CONVERGENT EVOLUTION

Convergent evolution of pain-inducing defensive venom components in spitting cobras

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Convergent evolution provides insights into the selective drivers underlying evolutionary change. Snake venoms, with a direct genetic basis and clearly defined functional phenotype, provide a model system for exploring the repeated evolution of adaptations. While snakes use venom primarily for predation, and venom composition often reflects diet specificity, three lineages of cobras have independently evolved the ability to spit venom at adversaries. Using gene, protein, and functional analyses, we show that the three spitting lineages possess venoms characterized by an up-regulation of phospholipase A_2 (PLA_2) toxins, which potentiate the action of preexisting venom cytotoxins to activate mammalian sensory neurons and cause enhanced pain. These repeated independent changes provide a fascinating example of convergent evolution across multiple phenotypic levels driven by selection for defense.

Convergent evolution, the independent emergence of similar traits across taxa, is a pervasive characteristic of biodiversity and provides natural replicates to enable understanding of key evolutionary processes (*1*). Given their discrete function and direct genotype-phenotype link, animal venoms are excellent systems for understanding the driving forces and underlying genetic mechanisms of molecular adaptation. Snake venoms consist of variable mixtures of proteinaceous components causing potent hemotoxic, neurotoxic, and/or cytotoxic pathologies in both prey and potential adversaries, including humans (*2*). Previous work suggests that venom variation is largely driven by dietary variation (*3*), but defensive drivers of snake venom evolution are rarely considered [although, see (*4*)].

The evolution of venom projection or "spitting" in cobras offers an ideal system for exploring the evolution of defensive toxins: This behavior plays no role in prey capture, targets specific sensory tissues, and is the only long-distance, injurious defensive adaptation among almost 4000 species of snakes. Unexpectedly, venom spitting evolved independently three times, all within a single clade of closely related elapid snakes (*5*, *6*): the African spitting cobras (*Naja*: subgenus *Afronaja*), Asian spitting cobras (*Naja*: subgenus *Naja*), and rinkhals (*Hemachatus*). All use fangs with modified orifices (*7*) to spray venom over distances of up to 2.5 m (*8*), targeting an aggressor's eyes (*9*) (fig. S1). These behavioral, morphological, and biochemical traits result in intense ocular pain and inflammation, which can lead to the permanent loss of eyesight (*10*). The three origins of spitting, solely within a clade more generally characterized by the visually defensive behavior of hooding (*5*, *6*), allow us to test whether similar selective pressures have resulted in convergent changes in venom composition coevolving with morphological and behavioral adaptations.

To investigate the evolution of venom spitting, we used a multidisciplinary approach consisting of transcriptomic, proteomic, functional, and phylogenetic comparisons of 17 widely distributed elapids: 14 *Naja* (true cobras); *Hemachatus haemachatus* (rinkhals); and two nonspitting immediate sister-group species, *Walterinnesia aegyptia* and *Aspidelaps scutatus*. First, we reconstructed the phylogeny of these snakes using a multilocus coalescent species tree approach based on two mitochondrial and five nuclear genes. Fossil-calibrated molecular dating suggests that spitting originated in African spitting cobras between 6.7 million and 10.7 million years ago (Ma) and ~4 million years later in the Asian spitting cobras (2.5 to 4.2 Ma) (Fig. 1A). The origin of spitting in *Hemachatus* could not be dated beyond having occurred <17 Ma, after divergence from true cobras (*Naja*) (Fig. 1A).

Next, we used a top-down proteomics approach underpinned by venom gland transcriptomic data (*11*) to characterize the venom composition of each species. All cobra venoms are dominated by three-finger toxins (3FTXs), while in many species phospholipases A_2 (PLA_2) are the second most abundant toxin family (Fig. 1A and fig. S2). Principal coordinates analysis (PCoA, Bray-Curtis) of a proteomic data–derived venom composition matrix separated the spitting lineages into three clusters that are distinct from the homogeneous cluster of venoms from nonspitters (Fig. 1B). The sole exception, *Naja philippinensis*, has a purely neurotoxic venom despite being able to spit (*12*), and its venom composition placed it alongside nonspitting species (see Fig. 1B). Nonetheless, these findings demonstrate that each spitting cobra lineage exhibits distinct venom compositions that collectively differ from those of nonspitting cobras—a finding that is consistent with differences in venom-induced pathology observed after bites to humans (*13*).

In many elapid snakes, 3FTXs are major venom components (*14*) (table S1). They are encoded by a multilocus gene family, resulting in numerous functionally distinct isoforms, including neurotoxins and cytotoxins that disrupt cell membranes to cause cytotoxicity (*15*). Proteomic data revealed that cytotoxic 3FTXs (CTXs) are typically the most abundant toxins in *Naja* and *Hemachatus* venoms (mean: 57.7% of all toxins), contrasting with the sister-group species *W. aegyptia* and *A. scutatus* (*16*) and other elapids (Fig. 1A, figs. S2 and S3, and table S1). Although cytotoxins likely inflict defensive ocular pain, we found no significant difference in the abundances of CTXs between spitting and nonspitting species [phylogenetic generalized least squares (PGLS); $t = -0.83$, degrees of freedom (df) = 15, $P = 0.42$] (table S2), and ancestral state estimations suggest that the origin of CTX-rich venom preceded that of venom spitting (fig. S4) (*5*). Moreover, PCoA analysis of a CTX Euclidean distance matrix derived from venom proteomic data revealed that all highly abundant cobra CTXs cluster tightly together (Fig. 1C). Measures of irritation, stimulated by high doses (100 μg) of cobra venoms applied topically to nonsentient chick embryos (tables S3 and S4), also revealed no association between cytotoxicity and spitting (PGLS; $t = 1.08$, df = 15, $P = 0.30$) (figs. S5 and

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S6), consistent with prior reports of comparable cytotoxicity to mammalian cells across all cobra venoms (*5*).

However, pain inflicted via slow-onset cytotoxicity may be less defensively relevant than rapid pain caused by direct algesic activity. To investigate venom-induced nociception, we assessed the activation of mammalian trigeminal neurons—sensory neurons derived from trigeminal ganglia that innervate the face and eyes. All cobra venoms activated sensory neurons, with an observed mechanism of action consistent with nonspecific disruption of cell membranes, although activity was limited in African nonspitting cobras and the Asian nonspitter *Naja kaouthia* (fig. S7). Half-maximal effective concentrations (EC_{50}) of each venom in sensory neuron–derived F11 cells demonstrated significantly higher activity in spitting cobra venoms (PGLS; $t = -4.48$, df = 15, $P = 0.0007$) (Fig. 2A, fig. S8, and table S2). These findings support the hypothesis that venom spitting is associated with convergent elevations in venom-induced activation of mammalian sensory neurons, and that spitting cobra venoms are more effective in causing pain than their nonspitting counterparts.

To determine the toxins responsible for this effect, we repeated these experiments using fractionated venom from three representative spitting species (*N. nigricollis*, African; *N. siamensis*, Asian; and *H. haemachatus*) (fig. S9). For each species, only fractions corresponding to CTXs activated sensory neurons, while those corresponding to other toxins (e.g., neurotoxins, PLA_2s, etc.) were inactive (fig. S9). However, none of the CTX fractions completely recapitulated the effects of whole venom or re-pooled venom fractions, suggesting synergy between multiple venom components.

PLA_2s are nearly ubiquitous, typically enzymatic, multifunctional toxin components of snake venoms (*14*, *15*). As the hemolytic activity of CTXs was previously shown to be potentiated by PLA_2 toxins (*17*), we hypothesized that venom PLA_2s potentiate sensory neuron activation by CTXs. Consequently, we quantified the activation of sensory neurons stimulated by CTXs in the presence or absence of a corresponding PLA_2 fraction. The proportion of viable sensory neurons activated by each CTX fraction was significantly increased when combined with a PLA_2 fraction, and this result was consistent across representatives of the three spitting lineages (unpaired *t* test; *N. nigricollis*, $t = 18.77$, df = 2, $P = 0.003$; *N. siamensis*, $t = 5.75$, df = 4, $P = 0.005$; *H. haemachatus*, $t = 4.18$, df = 4, $P = 0.01$) (Fig. 2B, and fig. S10). Moreover, significant reductions in sensory neuron activation occurred in the presence of the PLA_2 inhibitor varespladib (unpaired *t* test; $t = 2.77$, df = 14, $P = 0.02$) (Fig. 2C and fig. S11), providing further compelling evidence that PLA_2s potentiate CTX effects on sensory neurons.

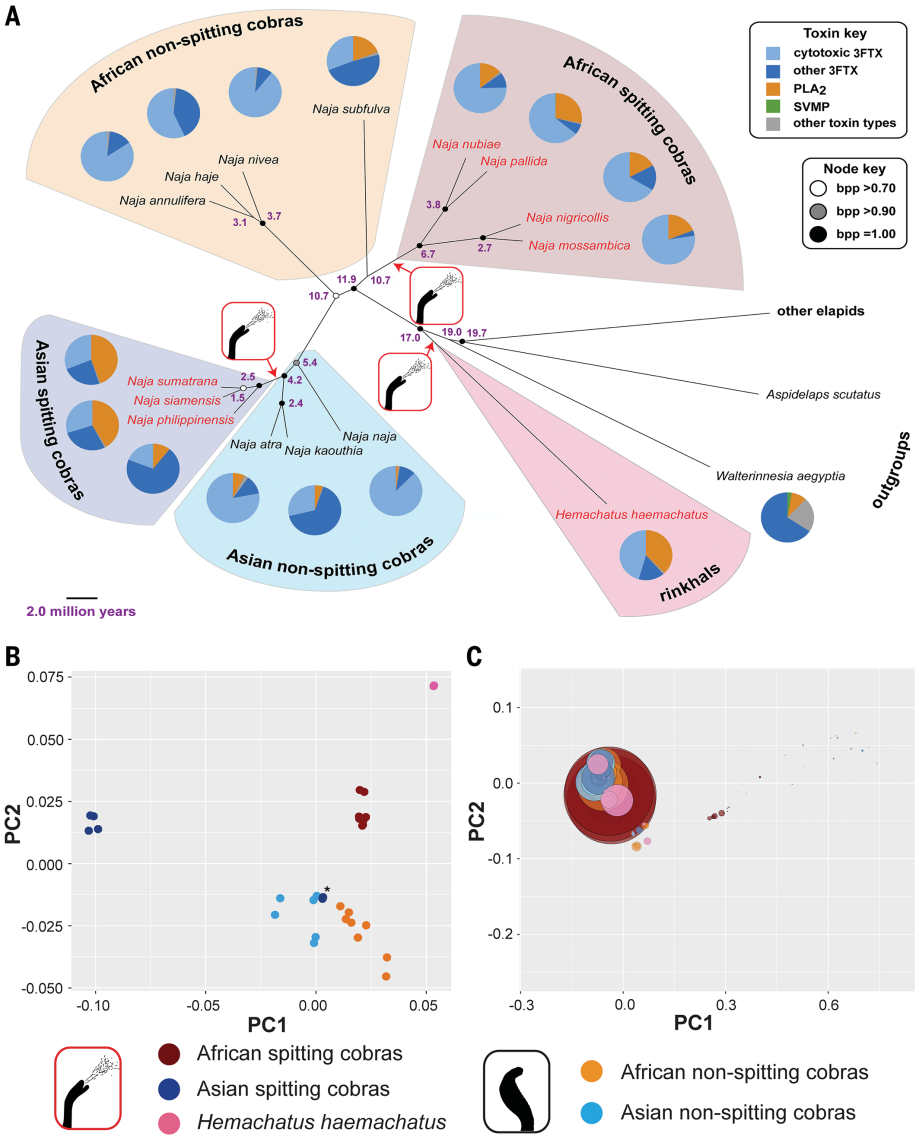

Fig. 1. Reconstruction of the evolutionary origin of venom spitting and comparative analysis of venom composition. (**A**) Multilocus-derived multispecies coalescent species tree, pruned to display the taxa whose venoms were analyzed in this study. Node support is indicated by colored circles, representing Bayesian posterior probabilities: black = 1.00, gray >0.90, white >0.70. Purple node labels indicate estimated divergence times (see fig. S14 for credibility intervals). Spitting species are highlighted by red tip labels, and the three independent origins of venom spitting are indicated by the red-boxed spitting images. Pie charts adjacent to tip labels represent proteomic toxin composition of each species as a percentage of total toxins. SVMP, snake venom metalloproteinases; bpp, Bayesian posterior probability. (**B**) Principal coordinates analysis (PCoA) of cobra (*Naja* spp.) and rinkhals (*H. haemachatus*) venom toxins reveal major distinctions between spitting and nonspitting lineages. Asterisk highlights the Asian spitting species *N. philippinensis*, which exhibits greater similarity to non-spitting species than to its nearest relatives. Each species is represented by two, typically overlapping, data points, which represent technical proteomic duplicates. (**C**) PCoA of cobra (*Naja* spp.) and rinkhals (*H. haemachatus*) cytotoxic three-finger toxins (CTXs) derived from top-down venom proteomics reveals that the most abundant CTXs detected in venom exhibit little sequence diversity among spitting and nonspitting lineages. Circle sizes reflect relative abundances of CTXs detected.

Consistent with the above findings, comparative analysis of the (i) proteomic abundance of PLA_2 toxins and (ii) enzymatic PLA_2 activity, determined via specific in vitro colorimetric assay, revealed that spitting cobra venoms have significantly higher PLA_2 abundance and activity than those of nonspitting species (PGLS; $t = 4.24$, df = 15, $P = 0.0007$, and

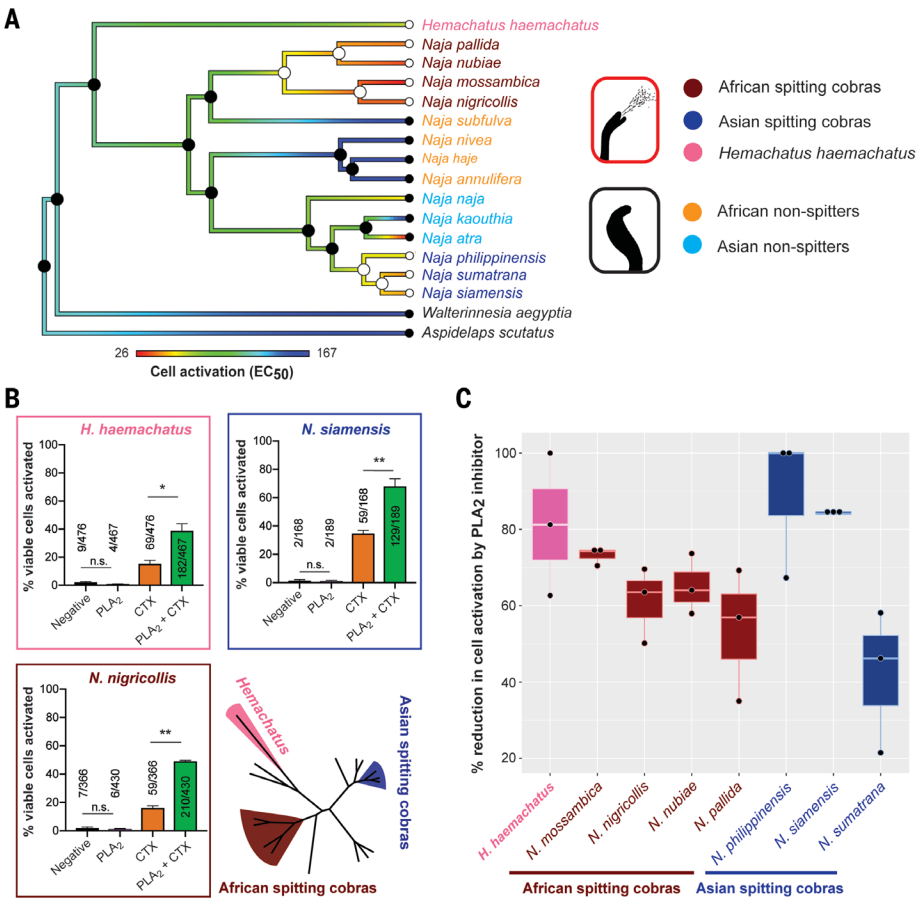

Fig. 2. Spitting cobra venoms cause significantly greater activation of sensory neurons than nonspitting cobras, mediated via potentiation by PLA_2 toxins. (**A**) Ancestral state estimation of half-maximal effective concentrations (EC_{50}) of venom-induced activation of neuronal cells shows a significant association between increased potency and venom spitting (PGLS, t = −4.48, P = 0.0004). EC_{50} values are expressed as the mean of triplicate measurements, and colored branches are scaled accordingly (red, low EC_{50} and thus high venom potency; blue, high EC_{50} and thus low venom potency). Filled or empty circles at nodes and tips represent estimated ancestral states of nonspitting or spitting, respectively, and colored tip labels correspond to the different lineages. (**B**) PLA_2 toxins in spitting cobra venoms potentiate the activating effect of CTXs on sensory neurons. A CTX fraction from each venom was added to dissociated mouse dorsal root ganglia neurons in the presence or absence of a corresponding PLA_2 fraction (added 1 min prior), neuronal activation (i.e., a rapid increase in $[Ca^{2+}]_i$) monitored, and data presented as mean ± SEM of the resulting percentage of viable cells from two to three independent experiments. The number of viable cells activated and the total number of cells used are also displayed above or within each bar. Statistical comparisons were performed using unpaired parametric t-tests; *P < 0.05; **P < 0.01; n.s., not significant. (**C**) The PLA_2 inhibitor varespladib reduces neuronal activation stimulated by spitting cobra venoms. Calcium influx in F11 cells was measured on a FLIPR instrument incubated in the presence of venom from spitting species (2.4 or 4.8 μg of venom in the case of *H. haemachatus* and *N. philippinensis*) and in the presence or absence of varespladib (13 μM). The data displayed represent the percentage of venom-only cell activation stimulated by treatment with venom and varespladib. Error bars represent SEM, and box midlines indicate median values.

t = 2.24, df = 15, P = 0.04, respectively) (Fig. 3, A and B; fig. S12; and table S2). Analysis of a PLA_2 Euclidean distance matrix derived from venom proteomic data revealed substantial differences between the different lineages of spitting and nonspitting cobras, particularly among the African species (Fig. 3C). Additionally, despite their divergence ~17 Ma, *H. haemachatus* PLA_2s clustered tightly with those from African spitting cobras, suggesting an element of molecular convergence. Interrogation of venom gland transcriptomic data revealed limited variation in PLA_2 gene number across the three spitting lineages, although all exhibited increased PLA_2 abundance compared with nonspitting cobras (fig. S13A). Notably, phylogenetic analyses revealed a PLA_2 gene duplication event that coincided with the origin of venom spitting in the ancestor of African spitting cobras (fig. S13B). These data, alongside the sensory neuron assays, demonstrate that independent evolution of spitting is tightly linked with convergent increases in PLA_2 toxins, which cause increased algesic activity. Our findings therefore imply that evolution has funneled defensive venom phenotypes along repeatable and predictable pathways, although different molecular mechanisms likely underpin this convergence.

To exclude the possibility that functional distinctions simply reflect general differences in venom potency (e.g., venom lethality for prey capture), we tested our venoms in murine lethality assays (table S5) and found no significant differences between spitting and nonspitting species (PGLS; t = 0.86, df = 15, P = 0.40) (table S2). These results suggest that enhanced pain caused by spitting cobras is explicitly associated with defensive venom use rather than being an evolutionary by-product of selection for prey subjugation. Moreover, reanalysis of our data with the Asian species *N. kaouthia* and *N. atra* scored as spitting cobras [on the basis of recent reports (*18*, *19*)], did not alter our key findings relating to venom spitting and PLA_2-mediated enhanced activation of sensory neurons (table S6).

Our results detail the molecular and functional correlates of the evolution of venom spitting and demonstrate that defense can be a major driver of snake venom composition. Spitting likely only evolved within a single, relatively small clade of elapid snakes owing to the integrated exaptation of a distinctive combination of preexisting behaviors and cytotoxic venom activities. Early evolution of cytotoxic venom activity in cobras and near relatives (~26 Ma; fig. S4) has previously been linked to defense, as cytotoxicity co-originates with "hooding" (*5*), a long-distance visual aposematic display. This elevated posture directed toward predators, coupled with preemptive striking and occasional premature releases of venom, may have provided a behavioral precursor for the evolution of more-targeted venom spitting. Preexisting CTXs, largely absent from the other elapid venoms (table S1), likely provided the baseline ocular toxicity that favored inception and retention of spitting (*19*). Subsequent independent increases in PLA_2 toxins, which act in synergy with preexisting CTXs, resulted in increased venom-induced activation of nociceptors (Fig. 2). This potentiating effect of PLA_2s may be crucial for causing immediate pain of sufficient

intensity to rapidly deter aggressors, allowing the snake to escape.

Rare but repeatedly evolved adaptations likely result from similar ecological circumstances. Most discussions of defensive behavior involve potential predators, and certain mammals and birds commonly eat snakes (*20*, *21*). However, predation on spitting cobras is evidently unremarkable in terms of evolutionary history and biogeography. Beyond predators, potential threats to snakes include inadvertent trampling and preemptive defensive killing. That spitting evolved to prevent snakes from being trampled by ungulates in African savannas (*22*) does not explain the existence of primarily forest-dwelling Asian spitting cobras (*23*). Moreover, large ungulates typically have lateral eyes, making them unlikely to be especially vulnerable to spitting.

Several considerations make ancient hominins a plausible and compelling candidate for favoring repeated evolution of spitting in the Afro-Asian cobras: (i) Growing evidence suggests that snakes have influenced primate neurobiology and behavior (*24*) and that interactions between these two lineages have been important throughout the 75-million-year history of primates (*25*, *26*). (ii) Compared with carnivorous mammals, anthropoid primates as a clade are visually acute, cognitively complex, and culturally sophisticated (*24*). (iii) Diverse anthropoids mob snakes, with some distinguishing between harmless and dangerous species, killing the latter from a distance with clubs or projectiles (*25*–*27*). (iv) The previous two characteristics are enhanced among bipedal, larger-brained hominins (*24*, *26*), which thus could have posed a considerable threat to snakes (*28*). The initial divergence of African spitting cobras as recently as 6.7 Ma (Fig. 1) occurred soon after the divergence of hominins from *Pan* (bonobos and chimpanzees) ~7 Ma (fig. S14), coinciding with early evolution of bipedalism, enlarged brains, tool use, and occupation of savannas by the former (*29*). Likewise, the origin of the Asian spitter clade ~2.5 Ma is approximately contemporaneous with the arrival in Asia of *Homo erectus* (*30*, *31*) (fig. S14). Although based on circumstantial evidence, additional fossils and more finely tuned dating of relevant cobra and primate divergences might allow further testing of this hypothesis.

Spitting cobras highlight how similar selection pressures and shared exaptations can drive convergence at molecular, morphological, behavioral, and functional levels. The outcome of these processes has resulted in the evolution of complex, integrated adaptations in ecologically important traits being funneled down repeatable pathways.

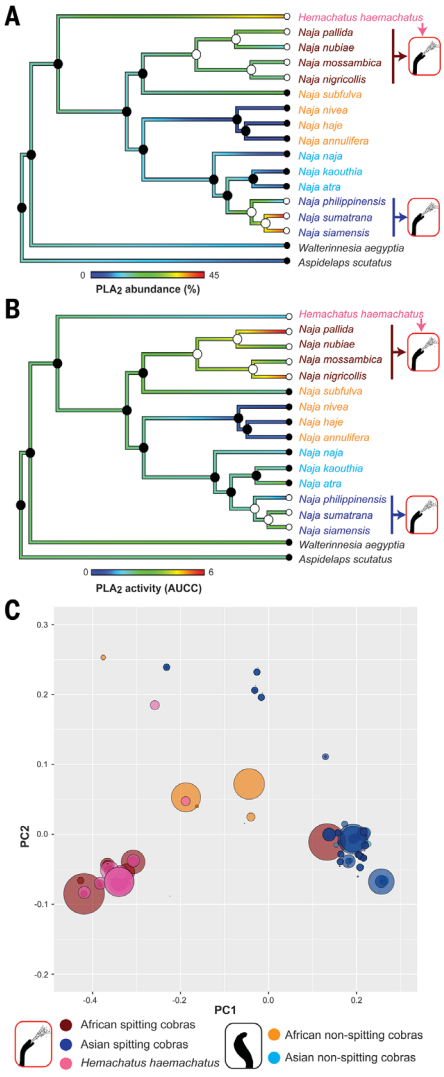

Fig. 3. The abundance, enzymatic activity, and diversity of PLA_2 toxins are associated with convergent evolution of venom spitting. (**A**) Ancestral state estimation of PLA_2 proteomic abundance, expressed as percentage of all toxins in venom proteomes, revealed a significant association with venom spitting (PGLS, t = 4.24, P = 0.0007). Colored branches are scaled according to PLA_2 abundance (blue, low abundance; red, high abundance), filled or empty circles at nodes/tips represent estimated ancestral states of nonspitting or spitting, respectively, and colored tip labels correspond to the different lineages. (**B**) Ancestral state estimation of enzymatic PLA_2 activity, expressed as area under the curve of concentration curves (AUCC) from kinetic in vitro colorimetric assay, revealed a significant association with venom spitting (PGLS, t = 2.24, P = 0.04). Colored branches are scaled according to PLA_2 activity (blue, low activity; red, high activity). Labels as in (A), and see fig. S12 for PLA_2 activity concentration curves. (**C**) PCoA of cobra (*Naja* spp.) and rinkhals (*H. haemachatus*) PLA_2 toxins derived from top-down venom proteomics reveals major variation between African spitting and nonspitting lineages but little variation between Asian cobras. Note the convergent placement of *Hemachatus* PLA_2 toxins with those of African spitting cobras. Circle sizes reflect relative abundances of PLA_2s detected in the venom proteomes.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S14
Tables S1 to S10
References (*32*–*126*)
MDAR Reproducibility Checklist
Data S1 to S5

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From offense to defense

Venom in snakes is largely used to subdue and/or kill prey, and most venoms have clear actions that facilitate death or paralysis. In one group of snakes, however, venom has evolved and shifted from predation to protection. Specifically, in three different lineages of "spitting" snakes, venom is used to deter predators. Kazandjian *et al.* show that similar adaptions have occurred within these lineages that transform cytotoxic components into a mixture that acts on mammalian sensory neurons and causes pain. The authors argue that increased predation on these lineages led to similar shifts in venom function.

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