# Adaptive evolution of a blood-clotting gene in venom-resistant opossums

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## Introduction

**Action of snake venom**

Hemolytic snake venoms—such as those found in rattlesnakes, moccasins, and lanceheads—are complex cocktails of proteases, phospholipases, and phospho-diesterases. One protein found in the venom of lancehead vipers (genus *Bothrops*) is botrocetin, which causes aggregation of blood platelets wherever **von Willebrand Factor (vWF)** is present in the bloodstream (**1**). By binding vWF, botrocetin promotes inappropriate systemic clotting, reducing the ability of vWF to respond to ruptures caused by proteolytic venom proteins and promoting hemorrhage.

**Resistance in opossums**

Several species of opossums in both North and South America are known to be resistant to lancehead venom—in fact, large opossums will even eat poisonous snakes (Figure 1). The mechanism by which they withstand snakebite is not completely known; our aim was to assess the possibility that venom resistance in these species resulted in part from adaptive evolution of the vWF gene.

**Hypothesis**

By calculating the rate of synonymous substitutions $d_S$ in the gene and the rate of replacement substitutions $d_N$ (*i.e.,* mutations that change the amino acid sequence of the resulting protein), we can test whether the vWF gene has experienced adaptive evolution in these opossum species. The ratio $\omega = d_N/d_S$ is a key statistic: if $\omega < 1$, purifying selection is acting on this gene (*i.e.,* mutations are purged); if $\omega = 1$, sites are neutral; if $\omega > 1$, positive, directional selection is acting on this gene. Our expectation is to find greater rates of substitution in the group of opossums highlighted in red in the phylogeny at right, because these are the species known to be resistant to snake venom (Figure 2).

## Methods

Using the gene sequence data at the vWF locus for 41 species of New World opossum (family Didelphidae), we calculated the rate of amino acid substitution along all branches in the phylogeny in Figure 2. To test for evidence of positive selection, we performed a likelihood ratio test, comparing the goodness-of-fit of two models to the data. The first model is a null model that assumes no difference in $\omega$ values across the tree; the second is a model that allows $\omega$ in the "foreground" group (the red branches in Figure 2) to differ from $\omega$ in the background lineages in the rest of the tree (**3**). In addition, this test allowed us to identify those sites in the locus that have a high posterior probability of being under positive selection.

To examine the possible functional effects of positive selection on this gene, we mapped the positively selected sites on an existing model of human vWF interacting with glycoprotein 1bα and botrocetin (a venom protein from the pit viper *Bothrops jararaca*). In particular, we compared how frequently selection was inferred for sites that bind botrocetin versus those that do not (**4**).

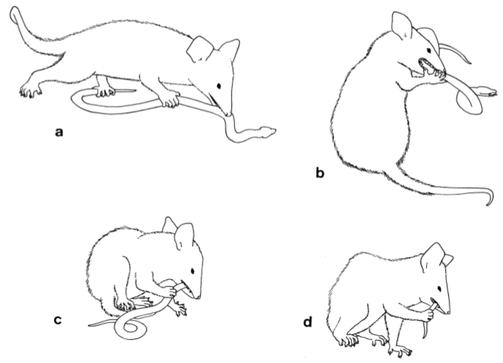


**Figure 1**: Illustration of white-eared opossum *Didelphis albiventris* feeding on a jararaca viper *Bothrops jararaca.* From Oliveira and Santori 1999.

## Phylogeny

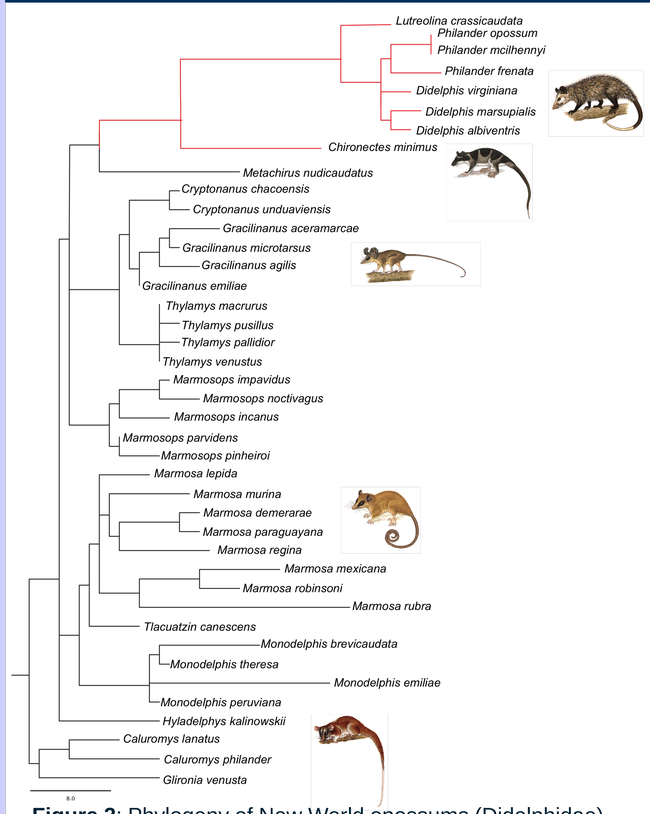


**Figure 2**: Phylogeny of New World opossums (Didelphidae) based on sequence data from five nuclear genes (BRCA, vWF, IRBP, DMP1, and RAG1). The horizontal length of each branch is proportional to the number of amino acid changes in the vWF protein sequence. The rate of apparent evolution is greater in the lineage comprising *Chironectes*, *Lutreolina, Didelphis,* and *Philander* (the clade *Didelphini*) than the average rate across the tree. Tree topology from Voss and Jansa 2009.

## Results

**Table 1**: The likelihood of competing models of molecular evolution applied to the opossum vWF data. The value $\omega_2$ represents the ratio of non-synonymous substitution to synonymous substitution in the lineage composed of *Didelphis, Lutreolina, Philander,* and *Chironectes;* $\omega_1$ represents the same ratio in the rest of the tree. The model in which a proportion of sites is allowed to assume values of $\omega_2 > 1$ in Didelphini is a significantly better fit to the data ($p < 0.05$).

| Model | P | likelihood |
|---|---|---|
| **Branch-site model A, with $\omega_2 = \omega_1$ (fixed)** | 3 | -5436.01 |
| **Branch-site model A, with $\omega_2 > 1$** | 4 | -5422.79 |

**Table 2**: Proportion of sites exhibiting estimated values of $\omega$. Values greater than 1 are interpreted as evidence for directional selection. These data suggest that there is much greater selective pressure on approximately 10% of sites in Didelphini.

| site class | proportion | $\omega_1$ | $\omega_2$ |
|---|---|---|---|
| **Purifying selection** | 0.69246 | 0.05442 | 0.05442 |
| **Neutral** | 0.20453 | 1 | 1 |
| **Positive selection** | 0.10302 | 0.05442 or 1 | 6.78643 |

**Table 3**: Of all amino acid residues with a >95% posterior probability of being subject to selection, 25% are involved in binding of botrocetin. In contrast, only 3% of residues not involved in binding botrocetin show such strong evidence of selection. This difference is highly significant (*G*-test, d.f. =1, $p$=0.01).

| PP>0.95 | selected | not selected |
|---|---|---|
| **Binding** | 3 | 9 |
| **Non-binding** | 1 | 36 |

## Crystal Structure

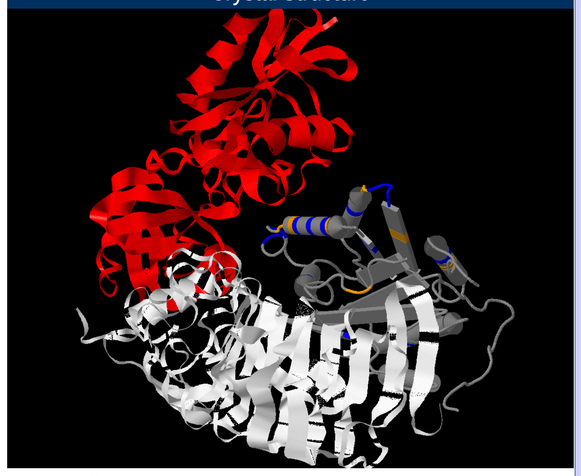

**Figure 3**: Crystal structure of botrocetin(red), human glycoprotein Ibα (white), and human vWF (gray). Sites that show evidence of selection in opossums are shown in blue and orange. Intriguingly, these sites are disproportionately concentrated in the region that interacts with botrocetin.

## Discussion

The likelihood ratio test (Tables 1 & 2) reveals evidence for positive selection in the lineage containing species known to be resistant to hemolytic snake venom. The sites that we identify with high posterior probability of being under directional selection are disproportionately clustered on sites that are known to confer botrocetin-binding ability (Table 3; Figure 3). These results strongly suggest that these mutations in vWF affect the interaction with botrocetin.

These lines of evidence reveal that evolution in the vWF gene is accelerated in opossums that are resistant to snake venom relative to all other opossums. In particular, the high density of amino acid changes in the region of vWF that interacts with botrocetin suggests that resistance may occur by denying botrocetin a binding site. This would keep vWF free to respond normally to clotting promoters while reducing venom-induced systemic clotting. Whereas elucidating the precise mechanism would require careful biochemical work beyond the purview of this project, we believe that this work suggests a clear path forward for future research.

An additional surprising result was the very long branch lengths (*i.e.,* high substitution rates) in at least one species each of *Monodelphis* and *Marmosa.* Neither of these species is known to be resistant to snake venom, but this is an ability that cannot be known unless it is tested. If vWF is responsible, at least in part, for venom resistance among opossums, these lineages are good candidates for exploring the extent of venom resistance.

Another fruitful line of investigation could explore the possibility of resistance to neurotoxins in opossums. Opossums of the genus *Philander* have been observed eating coral snakes (genus *Micrurus*). Whether this ability is attributable to agility alone or to resistance to coral snakes' neurotoxic venom is not known, and warrants further research.

**Figure 4**: *Bothrops jararaca*, the jararaca pit viper from South America. It produces a complex cocktail of venom proteins, including botrocetin.

## References

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