

# Evolution of CRISPs Associated with Toxicofera-Reptilian Venom and Mammalian Reproduction

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## Abstract

Cysteine-rich secretory proteins (CRISPs) are glycoproteins found exclusively in vertebrates and have broad diversified functions. They are hypothesized to play important roles in mammalian reproduction and in reptilian venom, where they disrupt homeostasis of the prey through several mechanisms, including among others, blockage of cyclic nucleotide-gated and voltage-gated ion channels and inhibition of smooth muscle contraction. We evaluated the molecular evolution of CRISPs in toxicofera reptiles at both nucleotide and protein levels relative to their nonvenomous mammalian homologs. We show that the evolution of CRISP gene in these reptiles is significantly influenced by positive selection and in snakes ( $\omega = 3.84$ ) more than in lizards ( $\omega = 2.33$ ), whereas mammalian CRISPs were under strong negative selection (CRISP1 = 0.55, CRISP2 = 0.40, and CRISP3 = 0.68). The use of ancestral sequence reconstruction, mapping of mutations on the three-dimensional structure, and detailed evaluation of selection pressures suggests that the toxicofera CRISPs underwent accelerated evolution aided by strong positive selection and directional mutagenesis, whereas their mammalian homologs are constrained by negative selection. Gene and protein-level selection analyses identified 41 positively selected sites in snakes and 14 sites in lizards. Most of these sites are located on the molecular surface (nearly 76% in snakes and 79% in lizards), whereas the backbone of the protein retains a highly conserved structural scaffold. Nearly 46% of the positively selected sites occur in the cysteine-rich domain of the protein. This directional mutagenesis, where the hotspots of mutations are found on the molecular surface and functional domains of the protein, acts as a diversifying mechanism for the exquisite biological targeting of CRISPs in toxicofera reptiles. Finally, our analyses suggest that the evolution of toxicofera-CRISP venoms might have been influenced by the specific predatory mechanism employed by the organism. CRISPs in Elapidae, which mostly employ neurotoxins, have experienced less positive selection pressure ( $\omega = 2.86$ ) compared with the “nonvenomous” colubrids ( $\omega = 4.10$ ) that rely on grip and constriction to capture the prey, and the Viperidae, a lineage that mostly employs haemotoxins ( $\omega = 4.19$ ). Relatively lower omega estimates in Anguimorph lizards ( $\omega = 2.33$ ) than snakes ( $\omega = 3.84$ ) suggests that lizards probably depend more on pace and powerful jaws for predation than venom.

**Key words:** CRISP, positive selection, toxicofera-reptilian venom evolution, adaptive evolution, cysteine-rich secretory proteins, mammalian reproduction, mammalian immune system.

## Introduction

Each year, thousands of people die worldwide from envenomation by various species, despite the fact that many of these animals have sophisticated warning behavior to avoid close encounters and that the resultant bites are mostly accidental or defensive in nature. Frequent bites from these animals in both developed and developing countries accentuate the importance of venom research. Intraspecific variation in venom composition, which is likely influenced by multiple factors, including their phylogenetic history, diet, predator pressure, etc. (Sasa 1999; Barlow et al. 2009), can be large (Daltry et al. 1996; Tsai et al. 2007), complicating the production of antivenom. Therefore, the assessment of diversifying mechanisms and selection pressures influencing the evolution of venom-encoding genes can potentially

provide valuable information for structure-based drug design and the production of life-saving antidotes of greater specificity.

Venomous predators often employ a concoction of polypeptides and other molecules with diverse biological activities in their venom to attack multiple homeostatic systems within the prey in very specific and targeted manners. Venom components can target numerous physiological pathways, tissues, and cell types that are accessible via blood and lymphatic systems or by direct injection into the musculature. Evolution of the venom arsenal in snakes has been studied considerably, with research recently focusing largely on venom-coding genes, including phospholipase A2, snake venom disintegrins, snake venom metalloproteases, and lectins (Kini and Chan 1999; Calvete et al. 2003; Fry 2005;

Morita 2005; Lynch 2007; Soto et al. 2007; Peichoto et al. 2010). However, there is a lack of understanding of the evolution of cysteine-rich secretory proteins (CRISPs) in toxiciferan reptiles, a hypothetical venomous clade comprising all the venomous and related nonvenomous reptile species of the suborder *Serpentes* and *Iguania* (snakes and lizards: *Anguillidae*, *Varanidae*, and *Helodermatidae*). CRISPs belong to a large family of proteins found extensively in vertebrates, and they participate in diverse biological processes (Kitajima and Sato 1999; Udby et al. 2002; Gibbs and O'Bryan 2007; Gibbs et al. 2007). CRISPs are particularly enriched in the pancreatic tissues, salivary glands, reproductive tracts (Kierszenbaum et al. 1981; Haendler et al. 1993; Schambony et al. 1998), and reptilian venom ducts (Hill and Mackessy 2000; Yamazaki et al. 2003; Fry et al. 2006). They have diverse biological activities including inhibition of various ion channels and the inducement of proteolysis and paralysis of prey. There are several venom CRISPs with no known acute toxic effects or functions (Chang et al. 1997). Scope and details of mammalian and reptilian in reproduction, immune system, and venom are poorly understood and will require increased evolutionary and functional analyses.

CRISPs are single-polypeptide proteins with molecular weights of ~20 to 30 kDa (Yamazaki and Morita 2004, 2007) and are known to have a high degree of amino acid sequence similarity and a highly conserved specific pattern of 16 cysteine residues. Ten of these cysteine residues form an integral part of the highly conserved cysteine-rich domain (CRD) at the c-terminus. The CRD is composed of two domains, a hinge region and an ion channel regulator (ICR) domain. A few reptilian venom CRISPs have been shown to interact with ryanodine receptors (RyRs), perhaps through the ICR, to inhibit the release of Ca<sup>2+</sup> ions, thus inhibiting smooth muscle fiber functions (Nobile et al. 1994; Morrissette et al. 1995). "Pseudechetoxin" isolated from the venom of Australian king brown snake (*Pseudechis australis*) has been shown to inhibit cyclic nucleotide-gated (CNG) ion channels. Many other targets such as potassium and calcium channels have also been suggested (Wang et al. 2005, 2006) and there could be many more targets yet to be discovered.

There are three types of CRISPs in most mammals (CRISP1: AEG or acidic epididymal glycoprotein, CRISP2: testis-specific protein 1, and CRISP3: specific granule protein). A fourth type has been described only in mice (CRISP 4). It is hypothesized that the ancestral salivary gland CRISP was modified as a venom component in the toxiciferan reptiles rather than the usual gene recruitment events that have led to the multiple copies of other venom proteins like PLA2, disintegrins, metalloproteases defensins, etc. (Fry 2005).

In this study, we assessed for the first time the evolutionary history and selection pressures influencing the toxiciferan CRISPs using both gene- and protein-level approaches. We tested the hypothesis of whether reptilian CRISP variation has accumulated under the regime of positive selection and if their mammalian homologs were constrained because of their importance in other functions. Finally, we investigate the implications of observed mutations in the

three-dimensional structure of the snake and lizard CRISP proteins to obtain further insight into the evolution of reptilian toxiciferan venom.

## Materials and Methods

### Sequence Retrieval and Alignment

To assess the molecular evolution of CRISPs, we compiled a data set of 119 nucleotide sequences (46 snakes + 21 lizards + 52 mammals). Nucleotide and protein sequences were downloaded from National Center for Biotechnology Information (NCBI: <http://www.ncbi.nlm.nih.gov/>) and UniProt databases (<http://www.uniprot.org/>), respectively. The sequences (identified by their GenBank accession numbers) were retrieved by using BLAST. Complete cDNA sequences from each lineage of toxiciferan reptiles (Elapidae: AY299475.1; Viperidae: AY181983.1; Colubridae: DQ139891.1; and Anguimorph lizards: EU790958.1) were used to retrieve the reptilian CRISPs, whereas one sequence from each type of mammalian CRISPs (CRISP1: GU985267.1; CRISP2: BT030687.1; and CRISP3: BC102058.1) was used for the retrieval of the mammalian sequences.

The translated nucleotide sequences were aligned using MUSCLE 3.8 (Edgar 2004). The alignments were manually inspected and edited by eye. We used the 16 universally conserved cysteine residues as anchors to refine the alignment. Gblocks (Talavera and Castresana 2007) was used to remove regions that had gaps in more than 50% of the sequences in the alignment.

### Phylogenetic Analyses

The best-fit model of nucleotide substitution for our data set was determined as TIM3 + G + I by jModeltest (Posada 2008), according to Akaike's information criterion. Model-averaged parameter estimates of gamma shape parameter (alpha) and the proportion of invariant sites (pinvar) were used for phylogenetic reconstruction. The phylogenetic relationships among the toxiciferan-reptilian CRISPs were determined using Bayesian and maximum likelihood (ML) approaches. MrBayes version 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was used for Bayesian inference. Tree searches were run using four Markov chains for a minimum of 10 million generations, sampling every 100th tree. The log-likelihood score of each saved tree was plotted against the number of generations to establish the point at which the log-likelihood scores of the analyses reached their asymptote. Twenty-five percent of the total trees sampled were discarded as burnin. The posterior probabilities (PPs) for clades were established by constructing a majority rule consensus tree for all trees generated after the completion of the burnin. The analyses were repeated three times to make sure that the trees generated were not clustered around local optima. An optimal ML phylogenetic tree was obtained using PhyML 3.0 (Guindon et al. 2010) and node support was evaluated with 1,000 bootstrapping replicates. Phylogenetic trees were rooted using the mouse (NM\_009639.2) and the human (X94323.1) CRISP3 sequences. Dendroscope (Huson et al. 2007) was used to prepare the phylogenetic tree.

Sequence divergence (F84 genetic distance) was plotted against the transition (*s*) and transversion (*v*) rates and a test of nucleotide substitution was carried out using DAMBE (Xia and Xie 2001; Xia et al. 2003) to evaluate the influence of saturation of nucleotide substitutions on the evolutionary inferences. Recombination analyses were done using Single Breakpoint Recombination and Genetic Algorithms for Recombination Detection implemented in the Datamonkey server (Pond et al. 2005; Kosakovsky Pond et al. 2006; Delpont et al. 2010).

### Selection Analyses

ML models of coding sequence evolution implemented in Codeml in the PAML (Yang 2007) package of programs version 4 were used to test the hypothesis that functional diversification of snake and lizard venom CRISP genes is driven by positive Darwinian selection. PAML compares the ML estimates of dN and dS across an alignment with a predefined distribution and uses empirical Bayes methods to identify individual positively selected site (Nielsen and Yang 1998; Yang and Bielawski 2000).

We evaluated the evidence for positive selection on CRISP genes in the toxicoferan reptiles and their homologous mammalian counterparts by employing branch models. The most simple one-ratio model assumes the same dN/dS for all the branches in the phylogenetic tree (Goldman and Yang 1994). The assumption of constant evolutionary selection pressure on all the lineages in the phylogenetic tree over millions of years of evolutionary time sounds quite unrealistic. Hence, the free-ratio model assumes separate dN/dS ratios for all branches in the tree. However, this model is parameter rich and is prone to inaccurate estimations (Yang et al. 2000). Both the one-ratio and the free-ratio models detect positive selection when the average of omega values over the entire length of protein is greater than one—that is, when the majority of the amino acids are under the influence of selection. To assess selection pressures acting upon individual lineages, we employed the two-ratio model as well as the optimized branch–site test (Yang and Nielsen 2002; Zhang et al. 2005). A likelihood ratio test (LRT) was conducted by comparing the two-ratio model that allows omega to be greater than 1 in the foreground branch, with the null model that does not. The branch–site model by comparison allows omega to vary both across sites of the protein and across branches in the tree and has reasonable power and accuracy to detect short bursts of episodic adaptations (Zhang et al. 2005).

Unlike the lineage-specific branch and branch–site models, the GA-Branch Test implemented in the HyPhy (Pond and Frost 2005) package does not require the foreground and background branches to be defined a priori. The algorithm works on the principle that there could be many models that better fit the data than a single a priori hypothesis and uses a robust multimodel inference to collate results from all models examined and provides confidence intervals on dN/dS for each branch.

Another drawback of most lineage-specific models is that they assume a single omega value for the entire length of the sequence. They lack the ability to identify sites in

proteins that might be under the influence of positive selection more than others, such as the surface of venom molecules. Thus, lineage-specific models can underestimate the degree of positive selection acting on biological sequences. To account for rate variation among sites, we used the site-specific models (Nielsen and Yang 1998; Yang 2000), which are powerful tools for detecting diversifying selection. Positive selection is detected statistically as a nonsynonymous-to-synonymous nucleotide substitution rate ratio ( $\omega$ ) significantly greater than 1. Because no a priori expectation exists for the distribution of  $\omega$  values, we compared likelihood values for three pairs of models with different assumed  $\omega$  distributions: M0 (constant  $\omega$  rates across all sites) versus M3 (allows the  $\omega$  to vary across sites within “*n*” discrete categories,  $n \geq 3$ ); M1a (a model of neutral evolution) where all sites are assumed to be either under negative ( $\omega < 1$ ) or neutral selection ( $\omega = 1$ ) versus M2a (a model of positive selection) which in addition to the site classes mentioned for M1a, assumes a third category of sites; sites with  $\omega > 1$  (positive selection) and M7 (beta) versus M8 (beta and  $\omega$ ) and models that mirror the evolutionary constraints of M1 and M2 but assume that  $\omega$  values are drawn from a beta distribution (Nielsen and Yang 1998). Only if the alternative models (M3, M2a, and M8: allow sites with  $\omega > 1$ ) show a better fit in LRT relative to their null models (M0, M1a, and M8: do not show allow sites  $\omega > 1$ ), are their results considered significant. LRT is estimated as twice the difference in ML values between nested models and compared with the  $\chi^2$  distribution with the appropriate degree of freedom—the difference in the number of parameters between the two models. The Bayes empirical Bayes (BEB) approach (Yang et al. 2005) was used to identify amino acids under positive selection by calculating the PPs that a particular amino acid belongs to a given selection class (neutral, conserved, or highly variable). Sites with greater PP ( $PP \geq 95\%$ ) of belonging to the “ $\omega > 1$  class” were inferred to be positively selected.

Some studies have suggested that the ML method of evaluating positive selection produces false-positive results even when no positively selected sites exist (Suzuki and Nei 2004) or when positively selected sites and negatively selected sites are mixed (Anisimova et al. 2002). Further support for the PAML results was obtained using a complementary protein-level approach implemented in TreeSAAP (Woolley et al. 2003). Models of reptilian and mammalian cysteine-rich proteins depicting the overall conservation of amino acids were built using the Consurf webserver (Ashkenazy et al. 2010).

Detection of positive Darwinian selection across lineages using branch and branch–site models requires the foreground branches (lineages tested to be under positive selection) and background branches (rest of the lineages) to be defined a priori. When a predefined biological hypothesis is unavailable or the functions of genes are not well understood, then it becomes difficult to define foreground branches. A possible approach then would be to treat each branch in the phylogeny alternately as the foreground lineage and test multiple hypotheses. A LRT can then conducted

by comparing a model that allows omega to be greater than 1 in the foreground branch, with the same model where foreground branches are constrained to have an omega equivalent to 1. It is suggested that when using the branch–site model to test multiple branches in the phylogenetic tree for positive selection, it is necessary to control the family wise error rate (FWER or Type I error) (Zhang et al. 2005). Bonferroni's correction is the easiest method to achieve this, which uses  $\alpha/n$  as the significance level to test each hypothesis; where “ $\alpha$ ” is the significance level and “ $n$ ” being the number of independent true null hypotheses.

If the lineages have different proportion of sites under selection, then comparing their omega estimates to assess the strength of selection could be misleading. This can be overcome by estimating the omega simultaneously for the data sets being compared. We employed the clade model analyses (Bielawski and Yang 2004) to facilitate an effective comparison between different lineages of reptiles. These models are based on the branch–site models allowing variations in the omega value among the sites with a proportion of sites evolving under divergent selection between the clades.

To clearly depict the proportion of sites under selection, an evolutionary fingerprint analysis was carried out using the ESD algorithm implemented in datamonkey (Pond et al. 2010).

### Ancestral Sequence Analyses

To understand the evolutionary pathway that shaped the extant CRISPs over millions of years, we reconstructed ancestral sequences using the ancestral sequence reconstruction (ASR) algorithm implemented on the Datamonkey server (Delpont et al. 2010) and used a ML-based joint reconstruction approach (Pupko et al. 2000). We evaluated selection pressures acting on the ancestral toxicofera-reptilian CRISPs and mapped the sites under positive selection on their three-dimensional crystal structure.

### Functional Divergence

We used the ML method implemented in Diverge 2.0 (Gu 1999) to test if there was any significant change in evolutionary rates at amino acid sites of the CRISPs after the reptilian lineages diverged. The method estimates the expected substitutions at each amino acid site and then calculates the coefficient of functional divergence ( $\theta$ ), which is the probability that evolutionary rate at a site is statistically independent between the two gene clusters. Such sites are referred to as Type I sites if they are conserved in one subfamily but vary greatly in another, implying that they have been subjected to different functional constraints and hence might have different functions. The coefficient of functional divergence ( $\theta$ ) and the PP for functional divergence were calculated for each position in the alignment.

### Structural Analyses

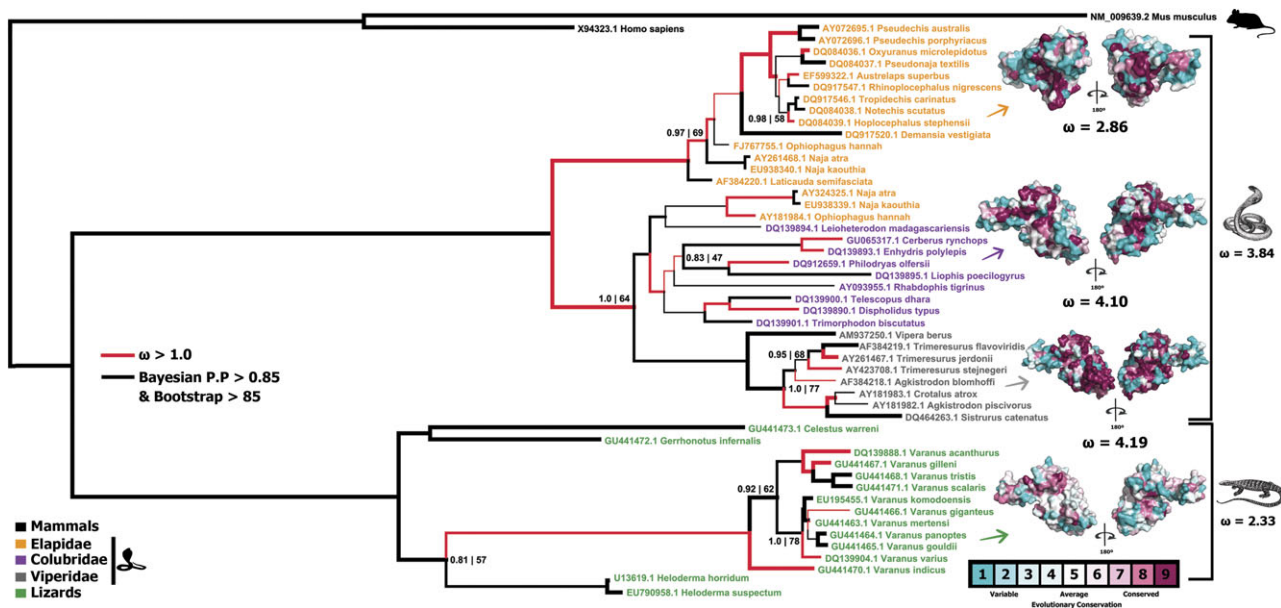
To depict the underlying effects of the selection pressures on reptilian and mammalian CRISPs, we mapped the sites under positive selection on their crystal structures. We

used the Swiss-model server (Arnold et al. 2006) to search for the homologous sequence with the highest identity, whose three-dimensional structure was already deduced empirically by X-ray crystallography or nuclear magnetic resonance. The crystal structures of 2gizA (Natrix: *Naja atra*) and 2ddbB (Pseudecin: *Pseudechis porphyriacus*) were obtained from the Swiss-model server as the best-fit template for the target CRISP sequences from *Ophopphagus hannah*: AY299475.1 (~86% identity) and *Varanus tristis*: GU441468.1 (~50% identity) respectively, whereas 1wvra (Triflin: *Trimeresurus flavoviridis*) was determined as the best-fit template for the mammalian CRISPs (~86% identity). Sequences of all these structures were used in the analyses. Pymol (DeLano 2002) was used to produce the images of the three-dimensional models of CRISPs. The program GETAREA (Fraczkiewicz and Braun 1998) was used to calculate the accessible surface area (ASA)/solvent exposure of amino acid side chains. It uses the atom co-ordinates of the Protein Data Bank file and indicates if a residue is buried or exposed to the surrounding medium by comparing the ratio between side chain ASA and the “random coil” values per residue. An amino acid is considered to be buried if it has an ASA less than 20% and exposed if ASA is more than or equal to 50%.

## Results and Discussion

Lizards are generally not associated with venom, not because they lack venom but because most lizards lack a specialized venom delivery apparatus to inject venom into the prey for maximum effect. Until recently, only two species of lizards (genus *Heloderma*) with venom potent enough to kill humans were considered venomous. Despite possessing an arsenal of deadly venom components, most colubrids cannot efficiently deliver venom in sufficient quantities and thus do not pose a serious medical threat to humans. However, it is now recognized that even the Anguimorph lizards and all colubrids possess venom-encoding genes (Fry et al. 2006).

Many venom components are hypothesized to evolve via the birth and death model of evolution, where new genes are created by repeated duplication events, and are subsequently either maintained in the genome or deleted or become nonfunctional by pseudogenization (Fry, Wüster, Kini, et al. 2003; Lynch 2007). Directional selection reinforces the functionally important toxin types through adaptive evolution, creating a venom gland–specific multi-gene family (Fry et al. 2008). The likelihood of neofunctionalization is increased through random mutation, gene conversion, and unequal crossing-over (Fry, Wüster, Kini, et al. 2003). The inevitable arms race between the predator and prey leads to precise biological targeting as a single amino acid change could alter the specificity or potency of the toxin. Toxicofera-reptilian CRISPs are unique, in that they are derived through the evolutionary modification of an ancestral CRISP present in the salivary gland rather than gene recruitment events (Fry 2005). Mammals, in contrast, have three types of CRISPs (and a fourth type in mice) that derive from mammalian lineage–specific gene duplication events.



**Fig. 1.** Molecular phylogeny of toxicoferan-reptilian CRISP genes. Branches with the Bayesian posterior probability (B.P.P) greater than 0.85 and 850 or more (of 1,000) bootstrap replicate support (BS) are presented as thick lines. Branches that have lower BS (<850) but a stronger B.P.P support (>0.85) are presented as “B.P.P | BS.” Branches under positive selection are represented as red lines in the online version of the publication). The three-dimensional models of CRISPs depicting the amino acid variability are also shown along with the omega estimate (Clade model C) of the respective lineages.

To assess how these venom proteins evolved in the reptilian lineage in comparison with their nonvenomous mammalian homologs, we employed both nucleotide and amino acid-level selection analyses. We conducted the site, branch, branch-site, and clade model tests to identify sites under positive selection and to assess its influence on the three-dimensional structure and function of the CRISP protein.

### CRISP Phylogeny

Bayesian and ML analyses of toxicoferan-reptilian CRISPs retrieved phylogenetic trees with similar topologies (fig. 1). The overall topology of these phylogenetic trees is not in concordance with the generally accepted reptilian phylogeny (Kelly et al. 2003). Viperidae is the most basal clade of Colubroids or the advanced snakes followed by Elapidae and Colubridae. However, the phylogenetic trees incorrectly depict Elapidae as the most basal clade, which reflects an often observed differences between the gene and species trees (Rosenberg 2002). We note that three elapid sequences form a clade within Colubridae, whereas other sequences from the same species group with other elapids as expected. The topology of the phylogenetic trees could be the result of ancestral polymorphisms given that CRISPs were recruited in reptiles before the divergence of the toxicoferan lineages.

When studying sequences that are separated by millions of years of evolutionary time, saturation of nucleotide substitution can bias evolutionary inferences. Substitution saturation can result in an underestimation of dS and an inflation of dN/dS. We plotted the sequence divergence (F84 genetic distance) against the transitions (s) and transversions (v) rates and found no evidence for nucleotide

saturation at the third position or any other position. The index score (ISS) remained significantly lower than the critical score (ISS.C). We also evaluated the effects of recombination and found no evidence for recombination in the data set.

### Selection Analyses

To assess the consequence of positive Darwinian selection on CRISP proteins, we used likelihood models of coding sequence evolution (Goldman and Yang 1994; Yang 1998) implemented in Codeml of the PAML (Yang 2007) package (tables 1 and 2).

The one-ratio model is the simplest of the branch-specific models and estimates a single  $\omega$  value for all branches in the phylogeny. The estimate of global  $\omega$  for the snake CRISP gene under this model is 1.14. This is an average over all codons and lineages and thus highlights the dominant role of positive selection in shaping snake venom CRISPs. Global  $\omega$  estimates for lizard CRISPs was 0.78 (table 2). In contrast, mammalian CRISPs exhibit significant negative selection (CRISPI: 0.42, CRISPII: 0.32 and CRISPIII: 0.50) (supplementary material, Supplementary Material online).

To evaluate the selection pressures on various reptilian lineages, we employed the lineage-specific two-ratio model (table 1). Similar to the one-ratio model, the lineage-specific two-ratio model tends to be very conservative as it can only detect positive selection if omega ratio averaged over all the sites along the lineage is significantly greater than 1. Estimates of omega for snake and lizard lineages under this model are 1.21 and 0.64, respectively, which also accentuates the influence of positive selection on snake CRISPs. Both these estimations were significant

**Table 1.** Lineage-Specific ML Parameter Estimates for Toxicoferan-Reptilian CRISPs.

Model	$\omega^a$	Likelihood ( $\ell$ )	Proportion of Sites with $\omega > 1^b$	Number of Sites with $\omega > 1^c$	Significance <sup>d</sup>
<b>Reptiles</b>					
<b>Snakes</b>					
Two-ratio model	1.21	-10370.88844	—	—	*P << 0.001
Branch-site model A	3.76	-9915.548688	25.3%	23 (PP ≥ 0.99)	*P << 0.001
Clade model C	3.84	-9876.069435	20.0%	13 (PP ≥ 0.95)	P << 0.001
<b>Lizards</b>					
Two-ratio model	0.64	-10465.99893	—	—	*P << 0.001
Branch-site model A	3.21	-10093.64418	18.1%	3 (PP ≥ 0.99)	*P << 0.001
Clade model C	2.33	-9876.069435	20.0%	12 (PP ≥ 0.95)	P << 0.001
<b>Snake lineages</b>					
<b>Colubridae</b>					
Site model 8	1.42	-3508.159988	28.0% ( $\omega_2 = 3.89$ )	22 (PP ≥ 0.99)	P << 0.001
Two-ratio model	1.16	-10382.84638	—	16 (PP ≥ 0.95)	NS
Branch-site model A	6.73	-9997.185149	8.1%	3 (PP ≥ 0.99)	*P << 0.001
Clade model C	4.10	-6578.072786	23.7%	3 (PP ≥ 0.95)	P << 0.001
<b>Viperidae</b>					
Site model 8	1.42	-2077.819161	22.7% ( $\omega_2 = 5.24$ )	18 (PP ≥ 0.99)	P << 0.001
Two-ratio model	1.05	-10383.8539	—	7 (PP ≥ 0.95)	NS
Branch-site model A	5.66	-9998.09742	15.0%	3 (PP ≥ 0.99)	*P << 0.001
Clade model C	4.19	-6578.072786	23.7%	6 (PP ≥ 0.95)	P << 0.001
<b>Elapidae</b>					
Site model 8	1.50	-2874.216899	25.3% ( $\omega_2 = 4.45$ )	15 (PP ≥ 0.99)	P << 0.001
Two-ratio model	1.28	-10381.65348	—	14 (PP ≥ 0.95)	NS
Branch-site model A	5.52	-9981.464478	16.1%	4 (PP ≥ 0.99)	*P << 0.001
Clade model C	2.86	-6578.072786	23.7%	6 (PP ≥ 0.95)	P << 0.001

NOTE.—NS, not significant.

<sup>a</sup> dN/dS (weighted average).<sup>b</sup> Proportion of sites with  $\omega > 1$ .<sup>c</sup> Number of sites with  $\omega > 1$  under BEB approach with a PP of more than or equal to 0.99 and 0.95.<sup>d</sup> Significance of the model in comparison with the null model.

\*Significant at 0.001 after Bonferroni correction.

compared with the strict branch model, which is essentially the same model but with omega constrained at 1. This again proves the dominance of positive selection on snake CRISPs. We further employed the model to evaluate the selection pressures along individual snake lineages. The omega estimate for the Elapidae lineage under this model was 1.28, whereas for the Colubridae and Viperidae lineages, the omega estimations were 1.16 and 1.05, respectively (table 1). These omega estimates were not significant when compared with the strict branch model estimates.

Because the branch model estimates omega for each lineage by averaging over all the branches and the site models by averaging over all the sites, they often fail to identify episodic adaptations that affect only few amino acids and/or lineages. Hence, we employed the branch site model (table 1) that allows omega to vary both across the lineages in the phylogenetic tree and across the sites in the genes and hence have a reasonable power and accuracy to detect short bursts of episodic adaptation targeting fewer amino acid residues (Zhang et al. 2005). The branch site model A

identified ~25% of sites ( $\omega = 3.76$ ) in snakes and only ~18% of the sites ( $\omega = 3.21$ ) in lizards as under positive selection. The omega estimates for individual snake lineages revealed greater evidence of positive selection in Viperidae (~15% of sites;  $\omega = 5.66$ ) than Elapidae (~16% of sites;  $\omega = 5.52$ ) and Colubridae (~8% of sites;  $\omega = 6.73$ ) considering both the number of positively selected sites and the strength of positive selection (table 1). We employed multiple test corrections using Bonferroni's test to keep the FWER less than alpha (at 0.1% significance).

The estimated  $\omega$  in snakes under the clade models is 3.84 in comparison with 2.33 in lizards (LRT = 295.3, degree of freedom (DF) = 3,  $P << 0.001$ ), again highlighting the dominant role of positive Darwinian selection on the snakes. The analyses of individual snake lineages revealed that the influence of positive selection on Viperidae ( $\omega = 4.19$ ) is more than that of Elapidae (2.86) and Colubridae lineages ( $\omega = 4.10$ ) (LRT = 231.39; DF = 3;  $P << 0.001$ ).

We further employed the site-specific models that account for rate variation across the sites (table 2). Estimates using the BEB approach implemented in M8

**Table 2.** Site-Specific ML Parameter Estimates for Toxicoforan-Reptilian CRISPs.

Model	Likelihood (L)	$\omega_0^a$	Parameters	Significance <sup>b</sup>	Number of Sites with $\omega > 1^c$
<b>Snakes</b>					
M0 (one ratio)	-6964.393838	1.14	= $\omega_0$		—
M0 (constrained)	-6965.771794	1.0	$\omega_0$ constrained to 1		—
			$P_0$ : 0.48460		
			$\omega_0$ : 0.07		
M1 (neutral)	-6711.896261	0.55	$P_1$ : 0.48460		—
			$\omega_1$ : 1.0		
			$P_0$ : 0.39687		
			$\omega_0$ : 0.05		
M2 (selection)*	-6595.953989	1.24	$P_1$ : 0.36260	$P \ll 0.001$	24 ( $PP \geq 0.99$ )
			$\omega_1$ : 1.0		13 ( $PP \geq 0.95$ )
			$P_2$ : 0.24054		
			$\omega_2$ : 3.58		
			$P_0$ : 0.43544		
			$\omega_0$ : 0.08		
M3 (discrete)*	-6593.227401	1.37	$P_1$ : 0.38890	$P \ll 0.001$	—
			$\omega_1$ : 1.0		
			$P_2$ : 0.17566		
			$\omega_2$ : 1.0		
M7 (beta)	-6713.705160	0.52	$p$ : 0.16753		—
			$q$ : 0.15459		—
			$p_0$ : 0.745		
			$p$ : 0.162		
M8 (beta and $\omega$ )*	-6598.579842	1.22	$q$ : 0.183	$P \ll 0.001$	26 ( $PP \geq 0.99$ )
			$p_1$ : 0.255		11 ( $PP \geq 0.95$ )
			$\omega$ : 3.43		
<b>Lizards</b>					
M0 (one ratio)	-3647.864377	0.78	= $\omega_0$		—
M0 (constrained)	-3650.682815	1.0	$\omega_0$ constrained to 1		—
			$P_0$ : 0.45529		
			$\omega_0$ : 0.10		
M1 (neutral)	-3588.206161		$P_1$ : 0.54471		—
			$\omega_1$ : 1.0		
			$P_0$ : 0.38959		
			$\omega_0$ : 0.10		
M2 (selection)*	-3566.671365	1.04	$P_1$ : 0.47093	$P \ll 0.001$	2 ( $PP \geq 0.99$ )
			$\omega_1$ : 1.0		8 ( $PP \geq 0.95$ )
			$P_2$ : 0.47093		
			$\omega_2$ : 3.73		
			$P_0$ : 0.23786		
			$\omega_0$ : 0.00		
M3 (discrete)*	-3566.221975	1.01	$P_1$ : 0.56320	$P \ll 0.001$	—
			$\omega_1$ : 0.68		
			$P_2$ : 0.19894		
			$\omega_2$ : 3.15		—
M7 (beta)	-3590.931288	0.59	$p$ : 0.18663		—
			$q$ : 0.12454		
			$p_0$ : 0.836		
			$p$ : 0.304		
M8 (beta and $\omega$ )*	-3566.284106	1.02	$q$ : 0.183	$P \ll 0.001$	6 ( $PP \geq 0.99$ )
			$p_1$ : 0.253		8 ( $PP \geq 0.95$ )
			$\omega$ : 3.44		

NOTE.—\*Models that allow  $\omega > 1$ .<sup>a</sup> dN/dS (weighted average).<sup>b</sup> Significance of the model in comparison with the null model.<sup>c</sup> Number of sites with  $\omega > 1$  under the BEB approach with a PP more than or equal to 0.99 and 0.95.

suggested that up to ~26% of the residues in snakes and 16% of the residues in the lizard CRISPs are under positive selection.

In snake CRISPs, site models M2a and M8 detected 37 and 38 positively selected amino acid residues, respectively, under the BEB approach. Model M2a detected 24 sites with  $PP \geq 0.99$  and 13 sites with  $PP \geq 0.95$ , whereas site model M8 detected 26 sites with  $PP \geq 0.99$  and 12 sites with

$PP \geq 0.95$ . In lizards, however, model 2 identified only ten amino acid sites (two sites with  $PP \geq 0.99$  and eight sites with  $PP \geq 0.95$ ) under selection, whereas model 8 identified 14 such sites (six sites with  $PP \geq 0.99$  + eight sites with  $PP \geq 0.95$ ). Clearly, Darwinian selection seems to be much more influential in snake than lizard CRISPs both in terms of number of amino acids under selection and in the strength of selection (tables 1 and 2).

**Table 3.** ML Parameter Estimates for Mammalian CRISPs.

	FEL <sup>a</sup>		REL <sup>b</sup>		SLAC <sup>c</sup>			Codeml	
	$\omega > 1^d$	$\omega < 1^e$	$\omega > 1^d$	$\omega < 1^e$	$\omega > 1^d$	$\omega < 1^e$	$\omega$	M8 ( $\omega$ )	$\omega > 1^f$
CRISP1	0	32	0	All	0	10	0.50	0.55 <sup>NS</sup>	5
CRISP2	3	49	3	49	0	5	0.40	0.40 <sup>NS</sup>	—
CRISP3	1	21	1	21	0	8	0.61	0.68 <sup>NS</sup>	4

NOTE.—NS, not significant in comparison with the null model M7 (beta).

<sup>a</sup> Fixed-effects likelihood.

<sup>b</sup> Random-effects likelihood.

<sup>c</sup> Single likelihood ancestor counting.

<sup>d</sup> Number of positively selected sites at 0.05 significance.

<sup>e</sup> Number of negatively selected sites at 0.05 significance;

$\omega$ : mean dN/dS.

<sup>f</sup> Number of positively selected sites under BEB approach with PP greater than 0.95.

The omega estimation under M8 for the mammalian CRISPs was 0.55, 0.40, and 0.68 for CRISP1, CRISP2, and CRISP3, respectively. The likelihood ratio of model 8 was not significant in comparison with model 7 (table 3), and none of the models of positive selection were significant in comparison with their null models (supplementary material, Supplementary Material online).

To evaluate if mammalian CRISPs evolve under the influence of negative selection, we employed the fixed-effects likelihood (FEL) and random-effects likelihood (REL) models that identify amino acids evolving under positive and negative selection. The results suggest that there is a strong influence of negative selection on the mammalian CRISPs (table 3), which may be attributed to functional constraints on these proteins, perhaps because of their hypothesized role in reproductive pathways and any genetic variation might easily affect homeostasis. Notwithstanding, a few positively selected amino acid sites were detected in CRISP3 with Codeml model 8, FEL and REL.

Selection analyses at the nucleotide level alone cannot distinguish events that may contribute to the diversification of the toxin molecules. The ML method of evaluating positive selection may sometimes retrieve false-positive results (Anisimova et al. 2002; Ashkenazy et al. 2010).

Hence, to provide additional support for the amino acid sites detected to be under positive selection by PAML, we employed a complementary protein-level approach implemented in TreeSAAP (Woolley et al. 2003) (table 4). TreeSAAP measures the selective influences on 31 structural and biochemical amino acid properties during cladogenesis and performs goodness-of-fit and categorical statistical tests based on ASR. The number of radical changes in the amino acid properties was used as a proxy for determining the strength of positive selection at a particular amino acid position (more radical changes in amino acid properties might indicate adaptive evolution). An empirical value of 6 amino acid property changes was set as a threshold. Thus, the residues that had lesser than six amino acid property changes were categorized as type I sites, whereas those that had more than six were categorized as type II sites.

There were 41 sites detected by both PAML (model 2 and model 8, BEB analysis,  $PP \geq 0.95$ ) and TreeSAAP ( $P \leq 0.001$ ) as under positive selection in snake CRISPs

(table 5). Ten of these sites were Type I (more than or equal to six radical changes in amino acid properties). However, in lizards, only 11 sites were detected by both PAML and TreeSAAP to be under positive selection, and of these, none were Type II. This further emphasizes that snake CRISPs not only have more number of positively selected sites but that they also experience greater selection pressures at these sites in comparison with their lizard homologs.

### Structural Analyses

To investigate selection patterns and to assess their influence on the structure and function of these molecules, we mapped the sites under selection on the crystal structure of CRISPs (figs. 2 and 3).

Previously studied snake venom proteins like PLA2, three-finger toxins, etc. have evolved via mutations targeted around their functional domains while the structural residues are constrained and maintain a highly conserved scaffold (Mackessy 2002; Kini 2004; Fry et al. 2006, 2008; Yamazaki and Morita 2007; Doley et al. 2008). Similarly, both snake and lizard CRISPs had a highly conserved structural scaffold, although most mutations were located in the functional domains. They have 16 cysteines that form eight disulphide bridges and are universally conserved in all CRISP proteins (Guo et al. 2005). Interestingly, ten of these cysteine residues are located in the c-terminus as part of the CRISP domain. This CRD has structurally flanking six cysteine repeats that exhibit a high degree of similarity with the K<sup>+</sup> channel-blocking venom from anemones (Alessandri-Haber et al. 1999; Guo et al. 2005; Shikamoto et al. 2005; Lange et al. 2006; Suzuki et al. 2008). The CRD domain in one of the mammalian CRISP has been shown to be associated with the ion channel regulatory activity (Gibbs et al. 2006). Hence, this region could mediate the interaction of CRISPs with ion-gated channels in other toxiciferan reptiles as well. In snake CRISPs, 44% (18) of the total amino acids (41) under positive selection were located on the CRD domain. Remarkably, of the 38 amino acids defining the CRD, 18 were under positive selection. However, some findings suggest that CRD alone might not be enough to mediate such interactions and other domains, particularly PR-1 might also be essential (Suzuki et al. 2008). We found 22 positively selected amino acids within the PR-1 domain. Thus, both CRD and PR-1 domains act as



**Table 4.** Amino Acid Sites Under Positive Selection.

Sites		PAML		TreeSAAP			
Position	A.A	M2A	M8	Radical Changes in Amino Acid Properties			
				Chemical	Structural	Total	A.S.A (%)
<b>Snakes</b>							
30	Q	3.495 ± 0.12	3.496 ± 0.095	—	$\alpha_C V^0$	2	<u>92.9</u>
73	N	3.392 ± 0.512	3.417 ± 0.453	—	$\alpha_C$	1	<u>57.6</u>
75	N	3.31 ± 0.664	—	—	$\alpha_C$	1	<u>35.0</u>
76	L	3.396 ± 0.444	3.421 ± 0.444	—	$\alpha_C$	1	<u>7.0</u>
81	D	3.501 ± 0.011	3.5 ± 0.011	—	$\alpha_C$	1	<u>75.9</u>
82	Y	3.501 ± 0.016	3.5 ± 0.016	—	$\alpha_C B_I$	2	<u>78.9</u>
83	S	3.454 ± 0.297	3.465 ± 0.297	—	$\alpha_C B_I$	2	<u>37.5</u>
87	E	3.499 ± 0.053	3.499 ± 0.053	—	$\alpha_C B_I$	2	<u>57.7</u>
100	N	3.496 ± 0.092	3.497 ± 0.092	pK'	$\alpha_C$	2	<u>82.6</u>
102	R	3.5 ± 0.031	3.5 ± 0.031	pK'	$\alpha_C$	2	<u>28.2</u>
103	A	3.472 ± 0.226	3.48 ± 0.226	pK'	$\alpha_C$	2	<u>64.3</u>
106	E	3.492 ± 0.121	3.494 ± 0.121	pK'	$\alpha_C$	2	<u>47.2</u>
110	L	3.501 ± 0.005	3.5 ± 0.005	pK'	$\alpha_C R_a N_s H_p$	5	<u>32.9</u>
115	Y	3.497 ± 0.081	3.497 ± 0.081	pK'	$\alpha_C R_a P_\beta N_s H_p$	6	<u>47.1</u>
119	V	3.471 ± 0.237	3.478 ± 0.237	pK'	$\alpha_C R_a P_\beta N_s H_p$	6	<u>53.2</u>
145	I	3.501 ± 0.011	3.5 ± 0.011	pK'	$\alpha_C R_a P_\beta H_p$	5	<u>0</u>
150	N	3.492 ± 0.124	3.494 ± 0.124	pK'	$\alpha_C R_a P_\beta H_p$	5	<u>22.9</u>
156	E	3.501 ± 0.005	3.5 ± 0.005	pK'	$\alpha_C R_a P_\beta H_p$	5	<u>63.9</u>
168	S	3.282 ± 0.706	—	pK'	$\alpha_C R_a P_\beta H_{nc} N_s H_p$	7	<u>21.7</u>
171	M	3.5 ± 0.025	3.5 ± 0.025	pK'	$A_C R_F R_a P_\beta H_{nc} N_s H_p$	8	<u>45.9</u>
172	R	3.501 ± 0.004	3.5 ± 0.004	—	$\alpha_C B_I R_F R_a P_\beta H_{nc} N_s H_p$	8	<u>87.0</u>
174	S	3.499 ± 0.057	3.499 ± 0.057	—	$\alpha_C B_I R_F R_a P_\beta H_{nc} N_s H_p$	8	<u>54.4</u>
186	G	3.386 ± 0.475	3.409 ± 0.475	—	$\alpha_C B_I P_C R_F R_a P_\beta H_{nc} N_s H_p$	9	<u>41.7</u>
202	T	3.402 ± 0.429	3.426 ± 0.429	—	$\alpha_C B_I P_C R_F R_a P_\beta N_s \alpha_m H_p$	9	<u>97.9</u>
203	L	3.501 ± 0.004	3.5 ± 0.004	—	$\alpha_C B_I P_C R_F R_a P_\beta N_s \alpha_m H_p$	9	<u>65.1</u>
204	Y	3.499 ± 0.058	3.499 ± 0.058	—	$\alpha_C B_I P_C R_F R_a P_\beta \alpha_m$	7	<u>33.6</u>
206	E	3.501 ± 0.004	3.5 ± 0.004	—	$\alpha_C B_I R_a P_\beta \alpha_m$	5	<u>40.0</u>
207	Y	3.45 ± 0.308	3.462 ± 0.308	—	$\alpha_C P_\beta \alpha_m$	3	<u>35.3</u>
211	D	3.445 ± 0.322	3.459 ± 0.322	—	$\alpha_C \alpha_m$	2	<u>88.7</u>
212	S	3.499 ± 0.05	3.499 ± 0.05	—	$\alpha_C \alpha_m$	2	<u>55.9</u>
214	V	3.356 ± 0.535	3.383 ± 0.535	—	$\alpha_C \alpha_m$	2	<u>47.6</u>
215	K	3.432 ± 0.36	3.448 ± 0.36	—	$\alpha_C \alpha_m$	2	<u>78.1</u>
217	S	3.501 ± 0.005	3.5 ± 0.005	—	$\alpha_C \alpha_m$	2	<u>45.0</u>
218	S	3.5 ± 0.035	3.5 ± 0.035	—	$\alpha_C$	1	<u>60.0</u>
220	Q	3.46 ± 0.278	3.469 ± 0.278	—	$\alpha_C$	1	<u>95.6</u>
222	E	3.254 ± 0.746	—	—	$\alpha_C$	1	<u>90.8</u>
223	W	3.488 ± 0.148	3.491 ± 0.148	—	$\alpha_C$	1	<u>55.7</u>
224	I	3.481 ± 0.19	3.486 ± 0.19	—	$\alpha_C$	1	<u>4.1</u>
226	S	3.465 ± 0.259	3.473 ± 0.259	—	$\alpha_C$	1	<u>60.2</u>
231	S	3.394 ± 0.445	3.42 ± 0.445	—	$\alpha_C$	1	<u>19.9</u>
235	H	3.501 ± 0.019	3.5 ± 0.019	—	$\alpha_C$	1	<u>84.3</u>
<b>Lizard CRISPs</b>							
18	H	3.634 ± 0.736	2.016 ± 1.129	—	$pH_I \alpha_C$	2	—
65	T	3.678 ± 0.661	3.086 ± 0.581	—	$\alpha_C$	1	<u>59.1</u>
85	T	3.684 ± 0.649	3.089 ± 0.575	pK'	$pH_I \alpha_C$	3	<u>86.3</u>
86	S	3.681 ± 0.657	3.087 ± 0.579	pK'	$c_p H_I \alpha_C$	3	<u>86.1</u>
130	T	3.586 ± 0.806	3.059 ± 0.623	—	$pH_I \alpha_C$	2	<u>91.5</u>
149	T	3.600 ± 0.790	3.060 ± 0.623	—	$\alpha_C$	1	<u>0.0%</u>
158	A	—	3.000 ± 0.703	—	$\alpha_C$	1	<u>80.3</u>
160	R	3.706 ± 0.605	3.096 ± 0.563	—	$\alpha_C$	1	<u>71.9</u>
176	E	3.671 ± 0.676	3.084 ± 0.585	—	$\alpha_C$	1	<u>100.0</u>
181	E	—	3.015 ± 0.680	—	$\alpha_C$	1	<u>33.3</u>
184	A	3.693 ± 0.633	3.091 ± 0.572	—	$\alpha_C$	1	<u>56.5</u>
187	E	—	2.987 ± 0.718	—	$pH_I \alpha_C$	2	<u>87.0</u>
207	H	—	3.017 ± 0.682	—	$pH_I \alpha_C$	2	<u>41.0</u>
210	Q	3.610 ± 0.778	3.060 ± 0.624	—	$pH_I \alpha_C$	2	<u>72.2</u>

NOTE.—Amino acid sites detected by PAML and TreeSAAP as under positive selection along with the  $\omega$  estimation and Bayesian (BEB) analysis PPs for sites with  $P \geq 95\%$  under M2 and M8 models. **TreeSAAP**: Radical changes in amino acid properties (chemical, structural, and other property changes) under category 6 and/or 7 and/or 8. Amino acid sites that belong to the Type II class (greater than 6 property changes) are represented in underlined letters. Amino acid property symbols used: Average number of surrounding residues ( $N_s$ ),  $\beta$ -structure tendencies ( $P_\beta$ ), bulkiness ( $B_I$ ), composition ( $c$ ), chromatographic index ( $R_F$ ), coil tendencies ( $P_c$ ), equilibrium constant for ionization of COOH ( $pK'$ ), isoelectric point ( $pH_I$ ), normalized consensus hydrophobicity ( $H_{nc}$ ), partial specific volume ( $V_0$ ), polar requirement ( $Pr$ ), power to be at C-terminus of the  $\alpha$ -helix ( $\alpha_C$ ), power to be in the middle of an  $\alpha$ -helix ( $\alpha_m$ ), solvent accessible reduction ratio ( $R_a$ ), surrounding hydrophobicity ( $H_p$ ).

**Table 5.** Functional Divergence.

Comparison	Theta ( $\theta$ ) <sup>a</sup>	SE ( $\theta$ )	LRT <sup>b</sup>	P <sup>c</sup>
Lizards versus Elapidae	0.0010	± 0.022361	0	NS
Lizards versus Colubridae	0.1552	± 0.081855	3.594956	NS
Lizards versus Viperidae	<u>0.3336</u>	± <u>0.130631</u>	<u>6.521658</u>	<u>P &lt; 0.05</u>
Colubridae versus Elapidae	<u>0.4080</u>	± <u>0.097791</u>	<u>17.406791</u>	<u>P &lt; 0.001</u>
Colubridae versus Viperidae	<u>0.3112</u>	± <u>0.103415</u>	<u>9.055442</u>	<u>P &lt; 0.01</u>
Elapidae versus Viperidae	<u>0.5048</u>	± <u>0.14939</u>	<u>11.418214</u>	<u>P &lt; 0.001</u>
CRISP1 versus CRISP2	<u>0.5032</u>	<u>0.115574</u>	<u>18.956533</u>	<u>P &lt; 0.001</u>
CRISP1 versus CRISP3	<u>0.7000</u>	<u>0.137197</u>	<u>26.031785</u>	<u>P &lt; 0.001</u>
CRISP2 versus CRISP3	<u>0.8064</u>	<u>0.122422</u>	<u>43.389136</u>	<u>P &lt; 0.001</u>

NOTE.—SE, standard error.

<sup>a</sup> Theta parameter or coefficient of functional divergence.<sup>b</sup> LRT between the alternate model that allows  $\theta > 1$  with the null model that does not.<sup>c</sup> *p*-value; significant values are highlighted in underlined letters.

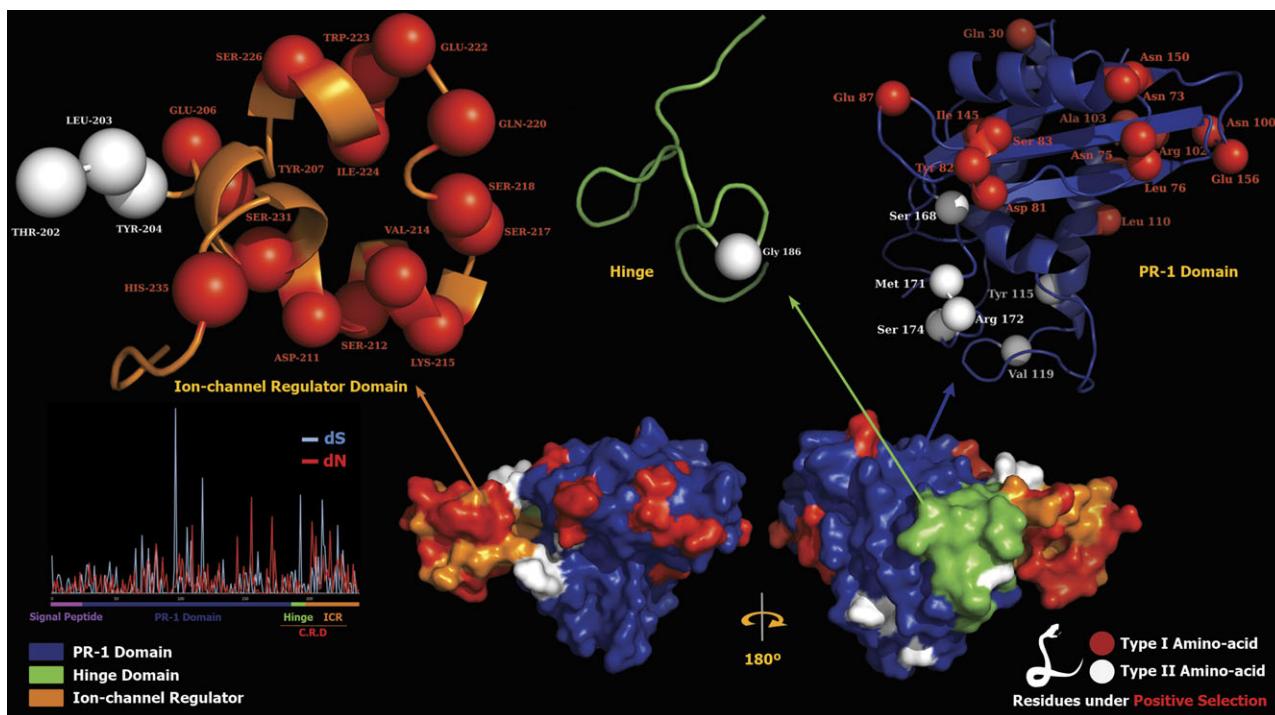
molecular hotspots for mutations which could facilitate the interaction of CRISPs with various ion-gated channels. Variations in PR-1 domain could also be due to the bifunctionality of these proteins with the CRD-mediated ion channel interactions, although PR-1 performs yet to be identified functions.

Venom components like phospholipase A2 (PLA2: belongs to a family of diversely distributed protein phospholipases) in snakes are known to accumulate numerous mutations on the outer surface of the molecule (Kini and Chan 1999). Our analysis reveals that CRISPs exhibit the same pattern (fig. 4). Of the 41 amino acid sites identified to be under positive selection by both PAML and TreeSAAP, 31 (75.6%) were located on the outer surface of the snake CRISP molecule with an ASA ratio of at least 40%, whereas the remaining ten are buried with an ASA

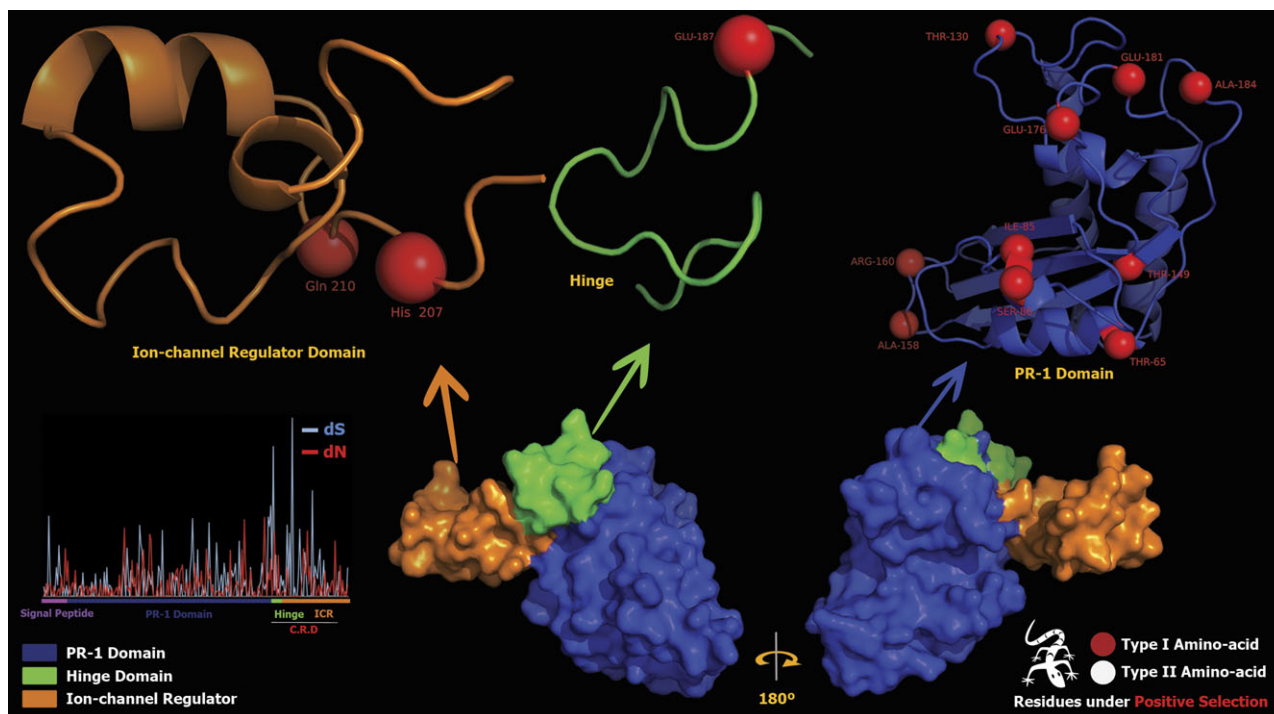
of less than 20%. In the lizard CRISPs, of the 14 amino acids under selection, 11 (78.5%) were exposed, whereas only two were buried. This is likely a general characteristic of venoms, where the molecular surface is diversified, whereas the structurally important residues are constrained. This preserves the venom function (especially if it is an enzyme) while simultaneously diversifying the range of target cells and tissues. This enables predators to more readily adapt to the new ecological niches by being able to rapidly exploit new prey species.

### Ancestral Sequence Analyses

We reconstructed the ancestral toxicoforan-reptilian CRISPs to assess patterns and strength of selection pressures and to detect shifts in evolutionary pressures during the course of evolutionary time (fig. 5). We further mapped the sites under



**Fig. 2.** Snake CRISPs. Three-dimensional model of snake CRISPs depicting the locations of positively selected amino acid sites detected by PAML (models 2 and 8, PP  $\geq 0.95$ ) and TreeSAAP ( $P < 0.001$ ). Type I ( $n \leq 6$  amino acid property changes) and type II amino acid sites ( $n \geq 6$ ) are shown in dark and light colors, respectively (red and white, respectively, in the online version of the manuscript).



**Fig. 3.** Lizard CRISPs. Three-dimensional model of lizard CRISPs depicting the locations of positively selected amino acids detected by PAML (models 2 and 8, PP  $\geq 0.95$ ) and TreeSAAP ( $P \leq 0.001$ ). Type I amino acids ( $n \leq 6$  amino acid property changes) are shown. There were no type II residues ( $n \geq 6$ ) in the lizard CRISPs.

positive selection on the crystal structure of CRISPs to depict their locations and to determine the effect of selection on the structure and function of these proteins. Our analysis reveals that the ancestral CRISPs of both snakes and lizards were influenced less by positive selection than modern forms. Many residues in the extant Viperidae CRISPs appear to show less variation compared with the ancestral forms. Thus, it is evident that these proteins undergo shifts in selection pressures across different time scales.

### Functional Divergence

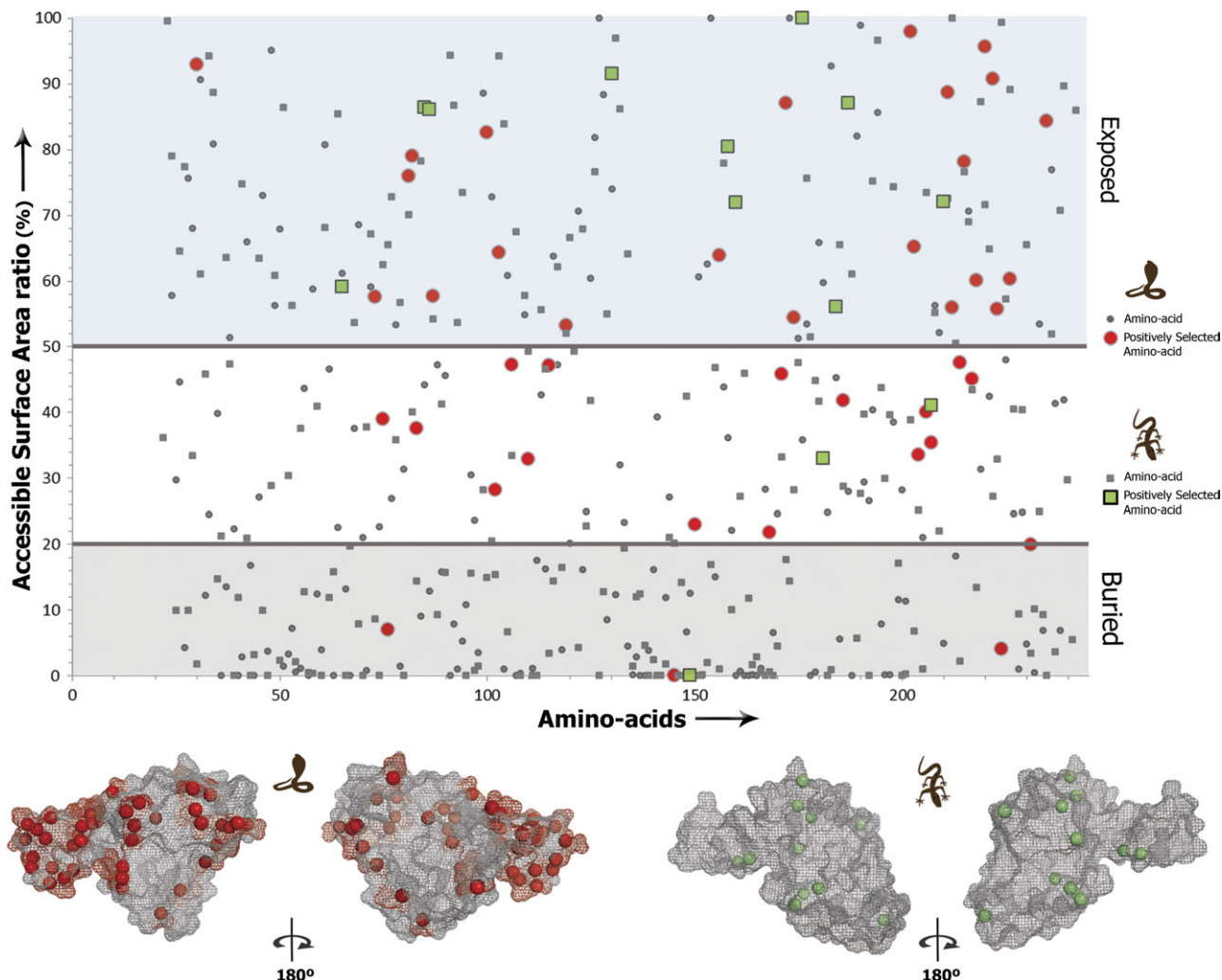
Greater magnitude of negative selection pressure at an amino acid position can imply a functional importance of that amino acid (Kimura 1983). Hence, the site-specific shifts in evolutionary rates of paralogous/orthologous proteins could imply functional divergence. The Gu99 LRT was used to detect if proteins had functionally diverged in different lineages of toxicoforan-reptilian and mammalian CRISPs (table 5). The theta-ML estimation indicates the level of functional divergence between proteins in different clusters of the tree. It also calculates the PP to detect the amino acids responsible for such a divergence. Significance of these comparisons is tested by a LRT between the alternate hypothesis that allows  $\theta$  to be more than zero with the null model that does not. The theta-ML estimate for the comparison between Colubridae and Elapidae ( $\theta = 0.408$ ), Viperidae and Elapidae ( $\theta = 0.5048$ ), Viperidae and lizards ( $\theta = 0.3336$ ), and Colubridae and Viperidae ( $\theta = 0.3112$ ) were all significant. These estimations suggest that there could be drastic differences in the way the CRISPs function in these lineages.

The comparative theta-ML estimates of CRISP1 versus CRISP2 (0.50), CRISP1 versus CRISP3 (0.70), and CRISP2 versus CRISP3 (0.80) indicate shifts in function after the duplication event. CRISP1 and CRISP2 seem to be drastically different from CRISP3 proteins. CRISP1 and CRISP2 (and CRISP4 in mice) are mostly found in mammalian reproductive system, whereas CRISP3 has a wide distribution in the body and is hypothesized to play a role in innate immune response.

### Evolutionary Fingerprint Analyses

Evolutionary fingerprint analysis that fits the general discrete bivariate model of site-to-site variation in selection and detects the number of selective classes, the dN/dS rates for each class, was conducted for reptilian and mammalian CRISPs. The intensities depicted in figure 6A and B correspond to the posterior density, whereas the width of the circle corresponds to the accuracy of estimations (compact circles represent most accurate estimations).

The plots reveal that the majority of amino acids in snake CRISPs have evolved under positive selection. In contrast, in lizards, most were under negative or neutral selection and only a handful were influenced by positive selection (fig. 6A). The lineage-specific plots indicate that in colubrids, the majority of amino acid sites were under positive selection, whereas other sites are under negative selection. In Elapidae, most sites were under either positive or neutral selection, whereas very few sites are evolving under negative selection. Although, many amino acid sites in the Viperidae lineage seem to be under both positive and negative selection, the algorithm could not confidently classify them into



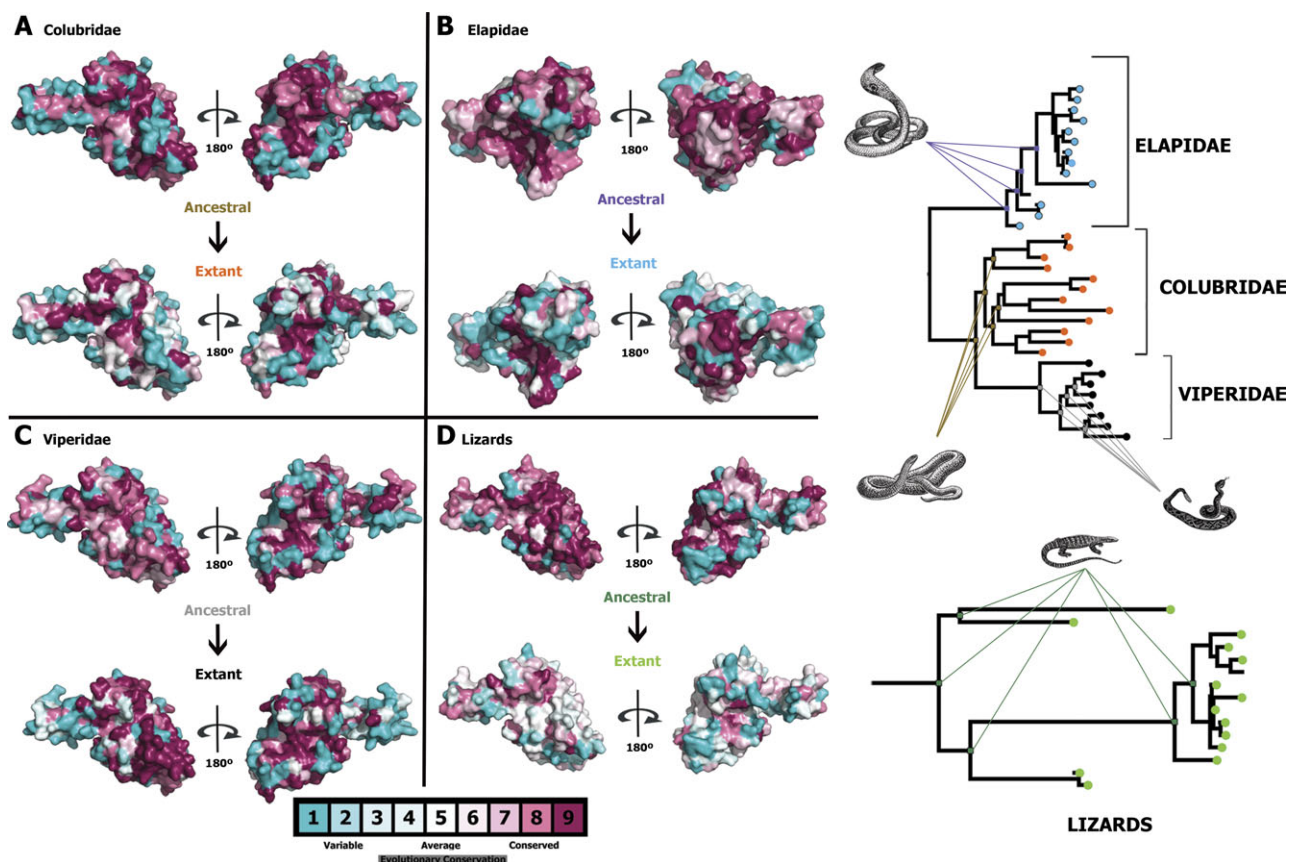
**FIG. 4.** Surface accessibility of CRISPs. A plot of amino acid positions ( $x$  axis) against ASA ratio ( $y$  axis) indicating the positions of amino acids (exposed or buried) in the crystal structure of toxiciferan-reptilian CRISPs is presented. The snake CRISP amino acids are represented as small and large (positively selected) dots, whereas the lizard CRISP amino acids are represented as small and large (positively selected) squares. Amino acids with an ASA ratio of more than 50% are considered to be exposed to the surrounding solvent, whereas those with a ratio lesser than 20% are considered to be buried. Three-dimensional models of snake and lizard CRISPs depicting the locations of positively selected sites are also presented.

these classes (fig. 6A). Almost all the sites were under negative selection in mammalian CRISP1 and CRISP2 proteins, whereas very few sites in CRISP3 were evolving under positive selection. A small proportion of sites in the CRISP2 protein also seemed to be under the influence of positive selection, but the results were inconclusive (fig. 6B).

## Conclusion

Snake venoms appear to have evolved via a strategy of gene recruitment (Fry and Wuster 2004), where, an existing gene is channelized to neofunctionalization by altered gene expression, followed by the accumulation of mutations, gene duplications, and the preservation of functional constraints (Todd et al. 1999; Miyata and Suga 2001). This often results in a multigene family where most proteins preserve their ancestral molecular scaffold while accumulating mutations on the outside of the molecule to amplify their biological targeting.

Reptilian CRISPs became involved in the venom functional pathway through the modification of the ancestral salivary gland CRISP (Fry 2005). The detailed evaluation of selection pressures at both nucleotide and amino acid level by the employment of the site, branch, branch-site, and clade models demonstrate that these modifications were significantly influenced by positive selection in all the lineages of toxiciferan reptiles, and in snakes more than in lizards, both in the number of residues under selection and in the magnitude/strength of the selection (tables 1 and 2). If the principle function of reptilian CRISPs is indeed subduing the prey by targeting various ion channels, then an accelerated accumulation of mutations guided by positive selection would enable them to target new ion channels and would be particularly useful for the reptiles venturing into new ecological niches. This should be distinguished from the arms race that exists between an adapting predator and prey.



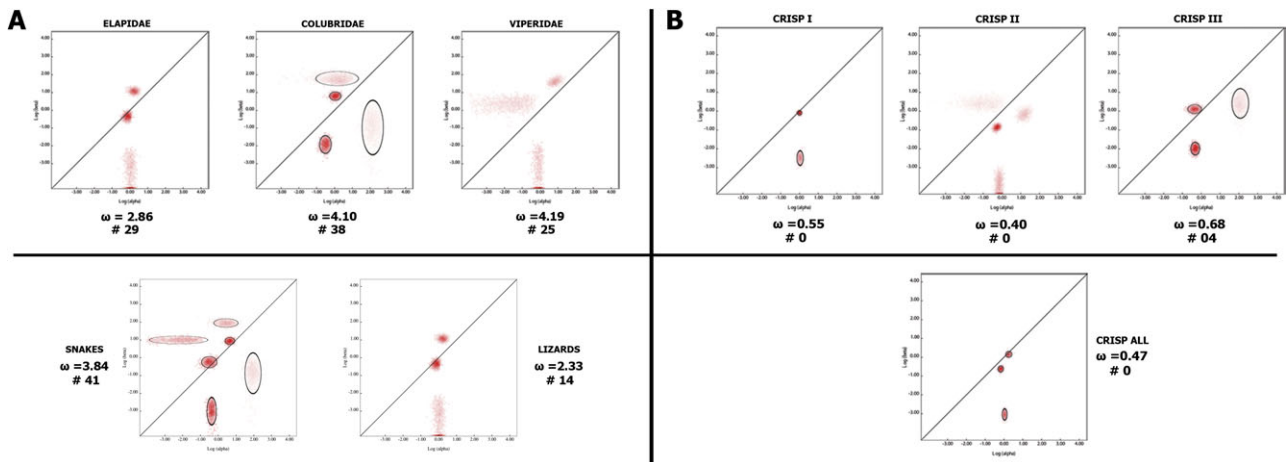
**Fig. 5.** Amino acid variability in ancestral and extant toxicoferan-CRISPs. Three-dimensional models depicting the amino acid variability in ancestral and extant toxicoferan-reptilian CRISPs. Phylogenetic trees showing the nodes that were sampled for creating the three-dimensional models of ancestral toxicoferan CRISPs and for the calculation of selection pressures are shown on the right.

Both snake and lizard CRISPs possess a highly conserved structural scaffold, although most of the mutations occur on the surface and in the functional regions. Although the main function of CRISPs is poorly understood, they possibly participate in the envenomation process by the blockage of various ion channels, such as CNG ion channels, potassium and calcium ion channels, and possible others yet to be discovered. The blockage of  $\text{Ca}^{2+}$  ion channel leads to inhibition of smooth muscle contraction and hypothermia, which could cause dizziness in the prey and thus further subdues the prey. This would be particularly helpful for snakes that ingest their prey whole. The paralysis of prey are vital especially for the front-fanged snakes (Elapidae, Viperidae, and Atractaspididae) as they could easily injure themselves or break fangs if the prey struggles while being swallowed. These snakes possess compressor muscles that squeeze the venom out of the venom gland into the grooves of the front fangs. Vipers are equipped with retractable fangs that can stab and retract in less than a second. Thus, the front-fanged snakes use a combination of venom glands and front fangs to deliver quick and deadly bites. They then wait for the victim to become completely paralyzed or die of envenomation before swallowing them whole. In contrast, rear-fanged colubrids have primitive venom glands with no or only rudimentary compressor muscles. Hence, they have to chew on their soft-skinned

prey to deliver the deadly venom. Sustained compression during biting likely deforms and helps the release of venom from the gland in the absence of well-developed compressor muscles.

Accumulation of variations in CRISP proteins likely facilitates the targeting of new ion channels in the prey, which further subdue the prey. Many elapids rely on other powerful neurotoxins to paralyze the prey, which might explain why selection pressures on elapid CRISPs ( $\omega = 2.86$ ) is not as high as in other lineages (table 1). Colubrids rely only on grip and constriction and hence CRISPs in them, which exploit variations guided by positive selection ( $\omega = 4.10$ ), may further assist these predators in subduing the prey. Most vipers possess a large proportion of haemotoxins as their principle venom component for the destruction of muscles, lymphatic systems, etc. But haemotoxins are generally considered to be less potent than neurotoxins as the latter can completely paralyze the prey in minutes, whereas haemotoxins are relatively slow acting. Perhaps, this is why Viperidae CRISPs ( $\omega = 4.19$ ) exhibit a strong positive selection pressure in comparison with the Elapidae CRISPs (table 1).

Anguimorph lizard CRISPs are the least selected genes among the toxicoferan reptiles ( $\omega = 2.33$ ), perhaps because these species depend mainly on speed and jaw strength to capture the prey rather than prey paralyzes.



**Fig. 6.** Evolutionary fingerprint of reptilian and mammalian CRISPs. Estimates of the distribution of synonymous ( $\alpha$ ) and nonsynonymous ( $\beta$ ) substitution rates inferred for reptilian (A) and mammalian (B) CRISPs. The ellipses reflect a Gaussian approximated variance in each individual rate estimate, and colored pixels show the density of the posterior sample of the distribution for a given rate. The diagonal line represents the idealized neutral evolution regime ( $\omega = 1$ ) and points above and below the line correspond to positive selection ( $\omega > 1$ ) and negative selection ( $\omega < 1$ ), respectively.

Moreover, it has been shown that CRISPs form the major portion of colubrid and Anguimorph lizard venom (Fry, Wuster, Ryan Ramjan, et al. 2003), further demonstrating the need for functional analyses of CRISPs in reptiles as they likely have multiple functions, similar to other snake venoms, such as the three-finger toxins (Fry, Wüster, Kini, et al. 2003).

The trends observed in our sequence analyses should also be studied in the future at population level to determine the importance of intraspecific and individual diversity. These predators do not rely on a single venom type for killing and subduing their prey. Instead, a cocktail of different venom types is employed where one component may successfully bring down a specific type of prey, whereas the same could be completely ineffective against the other. Moreover, the type of venom employed is not always consistent along lineages. Many vipers like the South American rattlesnake (*Crotalus durissus*) employ neurotoxins (in contrast to the typical haemotoxins employed by most vipers), whereas some elapid venoms like that of a red-bellied black snake (*Pseudechis porphyriacus*) are chiefly composed of haemotoxins (rather than the usual elapid neurotoxins). Some colubrids like the brown tree-snake (*Boiga irregularis*) employ neurotoxins, whereas others like the Boomslang (*Dispholidus typus*) use deadly haemotoxins.

The presently known forms of mammalian CRISPs resulted from mammalian lineage-specific duplication events. New copies of genes are relieved of any negative selection pressures after the duplication event and begin accumulating variations. In contrast, mammalian CRISP duplicates remained under negative selection pressures (CRISP1 = 0.55, CRISP2 = 0.40, and CRISP3 = 0.68) owing to the likely vital functional roles they play in the maintenance of homeostasis. Nonetheless, the branch-site model A, clade model analyses, FEL and REL tests, and the evolutionary fingerprint analyses detect a small number of sites under selection in CRISP3 proteins (table 3, fig. 6B, and supplementary material,

Supplementary Material online). CRISP3 is hypothesized to be associated with the innate immune response (Pfisterer et al. 1996; Haendler et al. 1997) and hence is likely to be benefited by such variation.

Reptilian CRISPs might derive their ability to interact with various ion channels by the virtue of their CRD, where almost half the amount of mutations ( $\sim 46\%$ ) of the whole protein occurs. As suggested previously (Suzuki et al. 2008), the pathogenesis-related group 1 (PR-1) domain might supplement this ability. This domain accumulates the rest of the positively selected sites. However, the possibility of the multifunctionality of these proteins cannot be rejected, with CRD and the PR-1 domains performing different functions. Such directional mutagenesis of venom where molecular surface and functional regions act as hotspots for mutations should have diversified the ability of CRISPs to target numerous cell and/or tissue types in the prey. The amino acid diversification influenced by a strong positive selection on these proteins suggests that they are one of the most indispensable components of reptilian venom. In contrast, the significant negative selection pressures observed in mammalian CRISPs highlights the important roles they play in mammalian systems.

## Supplementary Materials

Supplementary material is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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