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Pairwise Comparison of Orthologous Olfactory Receptor Genes Between Two Sympatric Sibling Sea Kraits of the Genus *Laticauda* in Vanuatu

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Olfaction-based reproductive isolation is widely observed in animals, but little is known about the genetic basis of such isolation mechanisms. Two species of sibling amphibious sea snakes, *Laticauda colubrina* and *L. frontalis* live in Vanuatu sympatrically and syntopically, but no natural hybrids have been reported. Adult females of both taxa possess distinctive lipids in the skin, and male *L. frontalis* distinguishes conspecific females based on olfactory cues. To shed light on the molecular basis of the evolution of olfaction-based isolation mechanisms, olfactory receptor (OR) gene repertoires of both taxa were identified using pyrosequencing-based technology, and orthologous OR gene sets were identified. Few species-specific gene duplications or species-specific gene losses were found. However, the nonsynonymous-to-synonymous substitution rate ratio was relatively higher between orthologous OR genes of *L. frontalis* and *L. colubrina*, indicating that *L. frontalis* and *L. colubrina* have evolved to possess different olfactory senses. We suggest that *L. frontalis* and *L. colubrina* have evolved allopatrically, and this may be a byproduct of the allopatric evolution, and that this dissimilarity may function as a premating isolation barrier, since *L. frontalis* has returned to the ancestral range (Vanuatu).

Key words: *colubrina* complex, *Laticauda frontalis*, olfaction, sea snake, speciation, yellow-lipped sea krait

INTRODUCTION

Sensory-based reproductive isolation is widely observed in animals, and is thought to play an important role as an isolation barrier between sympatric sibling species. There have been many studies focusing on the molecular basis of the evolution of such isolation mechanisms, especially focusing on vision-based isolation mechanisms (e.g., Seehaussen et al., 2008). However, despite the indications that odor-based reproductive isolation is likely to be important in many metazoan taxa including vertebrates (Coyne and Orr, 2004), little is known about the molecular basis of the evolution of isolation barriers based on pheromones and other chemicals, which is due in part to the fact that, unlike in the case of photoreceptors (opsins), there are too many chemosensory receptors (CRs) to be investigated in detail.

Sea snakes of the genus *Laticauda* (Reptilia; Squamata; Serpentes; Elapidae) are a group of monophyletic amphibious snakes, including the yellow-lipped sea krait *Laticauda colubrina*. This species spends half of its lifetime on land (Shetty and Shine, 2002) and is widely distributed around the tropical Pacific Ocean (Heatwole et al., 2005). From 1983 to 1996, extensive field research on sea snakes was conducted in the western Pacific under the leadership of Drs. N. Tamiya (Tohoku Univ.) and T. Tamiya (Sophia Univ.), leading to the discovery that two sympatric sibling species are included in the populations of the yellow-lipped sea krait in Vanuatu (details on this research are described by Cogger et al. (1987) and Shine et al. (2002)). One species is *L. colubrina*, and the other, named *L. frontalis* (de Vis, 1905), is available (Cogger et al., 1987). The two species are morphologically nearly identical, except that *L. colubrina* grows larger than *L. frontalis* (Fig. 1, Cogger and Heatwole, 2006). They exist sympatrically and syntopically in Vanuatu, but no natural hybrids have been reported, despite the fact that these two species breed at the same time (Shine et al., 2002). Shine et al. (2002) showed that adult females of both taxa possess distinctive lipids in the skin, and *L. frontalis* males distinguish conspecific females through their olfactory sense by tongue-flicking on the skin of females. Interestingly, *L. colubrina* males cannot distinguish conspecific females using olfactory cues, leading Shine et al. (2002) to speculate that male snakes prefer courting larger females, meaning that *L. colubrina* males would be unlikely to court *L. frontalis*-sized (small) females even in the absence of pheromonal barriers. Based on these studies, *L. frontalis* was formally elevated to full species status in 2006 (Cogger and Heatwole, 2006). In contrast to *L. colubrina*, the distri-

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bution of *L. frontalis* is limited to Vanuatu (Cogger and Heatwole, 2006; Lane and Shine, 2011). There is one more *Laticauda* species, *L. laticaudata*, distributed in Vanuatu, this species is morphologically and phylogenetically not so closely related to the other two species. The phylogenetic relationships of these three *Laticauda* species were described in Lane and Shine (2011).

Studies of how such olfaction-based isolation mechanisms have been achieved, and how *L. frontalis* and *L. colubrina* speciated, are of potentially great interest. Shine et al. (2002) discussed the possibility that *L. frontalis* arose in sympathy with *L. colubrina* in Vanuatu. In contrast, Lane and Shine (2011) suggest that allopatric speciation occurred, in which *L. frontalis* originated in New Caledonia and re-invaded the ancestral (*L. colubrina*) range approximately 180,000 years ago. Following the view of Lane and Shine (2011), a hypothesis has been suggested that olfaction-based isolation mechanisms are achieved through sensory drive (Endler, 1992; Bougman, 2002), which predicts that adaptation of signaling (i.e., lipid chemicals) and sensory (i.e., CRs) systems of allopatric populations to different environments may cause premating isolation upon secondary contact of these populations.

Olfaction is an important sensory modality for animals to perceive surrounding odors, such as environmental odors and conspecific pheromones. Seven trans-membrane (7TM) G-protein coupled receptors are known to function as CRs across vertebrate species (Nei et al., 2008). Especially notably among such CRs, olfactory receptors (ORs) are present in all vertebrates and are considered to play the primary role in olfaction in amniotes (Nei et al., 2008). The repertoire of OR genes varies greatly among amniote species, and the ecological niche that an animal inhabits is directly associated with the OR repertoire of the species (Niimura, 2009; Hayden et al., 2010). In fact, it has been reported that OR gene repertoires are quite different between the two closely-related species, humans and chimpanzees, mainly because of species-specific gene losses in both the human and chimpanzee branches (Go and Niimura, 2008) and that this may reflect the fact that these two species speciated allopatrically (Webster, 2009) and have evolved in different environmental niches (Adipietro et al., 2012).

At present, in silico screening of whole-genome sequences is the best and indeed the only way to obtain nearly complete OR gene repertoires, but no genomic databases are available for *Laticauda* sea snakes, or even for elapid snakes. Dehara et al. (2012) suggested that a large number of OR genes can be obtained for a species without any genome databases using pyrosequencing-based OR gene identification methods. In this study, we sampled three *Laticauda* species, *L. colubrina*, *L. frontalis* and *L. laticaudata* (as an outgroup) in Vanuatu, identified their OR genes using pyrosequencing-based technology, and compared the OR gene repertoires in *L. frontalis* and *L. colubrina* in order to investigate how olfactory abilities differentiated between these two species after their genetic split.

**MATERIALS AND METHODS**

**Sampling sea snake specimens**

A male *L. colubrina* and a male *L. frontalis* were sampled at Ngioriki Islet, Paonangisu village, Vanuatu (17°30′S, 168°25′E). These two taxa were discriminated in the field by the lateral head patterns, as Cogger and Heatwole (2006) suggested. This discrimination was confirmed by sequencing mitochondrial genes in the laboratory (data not shown). However, no *L. laticaudata* were found on the islet. Therefore, a male *L. laticaudata* was sampled at the southeast coast of the island of Efate (17°49′S, 168°26′E) at night.

**Sequencing OR genes**

The procedures for DNA extraction and OR gene amplification of the three *Laticauda* species followed Kishida and Hikida (2010) with ORSB and OR3B primers (Ben-Arie et al., 1994) and AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen). The amplicons are expected to contain part of the open reading frames of OR genes between TM2 and TM7, which are approximately 650 bp in length without primers. The amplicons were purified using a PCR purification kit (QIAGEN), and then tagged using a GS Titanium Rapid Library MID Adaptors Kit (Roche). The OR amplicon libraries of *L. frontalis*, *L. colubrina* and *L. laticaudata* were tagged with MID6, MID7, and MID8, respectively. Equal amounts of the amplicon libraries of *L. frontalis* and *L. colubrina* were mixed and sequenced together on a GS Junior sequencer (Roche). The amplicon library of *L. laticaudata* was also sequenced on a GS Junior sequencer together with an equal amount of an amplicon library, which was not analyzed in this study. Raw reads data obtained using the GS Junior sequencer have been deposited to the DDBJ Sequence Read Archive (DRA) under accession numbers DRA000729–DRA000731.

We then prepared OR gene amplicons of *L. frontalis* and *L. colubrina* once more, and identified OR genes following procedures used in a previous study (Kishida and Hikida, 2010) with an ABI3130 capillary sequencer (Applied Biosystems). Approximately 100 colonies were sequenced for each species.

**Identification of OR genes**

Reads obtained from the GS Junior sequencer were divided into the three species on the basis of the MID tags, and then assembled into contigs using GS De Novo Assembler ver. 2.5p1 (Roche) with the following settings; expected depth: 0, minimum read length: 45, minimum overlap length: 50, minimum overlap identity: 99, heterozygotic mode, other parameters: default. Each contig was searched against the entire mouse protein database, which was retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/protein?term=\%22
Identification of orthologous gene sets

We modified the methods of Go and Niimura (2008) to identify orthologous OR gene sets. As we suggested previously, the elapid snakes OR repertoires include a subfamily named squamate-specific ORs, which seems to have diverged rapidly (Kishida and Hikida, 2010). Among this subfamily, for example, L. frontalis OR LfrOR25 and L. colubrina OR LcoOR26, which are similar to each other in overall bases and are considered to be orthologous, are included in the squamate-specific OR sets. We identified several OR sequences following the methods of Kishida and Hikida (2010). Sanger sequencing-based OR sequences that were obtained are available in the DDBJ/EMBL/GenBank databases under the following accession numbers: AB754502–AB754548.

In the case of L. laticaudata, previously reported Sanger sequencing-based OR sequences were retrieved from GenBank under the following accession numbers (ABS24695–AB524714). These Sanger sequencing-based sequences were mixed with the pyrosequencing-based conspecific contigs, and aligned using the L-INS-i program in the MAFFT package (Katoh et al., 2005) with manual adjustments. Primer regions and low quality regions (scores < 32 in the sequence quality file) were cut off from the sequences. Two sequences that shared > 99.5% similarity with > 50 bp overlaps were considered to encode the same OR sequence and were merged. Finally, the OR gene repertoires of these three species were mixed together, and aligned using the L-INS-i program with manual adjustments. When we found a clearly orthologous gene pair of two species without species-specific duplications and we found two or more fragment sequences from the third species that strongly resembled the sequences of the two species and did not share overlapping aligned regions, we merged these fragments into a single sequence and filled gaps with ‘n (base unknown). The assembled OR sequences of L. frontalis, L. colubrina and L. laticaudata are available as Supporting Data.

OR sequences which are not included in the squamate-specific ORs

By using L. frontalis sequences as queries, we conducted BlastN searches (Altschul et al., 1997) against all Laticauda sequences obtained in this study, with the cutoff of e-value < 1.0 × 10−10. For each result, the query sequence and the hit sequences (excluding the query sequence itself) were aligned using L-INS-i with manual inspection, and the nucleotide sequence identities, excluding gaps, between the query sequence and the hit sequences were calculated. When the nucleotide identity was > 98.5% (frontalis—colubrina) or was > 96% (frontalis—laticaudata), and that the sequences were similar to each other over the entire length, they were assumed to be orthologous. These cutoff values were chosen because, to our knowledge from experiments, nucleotide identities of orthologous genomic DNA sequences for L. frontalis-L. colubrina and L. frontalis-L. laticaudata comparisons are normally > 98.5% and > 96%, respectively. Then, by using L. colubrina sequences as queries, we conducted the same procedures and confirmed that we obtained the same results.

OR sequences included in the squamate-specific ORs

We first inferred a phylogenetic tree using all Laticauda OR sequences included in the squamate-specific ORs (Fig. 2B). Whole-genome sequenced python OR sequences, identified by Dehara et al. (2012), were also included. In this phylogenetic tree, eight sets of putative orthologous trios (frontalis—colubrina—laticaudata) and three sets of duos (frontalis—colubrina, laticaudata orthologous gene not found) were found with bootstrap values > 90%. In all cases, we confirmed that the nucleotide identity was > 98.5% between L. frontalis and L. colubrina, and > 96% between L. frontalis and L. laticaudata, and that the sequences were similar to each other over the entire length.

Sequence analyses

We analyzed each ortholog gene set of Laticauda OR sequences separately. The numbers of nonsynonymous substitutions (N_d) and synonymous substitutions (S_d) in each branch, as shown in Fig. 3A, were calculated by the method of Nei and Gojobori (1986) based on the ancestral nucleotide sequences inferred by the Bayesian method (Yang et al., 1995). Numbers of nonsynonymous sites (N) and synonymous sites (S) were estimated by the maximum likelihood method (Goldman and Yang, 1994). These calculations were carried out using the CODEML program in the PAML4.4 package (Yang, 2007). In cases in which no L. laticaudata OR was found as an orthologous sequence, N_d and S_d between the L. frontalis and L. colubrina OR sequence were counted based on the method of Nei and Gojobori (1986). A sequence was judged to be a pseudogene if a termination codon and/or frame shift was found in its open reading frame. However, if these frame shifts were caused where three or more repetitions of the same base and the sequence had not been confirmed by Sanger sequencing, we did not judge the sequence to be a pseudogene, as such regions tend to be misread using the Roche 454 sequencing system. A test of the homogeneity of nonsynonymous/synonymous change ratios (Kishida and Thewissen, 2012) was applied to examine whether the number of nonsynonymous substitutions in particular branches could be considered homogeneous in comparison with that in a compared branch.

RESULTS

Orthologous relationships of OR genes between L. frontalis and L. colubrina

Sixty-two, 59, and 60 OR sequences were obtained from L. frontalis, L. colubrina and L. laticaudata, respectively. The sequence diversity of these ORs is shown in Fig. 2A. Among these sequences, we found 39 sets of orthologous trios (frontalis—colubrina—laticaudata) and nine sets of duos (frontalis—colubrina). All orthologous relationships of OR genes are listed in Supplementary Table S1 online. No L. frontalis-specific OR gene duplications were found, but one putative L. colubrina-specific OR gene duplication was found; L. frontalis LfrOR55 was similar to both L. colubrina LcoOR55 (nucleotide identity 99.67%) and LcoOR53 (nucle-
We found 11 \textit{L. frontalis} OR pseudogenes among

![Fig. 2. A neighbor-joining tree of OR genes identified in this study. \textit{L. frontalis} ORs are indicated by red font; \textit{L. colubrina}, green; \textit{L. laticaudata}, blue. Genome-determined python OR genes (indicated by black font), taken from Dehara et al. (2012), were added to the tree. (A) The FASTA3.5 program (Pearson and Lipman, 1988) was used to calculate opt scores pairwise between all combinations of OR sequence pairs, and the opt score matrix thus obtained was used as the distance matrix. Note that this tree is unrooted. (B) Details of a subtree named squamate-specific ORs (Kishida and Hikida, 2010). Distance matrix was calculated based on Kimura’s 2-parameter method. Bootstrap values were obtained by 500 resamplings, and values > 90% are shown. Sets of putative orthologous OR genes trios/duos, supported with > 90% bootstrap values, are indicated with brown vertical bars.]

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the other 47 sets of orthologous trios/duos, and in all cases, the *L. colubrina* ortholog of each *L. frontalis* OR was also a pseudogene, sharing the same pseudogenization mutations with its orthologous *L. frontalis* OR. Note that we analyzed only between the TM2 and TM7 regions, thus genes that seem to be intact may in fact be pseudogenes. For example, it is still possible that both *LfrOR4* and *LcoOR4* are pseudogenes and share identical pseudogenization mutations in the region between TM1 and TM2. In any case, these data indicate that, unlike in the case of humans and chimpanzees (Go and Niimura, 2008), *L. frontalis* and *L. colubrina* maintain similar OR gene repertoires to each other. These 11 sets of pseudogene orthologs were excluded from further analyses.

**Nonsynonymous substitutions within orthologous gene sets**

As shown in Fig. 3, in many cases (28/37 = 76%), nonsynonymous differences are found between *L. frontalis* OR genes and their *L. colubrina* orthologs, and the actual rate would be even higher because we sequenced only the region between TM2 and TM7 of the OR genes. Nonsynonymous substitutions occurred at nearly equal frequencies in the *frontalis* and *colubrina* branches (Fig. 3B), and the nonsynonymous to synonymous substitution rate ratios were higher in both branches compared with that in the *laticaudata* branch (Fig. 3C). This tendency was weakly significant according to the test of homogeneity of nonsynonymous/synonymous change ratios (P = 0.065, Table 1).

**DISCUSSION**

Approximately 60 OR genes were identified for each species, and these sequences did not seem to be concentrated extremely in any specific subtrees (Fig. 2A). Dehara et al. (2012) reported that 280 OR genes were identified based on the in silico screening of the python draft genome database. We thus may have obtained approximately 20% of the total OR gene repertoires, based on the assumption that pythons and Laticauda sea snakes possess almost equal numbers of OR genes, though we cannot judge whether this assumption is valid or not. Dehara et al. (2012) identified 96 OR genes from rat snakes following nearly the same protocols as used here, but using another primer sets.

Their primer sets seem to be more efficient compared with ours, but they obtained only approximately 330 bp for each OR gene, which was too short to conduct further sequence analyses. In any case, the OR gene repertoires obtained by the PCR-based pyrosequencing method were far from complete, but this method is nonetheless one of the best ways to identify a large number of OR genes without any reference genome databases and at a reasonable cost. In particular, this method would work efficiently for identifying orthologous gene sets from multiple species, as the sequences were obtained under the same primer bias for all species and thus if a sequence was obtained from a species, its ortholog would also be expected to be sequenced from the other species. Actually, 62 OR sequences were...
obtained from *L. frontalis* in this study, and among 77% (48/62) of them, the orthologous genes were obtained from *L. colubrina*.

As shown in Results, there were few species-specific gene duplications or species-specific gene losses when the OR gene repertoire of *L. frontalis* was compared with that of *L. colubrina*. This means that, both *L. frontalis* and *L. colubrina* are expected to have maintained the size of their OR repertoire after their genetic split. The OR repertoire has been shown to undergo extensive gains and losses of genes during vertebrate evolution (Nei et al., 2008), and it has long been discussed that the number of OR genes can serve as an indicator for assessing the olfactory ability of the animal (e.g., Dehara et al., 2012). In addition, orthologous genes are assumed to perform equivalent functions (Go and Niimura, 2008; Nehrt et al., 2011). According to these views, the olfactory abilities of both *L. frontalis* and *L. colubrina* are expected to be nearly equivalent to that of their last common ancestor, and no significant changes have occurred after their genetic isolation. However, as Nei et al. (2008) discussed, even a small number of amino acid changes could alter the function of OR genes. Keller et al. (2007) showed that only two amino acid changes on a human OR OR7D4 alter the human odor perception drastically. Adipietro et al. (2012) extended this view and showed that even a small number of nonsynonymous substitutions can change the ligand potency and efficiency of ORs dramatically. Our data shows that in many cases (76% or more), the amino acid sequences were different between *L. frontalis* and *L. colubrina* orthologs, suggesting that their olfactory abilities are different from each other. Unexpectedly, nonsynonymous substitutions occurred almost equally in both the *frontalis* and *colubrina* branches, indicating that both *L. frontalis* and *L. colubrina* has changed its olfactory sense. This may suggest that the surrounding environmental odors have changed, even in the same place since the time when their ancestors lived.

Most of the OR genes possessed by *Laticauda* sea snakes are expected to function only on land (Kishida and Hikida, 2010). The third *Laticauda* species, *L. laticaudata* spends more time in the sea, while the *L. colubrina* clade (*L. frontalis* and *L. colubrina*) relies more on terrestrial habitats (Lane and Shine 2011). Therefore, it can be expected that the selective pressures of purifying selection on ‘terrestrial-specific’ OR genes would be more strict in the *frontalis* and *colubrina* branches than in the *laticaudata* branch. However, as shown in Fig. 3C, the nonsynonymous to synonymous rate ratio \( \omega \) was higher in the *frontalis* and *colubrina* branches than in the *laticaudata* branch, and this tendency was weakly significant (Table 1). Higher \( \omega \) ratios indicate the relaxation of purifying selection (Yang, 2006). In the case of primates, \( \omega \) ratios of the orthologous OR genes are much higher between humans and chimpanzees (0.94 on average) compared to that between humans and macaques (0.44 on average), reflecting the fact that humans and chimpanzees have evolved to possess different senses of smell (Go and Niimura, 2008). Considering these things, at least, we can reject the hypothesis that *L. frontalis* and *L. colubrina* have evolved under the strict selective pressures to maintain similar OR genes. This may suggest that *L. frontalis* and *L. colubrina* have evolved to possess different olfactory senses. It is expected that most of the ORs analyzed in this study have been evolved to adapt the chemical environments surrounding snake habitats. Therefore, if these two *Laticauda* species have evolved sympatrically and syntopically on land, these two species should maintain similar OR gene repertoires. In contrast, our results can be explained easily if these two species have evolved allopatrically, as Lane and Shine (2011) suggested.

The present work is relatively descriptive about the genomic basis of the sense of smell among *Laticauda* sea snakes living in Vanuatu. It remains unclear whether ORs are involved in conspecific recognition or not, and whether such ORs were included in this analysis or not. In this study, we simply showed that *L. frontalis* and *L. colubrina* have evolved to maintain similar numbers of OR genes, but that they may possess different senses of smell to each other. It is possible that this dissimilarity functions as a premating isolation barrier since *L. frontalis* has returned to the ancestral range, but this has not been confirmed yet. Further studies will be required to reveal the evolution of odor-based isolation mechanisms between *L. frontalis* and *L. colubrina*.

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