Universidad de los Andes
Facultad de Ciencias
Departamento de Ciencias Biológicas

Evolution of the muscular voltage-gated sodium channel in relation to toxicity in poison-dart frogs (Dendrobatidae: *Phyllobates*)

Tesis de grado como requisito parcial para optar al título de Magister en Ciencias Biológicas - Área Biología

Roberto Márquez Pizano
Código 200722652

Director: Adolfo Amézquita Torres, PhD
Departamento de Ciencias Biológicas. Universidad de Los Andes. Bogotá, Colombia

Codirector: No Aplica
Asesor/es: No aplica

Bogotá, Colombia
Mayo de 2013

Evolution of the muscular voltage-gated sodium channel in relation to the toxicity in poison-dart frogs (Dendrobatidae: *Phyllobates*)

Roberto Márquez, BSc

Advisor: Adolfo Amézquita, PhD

Group of Behavioral Ecophysiology and Herpetology. Biological Sciences Department, Universidad de los Andes. Bogotá, Colombia.

Abstract. Although the evolutionary dynamics between the behavioral-ecological phenotypes that comprise aposematism, the use of conspicuous signals to advertise secondary defenses to predators, other important traits, such as the resistance to autotoxicity have received less attention. In toxicity-based aposematism, resistance of an animal to its own toxins is an important factor, since, without it, the adverse effects of the toxin would make it unprofitable to bear. Hypertoxic poison-dart frogs, *Phyllobates* spp., possess the powerful alkaloid batrachotoxin, which renders voltage-gated sodium channels useless by irreversibly opening them, and is synapomorphic to *Phyllobates* among frogs. In an effort to understand the role of batrachotoxin resistance in the evolution of toxicity in this genus, we sequenced two fragments of the muscular voltage-gated sodium channel gene that are involved in batrachotoxin binding and performed molecular docking simulations to assess the functional effects of the mutations found. Three amino acid mutations in sites important to batrachotoxin binding were found to have originated in the most recent common ancestor of all *Phyllobates*, and at least one of the sites was found to have been under positive selection. Docking simulations, however, showed no effect of these mutations on the interaction of batrachotoxin and the sodium channel. We propose that resistance to BTX may have been acquired through the action of several small-effect mutations, rather than a few with large effects, which could explain the docking results, and suggest that this resistance played a role in the evolutionary origin of batrachotoxin as a defense mechanism in the ancestor of all *Phyllobates*, but not further in the evolution of toxicity within the genus.

Introduction

It is common for a group phenotypic traits to evolve in a correlated manner, since specific combinations of traits should result in higher fitness than others in a given environment (Wright 1932; Gavrilets 2004; Svenson 2012). When a trait is part of a suite of correlated traits (a phenotypic syndrome), its evolution can strongly influence the evolution of the whole syndrome. Aposematism, the use of conspicuous signals to advertise secondary defense mechanisms to predators (Cott 1940; Ruxton et al. 2005), has been shown to covary and interact with several other traits at an evolutionary scale (Vogler 1998; Mappes et al. 2005; Santos & Cannatella 2011). Whereas the evolution of ecological and behavioral components of aposematism have been investigated in detail (Alatalo & Mappes 1996; Ruxton et al. 2005; Mappes et al. 2005), little attention has been given to other factors influencing the evolution of aposematic phenotypes. An especially important and unexplored example is resistance to autotocicity: In species where secondary defenses consist of toxic or poisonous chemicals, the lack of resistance to their own chemicals should severely constraint the evolution of aposematic forms, and resistance to these chemicals should evolve prior or in conjunction with toxicity.

Hypertoxic poison-dart frogs (genus *Phyllobates*, Anura: Dendrobatidae), are well known for secreting Batrachotoxin (BTX; Silverstone 1976), a potent steroidal alkaloid that binds irreversibly to voltage-dependent sodium channels on nerve and muscle cells. The affected channels are left permanently open, and their selectivity for Na^+ ions is reduced, which prevents the occurrence and propagation of action potentials in the affected neurons and muscle fibers (Daly et al. 1965; Myers et al. 1978; Strichartz et al. 1987). BTX is allegedly stored in vesicles, within the granular glands of the frogs' skin. Although vesicles probably prevent the translocation of BTX to other tissues (Neuwirth et al. 1979), the toxin could easily enter the bloodstream, especially if secretion is triggered by events that result in cuts or lacerations of the frog's skin, such as combats or predation attempts. Moreover, BTX, or its precursors, are probably sequestered from dietary arthropod sources in *Phyllobates* species (Daly et al. 1994). If so, BTX could contact neural and muscular sodium channels on its way to the storage vesicles.

The presence of cutaneous BTX is synapomorphic to *Phyllobates* within frogs (Maxson & Myers 1985). However, other species of poison frogs in the family Dendrobatidae, for example some

members of the genera *Andinobates, Dendrobates* and *Oophaga*, share microhabitat and prey (Esquivel 2012) with *Phyllobates* species. BTX is, by far, more toxic than other alkaloids secreted by dendrobatid frogs (Daly et al. 1965; Daly 1998). Considering the unusually high toxicity of batrachotoxin and its concomitant value as reinforcer of aposematic signals, an obvious questions is why is BTX not present as secondary defense in other lineages of poison frogs. Two non-exclusive hypotheses arise: first, other species may lack the physiological or biochemical mechanisms to transport and/or modify BTX or its precursors across epithelia, from the gut to the storing vesicles. Second, other species may lack physiological resistance to BTX and are, therefore, unable to sequester, store, and use it as an anti-predator toxin. Indeed, BTX seems to have no effect on the nerve and muscle cells of two species of *Phyllobates* (*terribilis* and *aurotaenia*), but inhibits the activity of the same cells on two other species of frog: *Lithobates pipiens* (Daly et al. 1980), and the dendrobatid *Oophaga histrionica* (cited as unpublished data in Daly et al. 1980). We test here two general hypotheses to resolve this issue: (H1a) that modifications in the sodium channel that confer them resistance to BTX preceded the origin of BTX, and (H1b) that only frog species in the genus *Phyllobates* evolved resistance to BTX, which *Phyllobates* but not other genera of Dendrobatids to sequester BTX as a defense mechanism.

Batrachotoxin concentration and lethality of skin extracts can vary in four and one order of magnitude, respectively, among the five currently recognized species of *Phyllobates* (Daly et al. 1980; Escovar 2009). A large portion of this variation can be explained by two major evolutionary changes in toxicity throughout the evolutionary history of the genus (Márquez 2011). Intrageneric variation in toxin concentration and lethality could also be influenced by evolutionary novelties that confer differential resistance to BTX, i.e. more toxic species should bear more resistant sodium channels. We thus tested the subordinate hypothesis (H2) that the more toxic lineages of *Phyllobates* evolved and perhaps share modifications in the amino acid sequence of their sodium channels.

The pore-forming $\boldsymbol{\alpha}$ subunit of the voltage-gated sodium channels is composed of four repeats or domains (DI-DIV), each containing six transmembrane helices (S1-S6), bound by extra or intracellular linkers. Segments S5 and S6 are part of the pore-forming domain of the alpha subunit,

and are connected by an extracellular linker, the P-loop, that contains the ion selectivity filter (Fig 1A-B). Directed mutagenesis and computer simulation studies have indicated that BTX interacts with fourteen residues in the four S6 transmembrane segments, and one in the DIII P-loop, close to the selectivity filter (Fig 1C). Specific amino acid changes in all of these sites have been experimentally and computationally shown to confer resistance to the effects of BTX (Trainer et al. 1996; Wang & Wang 1998; Linford et al. 1998; Wang & Wang 1999; Wang et al. 2000; Li et al. 2002; Tikhonov & Zhorov 2005a; Tikhonov & Zhorov 2005b; Wang et al. 2006; Wang et al. 2007a; Wang et al. 2007b; Du et al. 2011).

The relatively simple and genetically determined mechanisms that could explain resistance to BTX provide a good opportunity to study the role of resistance to toxins in the evolution of toxicity. We managed to sequence two of the four S6 segments of the muscular voltage-gated sodium channel (NaV1.4) of several species of *Phyllobates* and other Dendrobatids, where seven out of the fifteen sites known to be involved in interaction with BTX are located, to test for associations between amino acid substitutions and significant changes in BTX-based toxicity among genera of poison frogs (H1) and among species of *Phyllobates* (H2). Our predictions are straightforward: functional amino acid substitutions that increase resistance to BTX should have predated (H1a), coincided with the most recent common ancestor of *Phyllobates* (H1b), where BTX secretion originated, and in the two clades of *Phyllobates* that attained highest toxicity (H2; Márquez 2011). Figure 2 shows a schematic representation of our hypotheses and predictions. Since we did not sequence all potentially important sites of the NaV1.4 gene, the absence of functionally important substitutions in our data cannot be interpreted as a lack of genetically-mediated BTX resistance.

Methods

Genetic Data

We obtained DNA sequences for segments S6 of domains I and IV of the gene coding for the muscular voltage-gated sodium channel from all (6; including *P. niche* sp. nov. Amézquita et al. *submitted*) species of *Phyllobates*, and 15 species from other genera of dendrobatid frogs (Table

S1). We focused on the S6 segments, since all but one of the amino acid sites known to be involved in BTX binding are located in these segments. Genomic DNA was extracted from toe-clips, mouth swabs, and, in a few cases, liver or muscle tissues using DNeasy spin columns (Qiagen ®). To maximize the DNA yield, mouth-swab samples were vortexed twice as long as recommended in the standard DNeasy protocol.

S6 fragments were amplified by PCR and Sanger-sequenced in both directions in order to corroborate base calls. Primers were designed using the sequence of the NaV1.4 gene from the frog *Siluriana tropicalis* (GenBank accession XM_002936749.1), which was the only publicly available anuran NaV1.4 sequence. Table S2 contains primer and PCR details. The resulting chromatograms were assembled and visually checked in Geneious v5.1 (Drummond et al. 2010). DNA sequences were translated to amino-acids, and sequences from conspecific individuals were aligned using MUSCLE (Edgar 2004), in order to obtain a consensus protein sequence for each species. No variation in protein sequence was found within species, so this procedure was completely straight-forward.

Ancestral Sequence Reconstructions and Tests of Positive Selection

To identify the nodes where non-synonymous mutations occurred in the NaV1.4 gene, we reconstructed ancestral sequences using the *codeml* and *baseml* programs of the PAML package (Yang 1997; Yang 2007), through the Lazarus set of python scripts (http://markov.uoregon.edu/software/lazarus/). The tree of Pyron & Wiens (2011) and the best model of protein evolution, chosen under the BIC and AICc criteria using ProtTest 3.2 (Abascal et al. 2005; Darriba et al. 2011), were used for these and subsequent analyses.

In order to assess whether positive selection has driven the evolution of specific codons of the NaV1.4 gene at specific branches, we compared the evidence for positive selection between two clades, one nested within the other. If positive selection was detected for a specific site in the nested clade but not in the one containing it, selection was considered to have acted on that site at the base of the nested clade. Comparisons were performed at two phylogenetic levels, corresponding to H1 and H2: the branch containing *Phyllobates* (H1b) compared to the clade containing the subfamily Dendrobatinae (H1a), and the two clades within *Phyllobates* where toxicity increased in comparison

to the branch containing all *Phyllobates* (H2). In each case, selection was detected by fitting Improved Branch-Site A models of DNA evolution (Zhang et al. 2005) under neutral (dN/dS ratio = 1) and positive selection (dN/dS > 1) scenarios at each clade, and assessing whether the positive selection scenario was a better fit for the data with a likelihood-ratio test.

Molecular Docking

We used molecular docking simulations to examine the effect of specific amino acid substitutions and their combinations on the affinity of BTX to the sodium channel. First, we modified the model of BTX bound to the pore of the cockroach voltage-gated sodium channel (BgNaV1-1) generated by Du et al. (2011; hereon referred as the original model) and kindly provided by Dr. Boris Zhorov, to match the ancestral states of S6 residues inferred for anurans. This modified version of NaV1-1 (the template model) was mutated to incorporate the substitutions of residues involved in BTX binding, if any, found to occur in the clades of interest. Additionally, we modified the template model to match the inferred ancestral sequences of *Phyllobates*, Dendrobatinae, and Dendrobatidae.

The mutated proteins were prepared for docking under standard parameters in DockPrep, as implemented in UCSF Chimera (Pettersen et al. 2004). The structure of BTX, extracted from the original model, was prepared in PyRx (http://pyrx.sourceforge.net), also under standard parameters, except for a complete torsion restriction set in order to conserve the horseshoe conformation of BTX (Du et al. 2011). Docking simulations were performed in Autodock Vina (Trott & Olson 2009), implemented in PyRx. Searches were conducted on a 20 $Å^3$ box constructed around the binding site of the original model, using an exhaustiveness parameter of 10. Out of the top ten conformations of BTX generated by Vina, the most similar to the conformation proposed by the original model (i.e. that with the lowest root mean square deviation (RMSD) from the original model) was considered the best conformation, and chosen for further analyses.

To validate the accuracy of our analyses in detecting the effect of mutations on BTX affinity, we used one negative and four positive controls: The negative control consisted on docking BTX to the template model, and positive controls consisted of docking four additional simulated mutants that incorporated substitutions known to confer resistance to BTX on the sites where substitutions were

found in *Phyllobates* (see results): $S^{1i15}K$, $S^{1i15}R$ (Wang et al. 2007a), $I^{1i19}K$ (Wang & Wang 1998), and $V^{4i19}C$ (Vendantham & Cannon 2000).

Results

We found several amino acid variants throughout all clades of Dendrobatidae. Remarkably, all members of *Phyllobates* yielded identical NaV1.4 protein sequences, including three substitutions that arose in the most recent common ancestor (MRCA) of the genus, in sites that are involved in BTX binding ($S^{1i15}A$, $I^{1i19}V$, and $V^{4i19}I$; Fig. 3). Other substitutions shared by Phyllobates were found in intra or extracellular linkers, in areas with no known functional importance for BTX binding (data not shown). Whereas mutation $S^{1i15}A$ is unique to *Phyllobates*, $I^{1i19}V$ was shared with the two included species of *Dendrobates* and with a phylogenetically distant aquatic frog, *S. tropicalis*. In turn, $V^{4i19}I$ was shared with the non-poisonous dendrobatid frog *Silverstoneia nubicola*. No other substitutions were found in BTX binding sites.

The positive selection model fit the data better than the model assuming neutral evolution of the NaV1.4 gene when testing for selection in the branch leading to *Phyllobates* (χ^2=5.22, p = 0.022), and site site S^{1i15} was found to be under positive selection in this same branch (post. prob.= 0.988). No evidence for selection was found in the clade containing all Dendrobatines (χ^2=0.0007, p = 0.999).

Most of the chosen conformations for the simulated *Phyllobates,* Dendrobatidae, and Dendrobatinae genotypes were very similar the original BTX conformation, indicating that the simulated mutations had small or no functional effects on BTX affinity. The only exception was the double mutant $I^{1i19}V$ +$V^{4i19}I$, which showed a somewhat higher deviation. On the other hand, the best conformations for three out of the four control mutants ($S^{1i15}K$, $I^{1i19}K$, and $V^{4i19}C$) showed larger deviations from the original model, generated mostly by a change in the orientation of the pyrrole ring of BTX, which is crucial to BTX binding (Warnick et al. 1976; Khodorov et al. 1992). These results suggest that, contrary to the positive controls, the substitutions we found in *Phyllobates* do not have a strong

effect on the affinity of BTX to sodium channels. Figure 4 shows examples of the conformational changes found, as well as the RMSDs for each simulated mutant.

Discussion

Three amino acid substitutions synapomorphic to *Phyllobates* were found to occur in sites of the NaV1.4 protein that are involved in BTX binding, and one of these sites (S^{1i15}) was found to be under selection in *Phyllobates*, but not in Dendrobatinae. Taken alone, these results support hypothesis H1b, since an evolutionary change in resistance to BTX would have coincided with the evolution of hypertoxicity in *Phyllobates* frogs. Previous studies have experimentally shown that other mutations at precisely these three sites confer resistance to BTX (Wang & Wang 1998; Vendantham & Cannon 2000; Wang et al. 2007a), and it seems very unlikely that three substitutions in residues that strongly interact with BTX coincide with the evolutionary origin of this alkaloid in *Phyllobates* by chance alone.

Molecular docking simulations, however, failed to find clear changes in the BTX binding conformation generated by the three observed mutations or by their combinations. We advance two major non-competitive explanations for this apparent contradiction: we might have not detected all the relevant mutations necessary to confer BTX resistance to frogs, or our docking simulations do not appropriately represent the BTX-NaV1.4 interaction for frogs, since it is based in too distant biological models.

Phyllobates frogs may have acquired resistance to BTX through several mutations with small and cumulative effects, distributed throughout the sodium channel pore, instead of a few with large effects. According to Fisher's geometric model of adaptation (Fisher 1930), several small-effect mutations would have better chances to bring a population to an optimal phenotype, because they produce smaller pleiotropic effects than large-effect mutations. Mutations in sodium channels that aid in toxin resistance, have been shown to produce antagonistic pleiotropic effects on the correct functioning of the sodium channel, especially affecting its ion permeation and voltage gating properties. In the case of BTX, most mutations known to confer resistance are very close to (and

sometimes at) the sodium channel gating hinge, located in the the S6 segment (position 14 in Fig. 1C), or the ion selectivity filter, and mutations of these residues have been shown to modify the sodium channel's gating properties (Zhao et al. 2004; Wang et al. 2006). A similar situation applies for resistance to tetrodotoxin (TTX), a voltage-gated sodium channel antagonist that interacts with the channel's outer pore to block the movement of ions (Narahashi et al. 1964; Takata et al. 1966). In this case, mutations that confer TTX resistance have been shown to have negative pleiotropic effects on the channel's permeability (Terlau et al. 1991; Chiamvimonvat et al. 1996; Feldman 2012), and negative correlation has been shown to exist between the affinity of BTX to mutant sodium channels and the magnitude of the negative pleiotropic effects of their mutations (Feldman 2012). If a similar correlation exists for mutations that reduce the affinity of BTX to sodium channels, then the fixation of several small-effect mutations should be favored over the fixation of a few large-effect ones. However, TTX-resistant animals vary in the amount and location of TTX-resistant substitutions that they present, ranging from several mutations across the four domains in puffer fishes (Venkatesh et al. 2005; Jost et al. 2008), which may be the case for *Phyllobates*, to as little as one mutation in some snakes (Feldman 2012). Further investigation of the pleiotropic effects of mutations involved in each specific case should help understand the reasons for this difference and the situations that favor adaptation mediated by small-effect rather than large-effect mutations.

A potential limitation of our docking simulation relies on the biological models used for Du et al.'s (2011) docking simulation. Although their model performs very well at its original purpose of generating a binding conformation of BTX to voltage gated sodium channels that is compatible with experimental data available. The structure of the cockroach sodium channel they used, was obtained by homology modeling, based on the crystal structure of the pore of a distantly related potassium channel (Long et al. 2005). Thus, although docking of BTX on mutated versions of this homology model can have enough precision to pick up the effect of large-effect mutations, it may not detect changes mediated by small effect mutations. The low of sensitivity would, in turn, explain the lack of functional consequences for the *Phyllobates* mutations observed in our docking simulations.

Regarding our second hypothesis (H2), we found no evidence of variation in resistance to BTX among the species of *Phyllobates*; all members of the genus showed identical amino acid sequences. As discussed before, the lack of evidence in our study model should be interpreted with caution. Aminoacid substitutions that cause differential resistance to BTX might still exist in the second and third domains of the muscular channel (DII and DIII) or in any of the other eight isoforms of the sodium channel present in vertebrates. Indeed, previous research has shown that aminoacid substitutions in DII and DIII alone can generate resistance to BTX (Wang et al. 2000; Wang et al. 2006; Du et al. 2011). If, however, the lack of additional substitutions were the case for all the relevant segments, then the observed variation in toxicity among the species of *Phyllobates* would be explained by other factors, such as the capacity to sequester, metabolize, and or store BTX. In other species of poison frogs, among-populations and among-species differences in toxicity have been attributed to (1) habitat differences in the availability of arthropod prey that represent the key source of the toxin or its precursors; (2) variable degrees of dietary specialization in the frogs; (3) differential predation pressures on different species (reviewed by Saporito et al. 2011). We disfavor the first two scenarios, since we have found no conspicuous differences in diet after examining stomach contents from <70 frogs of all the species of *Phyllobates* (A. Amézquita , LM Arenas, MC González, C Esquivel, unpublished data).

Taken together, our results suggest that the changes in the muscular voltage-gated sodium channel played an important part in the evolutionary origin of BTX-mediated aposematism among poison frogs but we found no evidence of a role in the subsequent evolution of toxicity within the genus *Phyllobates*. Interestingly, we found partial convergence in amino acid substitutions with three other species of poison frogs (Figure 2) that lack BTX. Several distantly related groups of frogs have convergently evolved the same alkaloids as defense mechanisms (Daly et al. 1999; Daly et al. 2005; Saporito et al. 2011). Constraints on the adaptation of ion channels and other transport proteins to resist autotoxicity (Jost et al. 2008; Feldman et al. 2012; Zhen et al. 2012), the convergent acquisition of specific toxins to which proteins are more adaptable. Lastly, resistance to BTX may have evolved by the accumulation of several small-effect mutations, as opposed to few large-effect ones. Whether this hypothesis holds and whether it is attributable to the detrimental effect of large-

effect mutations deserve additional research on a wider spectrum of taxa and the whole sequence of several channel isoforms.

References

Abascal, F., Zardoya, R., & Posada, D. 2005. ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics*, **21**, 2104–2105.

Alatalo, R., & Mappes, J. 1996. Tracking the evolution of warning signals. *Nature*, **372**, 708–710.

Daly, J. W. 1998. Thirty years of discovering arthropod alkaloids in amphibian skin. *Journal of Natural Products*, **61**, 162–172.

Chevin, L.M., Martin, G., & Lenormand, T. 2010. Fisher's model and the genomics of adaptation: Restricted pleiotropy, heterogenous mutation, and parallel evolution. *Evolution*, **64**, 3213–3231.

Chiamvimonvat, N., Pérez-García, M. T., Tomaselli, G. F., & Marban, E. 1996. Control of ion flux and selectivity by negatively charged residues in the outer mouth of rat sodium channels. *The Journal of physiology*, **491**, 51–59.

Cott, H. B. 1940. *Adaptive coloration in animals.* London: Methuen & Co.

Daly, J., Garraffo, M., & Spande, T. 1999. Alkaloids from amphibian skins. In: *Alkaloids: Chemical and Biological Perspectives*, Vol 13 (Ed. by S. Pelletier), pp. 1–161. Pergamon.

Daly, J. W., Witkop, B., Bommer, P., & Biemann, K. 1965. Batrachotoxin. The active principle of the Colombian arrow poison frog, *Phyllobates bicolor*. *Journal of the American Chemical Society*, **87**, 124–126.

Daly, J. W., Myers, C. W., Warnick, J. E., & Albuquerque, E. X. 1980. Levels of batrachotoxin and lack of sensitivity to its action in poison-dart frogs (*Phyllobates*). *Science*, **208**, 1383–1385.

Daly, J. W., Secunda, S. I., Garraffo, H. M., Spande, T. F., Wisnieski, A., & Cover, J. F., Jr. 1994. An uptake system for dietary alkaloids in poison frogs (Dendrobatidae). *Toxicon*, **32**, 657–663.

Daly, J. W., Spande, T. F., & Garraffo, H. M. 2005. Alkaloids from Amphibian Skin: A Tabulation of Over Eight-Hundred Compounds. *Journal of Natural Products*, **68**, 1556–1575.

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

Drummond, A., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Kearse, M., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., & Wilson, A. 2010. Geneious v5.1. Available from <http://www.geneious.com/>.

Du, Y., Garden, D. P., Wang, L., Zhorov, B. S., & Dong, K. 2011. Identification of New Batrachotoxin-sensing Residues in Segment IIIS6 of the Sodium Channel. *Journal of Biological Chemistry*, **286**, 13151–13160.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

Escovar, T. 2009. *Evolución de la toxicidad en las especies y poblaciones colombianas del género Phyllobates.* Universidad de Los Andes.

Feldman, C. R. 2008. *Evolutionary Genetics of Tetrodotoxin (TTX) Resistance in Snakes: Tracking a Feeding Adaptation from Populations Through Clades.* Utah State University.

Feldman, C. R., Brodie, E. D., Jr, Brodie, E. D., III, & Pfrender, M. E. 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 4556–4561.

Fisher, R. A. 1930. *The genetical theory of natural selection.* Oxford: Oxford University Press.

Gavrilets, S. 2004. *Fitness landscapes and the origin of species.* Princeton Univ Press.

Jost, M. C., Hillis, D. M., Lu, Y., Kyle, J. W., Fozzard, H. A., & Zakon, H. H. 2008. Toxin-Resistant Sodium Channels: Parallel Adaptive Evolution across a Complete Gene Family. *Molecular Biology and Evolution*, **25**, 1016–1024.

Khodorov, B. I., Yelin, E. A., Zaborovskaya, L. D., Maksudov, M. Z., Tikhomirova, O. B., & Leonov, V. N. 1992. Comparative analysis of the effects of synthetic derivatives of batrachotoxin on sodium currents in frog node of Ranvier. *Cellular and molecular neurobiology*, **12**, 59–81.

Li, H.-L., Hadid, D., & Ragsdale, D. S. 2002. The batrachotoxin receptor on the voltage-gated sodium channel is guarded by the channel activation gate. *Molecular Pharmacology*, **61**, 905–912.

Linford, N. J., Cantrell, A. R., Qu, Y., Scheuer, T., & Catterall, W. A. 1998. Interaction of batrachotoxin with the local anesthetic receptor site in transmembrane segment IVS6 of the voltage-gated sodium channel. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 13947–13952.

Long, S. B., Campbell, E. B., & MacKinnon, R. 2005. Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. *Science*, **309**, 89–903.

Mappes, J., Marples, N., & Endler, J. 2005. The complex business of survival by aposematism. *Trends in Ecology & Evolution*, **20**, 598–603.

Maxson, L., & Myers, C. 1985. Albumin evolution in tropical poison frogs (Dendrobatidae): a preliminary report. *Biotropica*, **17**, 50–56.

Maynard Smith, J., Burian, R., Kauffman, S., Alberch, P., Campbell, J., Goodwin, B., Lande, R., Raup, D., & Wolpert, L. 1985. Developmental constraints and evolution: a perspective from the Mountain Lake conference on development and evolution. *The Quarterly Review of Biology*, **60**, 265–287.

Márquez, R. 2011. *¿Evolución paralela del síndrome aposemático en ranas venenosas del género Phyllobates?* Bogotá: Universidad de Los Andes.

Miller, S. P., Lunzer, M., & Dean, A. M. 2006. Direct Demonstration of an Adaptive Constraint. *Science*, **314**, 458–461.

Myers, C., Daly, J., & Malkin, B. 1978. A dangerously toxic new frog (*Phyllobates*) used by Emberá Indians of western Colombia, with discussion of blowgun fabrication and dart poisoning. *Bulletin of the American Museum of Natural History*, **161**, 307–366.

Narahashi, T., Moore, J. W., & Scott, W. R. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *The Journal of General Physiology*, **47**, 965–974.

Neuwirth, M., Daly, J. W., Myers, C. W., & Tice, L. W. 1979. Morphology of the granular secretory glands in skin of poison-dart frogs (Dendrobatidae). *Tissue and Cell*, **11**, 755-771.

Olson, M. E. 2012. The developmental renaissance in adaptationism. *Trends in Ecology & Evolution*, **27**, 278–287.

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. 2004. UCSF Chimera: A visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, **25**, 1605–1612.

Pigliucci, M., & Kaplan, J. 2000. The fall and rise of Dr Pangloss: adaptationism and the Spandrels paper 20 years later. *Trends in Ecology & Evolution*, **15**, 66–70.

Pyron, R. A., & Wiens, J. J. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution*, 1–39.

Ruxton, G. D., Sherratt, T. N., & Speed, M. P. 2005. *Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals and Mimicry.* Oxford University Press, USA.

Santos, J. C., & Cannatella, D. C. 2011. Phenotypic integration emerges from aposematism and scale in poison frogs. *Proceedings of the National Academy of Sciences*, **108**, 6175–6180.

Saporito, R. A., Donnelly, M. A., Spande, T. F., & Garraffo, H. M. 2011. A review of chemical ecology in poison frogs. *Chemoecology*, **22**, 159-168.

Schlichting, C. D., & Pigliucci, M. 1998. *Phenotypic Evolution: A Reaction Norm Perspective.* 1st edn. Sinauer Associates.

Silverstone, P. A. 1976. A revision of the poison-arrow frogs of the genus *Phyllobates* Bibron *in* Sagra (Family Dendrobatidae). *Natural History Museum of Los Angeles County*, **27**, 1–53.

Stern, D. L., & Orgogozo, V. 2009. Is genetic evolution predictable? *Science*, **323**, 746–751.

Streisfeld, M. A., & Rausher, M. D. 2010. Population genetics, pleiotropy, and the preferential fixation of mutations durring adaptive evolution. *Evolution*, **65**, 629–642.

Strichartz, G., Rando, T., & Wang, G. K. 1987. An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annual review of neuroscience*, **10**, 237–267.

Svensson, E., & Calsbeek, R. 2012. *The Adaptive Landscape in Evolutionary Biology.* Oxford University Press.

Takata, M., Moore, J. W., Kao, C. Y., & Fuhrman, F. A. 1966. Blockage of sodium conductance increase in lobster giant axon by tarichatoxin (tetrodotoxin). *The Journal of General Physiology*, **49**, 977–988.

Terlau, H., Heinemann, S. H., Stünhmer, W., Pusch, M., Conti, F., Imoto, K., & Numa, S. 1991. Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel HI. *FEBS Letters*, **293**, 93–96.

Tikhonov, D. B., & Zhorov, B. S. 2005a. Modeling P-Loops Domain of Sodium Channel: Homology with Potassium Channels and Interaction with Ligands. *Biophysical Journal*, **88**, 184–197.

Tikhonov, D. B., & Zhorov, B. S. 2005b. Sodium channel activators: Model of binding inside the pore and a possible mechanism of action. *FEBS Letters*, **579**, 4207–4212.

Trainer, V. L., Brown, G. B., & Catterall, W. A. 1996. Site of Covalent Labeling by a Photoreactive Batrachotoxin Derivative near Transmembrane Segment IS6 of the Sodium Channel α Subunit. *The Journal of biological chemistry*, **271**, 11261–11267.

Trott, O., & Olson, A. J. 2009. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, **31**, 455-461.

Vendantham, V., & Cannon, S. C. 2000. Rapid and Slow Voltage-Dependent Conformational Changes in Segment IVS6 of Voltage-Gated Na+ Channels. *Biophysical Journal*, **78**, 2943–2958.

Venkatesh, B., Lu, S. Q., Dandona, N., See, S. L., Brenner, S., & Soong, T. W. 2005. Genetic Basis of Tetrodotoxin Resistance in Pufferfishes. *Current Biology*, **15**, 2069–2072.

Vogler, A. 1998. Covariation of defensive traits in tiger beetles (genus Cicindela): a phylogenetic approach using mtDNA. *Evolution*, **52**, 529–538.

Wake, D. B. 1991. Homoplasy: The result of natural selection or evidence of design? *The American Naturalist*, **138**, 543–567.

Wang, S. Y., & Wang, G. K. 1998. Point mutations in segment I-S6 render voltage-gated Na+ channels resistant to batrachotoxin. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 2653–2658.

Wang, S.-Y., & Wang, G. K. 1999. Batrachotoxin-Resistant Na+ Channels Derived from Point Mutations in Transmembrane Segment D4-S6. *Biophysical Journal*, **76**, 3141–3149.

Wang, S.-Y., Nau, C., & Wang, G. K. 2000. Residues in Na+ Channel D3-S6 Segment Modulate both Batrachotoxin and Local Anesthetic Affinities. *Biophysical Journal*, **79**, 1379–1387.

Wang, S.-Y., Mitchell, J., Tikhonov, D. B., Zhorov, B. S., & Wang, G. K. 2006. How batrachotoxin modifies the sodium channel permeation pathway: computer modeling and site-directed mutagenesis. *Molecular Pharmacology*, **69**, 788–795.

Wang, S.-Y., Tikhonov, D. B., Zhorov, B. S., Mitchell, J., & Wang, G. K. 2007a. Serine-401 as a batrachotoxin- and local anesthetic-sensing residue in the human cardiac Na+ channel. *Pflügers Archiv - European Journal of Physiology*, **454**, 277–287.

Wang, S.-Y., Tikhonov, D. B., Mitchell, J., Zhorov, B. S., & Wang, G. K. 2007b. Irreversible block of cardiac mutant Na+ channels by batrachotoxin. *Channels*, **1**, 179–188.

Warnick, J. E., Alburquerque, E. X., Onur, R., Jansson, S. E., Daly, J., Tokuyama, T., & Wiktop, B. 1976. The pharmacology of batrachotoxin. VII. Structure-activity relationships and the effects of pH. *The Journal of Pharmacology and Experimental Therapeutics*, **193**, 232–245.

Weinreich, D. M. 2006. Darwinian Evolution Can Follow Only Very Few Mutational Paths to Fitter Proteins. *Science*, **312**, 111–114.

Wright, S. 1932. The roles of mutation, inbreeding, crossbreeding, and selection in evolution. *Proceedings of the Sixth International Congress of Genetics*, **1**, 356–366.

Yang, Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Computer applications in the biosciences. CABIOS*, **13**, 555–556.

Yang, Z. 2007. PAML 4: Phylogenetic Analysis by Maximum Likelihood. *Molecular Biology and Evolution*, **24**, 1586–1591.

Yang, Z., Wong, W. S. W., & Nielsen, R. 2005. Bayes Empirical Bayes Inference of Amino Acid Sites Under Positive Selection. *Molecular Biology and Evolution*, **22**, 1107–1118.

Zhang, J., Nielsen, R., & Yang, Z. 2005. Evaluation of an Improved Branch-Site Likelihood Method for Detecting Positive Selection at the Molecular Level. *Molecular Biology and Evolution*, **22**, 2472–2479.

Zhao, Y., Yarov-Yarovoy, V., Scheuer, T., & Catterall, W. A. 2004. A Gating Hinge in Na^+ Channels: A Molecular Switch for Electrical Signaling. *Neuron*, **41**, 859–865.

Zhen, Y., Aradema, M. L., Medina, E. M., Schumer, M., & Andolfato, P. 2012. Parallel Molecular Evolution in an Herbivore Community. *Science*, **337**, 1643–16.

Figures

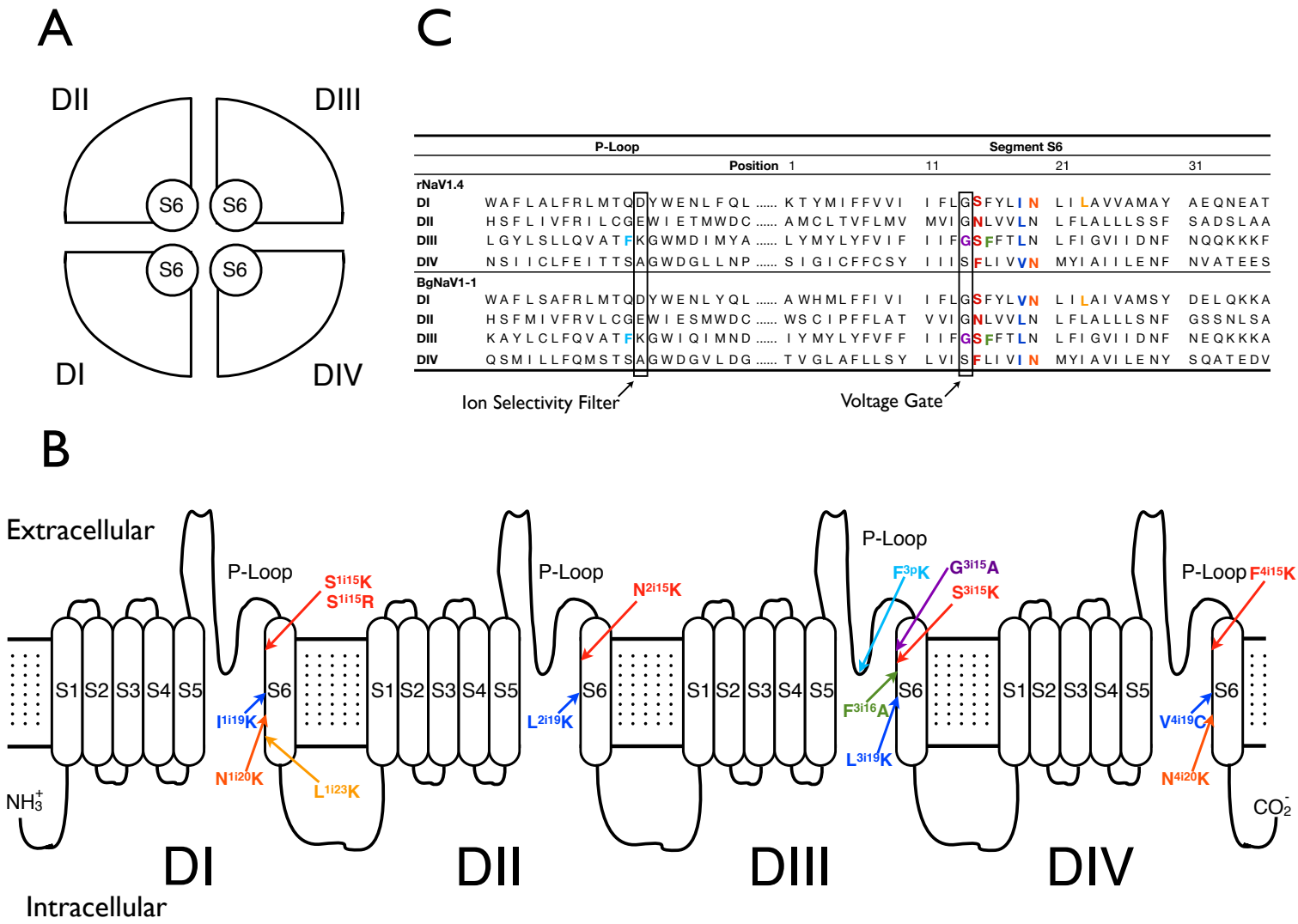

Figure 1. Top-down (A) and lateral (B) views of the two-dimensional structure of voltage-gated sodium channels, showing the four domains, each divided in six transmembrane segments linked by extra and intracellular loops. Panel C shows an alignment of the P-Loops and S6 segments of the four domains of the rat muscular channel (rNaV1.4) and the cockroach channel (BgNaV1-1). The residues that make up ion selectivity filter and voltage gate are enclosed in black rectangles, and the approximate location of mutations known to decrease BTX affinity for sodium channels is shown on panel B, and these sites are highlighted with matching colors on panel C.

Figures

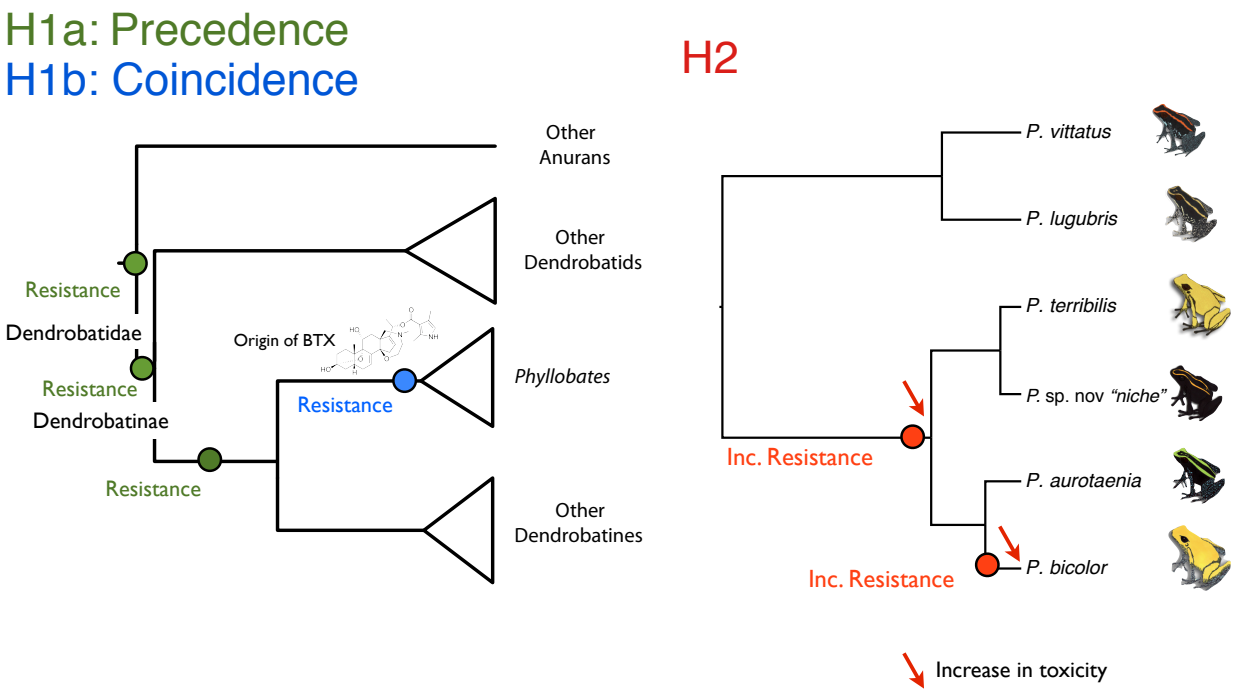

Figure 2. Schematic representation of the expected results under our two working hypotheses (see the introduction for details). H1a predicts that functional substitutions in the NaV1.4 gene that confer resistance to BTX (green dots) should predate the branch basal to all *Phyllobates*, where BTX toxicity is thought to have originated (Maxson & Myers 1985); H1b predicts that such changes (blue dot) coincided with the acquisition con BTX. H2 predicts similar substitutions (red dots) in branches where toxicity has increased within *Phyllobates*, after the acquisition of BTX (red arrows). The general topology of Dendrobatidae (H1) follows Pyron & Wiens (2011), and that of *Phyllobates*, as well as changes in toxicity follow Márquez (2011). Frog illustrations were made by Lina M. Arenas.

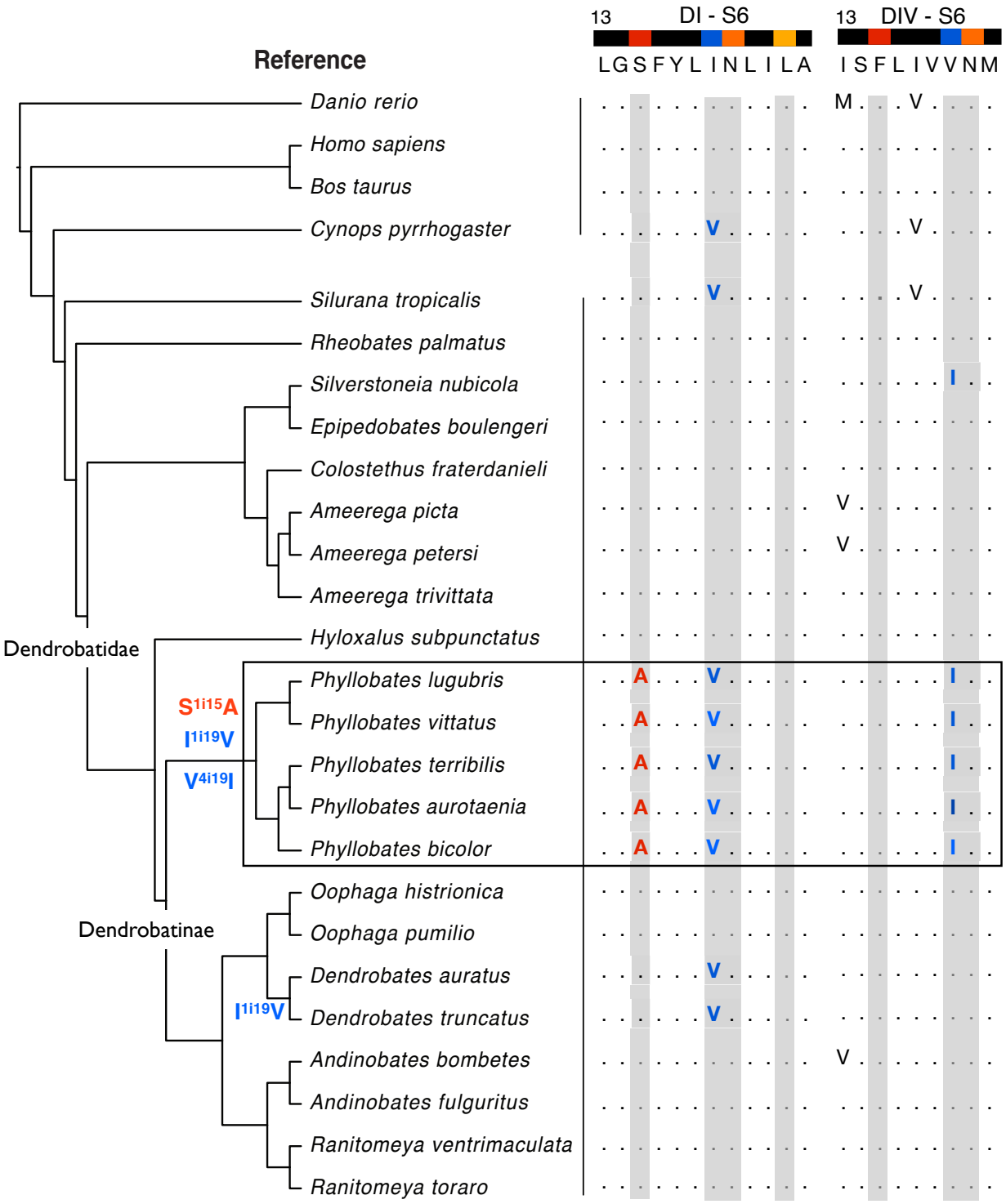

Figure 3. Phylogenetic relationships and amino acid sequences of the DI and DIV S6 segments of the muscular voltage-gated sodium channel (NaV1.4) for the studied taxa. Sites of known functional importance for BTX binding are shaded and marked with colors matching those of Fig. 1. Polymorphic sites far from those known to interact with BTX are not shown. The nodes where mutations occurred at functionally important sites are labeled with the specific mutation, also colored to match Fig. 1; only nodes within Dendrobatinae are labeled. The sequences of the Zebrafish, two mammals, and a salamander (*C. pyrrhogaster*) are shown only for comparison, and were not used in any analyses. The sequences of *Phyllobates* species are enclosed in a black rectangle. The topology is in accordance with Pyron & Wiens (2011), and branch lengths are not meaningful.

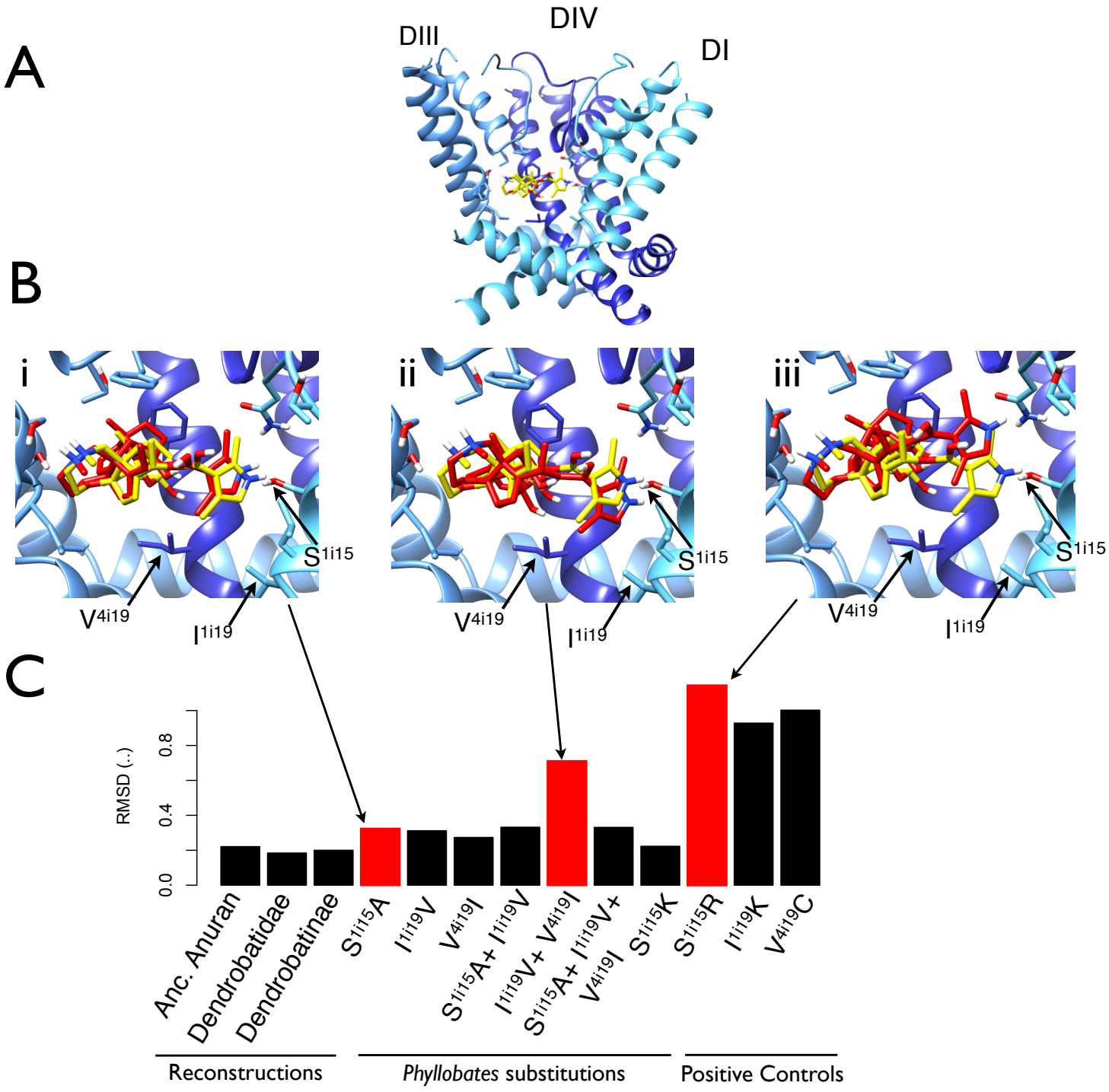

Figure 4. Results from molecular docking simulations. Panel A shows a general view of the binding conformation of BTX proposed by Du et al. (2011). B shows a detailed view of the binding site, and the conformation of three of the simulated mutants (**i:** $S^{1i15}A$, **ii:** $I^{1i19}V+V^{4i19}I$ **iii:** $S^{1i15}R$, colored red) in comparison to the original conformation, colored yellow. The view is oriented to highlight differences in the conformation of the pyrrole ring of BTX, which plays an important role in BTX binding, and domain II is not shown for better visualization. Panel C shows the RMSDs to the original BTX conformation. red bars correspond to the conformations illustrated in panel B.

Supplementary Information

Table S1. Vouchers and localities of individuals sequenced for this study. GECOH codes are from the tissue collection of the Group Behavioral Ecophisiology and Herpetology at Universidad de Los Andes, and TNHFS codes are from the Texas Natural History Collection.

Species	Voucher	Locality
Ameerega petersi	GECOH1407	Perú, Puerto Inca, Yuyapichis, Panguana Biological Station
Ameerega picta	GECOH1406	Perú, Puerto Inca, Yuyapichis, Panguana Biological Station
Ameerega trivittata	TNHCFS4966	Colombia, Amazonas, Leticia
Andinobates bombetes	GECOH1060	Colombia, Chocó, San José del Palmar
Andinobates bombetes	GECOH297	Colombia, Valle del Cauca, Bosque Yotoco
Andinobates bombetes	GECOH1495	Colombia, Risaralda, La Celia
Andinobates fulguritus	GECOH1314	Colombia, Chocó, Lloró
Andinobates fulguritus	GECOH1615	Colombia, Chocó, Cantón de San Pablo
Colosthetus fraterdanieli	N/A	N/A
Colosthetus fraterdanieli	N/A	N/A
Colosthetus fraterdanieli	GECOH193	Colombia, Quindío, Filandia
Dendrobates auratus	GECOH1844	Panamá, Veraguas
Dendrobates auratus	GECOH1824	Panamá, Panamá, Bayano Lake
Dendrobates truncatus	GECOH1589	Colombia, Chocó, Nuquí
Dendrobates truncatus	GECOH2035	Colombia, Tolima, Lérida
Epipedobates boulengeri	GECOH594	Colombia, Valle del Cauca, Buenaventura
Epipedobates boulengeri	GECOH640	Colombia, Valle del Cauca, Buenaventura
Epipedobates boulengeri	GECOH691	Colombia, Cauca, Guapi
Hyloxalus subpunctatus	TNHCFS4957	Colombia, Boyacá, Chiquinquirá
Oophaga histrionica	GECOH1002	Colombia, Valle del Cauca, Buenaventura
Oophaga histrionica	GECOH1008	Colombia, Valle del Cauca, Buenaventura
Oophaga pumilio	GECOH2007	Nicaragua, Kilambé
Oophaga pumilio	N/A	Panamá, Panamá, Bocas del Toro
Phyllobates aurotaenia	GECOH705	Colombia, Chocó, Tadó
Phyllobates aurotaenia	GECOH706	Colombia, Chocó, Tadó
Phyllobates aurotaenia	GECOH1145	Colombia, Chocó, Nuquí
Phyllobates aurotaenia	GECOH1155	Colombia, Chocó, Nuquí
Phyllobates aurotaenia	GECOH1307	Colombia, Chocó, Lloró
Phyllobates aurotaenia	GECOH1484	Colombia, Chocó, Tadó
Phyllobates bicolor	GECOH523	Colombia, Risaralda, Pueblo Rico
Phyllobates bicolor	GECOH1039	Colombia, Chocó, San José del Palmar
Phyllobates bicolor	GECOH1174	Colombia, Risaralda, Pueblo Rico
Phyllobates bicolor	GECOH1176	Colombia, Risaralda, Pueblo Rico
Phyllobates bicolor	GECOH1485	Colombia, Risaralda, Pueblo Rico
Phyllobates niche sp. nov.	GECOH1170	Colombia, Valle del Cauca, Buenaventura
Phyllobates niche sp. nov.	GECOH1189	Colombia, Valle del Cauca, Buenaventura
Phyllobates niche sp. nov.	GECOH1190	Colombia, Valle del Cauca, Buenaventura
Phyllobates niche sp. nov.	GECOH1194	Colombia, Valle del Cauca, Buenaventura
Phyllobates lugubris	GECOH963	Costa Rica, Limón, Puerto Viejo de Talamanca
Phyllobates lugubris	GECOH964	Costa Rica, Limón, Puerto Viejo de Talamanca
Phyllobates lugubris	GECOH966	Costa Rica, Limón, Puerto Viejo de Talamanca

Species	Voucher	Locality
Phyllobates lugubris	GECOH1865	Panamá, Panamá, Isla Vergauas
Phyllobates lugubris	GECOH1866	Panamá, Panamá, Isla Colon
Phyllobates terribilis	GECOH646	Colombia, Valle del Cauca, Buenaventura
Phyllobates terribilis	GECOH1165	Colombia, Valle del Cauca, Buenaventura
Phyllobates terribilis	GECOH1183	Colombia, Cauca, TImbiquí
Phyllobates vittatus	GECOH976	Costa Rica, Puntarenas, Gulf of Dulce
Phyllobates vittatus	GECOH979	Costa Rica, Puntarenas, Gulf of Dulce
Ranitomeya toraro	GECOH1698	Colombia, Amazonas, Leticia
Ranitomeya toraro	GECOH1702	Brazil, Amazonas, Atalaia do Norte
Ranitomeya ventrimaculata	GECOH1699	Colombia, Amazonas, Leticia
Rheobates palmatus	GECOH2050	Colombia, Cundinamarca, Ubaque
Rheobates palmatus	GECOH2051	Colombia, Cundinamarca, Ubaque
Silverstoneia nubicola	GECOH2039	Colombia, Valle del Cauca, Buenaventura
Silverstoneia nubicola	GECOH2040	Colombia, Valle del Cauca, Buenaventura

Table S2. Details of primers designed for this study. Primer names give information on the exon or intron where they are located, and the direction of the primer (for example E9F is a forward primer on exon 9).

Name	Location	Sequence (5' - 3')	T_A
E9F	DI S5-S6 Linker	ACCCTAAGAGCTGCAGGTAA	50ºC
E9R	DI-DII Linker	TGTTTCTTTAGCTGTTCTAGCATGT	50ºC
E24F	DIV S5	TGCCYTGATGATGTCWCTCCCWGC	58ºC
E24R	C-terminal Intracellular loop	TCCAAYCACCATYGGGAGRTCCA	58ºC