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ORIGINAL ARTICLE

Ancient Diversification of Three-Finger Toxins in *Micrurus* Coral Snakes

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Abstract
Coral snakes, most notably the genus *Micrurus*, are the only terrestrial elapid snakes in the Americas. Elapid venoms are generally known for their potent neurotoxicity which is usually caused by Three-Finger Toxin (3FTx) proteins. These toxins can have a wide array of functions that have been characterized from the venom of other elapids. We examined publicly available sequences from *Micrurus* 3FTx to show that they belong to 8 monophyletic clades that diverged as deep in the 3FTx phylogenetic tree as the other clades with characterized functions. Functional residues from previously characterized clades of 3FTx are not well conserved in most of the *Micrurus* toxin clades. We also analyzed the patterns of selection on these toxins and find that they have been diversifying at different rates, with some having undergone extreme diversifying selection. This suggests that *Micrurus* 3FTx may contain a previously underappreciated functional diversity that has implications for the clinical outcomes of bite victims, the evolution and ecology of the genus, as well as the potential for biodiscovery efforts focusing on these toxins.

Keywords Coral snake · Elapid · Micrurus · Venom · Three-finger toxin · 3FTx

Introduction

Coral snakes are a clade of elapid snakes that are known for their striking colors and patterns as well as their life-threatening bites. After the genus *Calliophis*, the coral snakes are the second-most basal branch of the elapid family (Pyron et al. 2013; Lee et al. 2016). The clade originated in Asia and are currently represented there by the genus *Sinomicrurus* (5 species), but migrated across the Bering land bridge to the Americas roughly 30 million years ago (Uetz et al. 2016; Kelly et al. 2009; Lee et al. 2016). Once in the Americas coral snakes divided into the genera *Micruroides* (1 species) and *Micrurus* (79 species, including the 4 species often placed in the genus *Leptomicrurus*) which rapidly diversified and can be found from the southern United States to central Argentina (Uetz et al. 2016; Roze 1996; Wallach et al. 2014).

Due to a combination of their small size, bright colors, and secretive habits, coral snakes are responsible for relatively few bites compared to other venomous snakes in the same area (Bucaretchi et al. 2016; Otero-Patiño 2014), but they nonetheless claim lives throughout their entire range (Norris et al. 2009; Rosenfeld 1971). Like those of many elapids, bites from coral snakes are often dangerously neurotoxic (Cañas et al. 2017; Bucaretchi et al. 2016; Norris et al. 2009; Manock et al. 2008). The classic neurotoxins of the elapids belong to the protein family of Three-Finger Toxins (3FTx) (Fry et al. 2003; Utkin et al. 2015). These small (60–90 amino acids) proteins are known to target many nerve receptors including nicotinic acetylcholine receptors (nAChRs), muscarinic acetylcholine receptors, calcium channels, acid-sensing ion channels, and more (Utkin et al. 2015). The toxin family gets its name from the three loops—or 'fingers'—that extend from the central core of the protein. Because the amino acids on these loops are far more exposed than those closer to the center, these domains almost always contain the sites that interact with the toxins' targets (Antil et al. 1999; Utkin et al. 2015). Analysis of various coral snake venoms has recently shown that many species produce more phospholipase A2 (PLA2) toxins than 3FTx (Lomonte et al. 2016). The enzymatic activities of *Micrurus* venoms vary widely, but for most species, the role each toxin family

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and the specific proteins within them plays remains largely unknown (da Silva and Aird 2001).

The ancestral forms of 3FTx contain ten cysteine residues, often antagonize acetylcholine receptors, and tend to be far more potent against diapsid targets (Fry et al. 2003; Utkin et al. 2015). In the elapid family, versions of the gene can be found with the 2nd and 3rd of these cysteines deleted. These 8-cysteine toxins have radiated dramatically and now dominate the venoms of most elapids; many of these toxins are more potent α-neurotoxins than the ancestral forms—especially against synapsid targets—or have undergone neofunctionalization and now exhibit a diverse array of biological activities (Sunagar et al. 2013; Utkin et al. 2015). Because of their broader range of functions and higher prevalence in the venoms of elapids, this study focuses on the 8-cysteine 3FTx from *Micrurus* and representatives from other elapid genera rather than the ancestral versions.

3FTx are interesting beyond their significant role in snakebites across the world because of their potential as a biodiscovery source for new laboratory tools or medications. For instance, α-bungarotoxin sees widespread use in neuroscience research because of its potent and specific inactivation of nAChRs (Chang 1999). 3FTx are also promising for drug development because of their small size and disulphide-bonded structural core which makes them easier to produce synthetically and increases the likelihood that they can be delivered to patients without the need for injection (Harvey 2014; King 2013). Both of these issues have challenged the development of existing venom-derived drugs (King 2015). In fact, several 3FTx from other elapid lineages have been studied for their therapeutic potential. Diochot et al. (2012) found that several 3FTx from black mamba (*Dendroaspis polylepis*) venom—which they called mambalgins—were non-toxic and just as effective against pain as morphine when injected into mice. What is more, these 3FTx did not cause respiratory distress or tolerance like opioids do.

Similarly, Pu et al. (1995) investigated the anti-nociceptive properties of hannalgesin, a 3FTx found in king cobra (*Ophiophagus hannah*) venom. Intriguingly, this toxin produced its pain-killing effects even when ingested orally, whereas most venom proteins are thought to be entirely ineffectual if they are not injected directly into the target organism (Nelsen et al. 2014). A comparable activity was found in the venom of *Micrurus lemniscatus*. Mice that ingested crude *M. leminscatus* venom were found to react much less strongly on various behavioral measures of pain (Leite dos Santos et al. 2012). The similarity to hannalgesin and the fact that the venom of *M. lemniscatus* is known to be dominated by 3FTx suggest that the venom component responsible for this effect might also be a 3FTx (Ciscotto et al. 2011).

In this study we examine the diversity and evolutionary history of known 3FTx from *Micrurus*. A better understanding of the molecular evolution of these toxins will be useful for those studying the ecology and medical significance of these snakes as well as providing guidance for any biodiscovery efforts focused on these venoms. Recent discoveries like that of calliotoxin, a 3FTx from *Calliophis bivirgatus* that is the first vertebrate toxin known to activate voltage-gated sodium ion channels, illustrate the potential of this toxin family (Yang et al. 2016). Examining the diversity of 3FTx in Micrurus may allow future studies to make similar discoveries and the location of the coral snakes near the base of the elapids may shed light on the impact that the evolution of 8-cysteine 3FTx had on the radiation of the elapid snakes.

Results

Our phylogenetic analysis of 8-cysteine 3FTx from across the elapids (Fig. 1) shows that those produced by *Micrurus* belong to eight distinct monophyletic clades (counting those with at least five sequences). These clades diverge from each other relatively early in the tree both in terms of branch length and overall topology. Many of the *Micrurus* clades are phylogenetically closer to sequences from other species than they are to other *Micrurus* clades.

Those 8-cysteine *Micrurus* sequences that have been previously characterized are marked in Fig. 1. The only reported activity for any of these sequences is α-neurotoxicity. Note that of the studies referenced, only Rey-Suárez et al. (2012) tested whether the toxin in question had any activity beyond nAChR antagonism. Carbajal-Saucedo et al. (2013) and Olamendi-Portugal et al. (2008) only investigated nAChR antagonistic activity while Moreira et al. (2010) relied on suppression of electrically stimulated neuromuscular response. The distribution of α-neurotoxicity suggests that phylogenetic position alone is not enough to assign putative function to *Micrurus* 3FTx sequences.

The clades from our phylogenetic analysis are largely corroborated by the independent similarity network and protein clustering analyses (see Fig. 2). This gives greater confidence that these are meaningful subdivisions within the 3FTx. In all three analyses many of the groups of *Micrurus* sequences are more akin to sequences from other elapids, including those with diverse modes of action, than they are to other groups of *Micrurus* toxins.

The sequences of the toxins also suggest that there may be biological activities beyond α-neurotoxicity. Figure 3 shows that only Clades E and G have greater than 50% identity at the residues homologous to the functional residues of erabutoxin-a, a well-characterized Type I α-neurotoxin (Antil et al. 1999). It must be noted that two of the *Micrurus* toxins with published α-neurotoxic activity belong to Clade B and only display 19% conservation of these sites (Moreira

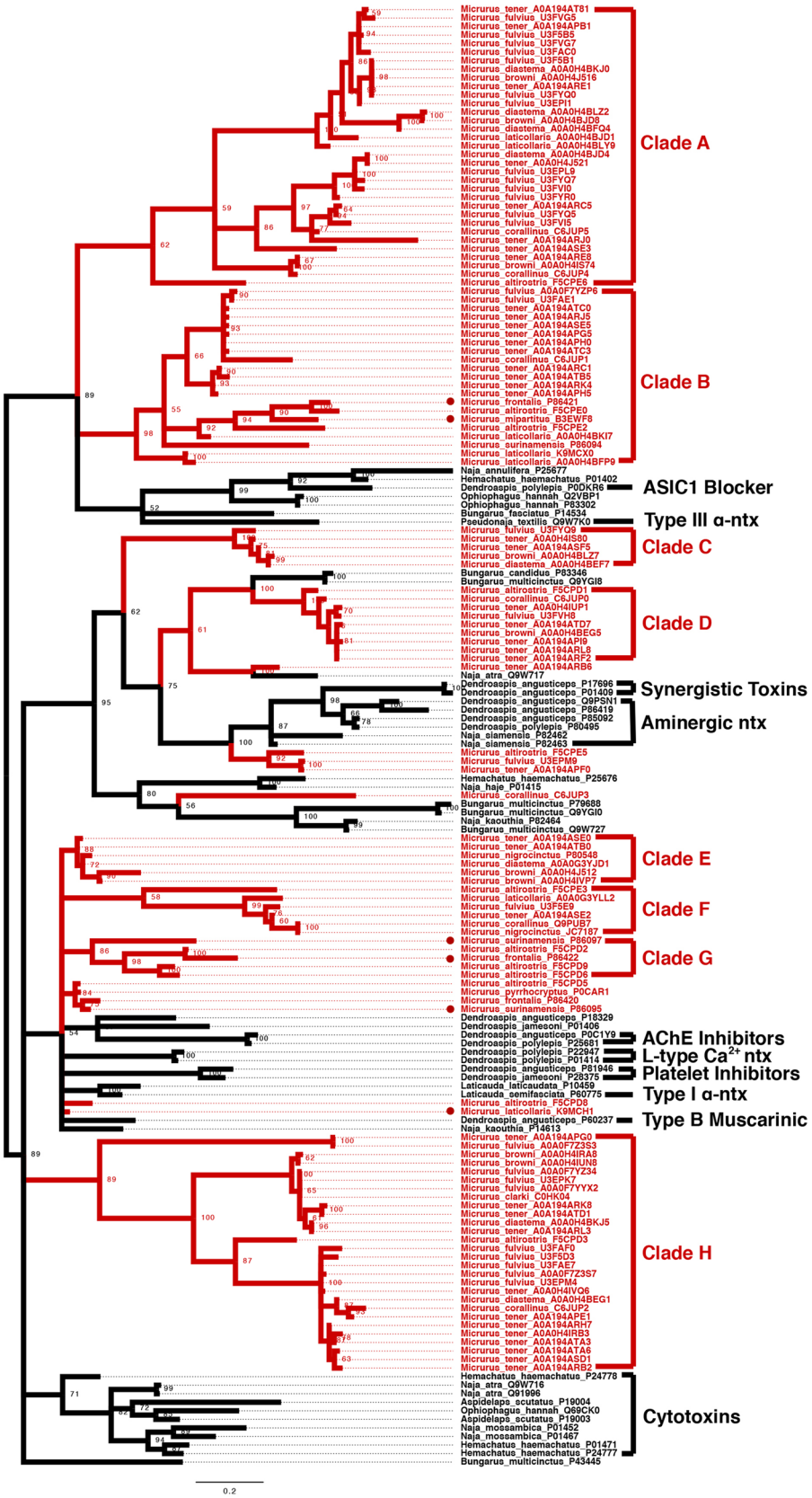

Fig. 1 Phylogenetic tree of publicly available 3FTx sequences. Clades with previously characterized activities and clades of Micrurus sequences are highlighted. Circle (•) indicates Micrurus toxins with previously observed α-neurotoxic activity (Olamendi-Portugal et al. 2008; Moreira et al. 2010; Rey-Suárez et al. 2012; Carbajal-Saucedo et al. 2013). Scale bar represents an average of 0.2 substitutions per site

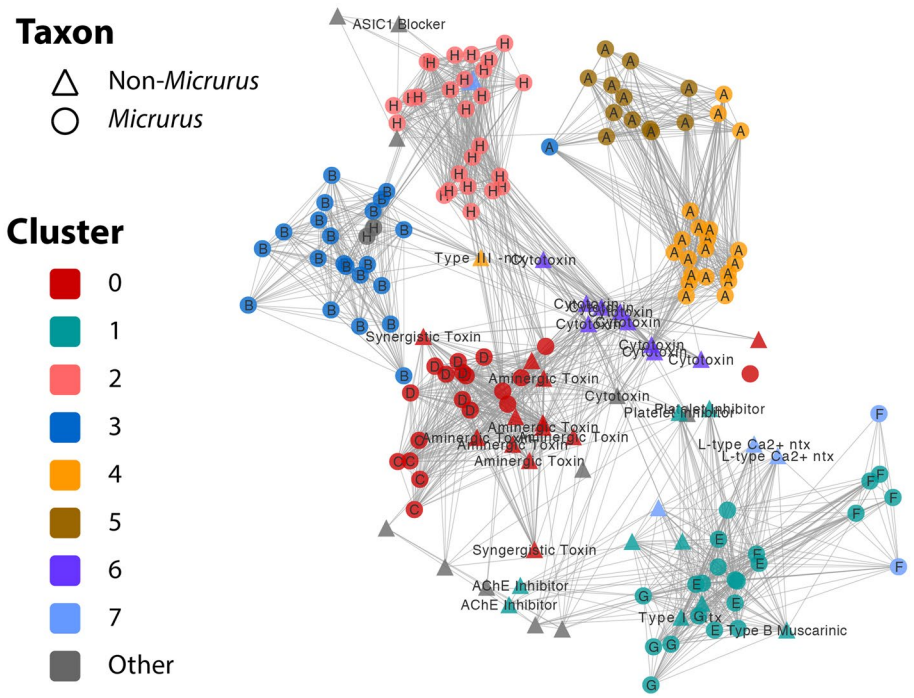

Fig. 2 Protein similarity network based on BLAST e-values. Colors correspond to CD-HIT clusters containing at least five sequences and labels that correspond to those found in Fig. 1

et al. 2010; Rey-Suárez et al. 2012). This could be due to Clade B's close relationship to the Type III α-neurotoxins (which do not conserve these residues, see Gong et al. 1999), a preservation of the putative ancestral activity despite these amino acid changes, or convergent evolution in function.

There are also clear differences in the rates and patterns of molecular evolution among the four of these clades for which sufficient nucleotide sequences were available for analysis (see Table 1). Clade A was the highest in terms of the overall ω value (a measure of the strength of selection based on rates of synonymous and non-synonymous mutations, also known as the $\frac{dN}{dS}$ ratio or $\frac{Ka}{Ks}$ ratio), the number of sites that FUBAR identified as being positively selected, the number of sites that MEME determined had been subject to episodes of diversifying selection, and the number of sites that were called by both FUBAR and MEME. On the other hand, Clade D had a much lower ω value and very few codons were indicated to be under positive selection by either site-by-site algorithm.

Figure 4 combines these tests for selection with protein structures predicted by the Phyre2 server and provides additional phylogenetic context. It should be noted that each clade is predicted to have a different structure than the others which once again suggests the potential for different activities as well. This is reinforced by the tendency for the diversifying selection to be more concentrated on the three loops, or fingers, of the proteins rather than the core. This is in spite of the fact that the eight structural cysteine residues, which are almost perfectly conserved across and within all eight clades, do not appear to be subject to strong negative selection. This somewhat paradoxical result comes from the fact that site-by-site selection algorithms will predict a very low rate of non-synonymous mutation for codons which have little to no synonymous mutations; when these codons also display very low rates of non-synonymous mutations, measures that compare the two—such as ω values or FUBAR's β-α—will tend to show neutral or weak negative selection.

Discussion

The pattern of evolution we see in the *Micrurus* 3FTx, where the amino acids in the core of the protein are subject to purifying selection while positive selection acts strongly on the rest is not a novel finding. This pattern of selection was previously reported and thoroughly discussed for other clades of 3FTx by Sunagar et al. (2013). What is notable are the extremely high overall ω values exhibited by some clades of *Micrurus* toxins (see Table 1). Of all the functional divisions studied by Sunagar et al. (2013) the highest reported ω value was 2.59 (belonging to the Type III α-neurotoxins, see Fig. 4). Margres et al. (2013) analyzed all of the *Micrurus* 3FTx—for which there were nucleotide sequences available at the time—together and found significant positive selection for over half the sites in their alignment averaging an ω value at those sites of 3.79. When averaged with the neutral and negatively selected sites from that analysis, the overall ω value for the *Micrurus* 3FTx was 2.43. This is in strong agreement with our results: the weighted mean of our ω values returns a value of 2.42. That being said, our phylogenetic

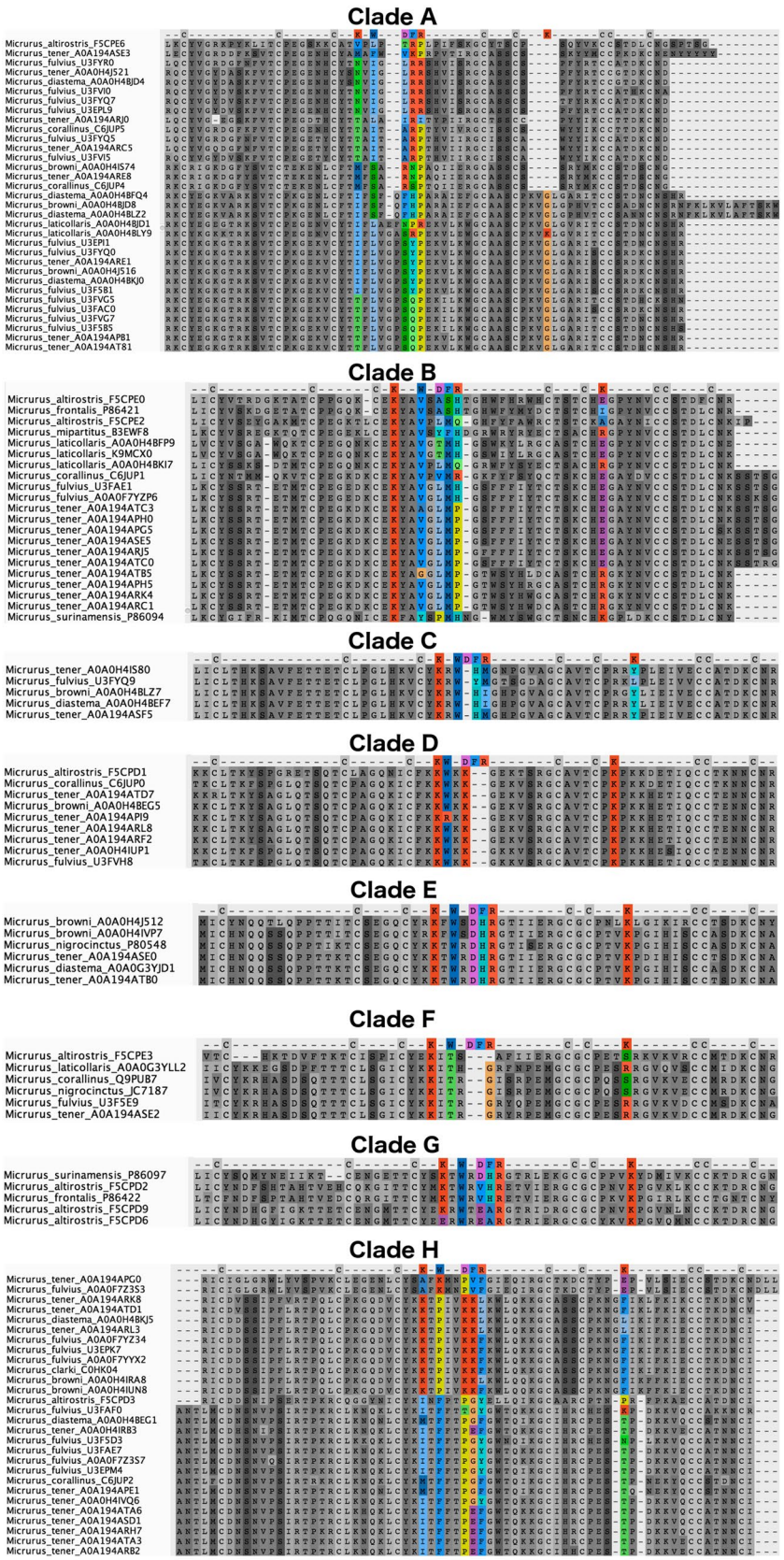

Fig. 3 Alignments of Micrurus 3FTx where the functional residues of α-neurotoxic 3FTx are highlighted. Amino acids are colored with AliView default colors: hydrophobic residues in blue, polar in teal and green, and charged residues in red and purple (chemically unique residues like C, H, G, and P are given unique colors). Percent identity at the functional sites: A—4%; B—19%; C—33%; D—48%; E—83%; F—17%; G—67%; H—7%

Table 1 Tests of selection on the various clades of *Micrurus* 3FTx

Clade	ω	FUBAR (−)[a]	FUBAR (+)[b]	MEME[c]	FUBAR and MEME[d]
A	3.39	0	24	19	16
B	2.16	1	11	10	6
D	1.51	1	2	0	0
H	1.78	2	12	10	6

[a]Number of codons under negative selection according to FUBAR

[b]Number of codons under positive selection according to FUBAR

[c]Number of codons under episodic diversifying selection according to MEME

[d]Number of codons that fit criteria [b] and [c]

analysis suggests that a clade-by-clade approach is more appropriate for these toxins and Clade A's ω value of 3.39 is considerably higher than any other that has been reported yet. For context, the highest reported ω value for a gene from the human male reproductive system (which "have evolved much more rapidly than other types of character") was 2.89 for Protamine 1 (Wyckoff et al. 2000, p. 304).

Many elapids produce 3FTx with a number of different activities and in Fig. 1 we can see that a given toxin is often more closely related to those from other species (including *Micrurus*) than they are to toxins with different activity from within the same species' venom. This suggests that the seeds of this functional diversity were sown before the coral snakes had diverged from the more derived elapids. Our results show that the toxins produced by *Micrurus* alone show a similar level of evolution (in terms of sequence diversity and diversifying selection) among themselves as do the 3FTx produced by all other elapids. When this is considered along with the fact that only two of the eight clades have been shown to have greater than 50% conservation of the functional α-neurotoxic residues, it seems that snakes of the genus *Micrurus* have evolved an array of 3FTx that are likely to have activities beyond α-neurotoxicity. It should be noted that it is still possible that the ancestral 8-cysteine 3FTx was α-neurotoxic and all of the *Micrurus* toxins have retained this activity in the face of rapid evolution at the interacting sites. This may seem unlikely, but those *Micrurus* sequences which have been shown to exhibit α-neurotoxic activity are not particularly closely related to each other (see Fig. 1). Most of these α-neurotoxins (P86095, P86422, K9MCH1, P86097) conserve all but one or two of the previously characterized functional residues, but two of the toxins (B3EWF8, P86421) do not conserve any of them (see Fig. 3). The discovery of two plesiotypic 10-cysteine 3FTx from *Micrurus mipartitus* that modulate $GABA_A$ activity, however, shows that novel activities are certainly possible within this particular toxin family and genus (Rosso et al. 2015). Functional testing of specific toxins must be performed across the full diversity of *Micrurus* 3FTx to settle the matter conclusively.

To date most studies of *Micrurus* 3FTx have not pursued the activities of individual toxins and those that have been specifically screened for the standard α-neurotoxicity (Rey-Suárez et al. 2011, 2016; Terra et al. 2015). This makes a

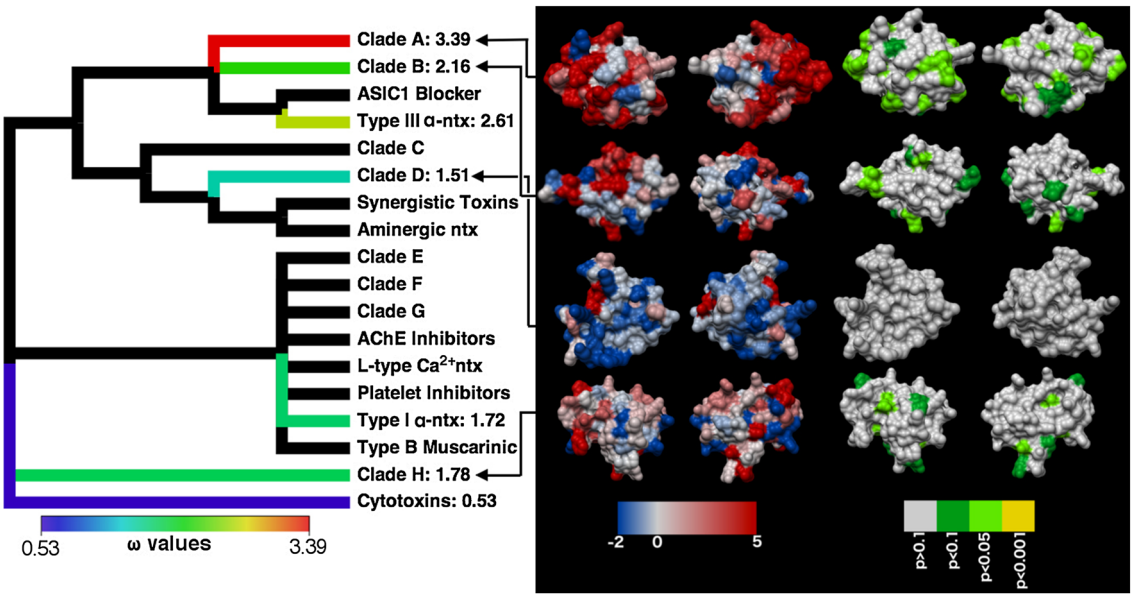

Fig. 4 Schematic phylogeny of Micrurus 3FTx clades and previously known functional divisions. Branches are colored according to ω values. Protein models show front and back views colored according to FUBAR's estimated strength of selection (β-α, left) and MEME's significance levels (right)

certain amount of sense given that the paralysis typical of α-neurotoxicity is the most significant clinical symptom of *Micrurus* bites (e.g., Norris et al. 2009). Unfortunately, these methods will never discover any toxins which exhibit activities beyond α-neurotoxicity. Since *Micrurus* mostly eat non-mammalian prey, an anthropocentric approach to studying their venom may overlook much of the relevant evolutionary history. In addition, it is these potential novel activities that hold the greatest possibilities for developing new scientific tools or laboratory techniques.

These results may even be relevant from a clinical perspective. If coral snake venoms do in fact contain 3FTx with a diverse range of activities, then simple regulatory changes could easily lead to major differences in the overall effects of the venoms on short time scales or between closely related populations. Even if this did not lead to a significant change in the function of the venom it could affect the cross-reactivity of antivenom against species that were not included in its production. Along with shifts between 3FTx and PLA2 dominated venoms, shifts within the 3FTx makeup could help explain the somewhat patchy cross-reactivity of *Micrurus* antivenoms (Lomonte et al. 2016; Yang et al. 2017).

In conclusion, most of the 3FTx produced in *Micrurus* venoms can be grouped into eight separate clades, which exhibit differences in their relationships to characterized 3FTx from other taxa, predicted structures, and rates of evolution. It was well known that *Micrurus* venoms contained a diversity of 3FTx, but the existence of these distinct clades, their affinity to better characterized toxins, and the difference in predicted structure was not known before now because other studies of *Micrurus* 3FTx had not included representative sequences from other genera to compare against. This variation in the sequence and structure of these toxins, especially in conjunction with evidence of diversifying selection, suggests that some of these clades may well have undergone neofunctionalization. Because of this we recommend that researchers use a wider variety of in vitro assays when characterizing the function of *Micrurus* 3FTx. Hopefully this will allow for the discovery of toxins with previously unknown functions within these venoms which would benefit our understanding of the evolutionary history of the genus as well as increasing their potential for biodiscovery.

Materials and Methods

Phylogeny

Protein sequences for *Micrurus* 3FTx and representative 3FTx with known functions were obtained from the UniProt database (Consortium 2017). This study was limited to 8-cysteine 3FTx because they account for the vast majority of reported sequences and analyses that included the 9- and 10-cysteine forms failed to converge properly (Sunagar et al. 2013). Only the mature peptide sequences were aligned and used as input due to the fact that the signal peptide was not available for all sequences. Details of the dataset can be found in Table 2. Many diverse, but monophyletic clades of 3FTx (such as those produced by Australian elapids) are represented by a single sequence. We reconstructed the phylogeny of these sequences using MrBayes 3.2 for `15,000,000` generations and `1,000,000` generations of burnin with `lset rates=invgamma` (allows rate to vary with some sites invariant and others drawn from a γ distribution) and `prset aamodelpr=mixed` (allows MrBayes to generate an appropriate amino acid substitution model by sampling from ten predefined models) (Ronquist et al. 2012). The run was stopped when convergence values fell below `0.01`. Nexus files containing the full alignment and MrBayes settings as well as the output tree can be found in the supplementary data.

Table 2 Composition of the 8-cysteine 3FTx dataset

Micrurus sequences	124
Species	13
M. altirostris	12
M. browni	9
M. clarki	1
M. corallinus	7
M. diastema	8
M. frontalis	3
M. fulvius	28
M. laticollaris	7
M. mipartitus	1
M. nigrocinctus	2
M. pyrrhocryptus	1
M. surinamensis	3
M. tener	42
Representative sequences	47
Genera	8
Aspidelaps	2
Bungarus	7
Dendroaspis	16
Hemachatus	5
Laticauda	2
Naja	11
Ophiophagus	3
Pseudonaja	1

Table 3 Phyre2 results for the various clades of Micrurus 3FTx

Clade	Query	PDB ID	%ID	Organism	Resolution
A	U3EPI1	1LSI	50	*Laticauda semifasciata*	NMR
B	A0A194APH0	4RUD	97	*Micrurus fulvius*	1.95 Å
D	A0A0H4BEG5	2VLW	42	*Dendroaspis angusticeps*	1.39 Å
H	A0A194ATA3	3PLC	47	*Ophiophagus hannah*	2.41 Å

Similarity Network

An all-vs-all BLAST search was conducted on the same dataset of protein sequences as was used for the phylogeny with `-outfmt "10 qacc sacc qcovs evalue"` (Altschul et al. 1990). The results of this search were filtered using a custom R script (see Supplementary Information) to remove self-to-self results, collapse bidirectional results into one entry, and create a *similarity* score defined as $-\log 10$ *e value* for each entry. Edges with *coverage* < 70% or *e value* $> 1 \times 10^{-10}$ were excluded from the analysis and the network was created in Cytoscape 3.5.1 using the `Prefuse Force Directed OpenCL` Layout on the `similarity` scores (Shannon et al. 2003).

Protein Clustering

Clustering was carried out using the CD-HIT 4.7 algorithm with options `-c 0.4 -n 2 -d 0 -sc 1 -g 1` (Li and Godzik 2006; Fu et al. 2012). This sets the similarity threshold of the clusters to 40% and sorts the clusters by the number of sequences they contain.

Functional Residues

The amino acid sequence of erabutoxin-a was aligned with each clade of *Micrurus* 3FTx in AliView 1.18 using the MUSCLE algorithm (Larsson 2014; Edgar 2004). All residues beside the structural cysteines and those identified by Antil et al. (1999) as functional residues were converted to gap characters for legibility. All resulting gap-only columns were then deleted.

Tests for Selection

Coding DNA sequences for all possible *Micrurus* 3FTx were compiled from GenBank (Benson et al. 2013). The sequences were trimmed to only include those codons which translate to the mature protein, translated, aligned, and reverse translated using AliView and the MUSCLE algorithm (Larsson 2014; Edgar 2004). The resulting codon alignments can be found in the supplementary data.

Phylogenetic trees for each clade were generated from the resulting codon alignments using similar methods as described above. This tree topology was used for all subsequent analyses.

We used several of the tests for selection implemented in HyPhy version 2.220150316beta due to their different emphases (Pond et al. 2005). The AnalyzeCodonData analysis generates overall ω values for an alignment while the FUBAR method gauges the strength of consistent positive or negative selection on individual amino acids (Murrell et al. 2013). In contrast, the MEME method identifies individual sites that were subject to episodes of diversifying selection in the past (Murrell et al. 2012).

Protein Modeling

Custom models for each clade of *Micrurus* 3FTx were generated by inputting representative sequences (Clade A—U3EPI1; Clade B—A0A194APH0; Clade D—A0A0H4BEG5; Clade H—A0A194ATA3) to the Phyre2 webserver using the Intensive option (Kelley et al. 2015). The details of the queries and results can be found in Table 3.

Alignments of each clade were trimmed to match these structures and attribute files were created from FUBAR and MEME results. Conservation scores were calculated using the default settings of AL2CO (Pei and Grishin 2001). The structures were rendered and colored according to these attributes in UCSF Chimera version 1.10.2 (Pettersen et al. 2004). Full images from each clade for all these attributes can be found in Supplementary Figs. 1–4.

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