Convergent Substitutions in a Sodium Channel Suggest Multiple Origins of Toxin Resistance in Poison Frogs

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Abstract

Complex phenotypes typically have a correspondingly multifaceted genetic component. However, the genotype–phenotype association between chemical defense and resistance is often simple: genetic changes in the binding site of a toxin alter how it affects its target. Some toxic organisms, such as poison frogs (Anura: Dendrobatidae), have defensive alkaloids that disrupt the function of ion channels, proteins that are crucial for nerve and muscle activity. Using protein-docking models, we predict that three major classes of poison frog alkaloids (histrionicotoxins, pumiliotoxins, and batrachotoxins) bind to similar sites in the highly conserved inner pore of the muscle voltage-gated sodium channel, Nav1.4. We predict that poison frogs are somewhat resistant to these compounds because they have six types of amino acid replacements in the Nav1.4 inner pore that are absent in all other frogs except for a distantly related alkaloid-defended frog from Madagascar, *Mantella aurantiaca*. Protein-docking models and comparative phylogenetics support the role of these replacements in alkaloid resistance. Taking into account the four independent origins of chemical defense in Dendrobatidae, phylogenetic patterns of the amino acid replacements suggest that 1) alkaloid resistance in Nav1.4 evolved independently at least seven times in these frogs, 2) variation in resistance-conferring replacements is likely a result of differences in alkaloid exposure across species, and 3) functional constraint shapes the evolution of the Nav1.4 inner pore. Our study is the first to demonstrate the genetic basis of autoresistance in frogs with alkaloid defenses.

Key words: **chemical defense, autoresistance, voltage-gated sodium channels, functional constraint, alkaloid docking, aposematism.**

Article

Introduction

Chemical defense has evolved in almost every major taxon, from protists to plants, insects, and vertebrates (Dumbacher et al. 2000; Mebs 2002; Wortman-Wunder and Vivanco 2011). Defended organisms synthesize their own chemicals, sequester them from the environment, or harbor toxic symbionts (Noguchi et al. 1986; Laurent et al. 2005). These organisms often have a complicated evolutionary history that requires tandem origins of toxin acquisition, unique morphological or physiological adaptations, and ecological transformations. Autoresistance, or the mechanism by which an organism avoids self-intoxication, is a component of this phenotype, predicted to evolve as a consequence of increased toxin exposure during the evolution of defense (Després et al. 2007; Petschenka and Dobler 2009; Aardema et al. 2012; Agrawal et al. 2012; Bramer et al. 2015; Hanifin and Gilly 2015). Molecular and physiological mechanisms that underlie the implied autoresistance of chemically defended organisms have been widely studied in invertebrates (e.g., Ffrench-Constant et al. 2004; Bricelj et al. 2005; Dobler et al. 2011; Petschenka, Pick, et al. 2013), but less so in vertebrates, with a few exceptions including tetrodotoxin (TTX) resistance in newts, snakes, and pufferfish (Kaneko et al. 1997; Jost et al. 2008; Feldman et al. 2012; Hanifin and Gilly 2015), and resistance to cobra and scorpion venoms in snakes and mammals (Barchan et al. 1992; Takacs et al. 2001; Rowe et al. 2013). Using transcriptome sequencing and computational modeling, we determine the genetic underpinnings of resistance to diet-derived alkaloids in poison frogs by identifying changes in one molecular target of their alkaloids and analyzing how such changes affect alkaloid binding.

Many salamanders (Caudata) and frogs (Anura) have some form of defense, including antimicrobial proteins (e.g., opioid peptides in *Phyllomedusa*), proteolytic enzymes (e.g., in the venomous *Aparasphenodon*), and toxic alkaloids (e.g., *Cynops*, *Taricha*, Dendrobatidae, and *Mantella*) (Erspamer et al. 1985; Garraffo et al. 1993; Hanifin et al. 1999; Tsuruda et al. 2002; Daly et al. 2004; Jared et al. 2015). Alkaloid defense is especially interesting as its origin involves a complex interplay among diet specialization, alkaloid sequestration, and accumulation, as well as autoresistance (Santos and Cannatella 2011; Saporito et al. 2012; Santos et al. 2016). One notable example is poison frogs (Dendrobatidae sensu Santos et al. 2009), a Neotropical clade of greater than 300 species in which alkaloid defense has evolved four times (Santos et al. 2003, 2014; Santos and Cannatella 2011). Poison frogs sequester lipophilic

alkaloids from arthropods into specialized skin glands (Neuwirth et al. 1979; Saporito et al. 2009, 2012). Many of these chemicals deter diverse predators (Darst et al. 2006) because of their bitter taste, and a subset can be lethal to vertebrates, fungi, parasites, and bacteria (reviewed by Santos et al. 2016). Mechanisms underlying sequestration of and resistance to dietary alkaloids in poison frogs are currently unknown. However, given the information available regarding origins of their alkaloid defense, diversity, and function, dendrobatids provide a framework for investigating the genetic basis of alkaloid resistance.

Three broad mechanisms underlie chemical resistance: 1) compartmentalization (e.g., in a specialized gland), 2) metabolic detoxification (e.g., increased expression of P450 enzymes), and 3) target-site insensitivity; that is, amino acid changes in the molecular target of the compound that alter the toxin's ability to bind (Li et al. 2002; Francis et al. 2005; Petschenka and Dobler 2009; Giannotti et al. 2013; Shear 2015). Organisms that are exposed to high levels of pesticides (e.g., cockroaches) or that accumulate chemicals for their own defense (e.g., danaine butterflies, *Taricha* newts) tend to have target-site insensitivity (Després et al. 2007; Aardema et al. 2012; Savitzky et al. 2012; Zakon 2012; Bramer et al. 2015; Hanifin and Gilly 2015). Hence, we expect that poison frogs that sequester defensive alkaloids should have target-site insensitivity.

Although dendrobatid alkaloid profiles vary intraspecifically and across genera (Daly et al. 2005; Saporito et al. 2007), their defensive chemicals mostly affect ion channels (Daly and Spande 1986). These are pore-forming proteins that open and close in response to ligand binding or voltage changes, allowing ions to pass through cell membranes. The voltage-gated sodium channel (VGSC, gene family Nav1) is highly conserved and physiologically critical because it initiates action potentials in the heart, skeletal muscle, and nervous system (Hille 2001). There are six Nav1 paralogs in frogs (Zakon 2012). Amino acid (AA) replacements in the pore of snake (*Thamnophis*) and newt (*Taricha*) VGSCs render them less sensitive to TTX, allowing them to sequester TTX or consume TTX-rich prey (Geffeney et al. 2005; Hanifin and Gilly 2015). In contrast to newts, poison frogs have hundreds of defensive alkaloids (Daly et al. 2005). Three major classes of their alkaloids affect VGSCs: batrachotoxins (BTX, 6 of 523 alkaloids known from poison frogs, also the most toxic class), histrionicotoxins (HTX, 16 of 523), and pumiliotoxins/allopumiliotoxins (PTX/aPTX, 55 of 523) (Saporito et al. 2012). These compounds either prevent the passage of sodium ions by blocking the channel pore (fig. 1A) or allow increased passage of sodium ions by preventing channel inactivation (Daly et al. 1985; Vandendriessche et al. 2008). Such effects can cause temporary paralysis and even death (Karalliedde 1995).

Given the lethality of these substances, our limited knowledge regarding how poison frogs resist their own alkaloid defenses is surprising. The only experiments were carried out in the 1970s and 1980s; these demonstrated that *Phyllobates* species are resistant to BTX and that *Dendrobates histrionicus* is somewhat resistant to HTX but not to BTX (Albuquerque et al. 1973; Albuquerque, Kuba, Daly, et al. 1974; Daly et al. 1980). Breeding experiments showed that the resistance to BTX in *Phyllobates* is heritable, suggesting that poison frog autoresistance has a genetic component (Daly et al. 1980). Indeed, certain genetic changes in VGSCs are known to decrease sensitivity to BTX (Wang S-Y and Wang GK 1999; Du et al. 2011). In spite of these results, the basis of resistance in poison frogs remains unstudied.

We focus on the skeletal muscle VGSC, Nav1.4, which is one of three Nav1 paralogs expressed outside of the central nervous system in frogs and is likely exposed to relatively high levels of alkaloids (Zakon 2012; McGlothlin et al. 2014). Based on preliminary sequence data showing AA replacements in the Domain I and IV inner pore regions (S6) of Nav1.4 in some poison frogs, we sequence and reconstruct these regions across three origins of chemical defense in Dendrobatidae. We then analyze the binding position and affinity of seven poison frog alkaloids (BTX, HTX, and five PTXs) to a model of Nav1.4 with and without AA replacements found in these frogs. We confirm the hypothesized inner-pore binding site of BTX (Du et al. 2011) and propose similar binding sites for HTX and PTX, which were previously unknown. We identify six types of AA replacements in the inner pore of the poison frog Nav1.4; comparative phylogenetic analyses and docking models predict these confer some alkaloid resistance and have likely evolved in response to alkaloid exposure. Our findings are the first step toward understanding how frogs resist multiple toxins and how chemical defense diversified in this remarkable group of amphibians.

Results

Amino Acid Replacement Identification

From our transcriptome assemblies and targeted polymerase chain reaction (PCR) sequencing we obtained Nav1.4 inner pore (S6) sequences for 24 dendrobatids and 6 other frog species; sequences from nine additional vertebrate outgroups were obtained from GenBank (see supplementary tables S1 and S2, Supplementary Material online, for accession numbers). We found six types of AA replacements in Nav1.4 at three sites in DI-S6: 429, 433, 446 and two sites in DIV-S6: 1583, 1584 (fig. 1, see supplementary table S3, Supplementary Material online, for DII- and DIII-S6 AA alignments). One of these replacements (A446D) has evolved independently in the aposematic Malagasy frog *Mantella*, which has PTX defense convergent with dendrobatids (Garraffo et al. 1993). All five sites are intriguing because these residues are highly conserved among vertebrates, and the replacements are unique to poison frogs and *Mantella* (fig. 1B). Moreover, the random occurrence of these AA replacements is unlikely because they evolved via a first- or second-position nucleotide transversion, and there are no transitions or transversions at the same positions in any other vertebrate taxa (table 1 and table S4, Supplementary Material online).

Evolutionary Analyses

Ancestral reconstruction predicted independent origins of the following replacements (fig. 2A): five origins of V1583I,

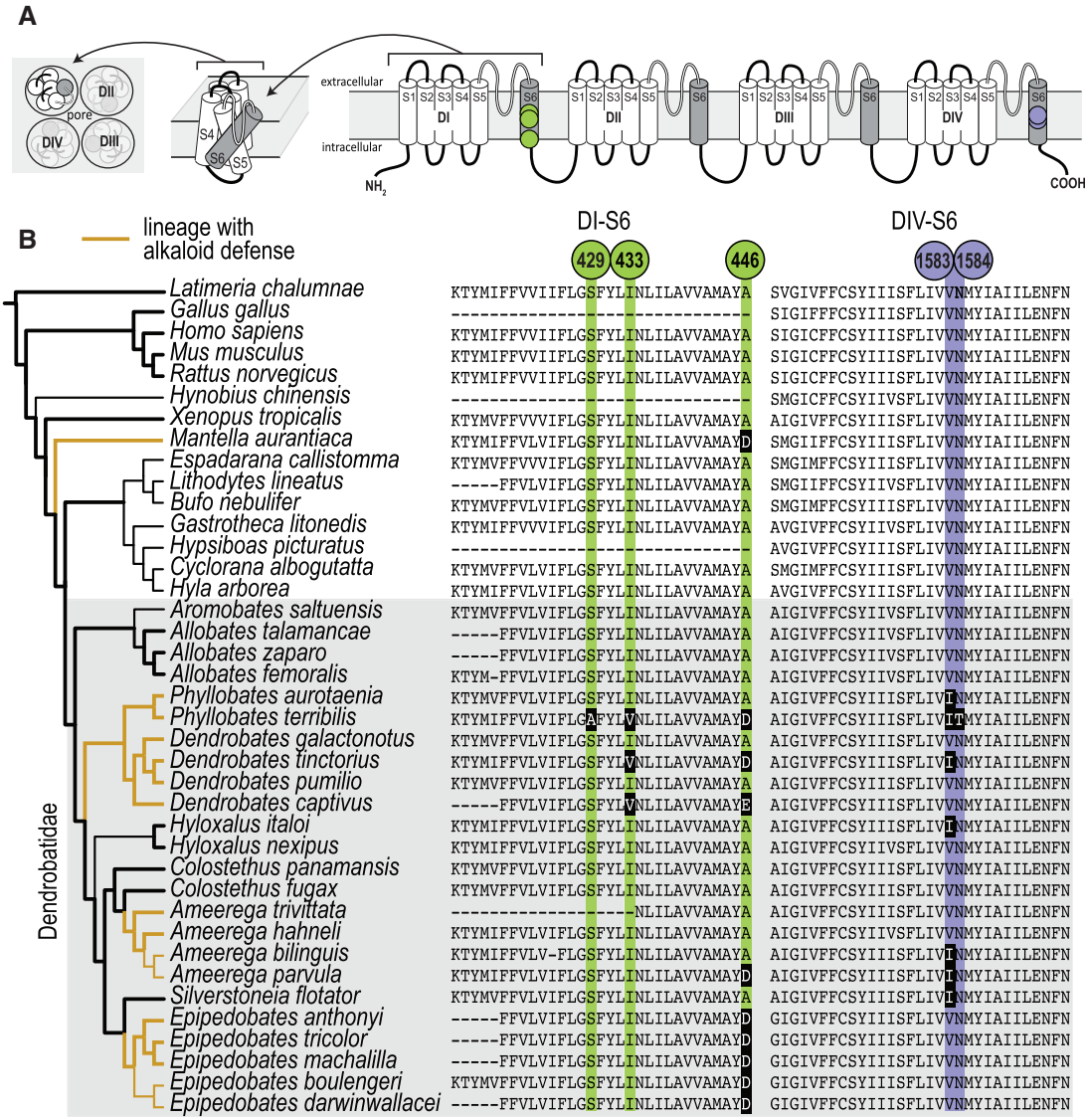

Fig. 1. (*A*) A schematic of Nav1.4 shows its four homologous domains (DI–DIV), each of which contains six transmembrane helices (S1–S6) that surround the pore through which sodium ions pass. The first four helices (S1–S4) are responsible for voltage-dependent conformation changes that open and close the channel, whereas the pore region (S5–S6) is involved in channel inactivation and responsible for selectivity in ion permeation. The outer pore is formed by DI–DIV pore loops (S5–S6 extracellular linkers). The suspected binding site of BTX and our hypothesized binding site of HTX and PTX is the inner pore, which is formed by the four DI-DIV S6 helices (dark gray). (*B*) Five residues in Nav1.4 S6 helices were identified to have AA replacements in poison frogs; one of these has evolved convergently in *Mantella*, the Malagasy poison frog. Thick branches indicate bootstrap support greater than 85%.

five origins of A446D/E, three origins of I433V, and one origin each of S429A and N1584T. The pattern of these replacements across the dendrobatid phylogeny supports seven origins of replacements in the poison frog Nav1.4 inner pore (fig. 2*A*).

The predicted ancestral states of these residues in the most recent common ancestor (MRCA) of Dendrobatidae were: serine, isoleucine, alanine, valine, and asparagine [P(S429) $\approx$ 1.000, P(I433) = 0.993, P(A446) = 0.994, P(V1583) = 0.649, P(N1584) $\approx$ 1.000]; they were the same for the MRCA of Dendrobatinae [includes all clades but *Aromobates* + *Allobates*; P(S429) $\approx$ 1.000, P(I433) = 0.996, P(A446) = 0.999, P(V1583) = 0.657, P(N1584) $\approx$ 1.000]. Our correlation analyses (Pagel's test and Monte Carlo simulations by Mesquite [Maddison and Maddison 2015]) suggested that two replacements were significantly correlated with alkaloid defense. There was an association of A446D/E with origins of chemical defense in *Ameerega*, *Epipedobates*, and *Phyllobates* + *Dendrobates* ($\Delta[-\log(L)]$ = 3.9, $P < 0.001$) and of I433V with chemical defense in *Phyllobates* + *Dendrobates* ($\Delta[-\log(L)]$ = 2.2, P = 0.0330). In contrast, V1583I was not significantly correlated with origins of defense ($\Delta[-\log(L)]$ = 0.5, P = 0.369). Finally, selection analyses did not identify any sites under positive selection, but three sites that were not associated with chemical defense were identified to be under strong purifying selection, namely: V422 (dN−dS = −7.00, P = 0.000457), A439 (dN−dS = −7.00, P = 0.000457), and V1582 (dN−dS = −3.00, P = 0.0370).

Table 1. Effect of (A) Single and (B) Multiple AA Replacements Found in Poison Frogs on Alkaloid Binding Affinities to Nav1.4.

AAReplacement Pattern[a]	Nucleotide Mutation(s)	Nucleotide Mutation Position(s)	Species[b] with Mutation(s)	BTX			HTX			aPTX 267A			aPTX 323B			PTX 307A			PTX 323A			PTX 251D		
				P[c]	*W*[c]	ΔΔG (kJ)	*P*	*W*	ΔΔG (kJ)	*P*	*W*	ΔΔG (kJ)	*P*	*W*	ΔΔG (kJ)	*P*	*W*	ΔΔG (kJ)	*P*	*W*	ΔΔG (kJ)	*P*	*W*	ΔΔG (kJ)
None[d]	—	—	—	1.000	113	0.000	1.000	113	0.000	1.000	113	0.000	1.000	113	0.000	1.000	113	0.000	1.000	113	0.000	1.000	113	0.000
(A) Single AA Replacements[e]																								
S429A	T → G	1	*P. terribilis*	*	63	0.226	0.796	106	0.0133	0.412	132	−0.0533	1.000	113	0.00667	0.510	128	−0.0200	1.000	113	−0.00667	0.432	93.5	0.0467
I433V	A → G	1	*D. captivus* *D. tinctorius* *P. terribilis*	0.157	143	−0.0267	0.366	134	−0.0533	0.659	123	−0.0200	0.763	120	−0.0600	0.757	106	0.00667	0.419	93	0.0333	**	187	−0.267
A446D	C → A	2	*M. aurantiaca* *P. terribilis* *D. tinctorius* *A. parvula* *E. spp*	0.906	116	0.0133	0.879	117	−0.00667	0.669	103	0.00667	0.438	95	0.0333	0.825	108	0.00667	0.702	122	−0.0400	0.949	111	0.00667
A446E	C → A, C → A	2, 3	*D. captivus*	1.000	113	0.000	0.931	110	0.00667	0.408	132	−0.0400	0.748	120	−0.00667	1.000	113	0.000	0.671	123	−0.0333	0.931	110	0.00667
V1583I	G → A	1	*H. italoi* *A. bilinguis* *A. parvula* *D. tinctorius* *P. aurotaenia* *P. terribilis* *S. flotator*	**	0.000	0.613	**	0.000	0.593	**	0.000	0.460	**	0.000	0.767	**	0.000	0.993	0.0523	66	0.247	**	44	0.173
N1584T	C → A	2	*P. terribilis*	1.000	113	0.000	1.000	113	0.000	1.00	113	−0.0133	0.875	109	0.00667	1.000	113	0.000	0.933	115	−0.0133	0.93	110	0.00667
(B) Species-Specific Multiple AA Replacement Patterns																								
A446D V1583I	—	—	*A. parvula*	**	0.000	0.653	**	0.000	0.647	**	0.000	0.420	**	0.000	0.800	**	0.000	1.03	*	62	0.267	**	27	0.213
I433V A446E	—	—	*D. captivus*	0.157	143	−0.0267	0.830	118	−0.0267	0.826	118	−0.0133	0.966	111	−0.0333	0.407	94	0.0267	0.254	85.0	0.0733	0.151	147	−0.113
I433V A446D V1583I	—	—	*D. tinctorius*	**	0.000	0.773	**	0.000	0.600	**	0.000	0.560	**	0.000	0.860	**	0.000	0.967	**	48	0.347	**	50	0.173
S429A I433V A446D V1583I N1584T	—	—	*P. terribilis*	**	0.000	0.740	**	0.000	0.613	**	0.000	0.387	**	0.000	0.873	**	0.000	0.967	**	48	0.340	**	39	0.207

[a]According to the rat Nav1.4 protein (NCBI accession number NM_013178.1).

[b]*A., Ameerega; D., Dendrobates; E., Epipedobates; H., Hyloxalus; M., Mantella; P., Phyllobates; S., Silverstoneia.*

[c]Test statistic, *W*, and p-value, *P*, are from unpaired Wilcoxon rank-sum tests.

[d]Walker1 model without replacements (Walker et al. 2012).

[e]This section includes species-level AA replacement patterns for species with only one replacement.

*$0.01 < P < 0.05$.

**$P < 0.01$.

Ecological Analyses

When accounting for phylogeny, there were no significant correlations between alkaloid quantity or diversity and resistance or number of replacements ($P > 0.100$; fig. 2B). Overall, our ecological analyses suggest that alkaloid profiles in poison frogs are independent from individual AA replacements in the Nav1.4 protein, except perhaps for BTX (but see Discussion). When we did not control for phylogeny, BTX quantity correlated with alkaloid resistance (nonparametric Spearman correlation tests; resistance to PTX: $S = 615$, $\rho = 0.538$, $P = 0.0144$; HTX: $S = 653$, $\rho = 0.509$, $P = 0.0219$; BTX: $S = 615$, $\rho = 0.538$, $P = 0.0144$; total resistance: $S = 615$, $\rho = 0.538$, $P = 0.0144$). BTX quantity also showed a tendency to be correlated with the number of replacements in Nav1.4, but this was not significant ($S = 783$, $\rho = 0.411$, $P = 0.0715$). Not surprisingly, BTX quantity was related to presence of the S429A and N1584T replacements ($W = 18.5$, $P = 0.00461$; $W = 18.5$, $P = 0.00461$), which only occur in *Phyllobates terribilis*, and also to V1583I ($W = 56.0$, $P = 0.0323$). PTX quantity was only marginally related to the presence of

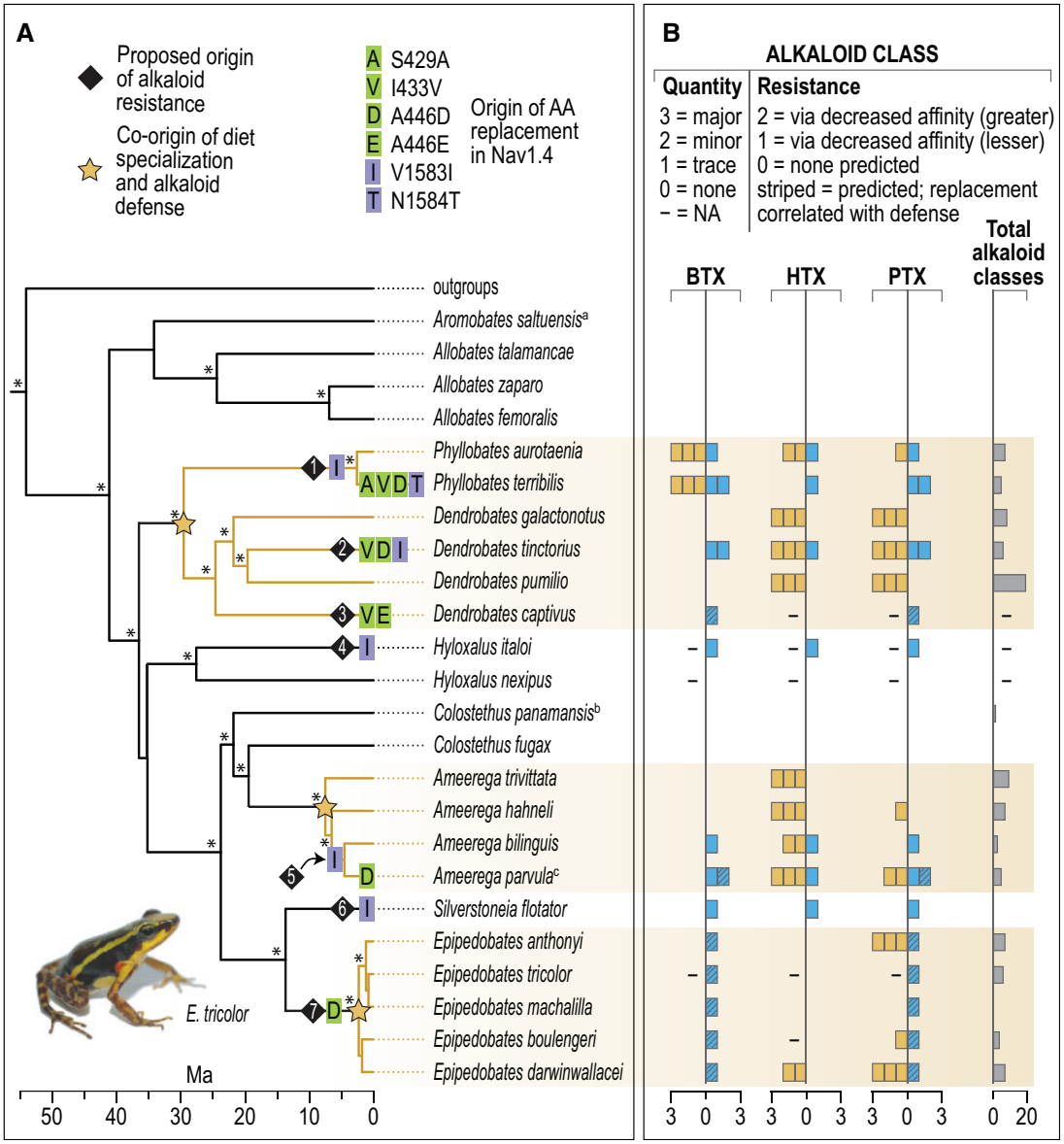

Fig. 2. (*A*) Ancestral state reconstruction shows that three replacements in Nav1.4 (I433V, A446D/E, V1583I) evolved multiple times independently in different poison frog clades; two more (S429A, N1584T) have each evolved once in the most toxic species, *Phyllobates terribilis*. In total, we predict seven origins of autoresistance in Dendrobatidae within approximately the last 10 My. (*B*) Our docking models predict that resistance to alkaloids is conferred by S429A and V1583I replacements in Nav1.4, which decrease alkaloid binding affinity (open boxes, right); phylogenetic correlation of A446D/E and I433V replacements with alkaloid defense predicts that these replacements also provide some resistance (striped boxes, right). Overall, there is no clear correlation between alkaloid quantity (boxes, left) and predicted resistance (boxes, right). Chronogram and alkaloid data are from Santos et al. (2009): Asterisks indicate 1.0 posterior probability and >85% bootstrap support; trace alkaloid ≤50μg/100mg skin, minor ≥50 and <150 μg/100mg skin, major ≥150 μg/100 mg skin; —, missing data. [a]Alkaloid data for *Aromobates saltuensis* are from *Aromobates nocturnus*. [b]Defended by TTX; diet generalist. [c]Some resistance predicted by docking analyses (open box); increased resistance predicted by the presence of A446D (striped box). Photograph by RDT.

A446D/E (W = 66.0, P = 0.0795), and HTX quantity was not related to any replacement.

Docking Analyses

We found that three alkaloid classes (i.e., PTX, HTX, and BTX) interacted with multiple residues in all four S6 regions of the Nav1.4 protein (fig. 3). The top three predicted docking positions for each alkaloid were consistent, except for PTX 323A, whose first docking position is different and likely more accurate than its second and third predicted docking positions (supplementary table S5, Supplementary Material online, see Discussion). Even though we did not find any AA replacements in the pore loop regions of the species sequenced, our models suggested that some alkaloids (e.g., HTX) interacted with residues in the p-loops of DI, DIII, and DIV (supplementary table S5, Supplementary Material online). These results imply that some regions where alkaloids dock (e.g., pore loops) might not allow AA replacements without seriously compromising VGSC function.

We modeled the effect on alkaloid binding of each single AA replacement and of each unique AA replacement pattern found in species with multiple replacements (table 1A and 1B, respectively). A model of Nav1.4 with the AA replacement pattern of *P. terribilis*, the species with the most replacements, shows that, together, these AA replacements altered the docking positions of all seven toxins (fig. 3). Two replacements independently decreased alkaloid-binding affinity: S429A in DI-S6 decreased the affinity of BTX and V1583I in DIV-S6 decreased the binding affinity of BTX, HTX, and all five PTXs (table 1). Another replacement in DI-S6 that we suspected to be related to resistance, I433V, actually increased the binding affinity of PTX 251D and had no significant effect on other alkaloid dockings. However, all three species-level patterns including this replacement (*Dendrobates tinctorius*, *D. captivus*, and *P. terribilis*) did not have a significantly higher PTX 251D binding affinity than the Nav1.4 model without mutations.

Our results support synergistic increases in alkaloid resistance in species with multiple AA replacements in Nav1.4: alkaloid resistance is higher in these species than would be predicted from total additive effect from individual changes. For example, only one of the three replacements found in *Dendrobates tinctorius* significantly affects BTX binding when modeled alone (V1583I), yet the full *D. tinctorius* species model (including replacements I433V and A446D) shows a greater decrease in BTX, aPTX 267A, and aPTX 323B binding

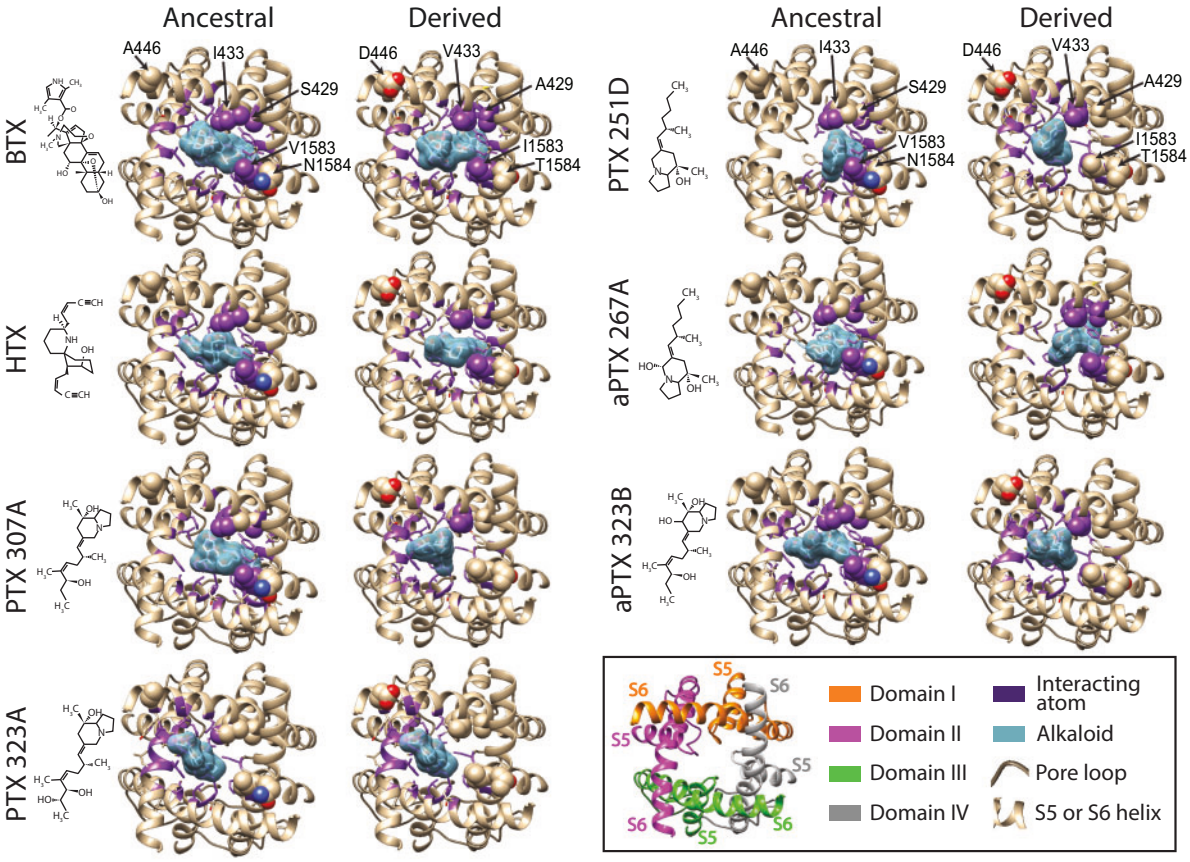

Fig. 3. Seven poison frog alkaloids were predicted to bind to the inner pore of the Walker1 Nav1.4 model (Walker et al. 2012 Dataset 1, "Ancestral") using Autodock Vina (Trott and Olson 2010). Models including the five inner pore amino acid replacements ("Derived") that were identified in the poison frog *Phyllobates terribilis* (shown in spherical form) altered the optimal docking sites of all seven alkaloids; two replacements (S429A and V1583I) significantly lowered their binding affinity.

affinity than predicted for V1583I alone (nonparametric Wilcoxon–Mann–Whitney tests; BTX: $\Delta\Delta G_{D.\ tinctorius} - \Delta\Delta G_{V1583I}$ = 0.160 kJ, W = 54.0, P = 0.0134; aPTX 267A: $\Delta\Delta G_{D.\ tinctorius} - \Delta\Delta G_{V1583I}$ = 0.100 kJ, W = 75.0, P = 0.0579; aPTX 323B: $\Delta\Delta G_{D.\ tinctorius} - \Delta\Delta G_{V1583I}$ = 0.0933 kJ, W = 68.0, P = 0.0569). The *Phyllobates terribilis* multiple mutation model (S429A, I433V, A446D, V1583I, N1584T) also decreased BTX and aPTX 323B binding affinity significantly more than did the V1583I replacement alone (BTX: $\Delta\Delta G_{P.\ terribilis} - \Delta\Delta G_{V1583I}$ = 0.127 kJ; W = 60.0, P = 0.0249; aPTX 323B: $\Delta\Delta G_{P.\ terribilis} - \Delta\Delta G_{V1583I}$ = 0.107 kJ; W = 62.0, P = 0.0277).

Discussion

With such a variety of diet-derived alkaloids, how do poison frogs avoid poisoning themselves? Based on observations from other systems, we predicted two sources of selection for alkaloid resistance in poison frogs: long-term low-level exposure to alkaloids via a generalist diet and shorter-term high-level exposure resulting from dietary specialization and alkaloid sequestration during the evolution of defense (Després et al. 2007; Dobler et al. 2011; Hua et al. 2013). Nav1.4 is a known molecular target of three classes of alkaloids found in poison frogs and their diets: BTX, PTX, and HTX (Daly and Spande 1986; Vandendriessche et al. 2008; Saporito et al. 2009). Hence, we expected to find AA replacements at alkaloid binding sites of Nav1.4 that evolved in the ancestor of dendrobatids, with additional replacements occurring in the lineages of chemically defended species.

Alkaloid Docking Sites on Nav1.4

Target-site insensitivity is caused by AA replacements that reduce the ability of a toxin to bind to its molecular target. For example, BTX inhibits inactivation of Nav1.4 by binding to its inner pore (S6 helices); substitutions in the inner pore of cockroach and rat Nav1.4 confer decreased sensitivity to BTX (Wang S-Y and Wang GK 1998, 1999; Du et al. 2011). However, the exact docking sites of HTX and PTX are unknown, so inferences about target-site insensitivity to these alkaloids are more speculative. Many PTX/aPTXs (e.g., 307A, 323A, 323B, 267A) inhibit the inactivation of VGSCs, so they may bind to the same site as BTX (Daly and Spande 1986; Daly et al. 1990; Wang et al. 2003). At least one PTX (251D) has the opposite effect and blocks sodium flux of VGSCs (Vandendriessche et al. 2008); nevertheless, it also shifts Nav1.4 activation and inactivation to more negative potentials and reduces ion selectivity. These effects are consistent with other toxins that bind to S6 regions (Cestèle and Catterall 2000; Wang S-Y and Wang GK 2003; Vandendriessche et al. 2008; Stevens et al. 2011). HTX blocks VGSCs at very high concentrations and inhibits the binding of BTX to VGSCs, suggesting that they may also interact with S6 segments close to the BTX binding site (Daly and Spande 1986).

Combining this physiological evidence and results from protein-docking analyses (fig. 3), we predict that HTX, BTX, and PTX all have binding sites in close proximity in the Nav1.4 inner pore (DI-DIV S6). We also confirm that the binding site for PTX 323A is allosteric to that of BTX (Gusovsky et al. 1988), as BTX interacted most strongly with DI-S6 and DIV-S6, whereas PTX 323A interacted more with DII-S6 and DIII-S6 (fig. 3, supplementary table S5, Supplementary Material online). We recognize that our docking analyses are subject to the accuracy of these predicted binding sites. However, our results predict that BTX interacts with 9 of the 11 AA sites suggested by Du et al. (2011) to be important for BTX binding. Therefore, we consider the Walker1 model (Walker et al. 2012) capable of estimating binding sites for HTX and PTX.

Origins of AA Replacements in the Poison Frog Nav1.4

Because BTX, HTX, and PTX were predicted to share close binding sites on Nav1.4, any replacement in the Nav1.4 S6 regions might provide resistance to a variety of alkaloids, facilitating evolution of broad alkaloid resistance by very few mutations. In Dendrobatidae, chemical defense and diet specialization have evolved in parallel at least three times (fig. 2A): approximately 30 Ma in the MRCA of *Phyllobates* + *Dendrobates*, approximately 10 Ma in the MRCA of *Ameerega*, approximately 7 Ma in the MRCA of *Epipedobates*, and perhaps also approximately 25 Ma in *Hyloxalus* (Darst et al. 2005; Santos et al. 2009, 2014). However, we found no single replacement that is ancestral to all origins. In fact, all replacements appear to have evolved relatively recently, within the last 10 My, and only one preceded or evolved in parallel to an origin of defense (fig. 2A, No. 7). This suggests that long-term low-level consumption of alkaloids in a generalist ancestor may not have played a driving role in the evolution of alkaloid resistance, at least in Nav1.4. Instead, we hypothesize that independent origins of diet specialization and alkaloid sequestration resulted in repeated selection for alkaloid resistance. Although we did not find support for positive selection in DI-S6 or DIV-S6 (small sample size and low mutation rates probably decreased statistical power and skewed estimations of expected rates of AA change), convergence of the same AA replacements is likely evidence of strong selection in the face of functional constraint. Indeed, target-site insensitivity often evolves predictably, resulting in identical genetic changes across divergent organisms exposed to the same toxin (Dobler et al. 2012; Zhen et al. 2012). This is likely mediated by a trade-off between benefit of resistance and physiological cost of channel efficiency (Jost et al. 2008; Lee et al. 2011; Feldman et al. 2012; Ujvari et al. 2015).

Species-Level AA Replacement Patterns and Their Relationship with Alkaloid Defenses

Although none of the replacements we identified evolved deep in the dendrobatid phylogeny, there is some evidence that they could have promoted the evolution of chemical defense in poison frogs. For example, one of the predicted origins of V1583I is in the ancestor of *Phyllobates* (fig. 2A, No. 1); because this replacement is suspected to provide broad alkaloid resistance, we hypothesize that it could have

facilitated the evolution of extreme toxicity in *P. terribilis*, the only species of poison frog that can kill large mammals including humans (Myers et al. 1978). A moderate level of resistance from the V1583I replacement could have allowed *P. terribilis* to consume higher levels of BTX, which would have increased selection for resistance, resulting in the accruement of four additional replacements in this species (S429A, I433V, A446D, and N1584T) and a concurrent possibility for increase in toxicity. S429A was indeed predicted to decrease BTX binding affinity by our docking analyses; A446D and I433V were predicted by phylogenetic correlation to also play a role in alkaloid resistance. Although N1584T did not significantly affect alkaloid binding affinity, site-directed mutagenesis suggests that it and residue 433 are both part of the BTX binding site (Wang S-Y and Wang GK 1998, 1999). That these additional replacements do not occur in *P. aurotaenia* is not surprising because *P. terribilis* is known to be much more toxic, and resistance tends to increase with toxicity (Feldman et al. 2009; Petschenka, Fandrich, et al. 2013). Such stepwise increases in resistance and toxicity have also been observed in newts and their garter-snake predators (Geffeney et al. 2005; Williams et al. 2012; Hanifin and Gilly 2015) and across many insect herbivores and their hosts (Després et al. 2007; Aardema et al. 2012). A more complete sampling of species in the *Phyllobates* clade may reveal genotypes with intermediate resistance.

Dendrobates tinctorius and *D. captivus*, part of the sister-clade of *Phyllobates*, show convergent origins of A446D/E and I433V replacements (fig. 2A); V1583I also evolved once in *D. tinctorius*. However, there are more than 30 *Dendrobates* species (sensu Santos et al. 2009, which include the proposed genera *Oophaga*, *Adelphobates*, *Excidobates*, *Ranitomeya*, *Andinobates*, and *Minyobates*), so we cannot adequately describe how resistance evolved in the *Dendrobates* clade. Nevertheless, we can make the following observations. *Dendrobates tinctorius* and *D. galactonotus* have similar alkaloid diversity (number of classes: 5 and 7) and it is known that *D. captivus* is defended, but its alkaloid profile has not been analyzed. *Dendrobates pumilio* has higher alkaloid diversity (17 classes), but this is probably a result of increased sampling (e.g., Saporito et al. 2007). Despite these differences, *D. tinctorius*, *D. pumilio*, and *D. galactonotus* all have PTX and HTX as major alkaloids, and none have BTX (Daly et al. 2005). Hence, there may be a similar selection regime across *Dendrobates* for alkaloid resistance in Nav1.4. However, it remains unexplained why *D. pumilio* and *D. galactonotus* lack any resistance-conferring replacements in Nav1.4, which would suggest that these species are not resistant to their own alkaloids. This is probably not true and warrants an alternative hypothesis, as we do know that a species closely related to *D. pumilio*, *D. histrionicus* (Nav1.4 mutation pattern unknown), is somewhat resistant to HTX (Albuquerque, Kuba, Lapa, et al. 1974). *Dendrobates tinctorius*, *D. pumilio*, and *D. galactonotus* are from distantly related subclades within *Dendrobates*; they span almost the entire size range of the genus (e.g., from 0.48 g in *D. captivus* to 5.0 g in *D. tinctorius*; Santos and Cannatella 2011), so even though they appear to have similar alkaloid profiles, they may be consuming vastly different diets. Denser sampling may reveal differences in alkaloid defense or replacements in other ion channels that compensate for the lack of replacements in Nav1.4. In addition, it is possible that other mechanisms besides AA replacements might prevent autotoxicity, such as increased expression of toxin targets like Nav1.4 or of detoxification enzymes like P450 (Georghiou 1990; Li et al. 2002). These mechanisms have not been explored in poison frogs.

The other two major origins of chemical defense in poison frogs occur in *Epipedobates* and *Ameerega*. *Epipedobates* has mostly PTX defenses while *Ameerega* tends to have more HTX than PTX; neither have BTX (Daly and Spande 1986; Santos et al. 2016). The A446D/E replacement evolved once in the MRCA of *Epipedobates*, once in *Ameerega parvula*, which has relatively higher PTX defense than its congeners, and three times in the *Phyllobates* + *Dendrobates* clade, in which all species have PTX defense (fig. 2B), suggesting that this replacement evolved in response to high alkaloid exposure (perhaps PTX in particular). However, alkaloid quantity was not correlated with this replacement and docking analyses did not show that it was associated with resistance. Nevertheless, its origins are highly correlated with chemical defense in Dendrobatidae; it also occurs in *Mantella aurantiaca*, which is a distantly related, PTX-defended frog (Garraffo et al. 1993; Daly et al. 2008), and in the snake *Erythrolamprus epinephelus*, the only known natural predator of BTX-defended *Phyllobates* (Ramírez et al. 2014). This change occurs in a Nav1.4 region proposed to be part of the inactivation gate (C-terminals of S6 move during inactivation), so replacements at this site may be involved in enhancing resistance to alkaloids that inhibit channel inactivation, such as PTX or BTX (Yu et al. 2005). If this is true, the occurrence of A446D/E in lineages with high PTX/BTX defense (*Epipedobates*, *M. aurantiaca*, *A. parvula*, *P. terribilis*, *D. tinctorius*, and likely *D. captivus*) supports our model of autoresistance evolution.

The dominant alkaloid class in *Ameerega* is HTX (fig. 2B), which only blocks VGSCs at extremely high concentrations (LD50 $>$ 1,000 μg/mouse) and is better known for its effect on nicotinic acetylcholine receptors (Daly and Spande 1986). Hence, HTX may not be a strong agent of selection for resistance in Nav1.4. However, the replacement that provides broad alkaloid resistance, V1583I, is present in at least two species of *Ameerega* (fig. 1B). In total, this replacement evolved three times in defended clades and, unexpectedly, twice in undefended clades *Hyloxalus* and *Silverstoneia* (fig. 2A). Recent evidence suggests that the MRCA of *Hyloxalus* was defended, but that this trait was lost in its descendants, although the ability to sequester alkaloids may have been retained (Saporito et al. 2009; Santos et al. 2014). If this pattern holds (increased sampling of alkaloids in this group is necessary), it would explain the presence of V1583I in *H. italoi*. Alternatively, it is not implausible that this residue has a much more complicated history, characterized by one origin followed by multiple losses instead of multiple gains. If there is a high cost to maintaining isoleucine at residue 1583 (i.e., if it impairs the normal function of Nav1.4), there may be selection for reversion to valine in frogs with fewer to no alkaloids that interact at this residue. There is some evidence

for this, as the adjacent residue, 1582, was one of three residues identified as being under purifying selection.

The Role of Autoresistance in the Evolution of Chemically Defended Lineages

A recent study suggested that chemically defended organisms experience increased rates of speciation and, surprisingly, also of extinction (Arbuckle and Speed 2015). If there are elevated extinction rates in chemically defended taxa, autoresistance may be a potential cause. In fact, toxin resistance has been shown to be maladaptive and lost in organisms no longer exposed to high levels of toxins (Jiang et al. 2011; Lee et al. 2011; Ujvari et al. 2015). The notion that chemical defense drives both speciation and extinction is intriguing. However, the accuracy of extinction rate estimation is tenuous given criticisms of the binary-state speciation and extinction model, so this pattern may not be real (Maddison and FitzJohn 2014; Rabosky and Goldberg 2015). Nevertheless, the idea is compatible with a recent description of the evolution of animal venom as "two-speed"; an initial period of expansion and diversification is followed by selection and fixation (Sunagar and Moran 2015). Perhaps organisms with chemical defense also evolve at two speeds, rapid radiation followed by partial extinction.

Conclusion

VGSCs originated before the split of invertebrates and vertebrates and remain an integral component of the nervous system in both clades (Zakon 2012). Hence, the VGSC is a perfect target for a toxin: a disruptive chemical would be broad-spectrum and potentially lethal. It is not surprising, then, how many organisms have acquired or evolved toxins that target this protein family (Wang S-Y and Wang GK 2003). The apparently permissive nature of alkaloid sequestration in frogs exposes them to hundreds of toxins that have various effects on VGSCs and other ion channels (Daly and Spande 1986; Daly et al. 2005). Such a diverse chemical arsenal sets alkaloid-defended frogs apart from other organisms with acquired chemical defenses. Dendrobatid alkaloid profiles vary considerably over time in one species (Saporito et al. 2007); as a corollary, selective pressures for autoresistance may also vary extensively. Taking into account our limited sampling of Dendrobatidae (25/~300 species), and given that resistance likely carries some cost (e.g., Ujvari et al. 2015), it is possible that reversions to a more sensitive genotype were not detected by our analyses. If this is true, alkaloid resistance in poison frogs is likely a dynamic trait that changes in response to environmental variation over relatively short evolutionary timescales.

In poison frogs, target-site insensitivity via resistance-conferring AA replacements in Nav1.4 has evolved at least seven times within the last 10 My. The convergence in replacement location and type suggests high costs of mutation and/or strong directed selection pressure by a variety of alkaloids that bind to the same site. It is possible to uncover the proximate results of selection on function using electrophysiological assays to test the effect of alkaloids on Nav1.4. These would enhance our understanding of how genetic changes in Nav1.4 provide alkaloid resistance by making functional connections between the autoresistant genotype and phenotype. They may also detect physiological costs associated with mutations in the Nav1.4 inner pore. The phylogenetic distribution of replacements across Dendrobatidae highlights the complexity of target-site insensitivity, which is likely a result of a long-term balance between selection for resistance and cost of maintaining resistance. Deeper phylogenetic sampling of dendrobatids and their ion channels will be necessary to further unravel the complex interplay between alkaloid resistance and defense in this group.

Materials and Methods

Sample Collection

Individuals were collected by J.C.S. and L.A.O. in Ecuador in 2013 under Ecuadorian permit No. 003-11 IC-FAU-DNB/MA to Luis A. Coloma and by R.D.T. under Texas Parks and Wildlife Department Permit No. SPR-1097-912 to Travis Laduc (see supplementary table S1, Supplementary Material online, for collection sites and species). Animals were euthanized with benzocaine (Orajel dental gel) under ethically approved protocols (Harvard University IACUC # 12-10, UT Austin IACUC # 2012-00032); tissue samples of brain and muscle were removed and stored in RNAlater (Life Technologies, Carlsbad, CA) at −20 °C.

RNA Library Preparation, Sequencing, and Transcriptome Assembly

Trizol (Life Technologies) was used to extract total RNA; rRNA was removed with the Poly(A) Purist kit (Life Technologies). Libraries of mRNA with RNA integrity numbers (RIN) $\geq$8.0 were prepared with the NEXTflex directional RNA-Seq dUTP-based kit (Bioo Scientific, Austin, TX). Following barcoding of cDNA libraries, 10–18 cycles of PCR were used to enrich cDNA. The resulting cDNA libraries were quantified and qualitatively analyzed with Bioanalyzer 2500 (Agilent Technologies, CA) and purified using AMPure XP beads (Beckman Coulter, Inc) to a total mean size of 300 bp. Libraries were sequenced with 100 bp paired-end sequencing on the Illumina HiSeq 2100 platform at the UT Austin Genomic Sequencing and Analysis Facility.

Raw sequences were evaluated using FastQC v 0.10.1 (Andrews 2010); low-quality reads were removed or trimmed using SnoWhite v 2.0.3 (Lassmann et al. 2009; Dlugosch et al. 2013) with default parameters except: -Q 20, -D T, -L T, -Y T, -a B, -t B, -b 6, -l 20. A custom script was used to verify that sequence reads were present in both paired-end files. These reads were then assembled into de novo transcriptomes with Trinity v2013-02-25, using default Trinity parameters for strand-specific paired data (–SS_lib_type RF) (Grabherr et al. 2011; Haas et al. 2013); Assemblies were run on large memory nodes of the Lonestar cluster at the Texas Advanced Computing Center (TACC).

Nav1.4 Identification from *De Novo* Transcriptomes

BLASTX (Altschul et al. 1990) was used to compare the transcriptomes to the Universal Protein Resource (UniProt)

database (Bairoch et al. 2005). Transcripts that matched scn4a (Nav1.4 protein) with an *E* value cutoff of 10^{-5} were retained for further analyses. Because some transcripts assembled by Trinity appeared to be chimeric (the small 25-bp kmers mapped ambiguously to multiple VGSC paralogs, resulting in transcripts that included reads from different genes), we used higher kmer sizes (K = 35, 45) in SOAPdenovo (Li et al. 2010) with default parameters for the final assemblies. Longer kmers decreased the chance of assembling chimeric reads because they are less likely to be identical across paralogs. After visual inspection and reevaluation with BLASTX and BLASTN, the final set of scn4a sequences were aligned with MAFFT v7.023b (Katoh and Standley 2013) and the alignment was adjusted to match the start and end codon of the *Rattus norvegicus* Nav1.4 protein (NCBI accession number NM_013178.1) in Mesquite (Maddison and Maddison 2015).

AA Replacement Identification

We reviewed the alignment site by site to identify residues that were highly conserved in nondendrobatid frogs and other vertebrates, but showed patterns of AA replacements that were associated with chemical defense in dendrobatids. We identified six such replacements at five residues in Nav1.4 S6 regions, caused by nucleotide transversions in the first or second nucleotide position. To determine their evolutionary patterns across Dendrobatidae, we designed primers from our RNAseq transcripts to sequence additional samples from the Genetic Diversity Collection of UT Biodiversity Collections (see supplementary table S2, Supplementary Material online, for species and museum numbers). Primers used for DI-S6 were +869F (5′-CTGCAGGYAAAACCTACATGG-3′) and +1032R (5′-GATGTTTCTTTAGCTGTTCC-3′). PCR parameters were: 2-min initial denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 46 °C, 20 s at 72 °C, and a final extension time of 7 min at 72 °C. Primers used for DIV-S6 were +2268F (5′-TCTCCCGGCCCTCTTCAATA-3′) and +2681R (5′-GAGCCTTTATGCGAGGACGA-3′). PCR parameters were: 2-min initial denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 57.5 °C, 45 s at 72 °C, and a final extension time of 7 min at 72 °C. We used TaKaRa Ex Taq Hot Start DNA polymerase (Clontech, Mountainview, CA) for all reactions. PCR products were run on a gel, purified with QIAquick Gel Extraction Kits (Qiagen, Valencia, CA), and sequenced using forward and reverse primers at the UT Austin Institute for Cellular and Molecular Biology Core Facility on an Applied Biosystems 3730 DNA Sequencer. The sequences reported are consensuses of forward and reverse reads.

Reference Phylogenies for figures 1 and 2

A reference molecular phylogeny of amphibians and outgroups was inferred for the 39 species in figure 1B using previously published mitochondrial sequences (see supplementary table S6 and figure S1, Supplementary Material online, for accession numbers and full phylogeny). Sequence alignment was performed under an iterative approach (i.e., simultaneous alignment and tree estimation) using SATé v 2.2.7 (Liu et al. 2009). The substitution model was determined to be GTR + G + I for all genes using jModelTest v 0.1.1 (Posada 2008). A maximum likelihood (ML) phylogeny was estimated using Garli v2.0 (Zwickl 2006) and support was assessed using 400 nonparametric bootstrap searches. For all comparative analyses, we used a previously published chronogram of poison frogs inferred using approximately 10 kb of nuclear and mitochondrial markers; information on divergence time and phylogeny estimation can be found in the methods section of Santos et al. (2009).

Evolutionary Analyses

We predicted ancestral states of AA replacements (coded as discrete characters) with Mesquite (Maddison and Maddison 2015) using ML in Mk1 (1-parameter) and AsymmMk (2-parameter) models. Both estimate marginal probabilities of ancestral states for each site independently. The models were not significantly different (likelihood-ratio test, $P > 0.05$) for any reconstruction, so we only report values from less-parameterized Mk1 models. All AA sites are numbered according to their homology with the rat (*R. norvegicus*) Nav1.4 protein.

We also tested for correlation between origins of defense and AA replacements using Pagel's test of correlated evolution in Mesquite (Pagel 1994; Maddison and Maddison 2015). This test compares two models: "correlated" evolution, with four parameters where transition rates (both forward and reverse) of both characters are equal, and "independent" evolution, with eight parameters where these rates are not equal. To determine statistical significance, we ran 1,000 Monte Carlo simulations in Mesquite and report the average difference in model likelihoods, $\Delta[-\log(L)]$, and the P value, P. Because these analyses cannot be run with missing data or more than two states, we coded site 433 for *Ameerega trivittata* parsimoniously as the predicted ancestral state for the closet node (isoleucine [I]), and site 446 of *Dendrobates captivus* as aspartic acid (D) instead of glutamic acid (E). Analyses of positive selection were performed in HyPhy (Pond et al. 2005) using the Single Likelihood Ancestor Counting method. All phylogenetic analyses were also performed including four nondendrobatid frog outgroups, but our results were consistent, so we report only analyses without outgroups.

Ecological Analyses

We used the pgls function in the R "caper" package (with lambda set to "ML" and all other settings as their defaults) to test for phylogenetic correlations between the number of AA replacements in Nav1.4 and predicted resistance (based on docking analyses) with alkaloid diversity and BTX/PTX/HTX quantity. Resistance was scored independently for BTX, HTX, and PTX as: 0 = none, 1 = somewhat resistant, 2 = significantly more resistant (*P. terribilis* and *D. tinctorius*, see Results). Total resistance was equal to the sum of scores for BTX, PTX, and HTX. Alkaloid diversity was roughly scored as the number of alkaloid classes that have been found in each species; BTX/PTX/HTX quantity was scored as 1, 2, or 3 based on whether it was found as a trace (<50 μg/100mg skin), minor (> 50 and

<150 μg/100mg skin), or major (> 150 μg/100mg skin) alkaloid in each species; these data are from Santos and Cannatella (2011) and have been updated to include alkaloid diversity of *Epipedobates tricolor* and *E. boulengeri* (Tarvin RD, Cannatella DC, Ron SR, and Fitch RW, unpublished data; Cipriani and Rivera 2009).

Docking Analyses

To test our hypothesis that the AA replacements are related to alkaloid resistance, we predicted optimal binding sites of seven alkaloids (ChemSpider IDs: BTX, 10310314; aPTX 267A, 4580699; PTX 307A, 9154941; PTX 323A, 4941919; aPTX 323B, 4518097; PTX 251D, 4944741; HTX 283A, 4941928) using the Nav1.4 homology model of Walker et al. (2012) (their Dataset 1). Alkaloids are denoted by class followed by molecular weight and an additional letter if multiple alkaloids with this mass exist (Daly et al. 2005). The seven alkaloids selected are known to affect VGSCs (Santos et al. 2016); we included a variety of PTX because their effects on ion channels vary (Daly and Spande 1986; Daly et al. 2003; Vandendriessche et al. 2008).

We used Python Molecule Viewer (Sanner 1999) to set up docking parameters, adding Gasteiger PEOE partial charges to alkaloids and Kollman United Atom charges to protein models. We then ran docking analyses in Autodock Vina (Trott and Olson 2010) on TACC, searching a 3D rectangular prism centered in the middle of the protein that encompassed both inner and outer regions of the channel pore (pore loop and S6) with the exhaustiveness parameter set to 10,000. For each permutation of mutation pattern and alkaloid we retained the top three docking positions from five different runs (N = 15). Finally, we used Chimera (Pettersen et al. 2004) to view the protein-alkaloid complex and identify atoms in close contact between protein and alkaloid. We ran contact analyses with a distance of 2.5 Å, the maximum length of a hydrogen bond, to identify potentially interacting atoms.

We then mutated the Nav1.4 model in Chimera (Pettersen et al. 2004) to contain each single AA replacement as well as unique species-specific multiple AA replacement patterns found in DI-S6 and DIV-S6 of dendrobatids. We reran the docking analysis to determine the effect of single and multiple AA replacement patterns on alkaloid binding affinity (table 1). If the Gibbs free energy (ΔG) of the new predicted docking site increased (became more positive), then the alkaloid had lower affinity for the mutated model, suggesting that the replacement pattern provides decreased sensitivity to that alkaloid. We performed nonparametric unpaired Wilcoxon rank-sum tests in R v3.0.2 (R Core Team 2013) [AQ8]comparing the free energy of alkaloid docking to ancestral ("Walker1") and derived Nav1.4 models; we report the test statistic, W, and its P value, P.

Supplementary Material

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

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