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Evolution of an Ancient Venom: Recognition of a Novel Family of Cnidarian Toxins and the Common Evolutionary Origin of Sodium and Potassium Neurotoxins in Sea Anemone

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Abstract

Despite Cnidaria (sea anemones, corals, jellyfish, and hydroids) being the oldest venomous animal lineage, structure–function relationships, phyletic distributions, and the molecular evolutionary regimes of toxins encoded by these intriguing animals are poorly understood. Hence, we have comprehensively elucidated the phylogenetic and molecular evolutionary histories of pharmacologically characterized cnidarian toxin families, including peptide neurotoxins (voltage-gated Na^+ and K^+ channel-targeting toxins: NaTxs and KTxs, respectively), pore-forming toxins (actinoporins, aerolysin-related toxins, and jellyfish toxins), and the newly discovered small cysteine-rich peptides (SCRiPs). We show that despite long evolutionary histories, most cnidarian toxins remain conserved under the strong influence of negative selection—a finding that is in striking contrast to the rapid evolution of toxin families in evolutionarily younger lineages, such as cone snails and advanced snakes. In contrast to the previous suggestions that implicated SCRiPs in the biomineralization process in corals, we demonstrate that they are potent neurotoxins that are likely involved in the envenoming function, and thus represent the first family of neurotoxins from corals. We also demonstrate the common evolutionary origin of type III KTxs and NaTxs in sea anemones. We show that type III KTxs have evolved from NaTxs under the regime of positive selection, and likely represent a unique evolutionary innovation of the Actinioidea lineage. We report a correlation between the accumulation of episodically adaptive sites and the emergence of novel pharmacological activities in this rapidly evolving neurotoxic clade.

Key words: **Cnidaria, sodium channel toxins, potassium channel toxins, phylogeny, disulfide-rich toxins, positive selection.**

Introduction

Venoms are among nature's most complex cocktails that are characterized by a diversity of molecules, such as large proteins, small peptides, polyamines, and salts, which disrupt the physiology of prey animals upon injection (Mebs 2002). Several gene families encoding venom components have been subjected to extensive duplication and have evolved under the influence of positive (diversifying) selection (Chang and Duda 2012; Casewell et al. 2013). The evolution of venom has been extensively studied in several venomous lineages that are of relatively younger evolutionary origin, such as advanced snakes (originated ~54 Ma) (Vidal et al. 2009) and cone snails (originated ~33–50 Ma) (Olivera 1997; Duda and Kohn 2005). However, to date, the evolution and diversification of ancient venom systems, such as those of cnidarians, remains understudied.

The phylum Cnidaria (sea anemones, corals, jellyfish, and hydroids) is composed of diploblastic predatory animals (Ruppert et al. 2004), where all members are venomous (Turk and Kem 2009). Cnidarians are typified by the unique venom delivery apparatus called nematocyst (stinging organelle), which they employ to inject a cocktail of toxins into animals for predation and defense (Kass-Simon and Scappaticci 2002; Ruppert et al. 2004; David et al. 2008). Molecular and fossil data place the origin of cnidarians in the Ediacaran Period, ~600 Ma (Erwin et al. 2011; Park et al. 2012; Menon et al. 2013), making them the oldest lineage of venomous animals and an ideal phylum to understand the origin and diversification of venom. Because certain cnidarians serve pivotal ecological roles (e.g., reef-building corals) and are important model organisms in the field of evolutionary developmental biology (sea anemones and hydroids),

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genomic and transcriptomic data for several cnidarian species are rapidly accumulating in recent times (Putnam et al. 2007; Chapman et al. 2010; Shinzato et al. 2011; Steele et al. 2011; Technau and Steele 2011). Moreover, certain cnidarian animals, such as box jellyfishes (Cubozoa), produce some of the world's most lethal venoms (Tibballs et al. 2011).

In this study, we have assessed the phylogenetic histories and the molecular evolutionary regimes of a diverse range of cnidarian toxin families, including 1) sodium channel modulators (NaTx: type I and type II families) (Moran et al. 2009; Wanke et al. 2009); 2) potassium channel toxins (KTx: type I and type III families) (Castaneda and Harvey 2009); 3) aerolysin-related pore-forming toxins (PFTs) (Sher et al. 2005; Moran, Fredman, et al. 2012); 4) actinoporin PFTs (Anderluh and Macek 2002); 5) the jellyfish toxins (JFTs) (Brinkman et al. 2014); and 6) the newly discovered family of small cysteine-rich peptides (SCRiPs), which have been previously implicated in biomineralization in corals (Sunagawa et al. 2009). Our results unravel fascinating insights into the evolutionary origin and diversification of venom in this ancient clade of animals.

Results

Cnidarians secrete a diversity of toxin types to facilitate predation and defense. Here, we present the phylogenetic histories and the molecular evolutionary regimes of eight pharmacologically characterized cnidarian toxin types.

Neurotoxins

Neurotoxins rapidly immobilize prey animals and play an extremely important role in the venoms of certain cnidarians. They disrupt ion conductance through the modification or blocking of the voltage-gated sodium (Na_v) and potassium (K_v) ion channels: NaTx and KTx, respectively. Among cnidarians, neurotoxins have only been retrieved from the venoms of sea anemones (class: Actiniaria). NaTxs are one of the best characterized cnidarian toxins (Beress et al. 1982; Moran et al. 2009; Wanke et al. 2009) and are classified into three types (type I–III) based on the cysteine arrangement, overall structure, amino acid composition, and immunological cross-reactivity (Moran et al. 2007, 2009; Smith and Blumenthal 2007). The distinction between type I and type II NaTxs has been artificial, considering the similarity they share in structure and function. However, whether they resolve into distinct phylogenetic clades remains to be investigated. Hence, we have analyzed type I and type II NaTxs together. Based on sequence identity, sequence length, number of disulfide bridges, and binding affinities toward various Kv ion channels (Honma and Shiomi 2006; Honma et al. 2008; Castaneda and Harvey 2009), sea anemone KTxs have been classified into five types: type I–V (Orts et al. 2013). Because type II KTxs cannot be distinguished from their physiological homologs (nontoxic body proteins) in the absence of biochemical characterization (Minagawa et al. 2008), and because not enough nucleotides of type IV and V KTxs have been sequenced to date, we have only analyzed KTxs of type I and type III families. It should be noted that the five KTx types are not homologous (Orts et al. 2013).

We investigated the nature of natural selection influencing the evolution of genes encoding various cnidarian toxin families using maximum-likelihood models (Yang 1998) implemented in PAML (see Methods). The site model 8 (M8) computed omega (ω) of 0.58 and 0.66 for NaTxs and type I KTxs, respectively, indicating a strong influence of negative selection on these toxin types, while highlighting the influence of positive selection on type III KTxs ($\omega = 1.33$) (fig. 1 and table 1). The Bayes Empirical Bayes (BEB) approach implemented in M8 failed to identify positively selected sites in NaTx. However, M8 identified one and six positively selected sites in type I and type III KTxs, respectively (figs. 1 and 2, table 1, and supplementary tables S1.1–S1.3, Supplementary Material online). The mixed effects model of evolution (MEME) identified as many as eight sites evolving under the influence of episodic diversifying selection in NaTxs, while identifying three and five sites in type I and type III KTxs, respectively (fig. 2 and table 1). These results indicate that certain regions in these neurotoxins have evolved rapidly under the episodic influence of adaptive selection.

To understand the surface accessibility of positively selected and episodically diversifying sites, we computed the accessible surface area (ASA) ratio for all sites in the homology models of these neurotoxins. Our analyses revealed that all four episodically diversifying sites, with an ASA ratio in the range of 60–100 (ASA $\geq$ 50 is characteristic of surface-exposed sites) in the secreted region of NaTxs, were surface exposed (supplementary table S2, Supplementary Material online). Six of the 8 positively selected and episodically diversifying sites in the mature sequence of type III KTxs had an ASA ratio in the range of 51–100, while only a single site was found to be buried (ASA $\leq$ 20 indicates buried residues). The remaining site (ASA = 35) in this toxin type could not be assigned to buried/exposed category. Similarly, the single positively selected site in KTx type I was surface exposed (ASA = 100), while the lone diversifying site in the mature region of this toxin had its side chain exposed (ASA = 47; ASA value ranging between 40 and 50 is indicative of residues with surface accessible side chains).

Several previously published KTx type III toxins were known to have a similar cysteine framework and activity as that of the sea anemone NaTxs (Diochot et al. 2004; van Vlijmen et al. 2004; Zaharenko et al. 2008; Peigneur et al. 2012). Hence, we analyzed them together in this study to explore the possibility of a common evolutionary origin of the two toxin types. Midpoint rooted nucleotide Bayesian and maximum-likelihood phylogenetic trees placed type III KTxs in the same clade as type I NaTxs (Bayesian posterior probability [PP] 0.997; bootstrap support 734/1,000), suggesting a common evolutionary origin of these two toxin types (fig. 3 and supplementary fig. S1, Supplementary Material online). Our phylogenetic analyses further resolved NaTxs into four distinct clades: 1) Type I NaTx (polyphyletic clades); 2) type II NaTxs; 3) the orphan NaTx clade (a unique clade of NaTxs that does not belong to either type I or type II NaTxs); and 4) a clade of Nv1 toxins from

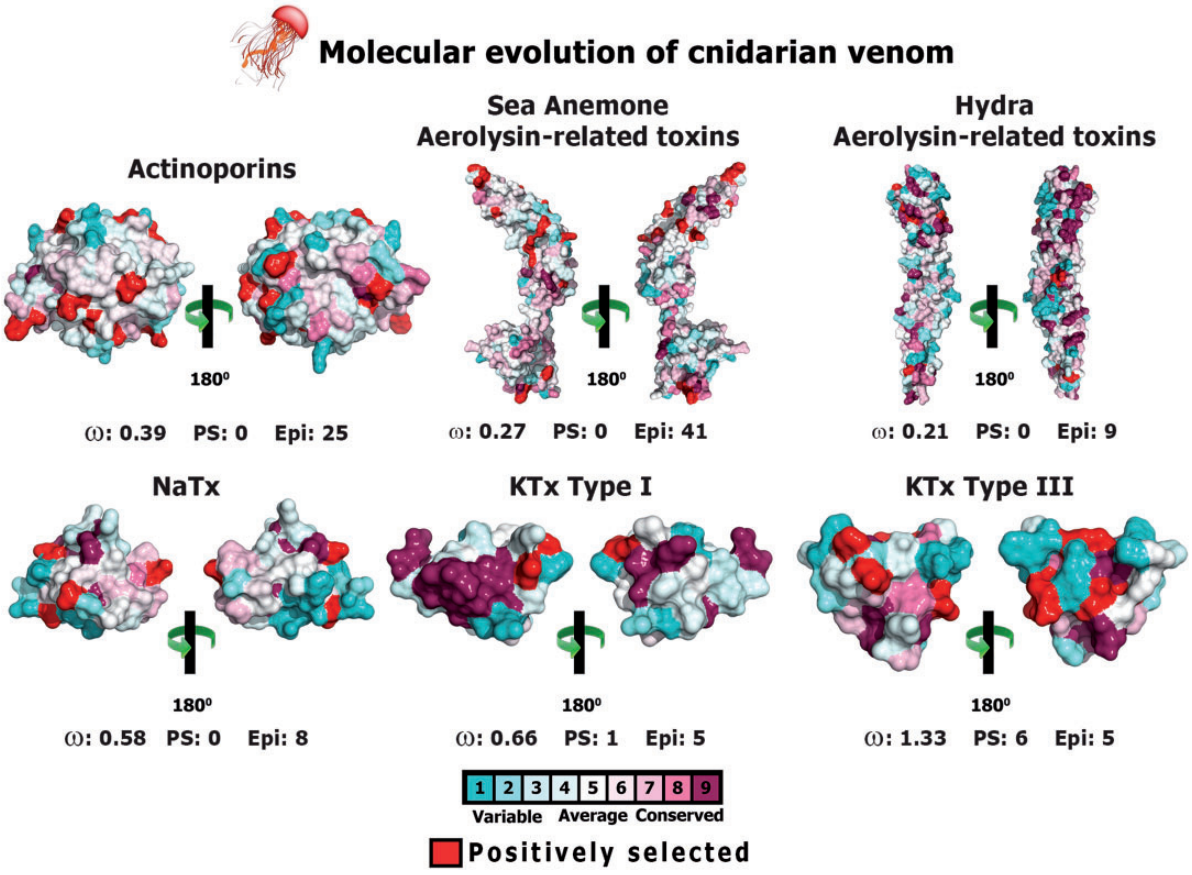

Fig. 1. Molecular evolution of cnidarian toxins. Three-dimensional models of various cnidarian toxins with their molecular surface colored according to the evolutionary conservation of amino acids are presented here (see color code). Positively selected/episodically adaptive sites are depicted in red. Omega values, the number of sites detected as positively selected (Model 8, PP $\geq$ 0.95, Bayes-Empirical Bayes approach) and episodically adaptive sites (MEME) are also provided for the respective toxin class. PDB codes: Actinoporin: 1IAZ; sea anemone aerolysin: 3C0N; *Hydra* aerolysin: 2ZTB; NaTx: 2SH1; KTx type I: 1ROO; and KTx type III: 1BDS.

Nematostella vectensis (fig. 3). Rooting the tree with toxin sequences from *N. vectensis* or *Halcurias carlgreni*, two basal sea anemone lineages (for sea anemone species phylogeny [Rodriguez et al. 2014], see supplementary fig. S2, Supplementary Material online), did not change the topology of the tree and supported the common origin of NaTx and KTx type III (supplementary fig. S1, Supplementary Material online). Because neurotoxins from *N. vectensis* (Nv1) and *H. carlgreni* (Halcurin) are characterized by domains similar to both type I and type II NaTxs, they have been previously suspected to be among the most basal sea anemone neurotoxins (Ishida et al. 1997; Moran and Gurevitz 2006). Because phylogenetic analyses in this study resolved type I and type II NaTxs into distinct clades, we computed omega values for each of these clades independently. These analyses suggested that both type I and type II NaTxs have evolved under the influence of negative selection (supplementary tables S3.1 and S3.2, Supplementary Material online).

Pore-Forming Toxins

Actinoporins

Actinoporins are arguably the best studied PFTs secreted by Cnidaria (Anderluh and Macek 2002). They typically interact with their target sphingomyelin membrane (SM) to form oligomeric pores, which result in osmotic imbalance and cell death (Garcia-Ortega et al. 2011). It was previously shown that the presence of the aromatic cluster, an array of basic residues, the phosphocholine (POC) binding site, and the N-terminal region containing an amphipathic α-helix are essential for the recognition and binding to the SM membrane and the cytolytic activity of actinoporins (Gutierrez-Aguirre et al. 2004; Kristan et al. 2009; Garcia-Ortega et al. 2011). Mapping the results of evolutionary selection analyses on the sequence alignment of actinoporins revealed the greater influence of negative selection on regions responsible for this cytolytic activity. Most sites implicated in pore-forming activity and the structural stability were found to have evolved under the influence of negative selection (fig. 4). The Fast, Unconstrained Bayesian AppRoximation (FUBAR) analysis, which detects sites evolving via pervasive diversifying and purifying selection, identified 7/9 POC binding sites, 4/7 sites in the array of basic residues, 6/8 sites in the array of aromatic residues, and 3/22 residues of α-helix as constrained by pervasive negative selection pressure (PP $\geq$ 0.90) (table 2). M8 computed ω of 0.39 for actinoporins, indicating a strong influence of negative selection on this toxin type, while the BEB approach implemented in this model failed to identify

Table 1. Molecular Evolution of Cnidarian Neurotoxins.

	FUBAR[a]	MEME Sites[b]	PAML[c] (M8)
Type I and Type II NaTx	$\omega>1^d$: 0 $\omega<1^e$: 25	8	0
			0.58
Type I KTx	$\omega>1^d$: 1 $\omega<1^e$: 16	5	1
			(0+1)
			0.66
Type III KTx	$\omega>1^d$: 3 $\omega<1^e$: 15	5	6
			(2+4)
			1.33

NOTE.—ω = mean dN/dS.

[a]Fast Unconstrained Bayesian AppRoximation.

[b] Sites detected as experiencing episodic diversifying selection (0.05 significance) by the MixedEffects Model Evolution (MEME).

[c]Positively selected sites detected by the Bayes Empirical Bayes approach implemented in M8. Sites detected at 0.99 and 0.95 significance are indicated in the parenthesis.

[d] Number of sites under pervasive diversifying selection at the posterior probability $\geq$0.9 (FUBAR).

[e] mber of sites under pervasive purifying selection at the posterior probability $\geq$0.9 (FUBAR).

positively selected sites (table 2). However, the MEME identified as many as 25 sites in actinoporins as evolving under the influence of episodic diversifying selection (figs. 1 and 4, table 2, and supplementary table S4.1, Supplementary Material online).

Aerolysin-Related Toxins in Sea Anemones and Hydroids

Aerolysin-related toxins are nonnematocystic paralytic toxins with β-pore-forming activity, secreted from the nonnematocystic digestive cells of various sea anemones and hydra species (hydralysins) (Sher et al. 2005; Mancheno et al. 2010; Moran, Fredman, et al. 2012). Because hydralysins were found to be secreted by *Hydra viridissima* during feeding, they have been theorized to play a role in the digestion of prey animals (e.g., crustaceans) (Sher et al. 2008). Hence, it has also been hypothesized that aerolysin-related toxin homologs in sea anemones, which are secreted by the ectodermal cells of the pharynx, play a similar role (Moran, Fredman, et al. 2012). Interestingly, hydralysins and the sea anemone aerolysin-related toxins have been suggested to originate from two independent recruitment events that involved horizontal gene transfers (Moran, Fredman, et al. 2012).

Evolutionary assessments revealed that aerolysin-related toxins in both sea anemones and *Hydra* have evolved under the extreme influence of negative selection (ω of 0.27 and 0.21, respectively) (table 2 and supplementary tables S4.2 and S4.3, Supplementary Material online). FUBAR revealed numerous amino acid sites in these toxin types that evolved under the pervasive influence of negative selection (300 and 120 sites, respectively). Although MEME detected only 9 sites as episodically adaptive in hydralysins, as many as 41 sites were detected in aerolysin-related homologs of sea anemones (figs. 1 and 5 and table 2).

Jellyfish Toxins

JFTs are among the most dangerous toxins secreted by jellyfish and are known for a diversity of immunological and toxicological activities, including hemolyticity, cardiotoxicity, cutaneous inflammation, and necrotic activities. It has been proposed that their pore-forming activity is responsible for toxicity associated with human envenomations (Tibballs et al. 2011). Originally, JFTs were reported to be limited to Cubozoa and Schyphozoa. However, homologs of JFTs were recently reported in *H. magnipapillata* (Brinkman et al. 2014). Through BLAST searches, we have retrieved such homologs from various sea anemones (*Aiptasia pallida*) and various hydroids (*Hydractinia symbiolongicarpus* and *H. vulgaris*), suggesting that JFTs originated within the common ancestor of all extant cnidarians more than 600 Ma. ω of 0.39 was computed by M8 for JFTs, highlighting the significant influence of negative selection on this toxin type (table 2, supplementary fig. S3 and supplementary table S4.4, Supplementary Material online). Although BEB failed to detect any site in this toxin as positively selected, MEME identified as many as 17 sites that evolved under episodic bursts of positive selection.

Small Cysteine-Rich Peptides

SCRiPs were originally identified as genes unique to reef-building corals (Scleractinia) that are downregulated during heat stress (Sunagawa et al. 2009). Given the similarity in the temporal expression pattern they share with galaxin, a key protein involved in the biomineralization process (Fukuda et al. 2003), SCRiPs were implicated in calcification of the coral skeleton (Sunagawa et al. 2009). However, a greater sequence diversity in SCRiPs (fig. 6) is uncharacteristic of homeostatically important proteins, such as those involved in the biomineralization process. Previously, a β-defensin domain was reported in Mfav-SCRiP1 (Sunagawa et al. 2009) and Interpro (Hunter et al. 2012) scanning performed in our study detected a domain that is very similar to the basic myotoxic domains of rattlesnake crotamine toxins (Interpro entry: IPR000881). Moreover, like SCRiPs, several peptide toxins in sea anemones have also been shown to exhibit downregulation in expression as a result of thermal stress (Richier et al. 2008). Considering these observations, we suspected that SCRiPs may represent a class of toxins and not calcifying proteins as claimed by earlier studies. BLAST searches in this study surprisingly retrieved SCRiP homologs in sea anemones, *Anemonia viridis* and *Metridium senile* (fig. 6), refuting the claim that these peptides are specific to scleractinian corals (Sunagawa et al. 2009). To gain further understanding regarding the bioactivity of SCRiPs, we expressed Amil-SCRiP1, Amil-SCRiP2, and Amil-SCRiP3 from the scleractinian coral *Acropora millepora* in a recombinant form (see Methods). Although the expression of Amil-SCRiP1 was unsuccessful, Amil-SCRiP2 and Amil-SCRiP3 were

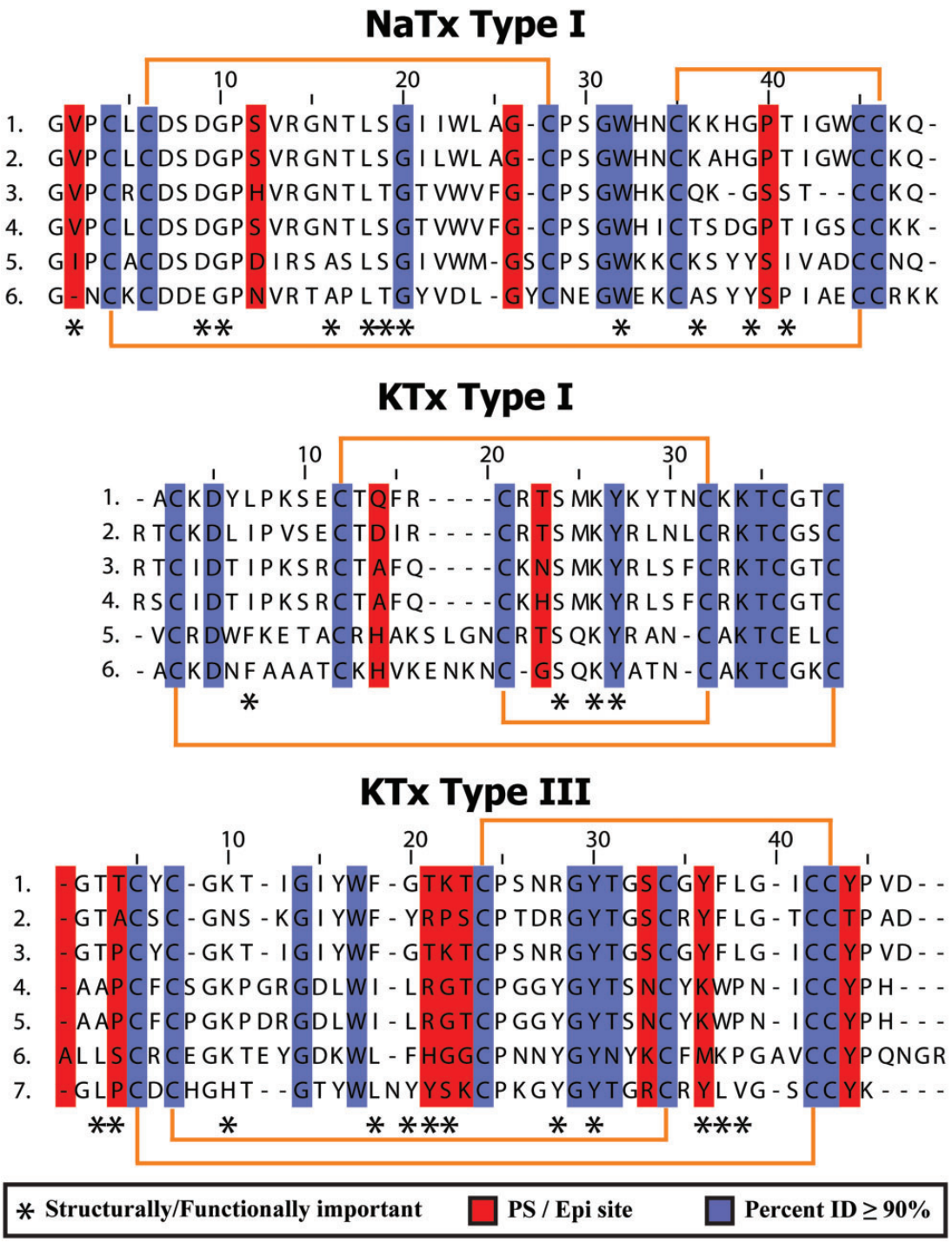

Fig. 2. Sequence alignment of NaTx, KTx type I, and KTx type III. Positively selected/episodically adaptive (PS/Epi), structurally/functionally important, and extremely well-conserved (percent ID ≥90%) sites have been depicted. Uniprot accession numbers and pharmacological names of sequences are as follows. NaTx: 1) P01528 *Anemonia viridis* (Av2); 2) P01532 *Anthopleura elegantissima* (ApC); 3) Q76CA3 *Stichodactyla gigantean* (Gigt II); 4) P86459 *Bunodosoma cangicum* (Bcg1a); 5) B1NWS1 *Nematostella vectensis* (Nv1); and 6) P30785 *Heteractis crispa* (RTX-V). KTx type I: 1) Q0EAE5 *Anemonia erythraea* (Aer1a); 2) O16846 *Heteractis magnifica* (Hm1a); 3) E2S064 *Cryptodendrum adhaesivum* (Sm1a); 4) P29187 *Stichodactyla helianthus* (She1a); 5) P29186 *Bunodosoma granuliferum* (BcsTx1); and 6) Q9TWG1 *A. viridis* (AsKS). KTx type III: 1) P61541 *A. elegantissima* (APETx1); 2) P61542 *A. elegantissima* (APETx2); 3) B3EWF9 *A. elegantissima* (APETx3); 4) P11494 *A. viridis* (BDS-1); 5) P59084 *A. viridis* (BDS-2); 6) P69930 *Antheopsis maculata* (Am-2); and 7) P84919 *B. caissarum* (BcIV).

expressed in relatively large amounts (1.5 mg protein per 1 l of bacterial culture). This enabled us to test the notion that the two peptides are toxic. Interestingly, the injection of these recombinantly expressed SCRiPs in blowfly larvae (*Sarcophaga falculata*) did not result in toxicity, while the incubation of zebrafish (*Danio rerio*) larvae at a concentration of 230 μg/ml resulted in severe neurotoxicity. In the first few minutes of incubation with SCRiPs, the fish exhibited

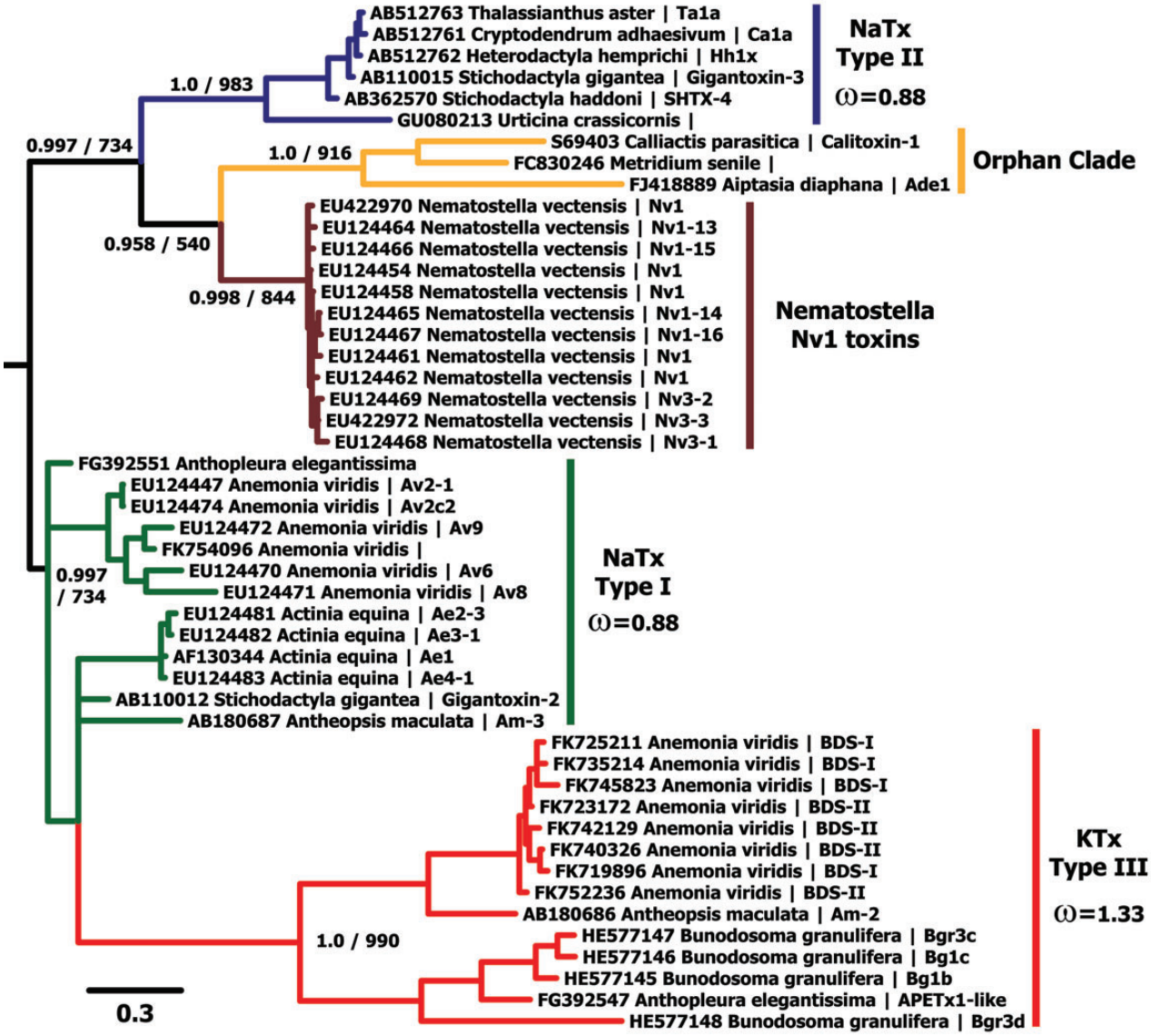

FIG. 3. The common origin of NaTx and KTx type III. The phylogenetic tree of NaTx and KTx type III built using nucleotide sequences is presented here. Node support (Bayesian posterior probability/bootstrap replicate support) for the major clades and clade-specific omega values are also provided.

abnormal tail twitching. In contrast to the control group incubated with bovine serum albumin, fish incubated with SCRiPs gradually exhibited frequent twitching and shivering, stopped reacting to touch, and eventually became completely paralyzed. All fish incubated with Amil-SCRiP2 died within 200 min of exposure, while Amil-Scrip3 killed fish within 16 h. These results support the notion that SCRiPs may serve as neurotoxins, making them the first peptide neurotoxin family described from scleractinian corals. M8 computed ω of 0.72 and the BEB approach failed to identify positively selected sites in SCRiPs (table 2, fig. 6, and supplementary table S4.5, Supplementary Material online). Thus, selection assessments indicated that coral SCRiPs have evolved under the influence of negative selection.

Discussion

Strong Negative Selection Governs the Evolution of Most Cnidarian Toxins

Molecular evolutionary assessments of cnidarian toxin families using the state-of-the-art methods revealed the extreme conservation of toxin-encoding sequences, despite their long evolutionary histories (figs. 1–6, tables 1 and 2, and supplementary fig. S3 and supplementary tables S1.1–S1.3, S3.1 and S3.2, S4.1–S4.5, Supplementary Material online). The computed ω values ranged between 0.21 and 0.72, indicating a strong influence of negative selection on a majority of sites in these proteins. This is in stark contrast to most predatory animal venoms that are known to rapidly evolve under the influence of positive selection (Lynch 2007; Casewell et al.

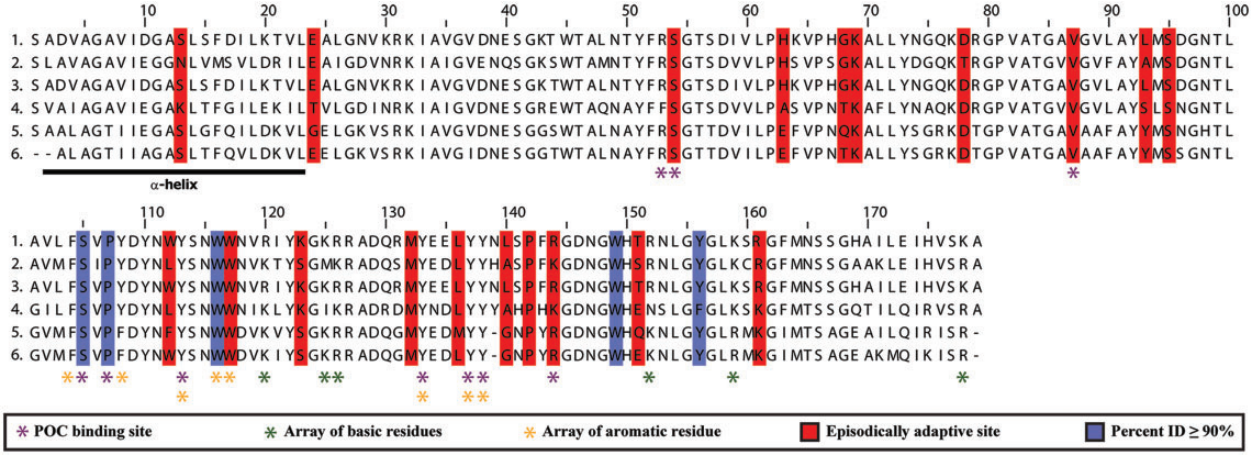

FIG. 4. Sequence alignment of actinoporins. Episodically adaptive, structurally/functionally important, and extremely well-conserved (percent ID ≥90%) sites have been depicted. Uniprot accession numbers and pharmacological names of sequences are as follows: 1) P61914 *Actinia equina* (EqTII); 2) C5NSL2 *Anthopleura asiatica* (bp-1); 3) P61915 *Actinia tenebrosa* (Tenebrosin-C); 4) C9EIC7 *Urticina crassicornis* (Urticinatoxin); 5) Q9U6X1 *Heteractis magnifica* (HMg III); and 6) P07845 *Stichodactyla helianthus* (StnII).

Table 2. Molecular Evolution of Cnidarian Pore-Forming Toxins and SCRiPs.

	FUBAR[a]	MEME Sites[b]	PAML[c] (M8)
Actinoporins	$\omega>1^{d}$: 0 $\omega<1^{e}$: 121	25	0 0.39
Sea Anemone Aerolysins	$\omega>1^{d}$: 0 $\omega<1^{e}$: 300	41	0 0.27
Hydralysins	$\omega>1^{d}$: 0 $\omega<1^{e}$: 120	9	0 0.21
JFTs	$\omega>1^{d}$: 0 $\omega<1^{e}$: 287	17	0 0.39
Coral SCRIPs	$\omega>1^{e}$: 0 $\omega<1^{e}$: 18	7	0 0.72

NOTE.—ω = mean dN/dS.

[a]Fast Unconstrained Bayesian AppRoximation.

[b]Sites detected as experiencing episodic diversifying selection (0.05 significance) by the MixedEffects Model Evolution (MEME).

[c] Positively selected sites detected by the Bayes Empirical Bayes approach implemented in M8. Sites detected at 0.99 and 0.95 significance are indicated in the parenthesis.

[d] Number of sites under pervasive diversifying selection at the posterior probability ≥0.9 (FUBAR).

[e]Number of sites under pervasive purifying selection at the posterior probability ≥0.9 (FUBAR).

2011, 2012; Sunagar et al. 2012, 2014; Brust et al. 2013; Sunagar, Jackson, et al. 2013; Dutertre et al. 2014). Among all the cnidarian toxins examined in this study, only the type III KTxs seem to have evolved rapidly ($\omega = 1.33$) under the influence of positive Darwinian selection. When the majority of lineages or sites evolve under the strong influence of negative selection, it becomes difficult to identify adaptive selection that occurs in short bursts and influences a small proportion of lineages or sites (Murrell et al. 2012). The application of MEME detected several sites (ranged from 5 to 41) in most toxin types that experienced episodic bursts of adaptive selection (tables 1 and 2). Interestingly, a similar phenomenon was also reported in scorpions (Sunagar, Undheim, et al. 2013), which represent another ancient venomous animal lineage that originated nearly 400 Ma (Dunlop and Selden 2009). This reinforces the dynamic nature of venom in ancient lineages, where variations in venom-encoding genes accumulate episodically, possibly under an evolutionary chemical arm's race scenario with their target sites in prey animals. When toxin sequences that increase the potency and efficacy of envenoming are generated, they get fixed in the population and experience purifying selection for long periods of time. In contrast, in evolutionarily younger lineages, such as cone snails and advanced snakes, the rapid evolution of genes under the positive Darwinian selection is much more pronounced.

Most Adaptive Sites in Cnidarian Neurotoxins Are Surface Accessible

It has been suggested that most venom proteins that are involved in predation evolve through *r*apid *a*ccumulation of *v*ariation in the *e*xposed *r*esidues (RAVER), where the molecular surface of the toxin accumulates bulk of the variations under the significant influence of positive Darwinian selection, while preserving the core residues involved in stability and/or catalytic activity (Sunagar, Jackson, et al. 2013). As synthesis and secretion of proteins is an energetically expensive process, over time, mutations that lead to the loss of

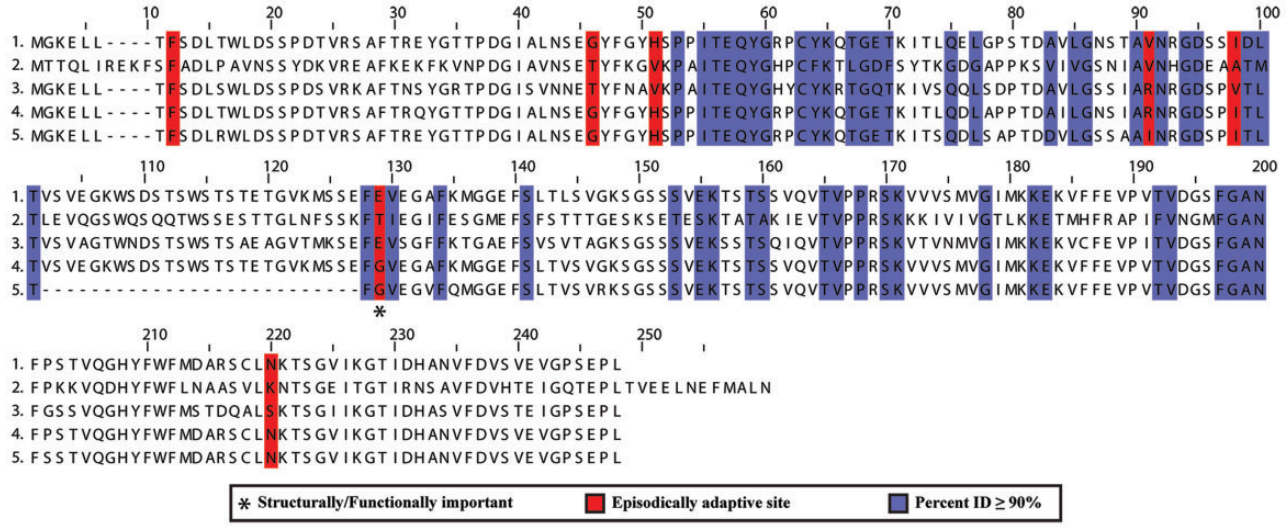

FIG. 5. Sequence alignment of hydralysins. Episodically adaptive, structurally/functionally important, and extremely well-conserved (percent ID ≥90%) sites have been depicted here. Uniprot accession numbers and pharmacological names of sequences are as follows: 1) Q86LR2 *Hydra viridissima* (Hln-1); 2) X2GKY3 *Lysinibacillus* sp. (Hln-2); 3) Q52SK7 *Hydra vulgaris* (Hln); 4) Q564A5 *H. viridissima* (Hln-2); and 5) Q52SK6 *H. viridissima* (Hln-3).

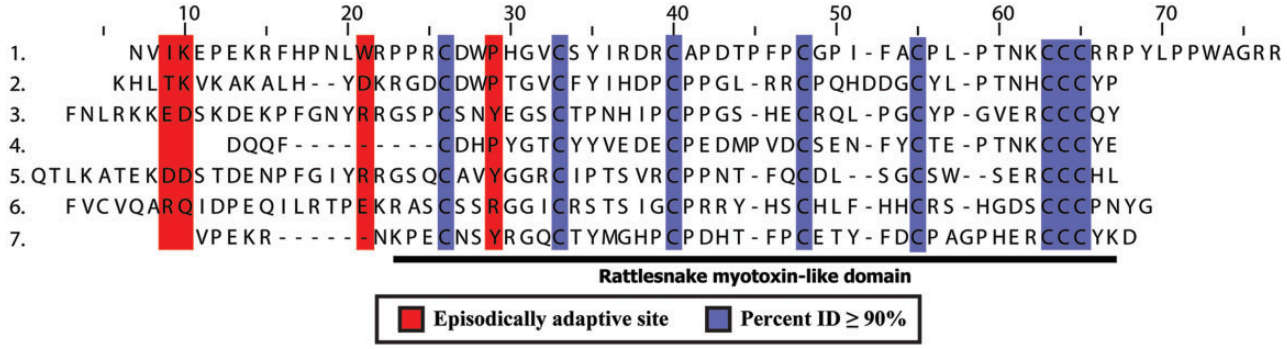

FIG. 6. Sequence alignment of SCRiPs. Episodically adaptive, structurally/functionally important, and extremely well-conserved (percent ID ≥90%) sites are depicted here. Uniprot (sequences 1–5) and NCBI (sequences 6 and 7) accession numbers and pharmacological names of sequences are as follows: 1) C0H690 *Acropora millepora* (SCRiP1); 2) C0H691 *A. millepora* (SCRiP2); 3) C0H692 *A. millepora* (SCRiP3); 4) C1KIZ0 *Montastraea faveolata* (SCRiP2); 5) C0H693 *Montipora capitata* (SCRiP1a); 6) FK725749 *Anemonia viridis*; and 7) FC835414 *Metridium senile*.

stable structure and function are filtered out of the population by purifying selection. Thus, favoring the conservation of structurally important and catalytic residues. Moreover, the accumulation of variations on the molecular surface of the toxin seems to be advantageous as the altered surface biochemistry might lead to neofunctionalization (generation of novel pharmacological properties) and assist in immunological evasion (Low et al. 2013).

Computation of ASA ratios revealed that all four episodically diversifying sites in the mature peptide of NaTxs, one positively selected and another episodically adaptive site in KTx type I, were surface exposed (supplementary table S2, Supplementary Material online). Six of the eight positively selected and episodically diversifying sites in the mature sequence of type III KTxs were also surface exposed, while only one site was buried. Thus, a majority of positively selected or episodically adaptive sites in neurotoxins are surface exposed. RAVER has been reported in a myriad of animal lineages and in a plethora of venom proteins, including scorpion neurotoxins (Kini and Chan 1999; Sunagar et al. 2012; Brust et al. 2013; Low et al. 2013; Ruder et al. 2013; Sunagar, Fry, et al. 2013; Sunagar, Jackson, et al. 2013; Pineda et al. 2014). Hence, it appears that even the toxins of the most ancient animal lineages adopt RAVER and favor the accumulation of variations on the molecular surface.

Negative Selection Constrains the Evolution of Pore-Forming Toxins

It is particularly interesting to note that most of the cnidarian PFTs have evolved under the extreme influence of negative selection (figs. 2 and 4, table 2, and supplementary fig. S3 and supplementary tables S4.1–S4.5, Supplementary Material online). This may be a result of their ability to utilize certain amino acids (e.g., charged, polar, or hydrophobic) to nonspecifically bind to the membranes of target cells in prey animals, before potentiating the deadly pore-forming activity. As a result of this nonspecific function, they probably do not experience the predator–prey chemical arm's race and

could act on a very wide range of species. Moreover, it becomes necessary to conserve a variety of residues on their molecular surface that are involved in binding to target cells (figs. 4 and 5). As outlined above, a majority of residues in actinoporins that are implicated in binding to target cells were found to have evolved under the pervasive influence of negative selection (fig. 4). Additionally, in order to gain their pore-forming function, actinoporins oligomerize into complex tetramers via the interaction of several residues (Kristan et al. 2004). Consequently, to ensure the proper organization of these toxins into complex tertiary structures, structurally important residues evolve under the constraints of negative selection. A similar phenomenon has also been noted in certain classes of snake venom toxins that exhibit nonspecific cytotoxicity (Sunagar, Jackson, et al. 2013; Sunagar et al. 2014), emphasizing the fact that not all toxins evolve rapidly.

The Common Evolutionary Origin of Sodium and Type III Potassium Neurotoxins in Sea Anemones

Bayesian and maximum-likelihood phylogenetic reconstructions in this study placed the type III KTxs in the same clade as type I NaTxs (fig. 3; Bayesian PP 0.997, bootstrap support 734/1,000), suggesting a common evolutionary origin of these two types. Although both type I and type II NaTxs remained constrained under the influence of negative selection, type III KTxs were found to have evolved rapidly ($\omega = 1.33$; six positively selected sites). Polytomy was observed at the node that leads to NaTx type I and KTx type III clades, suggesting that these proteins likely underwent rapid radiation in a short time. The pharmacological diversity in this neurotoxic lineage is probably reflective of an adaptive radiation. The examination of various sea anemone transcriptomes (Edwardsioidea: *N. vectensis* [Helm et al. 2013] and *Edwardsiella lineata* [Stefanik et al. 2014]; Acuticulata: *M. senile* and *A. pallida* [Lehnert et al. 2012]) failed to retrieve type III KTxs from these lineages. Hence, type III KTxs probably represent a unique evolutionary innovation of actinioids, such as *Anemonia*, *Bunodosoma*, and *Anthopleura* (for sea anemone species phylogeny [Rodriguez et al. 2014], see supplementary fig. S3, Supplementary Material online). Thus, it is likely that a subset of sodium channel targeting toxins experienced episodic bursts of adaptive selection in Actinioidea and accumulated mutations at an elevated rate, resulting in the origination of a novel toxin type that can target potassium ion channels. Interestingly, several KTx type III toxins still retain the ability to modulate sodium ion channels in prey animals. Thus, the evolution of neurotoxins in sea anemones is fascinating, because a subtype of potassium ion channel targeting toxin evolved within the sodium ion channel targeting toxins, and the potassium channel toxins evolved on at least five independent occasions (type I–V KTxs). It is also interesting to note that Actinioidea, which is likely the only sea anemone superfamily to have rapidly evolving type III KTxs, include several species with highly potent venoms such as *A. virids*, *Anthopleura elegantissima*, and *Anthopleura xanthogrammica* (Norton 1981; Schweitz et al. 1981). Thus, the evolution and diversification of several toxin types including type III KTxs, within Actinioidea, may have facilitated the emergence of a highly potent venom in this lineage.

Functional Diversification of Sea Anemone Neurotoxins

It has been documented that a single point mutation can alter the biochemical activities of toxins. APETx3, a type III KTx from *A. elegantissima*, differs from its homolog APETx1, a selective toxin modulator of human ether-á-go-go related gene (hERG) voltage-gated potassium channels, only in having a threonine instead of proline at position 3 (position 4 in fig. 2). Fascinatingly, this single point mutation has been demonstrated to be responsible for the loss of APETx3's ability to modulate hERG channels (Peigneur et al. 2012). This change has also been correlated with the origination of a different biochemical activity: the ability to modulate voltage-gated sodium ion channels (Peigneur et al. 2012). Strikingly, our evolutionary assessments detected this site as experiencing episodic influence of adaptive selection (fig. 2). Recently, several other sites were identified in APETx2 from *A. elegantissima*, as important for inhibiting hERG and the unrelated acid-sensing ion channel 3 (ASIC3) (Jensen et al. 2014). Evolutionary analyses in this study identified many of these functionally important sites as episodically adaptive: 1) Sites 21 and 36 (important for ASIC3 inhibition) and 2) site 22 (important for interacting with hERG; fig. 2). However, none of the positively selected sites we identified in type III KTxs coincide with sites in APETx2 that are responsible for targeting both hERG and ASIC3 channels (Jensen et al. 2014). Thus, positive selection has strongly shifted the pharmacological properties of the toxin between targets but does not result in the complete loss of activity, which could have a deleterious effect on the fitness of the animal.

We also identified a few functionally important sites in other classes of neurotoxins and PFTs that experienced episodic adaptation (figs. 2–5): 1) Site 2 in NaTxs which was shown to be responsible for a moderate decrease in the binding affinity to insect Na_v channels upon mutagenesis (V2A) (Moran et al. 2006); 2) sites 54, 117, and 144 in actinoporins that are responsible for binding to cell membranes; and 3) the site in hydralysins shown to result in slight reduction (2.5-fold) of paralytic and hemolytic activity upon mutation (G129E) (Sher et al. 2005). Additionally, sites that were shown to have minor effects on toxicity were also found to be positively selected (e.g., sites 12 and 40 in NaTx; fig. 1; Moran et al. 2006). These findings suggest that sites that are relieved of purifying selection pressures and those responsible for toxicity constitute the evolutionary hotspots in cnidarian toxins, as long as mutations do not result in the complete loss of toxicity. Such sites may also promote changes in toxin selectivity and expand the range of molecular targets (Weinberger et al. 2010).

SCRiPs May Represent the First Neurotoxin Family from Corals

Because SCRiPs cause profound neurotoxic effects in fish, it is most likely that they are employed as neurotoxins. Moreover,

SCRiPs have been reported to be widely expressed in the ectoderm of *A. millepora* (Grasso et al. 2011), which further support their likely role in prey envenomation, as ectoderm is chiefly lined by nematocytes and gland cells in anthozoans. In contrast to previous conclusions that SCRiPs are unique innovations of scleractinian corals (Sunagawa et al. 2009), the retrieval of SCRiP homologs from sea anemones in our study (fig. 6) indicates that these proteins evolved nearly 500 Ma in the common ancestor of sea anemones and scleractinian corals (Shinzato et al. 2011). Because reef-building corals form habitats for numerous species of marine animals, the understanding of their ecology and the evolutionary diversification of proteins, such as toxins that affect their ecological interactions, are important. Although peptide toxins from other cnidarians, particularly sea anemones, are well studied, practically nothing is known about venom in corals. Hence, the discovery of a novel family of neurotoxic peptides in this lineage is fascinating and may prove to be an important step forward in the understanding of the evolution of venom in this lineage.

To conclude, venom research in the past has chiefly focused on relatively evolutionarily younger animal lineages, such as the venoms of advanced snakes and cone snails. Our findings provide fascinating insights into the evolution of venom in Cnidaria: **1**) **T**he common origin of sodium channel toxins and a subtype of potassium channel toxins in sea anemones; **2**) the discovery of the first neurotoxin family from corals; **3**) identification of strong evolutionary constraints on most cnidarian toxin types, especially PFTs; and **4**) insights into the functional diversification of various toxin types. These results emphasize the importance of understanding the molecular evolution, diversification**,** and phylogenetic histories of venom components, particularly in the ancient venomous lineages.

Materials and Methods

Phylogenetic Analyses

Translated nucleotide sequences were aligned using MUSCLE 3.8 (Edgar 2004). Sequence alignments in FASTA format have been provided in the form of a zipped file (supplementary file 1, Supplementary Material online). The best-fit model of nucleotide substitution and amino acid replacement for individual toxin data sets were determined according to the Akaike's information criterion using jModeltest 2.1 (Darriba et al. 2012) and Prottest 3.0 (Darriba et al. 2011), respectively (supplementary table S5, Supplementary Material online). Model-averaged parameter estimates of the proportion of invariant sites (pinvar) and the gamma shape parameter (α) were used for reconstruction of trees. Maximum-likelihood and Bayesian phylogenetic analyses performed on the nucleotide data sets allowed the reconstruction of the molecular evolutionary histories of various cnidarian toxins. Bayesian inference implemented in MrBayes 3.2.3 (Ronquist et al. 2012) was used and a minimum of 15×10^6 generations in 4 chains were run, saving 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 reached their asymptote. After the completion of burn-in phase, posterior probabilities for clades were established by constructing a majority-rule consensus tree for all trees generated. The maximum-likelihood trees were generated using PhyML 3.0 (Guindon et al. 2010). Node support was evaluated with 1,000 bootstrapping replicates.

Selection Analyses

Maximum-likelihood models (Yang 1998) implemented in Codeml of the PAML (Yang 2007) were employed to assess the nature of natural selection on various cnidarian toxin families examined in this study. Site-specific models, which estimate positive selection statistically as a nonsynonymous-to-synonymous nucleotide substitution rate ratio (ω) significantly greater than 1, were employed. Because no a priori expectation exists, we compared likelihood values for three pairs of models with different assumed ω distributions: M0 (constant ω rates across all sites) versus M3 (allows ω 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 (β) versus M8 (β and ω) (Norton and Pallaghy 1998). The results are considered significant only if the alternative models (M3, M2a, and M8 that allow sites with $\omega > 1$) show a better fit in the likelihood ratio test (LRT) relative to their null models (M0, M1a, and M7: Do not allow sites $\omega > 1$). LRT is estimated as twice the difference in maximum-likelihood values between nested models and compared with the χ^2 distribution with the appropriate degree of freedom—the difference in the number of parameters between the two models. The BEB approach (Yang et al. 2005) is employed to identify sites under positive selection by calculating the posterior probabilities that a particular site belongs to a given selection class (neutral, conserved, or highly variable). Sites with greater PP ($\geq$95%) of belonging to the "$\omega > 1$ class" were inferred to be positively selected. FUBAR (Murrell et al. 2013) implemented in HyPhy (Pond et al. 2005) was utilized to identify sites evolving under the influence of pervasive diversifying and purifying selection pressures. Additionally, MEME (Murrell et al. 2012) was used to efficiently detect episodically diversifying sites.

Structural Analyses

Consurf webserver (Armon et al. 2001) was used to map the evolutionary variability of amino acids onto the crystal structures of various cnidarian toxins. Furthermore, we calculated the ASA or the solvent exposure of amino acid side chains using GETAREA (Fraczkiewicz and Braun 1998), which uses the atom co-ordinates of the Protein Data Bank (PDB) 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 with an ASA ratio of $\leq$20% is considered to be buried, while an amino acid with a ratio of 50% or more is likely exposed to the

surrounding medium. Pymol 1.3 (DeLano 2002) was used for visualizing three-dimensional structures.

Cloning, Recombinant Expression, and Purification of Toxins

Primers carrying *Nco*I and *Bam*HI restriction sites and corresponding to the mature Amil-SCRiP1, 2, and 3 were designed according to the available transcript sequences (Sunagawa et al. 2009). The primers were used in a polymerase chain reaction (94 °C for 2 min, 34 times [94°C for 20 s, 55 °C for 20 s, 72 °C for 30 s] and 72 °C for 5 min) with *A. millepora* complementary DNA made from mixed developmental stages (kindly provided by Drs D. Hayward and E. Ball, Australian National University, Canberra). The resulting PCR fragment was digested with *Nco*I and *Bam*HI (New England Biolabs, USA) and cloned with T4 DNA Ligase (Takara, Japan) into a pET-32b vector (Novagen, USA) digested with the same enzymes. Toxins were expressed fused to thioredoxin in the *Escherichia coli* Rosettagami strain (DE3, pLys; Novagen). Toxins were released from their thioredoxin tag and purified on HisTrap and Resource RPC columns connected to an AKTA fast protein liquid chromatography machine (GE Helathcare) as previously described for the Av3 toxin (Moran et al. 2007).

Toxicity Assays

Toxicity assays on blowfly and 3-days-old zebrafish larvae were performed as described before (Moran et al. 2007; Moran, Genikhovich, et al. 2012). The zebrafish larvae were kindly provided by Dr Y Gothilf (Tel Aviv University).

Supplementary Material

Supplementary figures S1 and S2, tables S1.1–S5, and file S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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References

Anderluh G, Macek P. 2002. Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). *Toxicon* 40:111–124.

Armon A, Graur D, Ben-Tal N. 2001. ConSurf: an algorithmic tool for the identification of functional regions in proteins by surface mapping of phylogenetic information. *J Mol Biol.* 307:447–463.

Beress L, Ritter R, Ravens U. 1982. The influence of the rate of electrical stimulation on the effects of the Anemonia sulcata Toxin ATX II in guinea pig papillary muscle. *Eur J Pharmacol.* 79:265–272.

Brinkman DL, Konstantakopoulos N, McInerney BV, Mulvenna J, Seymour JE, Isbister GK, Hodgson WC. 2014. *Chironex fleckeri* (box jellyfish) venom proteins: expansion of a cnidarian toxin family that elicits variable cytolytic and cardiovascular effects. *J Biol Chem.* 289:4798–4812.

Brust A, Sunagar K, Undheim EA, Vetter I, Yang DC, Casewell NR, Jackson TN, Koludarov I, Alewood PF, Hodgson WC, et al. 2013. Differential evolution and neofunctionalization of snake venom metalloprotease domains. *Mol Cell Proteomics.* 12:651–663.

Casewell NR, Huttley GA, Wuster W. 2012. Dynamic evolution of venom proteins in squamate reptiles. *Nat Commun.* 3:1066.

Casewell NR, Wagstaff SC, Harrison RA, Renjifo C, Wuster W. 2011. Domain loss facilitates accelerated evolution and neofunctionalization of duplicate snake venom metalloproteinase toxin genes. *Mol Biol Evol.* 28:2637–2649.

Casewell NR, Wuster W, Vonk FJ, Harrison RA, Fry BG. 2013. Complex cocktails: the evolutionary novelty of venoms. *Trends Ecol Evol.* 28:219–229.

Castaneda O, Harvey AL. 2009. Discovery and characterization of cnidarian peptide toxins that affect neuronal potassium ion channels. *Toxicon* 54:1119–1124.

Chang D, Duda TF Jr. 2012. Extensive and continuous duplication facilitates rapid evolution and diversification of gene families. *Mol Biol Evol.* 29:2019–2029.

Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, Rattei T, Balasubramanian PG, Borman J, Busam D, et al. 2010. The dynamic genome of Hydra. *Nature* 464:592–596.

Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* 27:1164–1165.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods.* 9:772.

David CN, Ozbek S, Adamczyk P, Meier S, Pauly B, Chapman J, Hwang JS, Gojobori T, Holstein TW. 2008. Evolution of complex structures: minicollagens shape the cnidarian nematocyst. *Trends Genet.* 24:431–438.

DeLano WL. 2002. The PyMOL molecular graphics system. San Carlos (CA): DeLano Scientific.

Diochot S, Baron A, Rash LD, Deval E, Escoubas P, Scarzello S, Salinas M, Lazdunski M. 2004. A new sea anemone peptide, APETx2, inhibits ASIC3, a major acid-sensitive channel in sensory neurons. *EMBO J.* 23:1516–1525.

Duda TF Jr, Kohn AJ. 2005. Species-level phylogeography and evolutionary history of the hyperdiverse marine gastropod genus *Conus*. *Mol Phylogenet Evol.* 34:257–272.

Dunlop JA, Selden PA. 2009. Calibrating the chelicerate clock: a paleontological reply to Jeyaprakash and Hoy. *Exp Appl Acarol.* 48:183–197.

Dutertre S, Jin AH, Vetter I, Hamilton B, Sunagar K, Lavergne V, Dutertre V, Fry BG, Antunes A, Venter DJ, et al. 2014. Evolution of separate predation- and defence-evoked venoms in carnivorous cone snails. *Nat Commun.* 5:3521.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792–1797.

Erwin DH, Laflamme M, Tweedt SM, Sperling EA, Pisani D, Peterson KJ. 2011. The Cambrian conundrum: early divergence and later ecological success in the early history of animals. *Science* 334:1091–1097.

Fraczkiewicz R, Braun W. 1998. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comput Chem.* 19:319–333.

Fukuda I, Ooki S, Fujita T, Murayama E, Nagasawa H, Isa Y, Watanabe T. 2003. Molecular cloning of a cDNA encoding a soluble protein in the coral exoskeleton. *Biochem Biophys Res Commun.* 304:11–17.

Garcia-Ortega L, Alegre-Cebollada J, Garcia-Linares S, Bruix M, Martinez-Del-Pozo A, Gavilanes JG. 2011. The behavior of sea anemone actinoporins at the water-membrane interface. *Biochim Biophys Acta.* 1808:2275–2288.

Grasso LC, Negri AP, Foret S, Saint R, Hayward DC, Miller DJ, Ball EE. 2011. The biology of coral metamorphosis: molecular responses of larvae to inducers of settlement and metamorphosis. *Dev Biol.* 353:411–419.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol.* 59:307–321.

Gutierrez-Aguirre I, Barlic A, Podlesek Z, Macek P, Anderluh G, Gonzalez-Manas JM. 2004. Membrane insertion of the N-terminal

alpha-helix of equinatoxin II, a sea anemone cytolytic toxin. *Biochem J.* 384:421–428.
Helm RR, Siebert S, Tulin S, Smith J, Dunn CW. 2013. Characterization of differential transcript abundance through time during *Nematostella vectensis* development. *BMC Genomics* 14:266.
Honma T, Kawahata S, Ishida M, Nagai H, Nagashima Y, Shiomi K. 2008. Novel peptide toxins from the sea anemone *Stichodactyla haddoni*. *Peptides* 29:536–544.
Honma T, Shiomi K. 2006. Peptide toxins in sea anemones: structural and functional aspects. *Mar Biotechnol (NY).* 8:1–10.
Hunter S, Jones P, Mitchell A, Apweiler R, Attwood TK, Bateman A, Bernard T, Binns D, Bork P, Burge S, et al. 2012. InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Res.* 40:D306–D312.
Ishida M, Yokoyama A, Shimakura K, Nagashima Y, Shiomi K. 1997. Halcurin, a polypeptide toxin from the sea anemone Halcurias sp., with a structural resemblance to type 1 and 2 toxins. *Toxicon* 35: 537–544.
Jensen JE, Cristofori-Armstrong B, Anangi R, Rosengren KJ, Lau CH, Mobli M, Brust A, Alewood PF, King GF, Rash LD. 2014. Understanding the molecular basis of toxin promiscuity: the analgesic sea anemone peptide APETx2 interacts with acid-sensing ion channel 3 and hERG channels via overlapping pharmacophores. *J Med Chem.* 57:9195–9203.
Kass-Simon G, Scappaticci AA. 2002. The behavioral and developmental physiology of nematocysts. *Can J Zool.* 80:1772–1794.
Kini RM, Chan YM. 1999. Accelerated evolution and molecular surface of venom phospholipase A2 enzymes. *J Mol Evol.* 48:125–132.
Kristan K, Podlesek Z, Hojnik V, Gutierrez-Aguirre I, Guncar G, Turk D, Gonzalez-Manas JM, Lakey JH, Macek P, Anderluh G. 2004. Pore formation by equinatoxin, a eukaryotic pore-forming toxin, requires a flexible N-terminal region and a stable beta-sandwich. *J Biol Chem.* 279:46509–46517.
Kristan KC, Viero G, Dalla Serra M, Macek P, Anderluh G. 2009. Molecular mechanism of pore formation by actinoporins. *Toxicon* 54:1125–1134.
Lehnert EM, Burriesci MS, Pringle JR. 2012. Developing the anemone *Aiptasia* as a tractable model for cnidarian-dinoflagellate symbiosis: the transcriptome of aposymbiotic *A. pallida*. *BMC Genomics* 13:271.
Low DH, Sunagar K, Undheim EA, Ali SA, Alagon AC, Ruder T, Jackson TN, Pineda Gonzalez S, King GF, Jones A, et al. 2013. Dracula's children: molecular evolution of vampire bat venom. *J Proteomics.* 89: 95–111.
Lynch VJ. 2007. Inventing an arsenal: adaptive evolution and neofunctionalization of snake venom phospholipase A2 genes. *BMC Evol Biol.* 7:2.
Mancheno JM, Tateno H, Sher D, Goldstein IJ. 2010. *Laetiporus sulphureus* lectin and aerolysin protein family. *Adv Exp Med Biol.* 677:67–80.
Mebs D. 2002. Venomous and poisonous animals: a handbook for biologists, toxicologists and toxinologists, physicians and pharmacists. Stuttgart (Germany): Medpharm.
Menon LR, McIlroy D, Brasier MD. 2013. Evidence for Cnidaria-like behavior in ca 560 Ma Ediacaran. *Aspidella. Geology* 41:895–898.
Minagawa S, Sugiyama M, Ishida M, Nagashima Y, Shiomi K. 2008. Kunitz-type protease inhibitors from acrorhagi of three species of sea anemones. *Comp Biochem Physiol B Biochem Mol Biol.* 150: 240–245.
Moran Y, Cohen L, Kahn R, Karbat I, Gordon D, Gurevitz M. 2006. Expression and mutagenesis of the sea anemone toxin Av2 reveals key amino acid residues important for activity on voltage-gated sodium channels. *Biochemistry* 45:8864–8873.
Moran Y, Fredman D, Szczesny P, Grynberg M, Technau U. 2012. Recurrent horizontal transfer of bacterial toxin genes to eukaryotes. *Mol Biol Evol.* 29:2223–2230.
Moran Y, Genikhovich G, Gordon D, Wienkoop S, Zenkert C, Ozbek S, Technau U, Gurevitz M. 2012. Neurotoxin localization to ectodermal gland cells uncovers an alternative mechanism of venom delivery in sea anemones. *Proc Biol Sci.* 279:1351–1358.
Moran Y, Gordon D, Gurevitz M. 2009. Sea anemone toxins affecting voltage-gated sodium channels—molecular and evolutionary features. *Toxicon* 54:1089–1101.
Moran Y, Gurevitz M. 2006. When positive selection of neurotoxin genes is missing. The riddle of the sea anemone *Nematostella vectensis*. *FEBS J.* 273:3886–3892.
Moran Y, Kahn R, Cohen L, Gur M, Karbat I, Gordon D, Gurevitz M. 2007. Molecular analysis of the sea anemone toxin Av3 reveals selectivity to insects and demonstrates the heterogeneity of receptor site-3 on voltage-gated Na+ channels. *Biochem J.* 406:41–48.
Murrell B, Moola S, Mabona A, Weighill T, Sheward D, Kosakovsky Pond SL, Scheffler K. 2013. FUBAR: a fast, unconstrained bayesian approximation for inferring selection. *Mol Biol Evol.* 30:1196–1205.
Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Kosakovsky Pond SL. 2012. Detecting individual sites subject to episodic diversifying selection. *PLoS Genet.* 8:e1002764.
Norton RS, Pallaghy PK. 1998. The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon* 36:1573–1583.
Norton TR. 1981. Cardiotonic polypeptides from *Anthopleura xanthogrammica* (Brandt) and *A. elegantissima* (Brandt). *Fed Proc.* 40: 21–25.
Olivera BM. 1997. E.E. Just Lecture, 1996. Conus venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology. *Mol Biol Cell.* 8:2101–2109.
Orts DJ, Moran Y, Cologna CT, Peigneur S, Madio B, Praher D, Quinton L, De Pauw E, Bicudo JE, Tytgat J, et al. 2013. BcsTx3 is a founder of a novel sea anemone toxin family of potassium channel blocker. *FEBS J.* 280:4839–4852.
Park E, Hwang DS, Lee JS, Song JI, Seo TK, Won YJ. 2012. Estimation of divergence times in cnidarian evolution based on mitochondrial protein-coding genes and the fossil record. *Mol Phylogenet Evol.* 62:329–345.
Peigneur S, Béress L, Möller C, Marí F, Forssmann WG, Tytgat J. 2012. A natural point mutation changes both target selectivity and mechanism of action of sea anemone toxins. *FASEB J.* 26:5141–5151.
Pineda SS, Sollod BL, Wilson D, Darling A, Sunagar K, Undheim EA, Kely L, Antunes A, Fry BG, King GF. 2014. Diversification of a single ancestral gene into a successful toxin superfamily in highly venomous Australian funnel-web spiders. *BMC Genomics* 15:177.
Pond SL, Frost SD, Muse SV. 2005. HyPhy: hypothesis testing using phylogenies. *Bioinformatics* 21:676–679.
Putnam NH, Srivastava M, Hellsten U, Dirks B, Chapman J, Salamov A, Terry A, Shapiro H, Lindquist E, Kapitonov VV, et al. 2007. Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* 317:86–94.
Richier S, Rodriguez-Lanetty M, Schnitzler CE, Weis VM. 2008. Response of the symbiotic cnidarian *Anthopleura elegantissima* transcriptome to temperature and UV increase. *Comp Biochem Physiol Part D Genomics Proteomics.* 3:283–289.
Rodriguez E, Barbeitos MS, Brugler MR, Crowley LM, Grajales A, Gusmao L, Haussermann V, Reft A, Daly M. 2014. Hidden among sea anemones: the first comprehensive phylogenetic reconstruction of the order Actiniaria (Cnidaria, Anthozoa, Hexacorallia) reveals a novel group of hexacorals. *PLoS One* 9:e96998.
Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol.* 61:539–542.
Ruder T, Sunagar K, Undheim EA, Ali SA, Wai TC, Low DH, Jackson TN, King GF, Antunes A, Fry BG. 2013. Molecular phylogeny and evolution of the proteins encoded by coleoid (cuttlefish, octopus, and squid) posterior venom glands. *J Mol Evol.* 76:192–204.
Ruppert EE, Fox RS, Barnes RD. 2004. Invertebrate zoology: a functional evolutionary approach. Belmont (CA): Thomson-Brooks/Cole.
Schweitz H, Vincent JP, Barhanin J, Frelin C, Linden G, Hugues M, Lazdunski M. 1981. Purification and pharmacological properties of eight sea anemone toxins from *Anemonia sulcata*, *Anthopleura xanthogrammica*, *Stoichactis giganteus*, and *Actinodendron plumosum*. *Biochemistry* 20:5245–5252.

Sher D, Fishman Y, Melamed-Book N, Zhang M, Zlotkin E. 2008. Osmotically driven prey disintegration in the gastrovascular cavity of the green hydra by a pore-forming protein. *FASEB J.* 22:207–214.
Sher D, Fishman Y, Zhang M, Lebendiker M, Gaathon A, Mancheno JM, Zlotkin E. 2005. Hydralysins, a new category of beta-pore-forming toxins in cnidaria. *J Biol Chem.* 280:22847–22855.
Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, Tanaka M, Fujie M, Fujiwara M, Koyanagi R, Ikuta T, et al. 2011. Using the *Acropora digitifera* genome to understand coral responses to environmental change. *Nature* 476:320–323.
Smith JJ, Blumenthal KM. 2007. Site-3 sea anemone toxins: molecular probes of gating mechanisms in voltage-dependent sodium channels. *Toxicon* 49:159–170.
Steele RE, David CN, Technau U. 2011. A genomic view of 500 million years of cnidarian evolution. *Trends Genet.* 27:7–13.
Stefanik DJ, Lubinski TJ, Granger BR, Byrd AL, Reitzel AM, DeFilippo L, Lorenc A, Finnerty JR. 2014. Production of a reference transcriptome and transcriptomic database (EdwardsiellaBase) for the lined sea anemone, *Edwardsiella lineata*, a parasitic cnidarian. *BMC Genomics* 15:71.
Sunagar K, Fry BG, Jackson TN, Casewell NR, Undheim EA, Vidal N, Ali SA, King GF, Vasudevan K, Vasconcelos V, et al. 2013. Molecular evolution of vertebrate neurotrophins: co-option of the highly conserved nerve growth factor gene into the advanced snake venom arsenalf. *PLoS One* 8:e81827.
Sunagar K, Jackson TN, Undheim EA, Ali SA, Antunes A, Fry BG. 2013. Three-fingered RAVERs: Rapid Accumulation of Variations in Exposed Residues of snake venom toxins. *Toxins (Basel)* 5: 2172–2208.
Sunagar K, Johnson WE, O'Brien SJ, Vasconcelos V, Antunes A. 2012. Evolution of CRISPs associated with toxicoferan-reptilian venom and mammalian reproduction. *Mol Biol Evol.* 29:1807–1822.
Sunagar K, Undheim EA, Chan AH, Koludarov I, Munoz-Gomez SA, Antunes A, Fry BG. 2013. Evolution stings: the origin and diversification of scorpion toxin peptide scaffolds. *Toxins (Basel)* 5: 2456–2487.
Sunagar K, Undheim EA, Scheib H, Gren EC, Cochran C, Person CE, Koludarov I, Kelln W, Hayes WK, King GF, et al. 2014. Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): biodiscovery, clinical and evolutionary implications. *J Proteomics.* 99:68–83.
Sunagawa S, DeSalvo MK, Voolstra CR, Reyes-Bermudez A, Medina M. 2009. Identification and gene expression analysis of a taxonomically restricted cysteine-rich protein family in reef-building corals. *PLoS One* 4:e4865.
Technau U, Steele RE. 2011. Evolutionary crossroads in developmental biology: Cnidaria. *Development* 138:1447–1458.
Tibballs J, Yanagihara AA, Turner HC, Winkel K. 2011. Immunological and toxinological responses to jellyfish stings. *Inflamm Allergy Drug Targets* 10:438–446.
Turk T, Kem WR. 2009. The phylum Cnidaria and investigations of its toxins and venoms until 1990. *Toxicon* 54:1031–1037.
van Vlijmen HW, Gupta A, Narasimhan LS, Singh J. 2004. A novel database of disulfide patterns and its application to the discovery of distantly related homologs. *J Mol Biol.* 335:1083–1092.
Vidal N, Rage J, Couloux A, Hedges SB. 2009. Snakes (serpentes) in the timetree of life. New York: Oxford University Press.
Wanke E, Zaharenko AJ, Redaelli E, Schiavon E. 2009. Actions of sea anemone type 1 neurotoxins on voltage-gated sodium channel isoforms. *Toxicon* 54:1102–1111.
Weinberger H, Moran Y, Gordon D, Turkov M, Kahn R, Gurevitz M. 2010. Positions under positive selection—key for selectivity and potency of scorpion alpha-toxins. *Mol Biol Evol.* 27: 1025–1034.
Yang Z. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol Biol Evol.* 15: 568–573.
Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol.* 24:1586–1591.
Yang Z, Wong WS, Nielsen R. 2005. Bayes empirical bayes inference of amino acid sites under positive selection. *Mol Biol Evol.* 22: 1107–1118.
Zaharenko AJ, Ferreira WA Jr, Oliveira JS, Richardson M, Pimenta DC, Konno K, Portaro FC, de Freitas JC. 2008. Proteomics of the neurotoxic fraction from the sea anemone *Bunodosoma cangicum* venom: Novel peptides belonging to new classes of toxins. *Comp Biochem Physiol Part D Genomics Proteomics* 3: 219–225.