

The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system

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Snakes are limbless predators, and many species use venom to help overpower relatively large, agile prey. Snake venoms are complex protein mixtures encoded by several multilocus gene families that function synergistically to cause incapacitation. To examine venom evolution, we sequenced and interrogated the genome of a venomous snake, the king cobra (*Ophiophagus hannah*), and compared it, together with our unique transcriptome, microRNA, and proteome datasets from this species, with data from other vertebrates. In contrast to the platypus, the only other venomous vertebrate with a sequenced genome, we find that snake toxin genes evolve through several distinct co-option mechanisms and exhibit surprisingly variable levels of gene duplication and directional selection that correlate with their functional importance in prey capture. The enigmatic accessory venom gland shows a very different pattern of toxin gene expression from the main venom gland and seems to have recruited toxin-like lectin genes repeatedly for new nontoxic functions. In addition, tissue-specific microRNA analyses suggested the co-option of core genetic regulatory components of the venom secretory system from a pancreatic origin. Although the king cobra is limbless, we recovered coding sequences for all *Hox* genes involved in amniote limb development, with the exception of *Hoxd12*. Our results provide a unique view of the origin and evolution of snake venom and reveal multiple genome-level adaptive responses to natural selection in this complex biological weapon system. More generally, they provide insight into mechanisms of protein evolution under strong selection.

genomics | phylogenetics | serpentes

Snake venom contains biologically active proteins (toxins) encoded by several multilocus gene families that each comprise several distinct isoforms (1, 2). Venom is produced in a postorbital venom gland (3) and associated in elapids (cobras and their relatives) and viperids (vipers and pit vipers) with a small downstream accessory gland of unknown function (Fig. 1). Understanding the origin and evolution of the snake venom system is not only of great intrinsic biological interest (3–5), but is also important for drug discovery (1, 2, 6), understanding vertebrate physiological pathways (7, 8), and addressing public health concerns about the enormous number of snake bites suffered in tropical countries (9, 10).

Significance

Snake venoms are toxic protein cocktails used for prey capture. To investigate the evolution of these complex biological weapon systems, we sequenced the genome of a venomous snake, the king cobra, and assessed the composition of venom gland expressed genes, small RNAs, and secreted venom proteins. We show that regulatory components of the venom secretory system may have evolved from a pancreatic origin and that venom toxin genes were co-opted by distinct genomic mechanisms. After co-option, toxin genes important for prey capture have massively expanded by gene duplication and evolved under positive selection, resulting in protein neofunctionalization. This diverse and dramatic venom-related genomic response seemingly occurs in response to a coevolutionary arms race between venomous snakes and their prey.

Author contributions: F.J.V., N.R.C., and M.K.R. designed research; F.J.V. acquired samples for sequencing and estimated genome size; H.J.J., and R.P.D. prepared sequencing libraries; M.B., and W.P. developed assembly software; C.V.H. assembled the genome; H.J.J., M.Y., D.C., and H.P.S. annotated the genome; J.M.C.R. assembled and annotated RNA-seq libraries; F.J.V., N.R.C., H.M.E.K., and A.S.H. analyzed RNA-seq libraries; A.M.H., D.S., and E.M. annotated and analyzed small RNA libraries; H.M.E.K., I.G., H.P.S., and D.D. annotated and analyzed *Hox* genes; F.J.V., N.R.C., C.V.H., R.J.R.M., H.M.E.K., A.S.H., R.P.D., R.M.K., and M.K.R. annotated venom toxin genes and performed synteny analyses; N.R.C., R.A.V., and W.W. analyzed gene family evolution; A.M.H. performed miRNA in situ hybridization; A.E.W., and J.M.L. performed lectin in situ hybridization; N.R.C., R.J.R.M., J.J.C., R.A.H., C.R., R.B.C., D.P., L.S., and R.M.K. analyzed the venom proteome; T.A.C., A.P.J.d.K., and D.D.P. contributed Burmese python genome data and assisted with comparative analyses; H.J.J., J.W.A., G.E.E.J.M.v.d.T., R.P.D., H.P.S., and M.K.R. organized sequencing platforms and facilities; F.J.V., N.R.C., W.W., and M.K.R. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The king cobra genome assembly and reads reported in this paper have been deposited in the GenBank database (bioproject no. [PRJNA201683](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA201683)). The transcriptome sequences reported in this paper have been deposited in the GenBank Short Read Archive database (bioproject no. [PRJNA222479](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA222479)). The microRNA sequences reported in this paper have been deposited in miRBase, www.mirbase.org.

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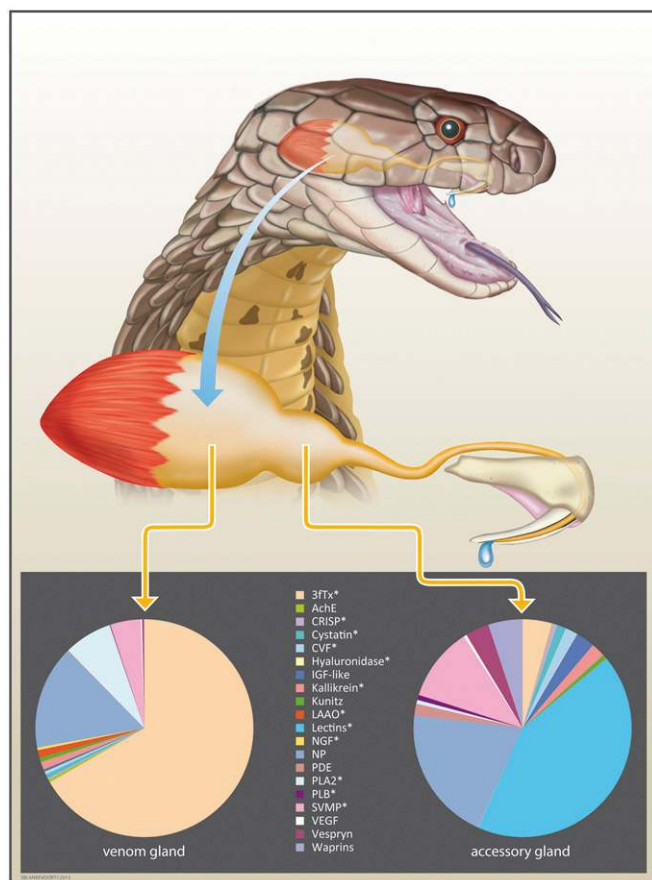


Fig. 1. The king cobra venom system with venom and accessory gland expression profiles. Pie charts display the normalized percentage abundance of toxin transcripts recovered from each tissue transcriptome. Three-finger toxins are the most abundant toxin family in the venom gland (66.73% of all toxin transcripts and 4.37% in the accessory gland), and they are represented in the genome by at least 21 loci. Lectins are the most abundant toxin family in the accessory gland (42.70% of all toxin transcripts and 0.03% in the venom gland), and they are represented in the genome by at least six loci. Asterisks indicate toxin gene families annotated in the genome. 3FTx, three-finger toxin; AChE, acetylcholinesterase; CRISP, cysteine-rich secretory protein; CVF, cobra venom factor; IGF-like, insulin-like growth factor; kallikrein, kallikrein serine proteases; kunitz, kunitz-type protease inhibitors; LAO, L-amino acid oxidase; NGF, nerve growth factor; PDE, phosphodiesterase; PLA2, phospholipase A₂; PLB, phospholipase-B; SVMP, snake venom metalloproteinase. Drawing made based on a photo by F.J.V.

The birth and death model of gene evolution is the canonical framework used to explain the evolutionary origin of snake venom toxins. Drivers of toxin diversification may include (i) directional selection for toxins that facilitate prey capture, (ii) the need to target a diversity of receptors in different prey, and (iii) the concomitant evolution of venom resistance in some prey as part of an evolutionary arms race (2). The lack of genome sequences for any venomous snake and the consequent dependence on transcriptome data have hampered our understanding of not only the tempo and mode of venom toxin evolution but also, the genomic mechanisms that regulate toxin-gene expression.

To address these issues, we have produced a draft genome of a venomous snake—that of an adult male Indonesian king cobra (*Ophiophagus hannah*). This iconic species is the longest venomous snake in the world. Native to tropical Asia, it feeds on other snakes, and it is a member of the family Elapidae. We also deep-sequenced transcriptomes and small RNAs of the venom gland, the accessory gland, and a pooled, multitissue archive and

characterized the king cobra venom proteome. These unique datasets provide an unprecedented insight into the evolution of venom.

Results and Discussion

King cobra genome sequence data (*SI Appendix, Table S1*) were first assembled de novo into contigs, which were subsequently oriented and merged into scaffolds. Haploid genome size was estimated by flow cytometry to be 1.36–1.59 Gbp (*SI Appendix, Fig. S1*). The assembled draft genome has an N50 contig size of 3.98 Kbp and an N50 scaffold size of 226 Kbp. The total contig length is 1.45 Gbp, and the total scaffold length (which contains gaps) is 1.66 Gbp.

As a genome quality check, we examined the *Hox* cluster, because it is well-characterized in other vertebrates (11). We annotated all 39 *Hox* genes, which we found clustered at four genomic regions, like in other vertebrates. However, the gene clusters are substantially larger than the *Hox* clusters observed in mammals (*SI Appendix, Fig. S2*). Of special interest is the absence of *Hoxd12* from the king cobra, the Burmese python (*Python molurus bivittatus*) (12), and other snake genomes (13) (*SI Appendix, Fig. S3*). *Hoxd12* is important for limb development in tetrapods (11) and thus, may have been lost along with limbs before the snake diversification. We also mapped microRNAs that had been previously located within mammalian and avian *Hox* clusters (*SI Appendix, Fig. S2* and *Dataset S1*).

We interrogated the king cobra genome and annotated the open reading frames of 12 venom toxin gene families (Fig. 1 and *SI Appendix, Fig. S4*). Venom toxins are thought to have been co-opted from gene homologs with nontoxic physiological functions that are expressed in tissues other than the venom gland (14, 15). Our analysis of tissue-specific transcriptomic data (12, 16–18) provides genome-scale confirmation that these venom genes have, indeed, been recruited from a wide variety of tissue types (*SI Appendix, Table S2*). Syntenic comparisons of king cobra genomic architecture with the genomes of other vertebrates revealed that toxin co-option has occurred by two distinct mechanisms: (i) gene hijacking/modification and (ii) duplication of nontoxin genes (*SI Appendix, Fig. S5*); they were followed in both cases by selective expression in the venom gland.

Sequencing and analysis of microRNA (miRNA) libraries made from a range of different tissues showed molecular similarities between the king cobra venom gland and known profiles of human and mouse pancreas (Fig. 2A). The most abundant miRNA in our venom gland library is *miR-375*, a canonical miRNA in the vertebrate pancreas. In the mouse, chicken, and zebrafish, *miR-375* expression is restricted to the pancreas and pituitary gland (19, 20). Here, we detected *miR-375* expression in the embryonic pancreas of the copperhead ratsnake (*Coelognathus radiatus*), the islet cell masses associated with the pancreas and spleen of the spitting cobra (*Naja siamensis*), and importantly, the venom gland of the king cobra (Fig. 2B–D and *SI Appendix, Fig. S6*). In the past, it has been hypothesized that the snake venom gland evolved by evolutionary modification of the pancreatic system (21–23), although this hypothesis has since been abandoned, because little evidence exists that toxins expressed in the venom gland have been co-opted from related proteins expressed in the pancreas (14). However, our results are consistent with *miR-375* being part of a core genetic network regulating secretion that has been co-opted during the evolution of the snake venom gland from an ancestral role in the pancreas and foregut secretory cells (24); it highlights an inherent link between these two secretory tissues, a link which was first suggested by Kochva et al. (21–23).

We identified 20 toxin families in the king cobra venom gland transcriptome (Fig. 1 and *Dataset S2*), including all toxin families annotated in the genome. Of the transcriptome hits, 14 toxin families were identified in the venom proteome (*SI Appendix,*

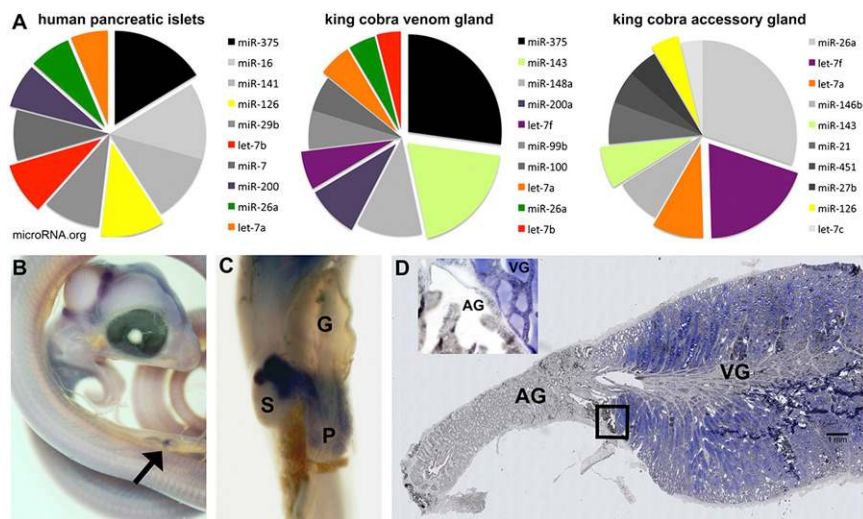


Fig. 2. MiRNA expression profiles of the king cobra venom gland and accessory gland and miRNA expression patterns by in situ hybridization. (A) The 10 most abundant miRNAs in the venom gland show similarities with the known expression profile of the vertebrate pancreas (shown here for human; microRNA.org). (B) In situ hybridization of *miR-375* in a *C. radiatus* embryo 27 d postoviposition with expression detected in the pancreas (arrow). (C) In situ hybridization of *miR-375* in an *N. siamensis* embryo 32 d postoviposition, showing expression in the islet cell masses of the pancreas and the intrasplenic islet tissue. (D) In situ hybridization of *miR-375* in a tissue section of the venom system of an adult *O. hannah* showing expression in the main venom gland. (Inset) Boundary of the venom gland (expression) and accessory gland (no expression) (*SI Appendix, Fig. S6*). AG, accessory gland; G, gallbladder; P, pancreas; S, spleen; VG, venom gland.

Figs. S7–S9 and Tables S3 and S4 and Dataset S3), and nerve growth factor, phospholipase-B, and cobra venom factor have not previously been reported in king cobra venom. We also identified a unique snake venom protein, insulin-like growth factor, which we found selectively expressed in the venom gland and the venom proteome (*SI Appendix, Fig. S10 and Table S4*). Recent findings have shown adaptive evolution in insulin-like growth factor genes in snakes, although the site of their expression was unknown (25). Evidence of selective venom gland expression combined with adaptive evolution is consistent with a function of these proteins as venom toxins. In addition, we discovered a unique independent recruitment event of L-amino acid oxidase into king cobra venom (*SI Appendix, Fig. S11*).

Comparisons of toxin expression in the venom gland, accessory gland, and pooled multitissue archive revealed that most toxins are expressed at high levels only in the venom gland (Fig. 1 and *SI Appendix, Fig. S10*). Our results indicate that toxin gene transcription in the venom gland is regulated independently from its expression in those other tissues. Most toxins observed in the venom gland transcriptome are expressed at low levels in the accessory gland. One exception was the lectin toxin family, with expression that was at least 40 times higher in the accessory gland (*SI Appendix, Fig. S10*). Our evolutionary analysis of the lectins shows that they have been recruited to the oral secretory glands before the radiation of the advanced snakes, followed by expansion of the gene family (*SI Appendix, Figs. S12 and S13*). Our king cobra data suggest a model in which venom-like lectin paralogs have then repeatedly become transcriptionally activated in the accessory gland and deactivated in the venom gland (*SI Appendix, Figs. S12 and S13*).

In situ hybridization showed that the expression of these recruited lectins is concentrated in the serous cells located in the proximal region (26) of the accessory gland (Fig. 3 and *SI Appendix, Fig. S14*). No lectins were detected in the king cobra venom proteome (*SI Appendix, Figs. S7–S9*), consistent with their low transcript abundance in the venom gland. These results suggest that lectins do not contribute to king cobra envenoming, which is in contrast to many other venomous snakes (1, 27), and that their repeated recruitment to the accessory gland is associated with the subsequent evolution of unidentified, nontoxic functions (15).

The venom gland transcriptome and venom proteome revealed multiple related venom isoforms for many different toxin families. To investigate the role of gene duplication in driving the genomic expansion of venom genes, we examined the evolutionary history of nine different toxin families by comparing gene orthologs and paralogs from other venomous snakes and the Burmese

python, and green anole lizard (*Anolis carolinensis*) genomes and tissue transcriptomes (12, 16–18) (*SI Appendix, Figs. S11 and S15–S22*). We then used these data to perform tests of directional selection. Our results reveal multiple distinct patterns of gene duplication and sequence evolution under positive selection in different protein-coding gene families both before and after their recruitment into venom-producing pathways (Fig. 4 *A* and *B* and *SI Appendix, Table S5*). Significantly, we found evidence of higher rates of duplication and selection in the most highly expressed,

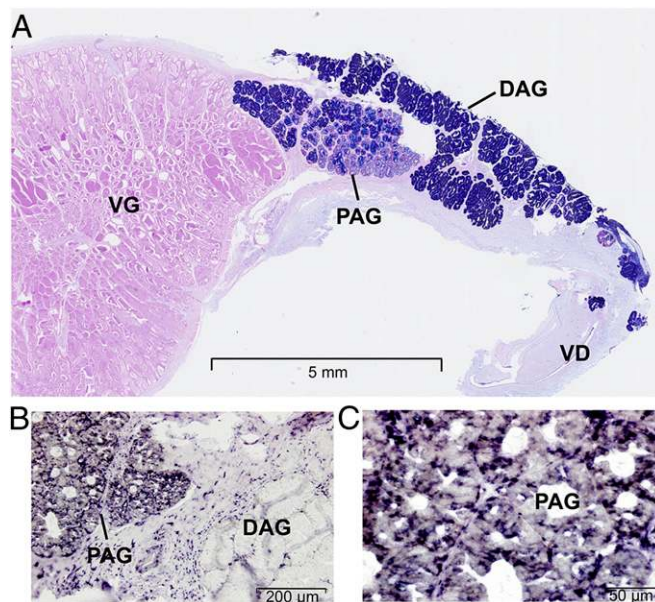


Fig. 3. Histological section of the complete venom apparatus of the king cobra and spatial expression of lectin genes in the accessory gland. (A) Longitudinal section of the venom system reveals the two regions of the accessory gland: the proximal portion (PAG) and the distal portion (DAG; consistent with a previous morphological study) (26). The venom system is stained by alcian blue and periodic acid-Schiff, in which the secretory epithelial cells and secretion of the venom gland are periodic acid-Schiff-positive and the seromucous acini of the PAG and the mucous acini comprising the DAG are stained with alcian blue. (B) In situ hybridization of lectin gene Oh-516 (genome ID s8808 gene 2) shows that lectin expression is restricted to the PAG. DAG shows no staining. (C) Detail of the PAG shown in *B* showing strong granular staining in the epithelium of the PAG (*SI Appendix, Fig. S14*). VD, venom duct; VG, venom gland.

proteomically abundant, and functionally important (28) gene families analyzed. The major lethal toxin family of the king cobra, the three-finger toxins (28, 29), is the most abundantly represented and isomerically diverse toxin family found in the venom gland transcriptome and venom proteome (Fig. 1 and *SI Appendix*, Fig. S7). This family has undergone massive expansion and shows high levels of positive selection and gene duplication (Fig. 4 B and C). In addition, phospholipase A₂, snake venom metalloproteinase, and kallikrein toxin families also exhibit substantial gene duplication (Fig. 4D), and evidence of positive selection was identified in two of these gene families (Fig. 4B).

Gene duplication coupled with positive selection is the mechanism underlying venom protein neofunctionalization (30–34). Our results are, therefore, consistent with a prominent role for prey-driven natural selection in generating the genetic diversity of the most pathogenic toxin families (28). By contrast, toxin families with ancillary functions show lower levels of gene expression, little to no evidence of gene duplication, and no evidence of directional selection (Fig. 4 B and E). For example, hyaluronidase, which possibly functions to break down prey tissue at the envenomation site (35), is not under positive selection. These results suggest that ancillary venom genes are less likely to generate resistance in

prey and therefore, likely to experience lower selection pressures. These gene families likely have conserved functional activities and do not participate in the evolutionary arms race seen in the more toxic venom protein families.

In conclusion, this study highlights the diversity of genomic responses to extrinsic selective factors (the imperative to overpower prey quickly). These responses include function-modulated patterns of transcript abundance, gene duplication, and protein evolution in different toxin families. In contrast with the only other venomous vertebrate genome sequenced to date [the platypus (36, 37)], gene duplication is apparently of fundamental importance in the adaptive evolution of the king cobra venom system. This distinction likely reflects the differences in selective pressures relating to the very different biological role of venom in these organisms. Platypus venom is implicated in male–male combat, and its evolution is driven by sexual selection, whereas snake venom is primarily used for predatory purposes. The requirement of snake venom to rapidly immobilize prey coupled with the concomitant evolution of resistance in some prey species apparently results in an evolutionary arms race that drives a diverse and dramatic genomic response in venomous snakes. Our study provides unique genome-wide perspectives on the

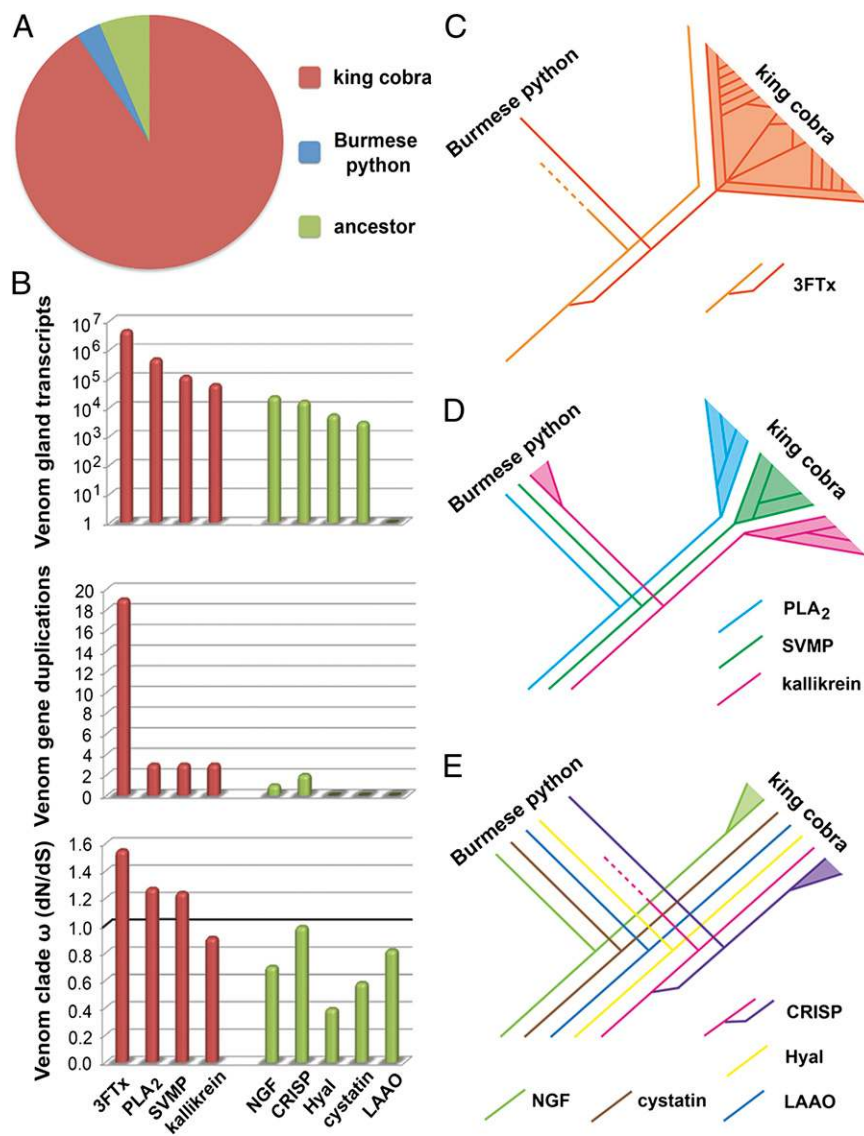


Fig. 4. Contrasting evolutionary histories of king cobra toxin gene families. (A) The vast majority of toxin family gene duplication events occurred in the king cobra lineage compared with the Burmese python and their common ancestor. (B) Comparisons of venom gland expression, venom-related gene duplication events, and rate of evolution of main toxin families (red) and ancillary toxin families (green). (C) Massive expansion of the three-finger toxin gene family and (D) moderate expansion of other pathogenic toxin families by duplication of venom-expressed genes after the split of the Burmese python from the advanced snakes. (E) Ancillary toxin families show reduced evidence of gene duplication. Colored lines indicate gene loci, with line splits representing gene duplication events and dotted lines indicating gene loss. Venom gene duplications are defined as duplications that occurred after the split of the Burmese python from the advanced snakes (king cobra). ω represents the dN/dS ratio identified for venomous gene clades. The boundary for directional selection is indicated by a bold line. Note the logarithmic scale in the normalized venom gland expression graph. 3FTx, three-finger toxin; CRISP, cysteine-rich secretory protein; Hyal, hyaluronidase; kallikrein, kallikrein serine proteases; LAAO, L-amino acid oxidase; NGF, nerve growth factor; PLA₂, phospholipase A₂; SVMP, snake venom metalloproteinase.

points of recruitment into venom-producing pathways were then reconstructed on these trees, thereby classifying tree branches into venomous and nonvenomous. The method of Yang and Nielsen (57) was implemented in the PAML software package to estimate ω_{venomous} and $\omega_{\text{nonvenomous}}$ for each toxin family.

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