Do Snakes Use Olfactory Receptors in the Nose to Detect Odors?: A Prediction Based on the Percentage of Nonfunctional Olfactory Receptor Genes Amplified in Four Species of Snakes

Taylor Byerly, Amanda Robinson and Michelle Vieyra

University of South Carolina Aiken, 471 University Parkway, Aiken, SC 29801

Received March 29, 2010

Though it is well known that snakes detect odors via the vomeronasal system, the study of their use of olfactory receptors to do so has been severely neglected. The primary purpose of this study was to estimate the reliance on an olfactory receptor system by Water Snake (*Nerodia fasciata)*, King Snake (*Lampropeltis getula)* Copperhead *(Agkistrodon contortrix)*, and Hognose (*Heterodon platirhinos*) by identifying and characterizing their olfactory receptor genes. Olfactory receptor (OR) genes from all four species were sequenced and screened for the presence of stop codons (making them pseudogenes). As pseudogenes are non-functional genes, the percentage of pseudogenes that accumulate within a given gene family should have an inverse relationship with reliance on the system coded for by that gene family. A total of 112 unique olfactory receptor genes were isolated: 36 Copperhead, 34 King Snake, 16 Water Snake, and 26 Hognose. Only one of the genes (belonging to a Copperhead) was identified as being a pseudogene. Based on the lack of olfactory receptor pseudogenes found in this study, it is predicted that these four species of snake rely heavily on the olfactory receptor system as a method of odor detection.

Introduction

A flickering tongue has long characterized the extent of our understanding of how a snake senses the odors around them. Perhaps the lack of further inquiry is due to the fact that this method is easily observed and provides an obvious explanation for how snakes detect odors. There are actually two methods of odor detection however: The vomeronasal system (characterized by tongue flickering and a vomeronasal organ on the roof of the mouth) and the olfactory receptor system (characterized by odors binding to proteins in the nose). Whether a snake has the ability to detect odors through the use of olfactory receptors, as humans and the majority of vertebrates do [1,2,3], has been severely under-researched.

Snakes are well known for the vomeronasal system of odor detection – a method comprised of their tongue picking up odor molecules and placing them upon the Jacobson's Organ located in the upper-back portion of the mouth. Within this organ are vomeronasal receptors which, when the tongue picks up an odor and places it onto the Jacobson's organ, bind with the odor molecules and relays signals to the brain [4,5,6]. An alternate and more common method of odor detection, however, would be facilitated by olfactory receptors [1,2,3]. Olfactory receptors are seven-transmembrane domain proteins found embedded in the olfactory cilia of the nostrils. When an animal utilizes this system, they need not actively work to touch an odor molecule as with the vomeronasal method, but can simply detect scents as they float through the air and bind to olfactory receptors of their own accord [4,2].

Why does a secondary method of scent collection seem a likely possibility for snakes? Snakes are known for being adept predators. If they relied solely upon the vomeronasal system, a snake would have to come directly across an animal's trail to hunt it, putting it at a severe disadvantage when compared to other animals that can sense floating odor molecules of *nearby* trails without coming in direct contact with them. A snake operating with a purely vomeronasal system could not, for instance, lie in wait for the scent of a passing animal to come wafting into their retreat. In matters of defense, a snake would also be chemically blind to the existence of local predators unless the snake happened upon a predator's trail by pure chance. From an evolutionary standpoint it would make sense that snakes would be best equipped with olfactory receptors as well as the Jacobson's Organ.

How can this hypothesis be tested? By studying the genes that code for olfactory receptor proteins and looking for the existence of interrupting stop codons. Stop codons prevent a gene from being fully translated and, therefore, functional. These non-functional genes are known as pseudogenes. Theoretically, there should be an inverse relationship between the number of pseudogenes and the reliance of a species upon the trait which that gene, if functional, codes for [7,8,3,9]. For example, a human's OR genes (a species not known for a particularly keen sense of smell) are more than half nonfunctional [10]. A mouse, on the other hand, relies more heavily upon the detection of odors, and so has only about twenty percent nonfunctional OR genes [11]. A snake's OR gene repertoire should likewise allow us to estimate its degree of olfactory receptor use.

The purpose of the following study was to progress our knowledge on this subject by analyzing the olfactory receptor (OR) genes of four species of snake: Water Snake (*Nerodia fasciata)*, King Snake (*Lampropeltis getula)* Copperhead *(Agkistrodon contortrix)*, and Hognose (*Heterodon platirhinos*). These particular snakes were chosen based on their representation of different habitats

and foraging strategy as these factors may affect a snake's use of an OR system. The Water Snake lives in an aquatic environment; and may either actively hunt, or wait for prey to come to it [12]. Both the Copperhead and King Snakes live on the land and do not actively hunt, but instead wait for passing prey [13]. Finally, the range of the Hognose is also on land, but the Hognosed actively hunts for its prey [14]. We hypothesized that that these four species of snakes would have a relatively low number of pseudogenes (and therefore, heavy reliance on an olfactory receptor system). Secondly, based on the different habitats and foraging strategies, we hypothesized that there would be a significant difference in the percentage of pseudogenes found across the species. Our objectives for the study were to 1) Identify OR genes from each species, 2) Characterize them as either functional or non-functional and, 3) Use our results to form a prediction about the use of olfactory receptors by the snake family as a whole.

Methods

Frozen blood samples from a single specimen each of Copperhead, Water, King, and Hognose snakes were obtained from staff at the Savannah River Ecology Laboratory. DNA was then isolated using a QIAgen extraction kit. Olfactory receptor genes were amplified from these samples using primers designed from aligned sequences of mammalian and bird OR genes (sens primer: 5'-CCYATGTAYTTBTTBCT-3'; antisens primer: 5' – GSHRCADGTNKARAADGCYT – 3') in a polymerase chain reaction (PCR). An Invitrogen cloning kit was used on the PCR results to isolate and replicate individual OR genes. These isolated samples were then purified using a PureLink Quick Plasmid Miniprep Kit before being sent to the University of South Carolina for sequencing (using an ABI 3730 Sequencer). The returned sequences were entered into the NCBI Network Blast Server, and all results matching olfactory receptor genes were recorded and studied for the presence of stop codons marking them as pseudogenes. Finally, Clustal X (a global alignment tool which analyzes the sequences for similarities) was used to align the genes, and their translated amino acids were entered into the Mega 4 program to create a neighbor joining phylogenetic tree; a visual representation of the relationships between sequenced OR genes.

Results

In all, 112 olfactory receptor genes were isolated and sequenced. When these sequences were interpreted in the correct reading frame, one (2.8% of the total) was found to have the interrupting stop codons characteristic of a pseudogene (see Table 1).

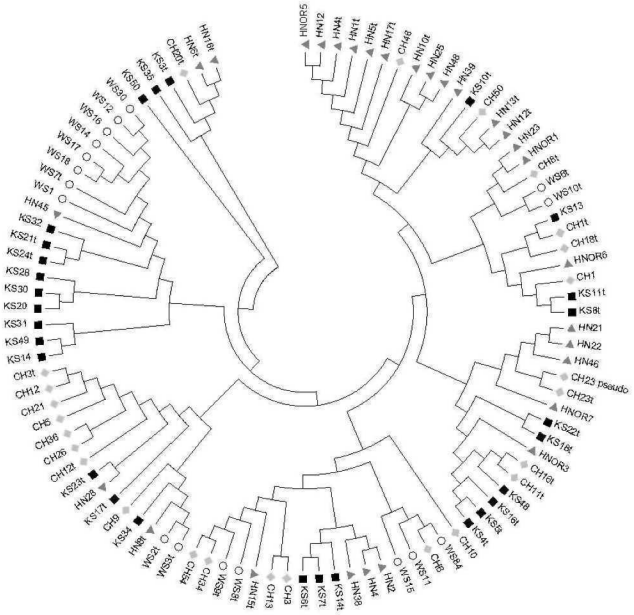

Figure 1: Neighbor joining tree of snake OR genes Key: Copperhead Snake (CH), King Snake (KS), Water Snake (WS), Hognosed Snake (HN)

Species	Olfactory Receptor Genes	Olfactory Receptor Pseudogenes
Copperhead	36	1
King Snake	34	0
Water Snake	16	0
Hognosed Snake	26	0

Table 1- Number of unique OR genes and Pseudogenes found in the four species of snake studied

Isolated snake OR gene sequences were used to create a phylogenetic tree (Figure 1). The diagram clusters gene sequences by similarity. Since OR gene sequences reflect protein structure and therefore function clustering represents functional not evolutionary similarity. Clusters on the phylogenetic tree therefore shows us which snake OR genes code for proteins that bind to similar odors. The majority of clusters on the tree contain sequences from all four species of snake. There are, however, several instances in the phylogenetic tree of clustering among a single species (particularly the Water, King, and Copperhead snakes).

Discussion

This study suggests that there is likely only a small percentage of olfactory receptor pseudogenes in the Copperhead, King, Water, and Hognosed snakes' full genetic repertoire. OR pseudogenes were identified in only one snake species and then at a relatively low 2.8% when compared with other species. Mice and dogs have been found to have approximately 20% non-functional OR genes

[11,15]; and in humans, a sizeable 67% of their OR genes can be called pseudogenes [16]. As one might guess from the percentages presented above, species tend to have a number of functional OR genes proportional to the evolutionary degree of need for an olfactory receptor system [1,17], and it therefore seems highly likely that these four snakes use olfactory receptors alongside their vomeronasal system. If the results of this study are considered representational of all snakes, we can then draw conclusions that all snake species rely upon the use of olfactory receptors.

In regards to the similarity of olfactory receptor genes among the four specific species that we studied; when represented by a phylogenetic tree, an inter-species mixing of OR genes is shown. This indicates a similar baseline of olfactory functioning among all four snakes. Instances of species specific grouping was also seen, however, which indicates the possibility that there are classes of odors that may be detected by some snakes but not others. Considering the different habits and environments of the snakes sampled, it seems worthy of investigation whether each has an OR system more discerning of the particular odors likely to be found within their habitat.

In summary, our hypothesis that the four species of snakes studied would have a low percentage of isolated OR genes was supported. The second part of our hypothesis, that there would be a notable difference of pseudogene percentage between the species, was refuted.

Due to the small sample size and incomplete sequencing of the snake's olfactory subgenome further study using additional primer sets is necessary to confirm our conclusions. Additional research questions are also brought up by the conclusions of this project. For instance, why do snakes require two methods of odor detection and how do these two systems blend to create a map of the olfactory world within the brain of the snake? Further characterization of the snake's olfactory receptor system is needed, however, before such inquires can realistically be made. It is hoped that this research will help to build the preliminary knowledge base needed to begin serious investigation into the details of snake olfaction.

Acknowledgements

Funding for this project was generously awarded by the University of South Carolina's Magellan Scholar program.

References

1. Glusman G., Yanai I., Rubin I., Lancet D. The complete human olfactory subgenome. *Genome Res*. 2001, **11**,685–702.

2. Lai P.C., Singer M.S., Crasto C.J. 2005. Structural activation pathways from dynamic olfactory receptor-odorant interactions. *Chem Sen*. 2005, **30(9)**, 81-792.

3. Steiger S.S., Fidler A.E., Valcu M., Kempenaers B. Avian olfactory receptor gene repertoires: evidence for a well-developed sense of smell in birds? *Proc Biol Sci B*. 2008, **275(1649),** 2309–2317.

4. Cooper Jr W.E., Burghardt G.M. Vomerolfaction and vomodor. *J. Chem. Ecol.* 1990, **16**,103-105

5. Halpern M. The organization and function of the vomeronasal system, Ann. Rev. *Neuro*. 1987, **10,** 325-362.

6. Halpern M., Kubie J.L. Snake tongue flicking behavior: clues to vomeronasal function, in D. Muller-Schwarze and R.M. Silverstein, eds., *Chem. Signals.* 1983 New York: Plenum; pp. 45-72.

7. Freitag J, Ludwig G, Andreini I, Rössler P, Breer H. Olfactory receptors in aquatic and terrestrial vertebrates. *J Comp Physiol [A]*. 1998, **183(5),** 635-50.

8. Niimura Y, Nei M. Extensive gains and losses of olfactory receptor genes in mammalian evolution. *PLoS ONE*. 2007, **2(8)**, e708

9. Young J.M., Trask B.J. The sense of smell: genomics of vertebrate odorant receptors. *Hum Mole Genet*. 11:1153-1160

10. Zozulya S, Echeverri F, Nguyen T. 2001. The human olfactory receptor repertoire. *Genome Biol*. 2002, **2(6),**1-12.

11. Zhang, X., Firestein S. The olfactory receptor gene superfamily of the mouse. *Nat. Neurosci*. 2002, **5**, 124-133

12. Mullin, S.J. and Mushinsky, H.R. Foraging ecology of the mangrove salt marsh snake, *Nerodia clarkia compressicauda*: effect of vegetational density. *Amph. Rep.* 1995, **16,** 167-175.

13. Miller, L.R. and Gutzke, W.H.N. The role of the vomeronasal organ of crotalines (Reptilia: Serpentes: Viperidea) in predator detection. *An. Beh.* 1999, **58,** 53-57.

14. Kroll, J.C. Feeding adaptations of hognose snakes. South. *Nat.* 1976, **20(4),** 537-557.

15. Olender, T., T. Fuchs, C. Linhart, Shamir, R. and Adams, M. 2004. The canine olfactory subgenome. *Gen.* 83(3): 361-372.

16. Rouquier, S., S. Taviaux , B.J. Trask, V. Brand-Arpon, and van den Engh, G. Distribution of olfactory receptor genes in the human genome. *Nat. Gen.* 1998, **18,** 243–250.

17. Niimura Y, Nei M. Evolutionary dynamics of olfactory receptor genes in fishes and tetrapods. *Proc. Natl. Acad. Sci* USA. 2005, **102(17)**, 6039-6044.