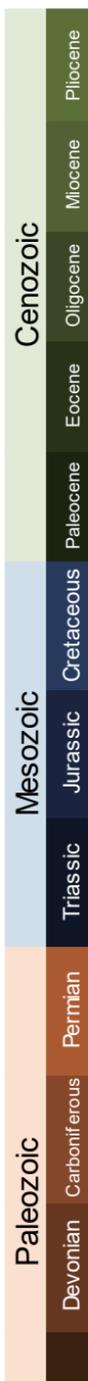
\*It remains unknown whether hydrophiins use the VNS for smelling in the air or not.

688

sea snakes

cetaceans

sirenians



140 | 40 | 20 (million years ago)

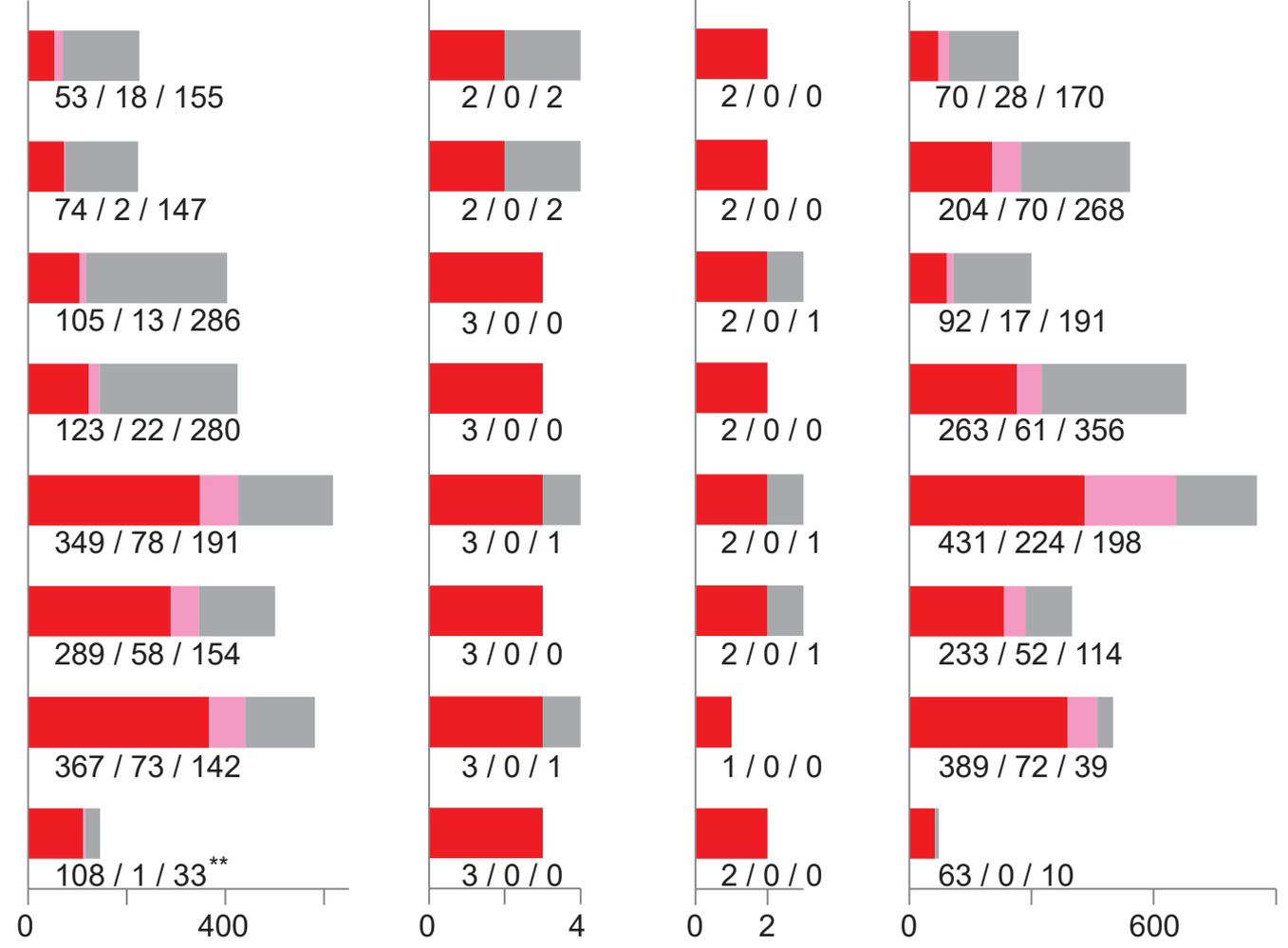
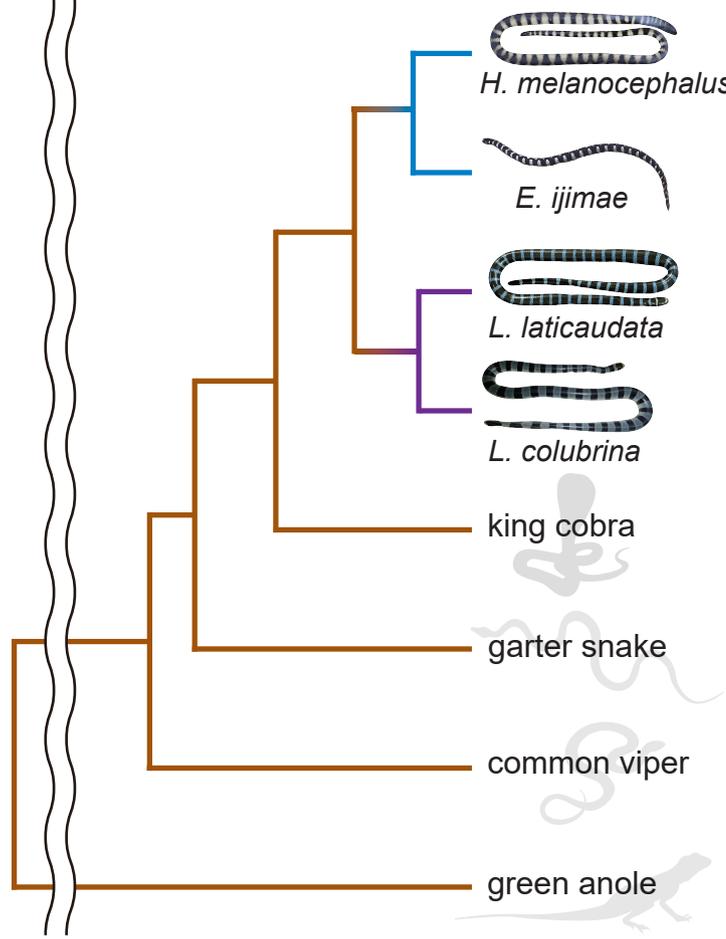
habitat  
land sea

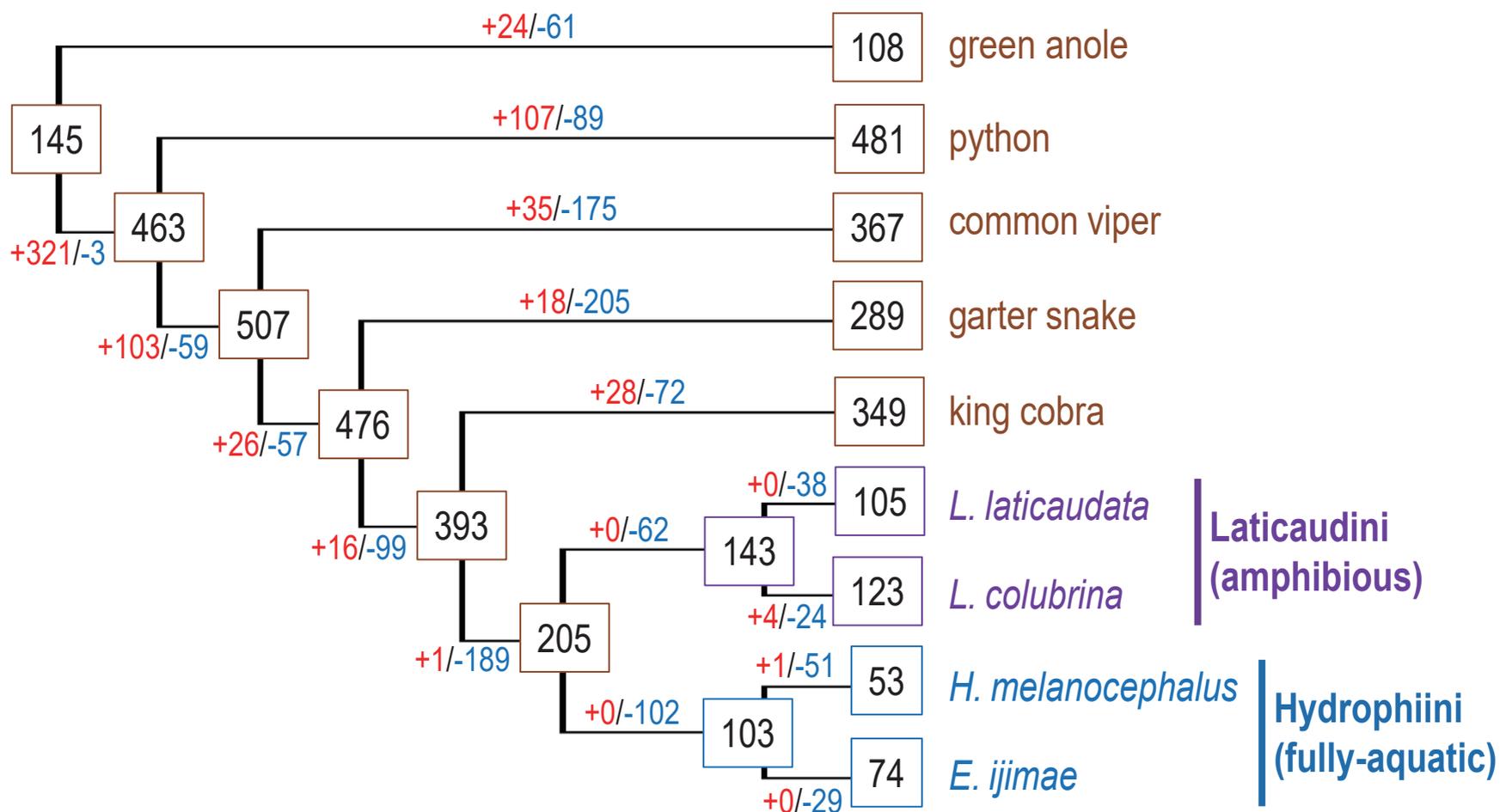
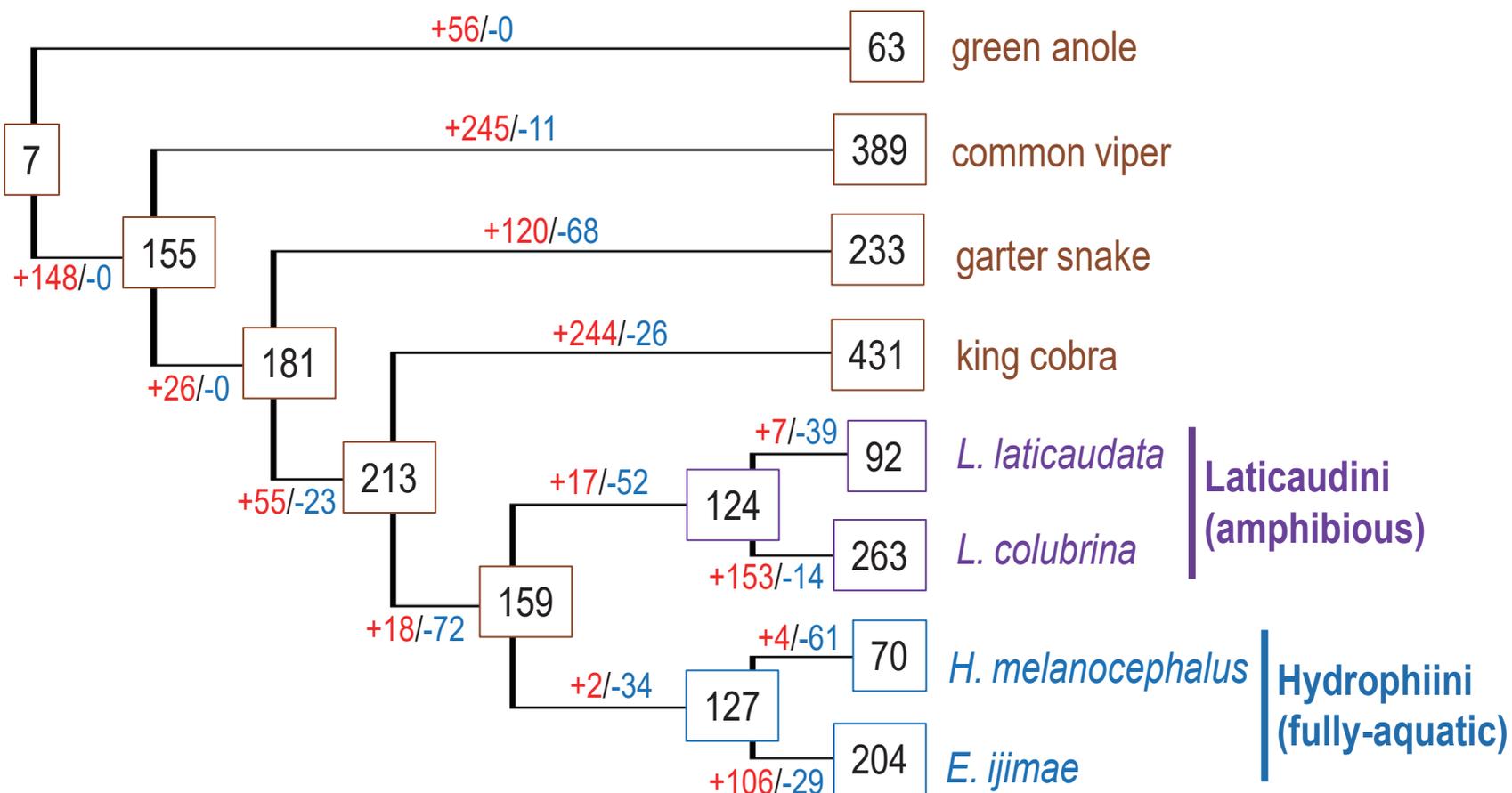
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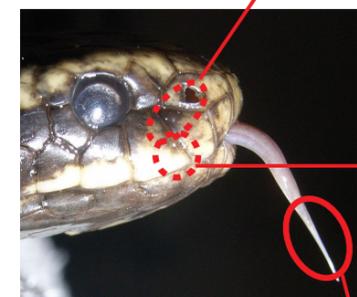
TAAR

V1R

V2R\*



**A****B**



NC

VNO

tongue

liver  
(control)

