

**Snakes (Colubridae: *Erythrolamprus*) with a complex toxic diet show convergent yet highly heterogeneous voltage-gated sodium channel evolution**

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**Abstract**

Chemical defense plays a crucial role in shaping ecosystems through selection for toxin resistance and has evolved convergently across multiple lineages. Research on toxin resistance has been pivotal in understanding trait evolution, as it often evolves through a simple genetic mechanism, target-site resistance (TSR), where mutations in target genes confer resistance. However, in tropical ecosystems, multiple selective pressures from prey with different toxins create complex chemical scenarios for predators that require more nuanced research. Royal ground snakes (*Erythrolamprus* spp.) are significant but understudied predators of poisonous frogs (families Bufonidae and Dendrobatidae), whose toxins affect voltage-gated sodium channels (VGSCs) and other neuromuscular system proteins. This study introduces *Erythrolamprus* snakes as a model for studying toxin resistance. We investigated the evolution of TSR in VGSC genes in relation to toxic frog predation, tracing the phylogenetic origin and geographic distribution of TSR-conferring genotypes across six *Erythrolamprus* species and outgroups. Our findings reveal convergent yet highly heterogeneous TSR evolution in at least two species known to predate poisonous frogs. Amino acid changes at nine resistance-related positions across eight VGSC genes were identified, suggesting coordinated evolution across this gene family. Four of these changes are known to provide tetrodotoxin resistance in other species.

We observed polymorphism in resistance-related sites across species, populations, and VGSC paralogs, possibly maintained through trade-offs among toxin resistance, VGSC function, and the snakes' wide geographic and ecological range. These findings offer new insights into adaptation mechanisms in predators with complex toxic diets, advancing our understanding of coevolution in tropical ecosystems.

**Keywords:** Toxin resistance, poison frogs, Tetrodotoxin, target-site resistance, convergent evolution

### **Significance Statement**

This study introduces a new evolutionary model for highly biologically and chemically diverse ecosystems where prey with multiple toxins and their predators interact. We show convergent origins of protein variants that confer toxin resistance in Royal Ground snakes (*Erythrolamprus* spp.) of the Colombian tropics. We found high variation in toxin-resistant protein variants within and among individuals, species, and populations of snakes, possibly because of their wide geographical and ecological range. Variation in available toxic prey likely trades off with the impact of toxin resistance on protein function, resulting in diversity in adaptations to toxic prey; thus, diversity begets diversity.

### **Introduction**

Chemical defense is an anti-predatory trait spread widely throughout the network of life. Along with chemical defenses evolves the ability to resist them, both in the organisms defending themselves (e.g., prey) and those that they target (e.g., predators). Three molecular resistance mechanisms have been proposed to counteract exposure to toxins, 1) detoxifying or clearing the toxin, 2) expressing toxin-binding proteins or transporters that prevent the toxin from reaching its target site, and 3) evolving insensitivity in the proteins targeted by the toxin, a mechanism called target-site resistance (TSR) (Geffeney et al. 2005; Després et al. 2007; Tarvin et al. 2017; Caty et al. 2019; Abderemane-Ali et al. 2021; Alvarez-Buylla et al. 2022; Chen et al. 2022; Tarvin et al.

2023). Among these, TSR is prevalent in the literature due to its ease of identification and quantification (see references in (van Thiel et al. 2022)). For example, a wide variety of animals that interact with tetrodotoxin (TTX) present a suite of convergent mutations in voltage-gated sodium channels (VGSCs), many of which have been shown to confer TTX resistance *in vitro* (Jost et al. 2008; Feldman et al. 2012; McGlothlin et al. 2016; Gendreau et al. 2021). Other amino acid substitutions in a nicotinic acetylcholine receptor encode TSR for epibatidine (Tarvin et al. 2017), and mutations in the sodium-potassium ATPase pump counteract cardiotonic steroids (Karageorgi et al. 2019; Mohammadi et al. 2021). The recurrent origin of similar or the same amino acid substitutions in the presence of toxins has shown to be a convergence that is molecularly constrained (Agrawal 2017). The widespread convergence in TSR suggests that screening DNA sequences is a powerful tool for investigating possible toxin-resistance mechanisms in previously unstudied taxa (Feldman et al. 2012).

Amphibians are renowned for their diverse array of toxic and antimicrobial skin secretions, making them a model group for studying the evolution of chemical defenses (Daly 1995; Daly 1998; Saporito et al. 2011). Their ecological interactions provide an exceptional framework to investigate mechanisms of toxin resistance, including TSR. The tropical rainforests of South America, a global amphibian biodiversity hotspot (Fritz and Rahbek 2012; Nori et al. 2018; Bolochio et al. 2020), present a unique opportunity to study these dynamics. In these ecosystems, interactions between toxic amphibians and their predators impose complex selection pressures across multiple trophic levels, driving the potential evolution of toxin-resistance mechanisms, including TSR, across diverse genes and species within the trophic network (Daly et al. 1999a; Asner et al. 2014; Salazar et al. 2018; González Montoya 2021). Among amphibian predators, the colubrid snake genus *Erythrolamprus* stands out for its ability to consume multiple poisonous frog species, raising questions about the molecular basis of their resistance. Although there are no comprehensive reports on the diet of this genus, a series of natural history observations support the hypothesis that these snakes are generalist predators that include

poisonous frogs in their diet (Santos et al. 2003; Acevedo et al. 2016; Pašukonis and Loretto 2020; Torres-Carvajal and Hinojosa 2020). For example, the Amazonian snake *Erythrolamprus reginae* and the Chocóan snake *Erythrolamprus epinephelus* coexist with and have been reported to prey on species of several chemically defended amphibian families, including the amine-defended Leptodactylidae, and cardiotonic-steroid (CTS) and TTX-defended Bufonidae (Michaud and Dixon 1989; Lynch et al. 1997; Jiménez-Ortega et al. 2004; Lynch 2005; Albarelli and Santos-Costa 2010; Pinto-Erazo et al. 2020; Gallardo et al. 2022; Instituto Humbolt 2022; Mosquera et al. 2022). *Erythrolamprus* snakes also prey on highly toxic frogs of the family Dendrobatidae: the diet of *E. reginae* includes *Ameerega trivittata*, which secretes several alkaloids including histrionicotoxin (HTX) and pumiliotoxin (PTX) (Albarelli and Santos-Costa 2010; Santos et al. 2016; Pašukonis and Loretto 2020); *E. epinephelus* is the only known predator of the highly toxic *Phyllobates terribilis* which secretes batrachotoxin (BTX), one of the most potent animal toxins (Myers et al. 1978). Populations of *E. epinephelus* from Costa Rica exhibit mutations that may allow them to consume TTX-defended prey, possibly frogs of the genus *Atelopus* (Feldman et al. 2012; McGlothlin et al. 2016; Pearson and Tarvin 2022). How *E. reginae* are able to prey on toxic frogs remains unknown. For decades, scientists have focused on the poisonous frogs, paying little attention to the predators involved in their evolution. Further, the consumption of toxic amphibians makes the genus *Erythrolamprus* an ideal system to study processes underlying adaptations to consume a highly diverse set of toxic prey.

*E. reginae* and *E. epinephelus* both consume multiple neurotoxic prey, possibly experiencing strong selective pressures on several different proteins. Many neurotoxins present in poisonous frogs interact with voltage-gated sodium channels (VGSCs), a family of proteins that govern the flow of sodium into neurons and muscle cells during an action potential (Daly et al. 1999a; Hille 2001; Santos et al. 2016). For example, highly toxic alkaloids such as batrachotoxin (BTX) and pumiliotoxin (PTX), and more mildly toxic molecules like histrionicotoxin (HTX) (Daly 2005; Santos et al. 2016), alter action potentials by impairing the function of VGSCs. In non-resistant organisms, exposure to these toxins leads to respiratory and cardiac arrest, pain, and/or death

(Daly et al. 1999a; Santos et al. 2016). Many of the aforementioned toxins bind several members of the VGSC family (Daly et al. 1999a; Santos et al. 2016). Therefore, the evolution of TSR should involve the adaptation of several VGSC genes. Given the broad diversity of compounds in poisonous frogs, other proteins involved in neuronal communication can also be targeted (Table S2) (Santos et al. 2016).

Although closely related, *E. epinephelus* and *E. reginae* are not sister species and they may have evolved the ability to resist toxic amphibians independently (Hurtado-Gomes 2016; Torres-Carvajal and Hinojosa 2020; Entiauspe-Neto et al. 2021). Such convergent adaptations can be categorized based on their underlying mechanisms, ranging from phenotypes produced by distinct molecular mechanisms to those produced by identical molecular pathways (Agrawal 2017). However, when comparing shared phenotypes, it is often important to consider them on a gradient between shared and distinct molecular mechanisms. Thus, the same toxin-resistance mechanism can be produced by fine-scale variations in the genotype, which could be fixed or could vary within species and populations (e.g., (Feldman et al. 2009a; Feldman et al. 2012; Hague et al. 2018)). For example, amino acid changes at different sites or different amino acid substitutions at the same site could lead to multiple segregating alleles across populations that cause the same or very similar TSR mechanisms (e.g., as (Karageorgi et al. 2019; Mohammadi et al. 2022)). Fine-scale variation in TSR genotypes and their frequencies could be used to trace microevolutionary processes. Although there are numerous examples of convergent evolution of TSR, there is less evidence regarding how microevolutionary processes such as population structure or ecology and community composition condition the evolution of the convergent phenotype. These factors offer insights into the mechanisms driving population-level biodiversity and their roles in speciation and ecosystem dynamics (Agrawal 2017).

Here we explore the degree to which TSR has evolved in VGSCs within and among populations, species, and ecosystems. Because we use convergence between species and paralogs as possible evidence for resistance, we conducted ancestral sequence reconstructions across the VGSC gene family tree, to test whether TSRs in both species evolved independently, and identify

amino acid changes linked to TSR phenotypes. We identify amino acid changes in VGSC paralogs that are likely associated with a TSR phenotype in *E. reginae*, *E. epinephelus*, and other snakes from the same community, and explore the phylogenetic origin, geographic structure, allelic variability, and linkage of these amino acid changes. Our findings provide an approximation of a resistance mechanism in predators with a complex toxic diet, and point to complex evolutionary and/or ecological dynamics as drivers of the coordinated evolution of toxin resistance across multiple genes and species, opening new research avenues to address evolutionary questions in chemically complex biotic interactions.

## Results

### VGSC Sequence Assembly & TSR Screening

We sequenced 74 genes related to neural communication using target enrichment. We assembled 9 VGSC genes of 8 *Erythrolamprus reginae* individuals and 9 *E. epinephelus* individuals from 3 and 7 different localities, respectively (Fig 1A & Table S2). In addition, we included 22 snake species that coexist in the same habitats but are not known to consume toxic prey, including four additional species of *Erythrolamprus*: five individuals of *E. melanotus*, one of *E. aesculapii*, one of *E. typhlus*, and one individual defined as *E. epinephelus* at the time of collection, but afterwards designated as *E. sp.* based on phylogenetic analyses and geographic location, which is outside the known range of *E. epinephelus* (Table S1, Fig 3). Although for this study we focus on the 9 genes that make up the VGSC alpha subunit family, as they are perhaps the best studied in the context of TSR, we make the reads containing data for all other sequenced ion channels publicly available as a rich resource for future research on the molecular mechanisms underlying toxin resistance in snakes (Table S1).

We assembled a complete coding sequence for six of the nine reptilian VGSC genes; for *SCN5A*, *SCN10A*, and *SCN11A*, we were only able to assemble partial sequences, mostly corresponding to domain IV (DIV), which has been shown to interact with multiple frog neurotoxins (Fig 2 & Fig S2) (van Thiel et al. 2022). To screen for possible sites underlying TSR in these species, we built

an amino acid alignment and constructed a phylogenetic and gene family tree with all paralogs, as well as other vertebrates and calcium channel outgroups (Supp results & Fig S1). Then, we narrowed down the set of all substitutions to only the positions that fulfilled all of the following three conditions: First, we focused only on specific regions that have been reported to be common toxin-target sites: the voltage-gate in segment 4 (S4), the channel pore (S5 & S6), and p-loops, where the ion selectivity filter is located (Stevens et al. 2011); of this set, we selected homologous positions where identical or similar amino acid substitutions were present in multiple VGSC paralogs in individuals of different or the same species; and finally, of these, we selected only the positions where amino acid substitutions were present in more than one *E. reginae* and/or *E. epinephelus* individual but were only present in a maximum of one ortholog in the outgroup species, which are not thought to consume toxic prey (Fig 1A). Nine homologous positions fulfilled these three conditions (for readability these sites will be referred to as “positions 1–9” or “P1–P9” throughout this study; Fig 1B). Amino acid substitutions at these nine positions were found in *E. reginae*, *E. epinephelus*, and notably, in *E. sp.*, which was previously identified as *E. epinephelus*. Four positions were previously reported as part of the TTX binding site (P4, P6, P7, and P8), and mutations at these sites are predicted to provide moderate (P4 & P6) or extreme (P7 & P8) TTX resistance based on computational models or electrophysiology experiments (Fig 1B) (Geffeney et al. 2005; Wang et al. 2005; Vandendriessche et al. 2008; Stevens et al. 2011).

Potential TSR substitutions were found in all VGSC genes except for *SCN5A*. The nine positions were located in the p-loops of domains II (DII), DIII, and DIV, in segment 5 of domain II (DII-S5), and in DI-S6 (Figs 2 & 4). Five of the nine positions are located in DIV, where several TSR mutations have been identified for multiple neurotoxins across animals (e.g. (Bricelj et al. 2005; Geffeney et al. 2005; Jost et al. 2008; Hanifin 2010)). Seven VGSC genes displayed mutations at one or more of the DIV positions (Fig 2). At five of the identified putative TSR positions, the TTX-resistant snake *Thamnophis sirtalis* also has substitutions (Fig 2). Using a Mixed Effects Model of Evolution (MEME) to analyze the evolutionary rates of VGSC codons in 22 snake

species, we showed that seven of the focal sites were found to be under positive selection along several branches of the VGSC family tree (in purple, Fig 2) (Kosakovsky Pond et al. 2019). Some additional sites that did not meet the conditions outlined above were also identified by MEME to be under positive selection; these are listed in Dataset S1-Sheet A.

Additionally, we found other interesting substitutions located in important functional regions that did not pass the TSR screening conditions. For example, two changes (A445D-SCN4A and V1576M-SCN11A) each present in only one gene were fixed in the *Erythrolamprus* genus but not shared with other snakes. The A445D-SCN4A change also convergently evolved in three branches of the poison frog family Dendrobatidae, as well as the distantly related genus *Mantella* (Tarvin et al. 2016). Although we don't focus on these mutations in the present study, they show interesting signatures for future studies and are reported in Dataset S1-Sheet B.

### **Geographic segregation and independent origin of TSR sites**

Samples from *E. reginae*, *E. epinephelus*, and *E. sp* were collected from three, seven, and one locality, respectively (Fig 3A). To interrogate the geographic segregation and phylogenetic origin of TSR sites relative to the history of *Erythrolamprus*, we reconstructed a phylogeny of our focal species using two mitochondrial (*COI*, *16S*) and two nuclear (*c-mos*, *RAG2*) gene sequences. Within the *Erythrolamprus* genus, we found *E. reginae* and *E. sp* to be the sister taxa of the rest of the *Erythrolamprus* samples (Fig 3A & Supp results). However, support for the position of *E. sp* is low (0.25), and further assessment of its position in the phylogeny will be necessary. We did not find evidence of genetic structure among the *E. reginae* populations despite our large geographic sampling region (Fig 3A & 4A). In contrast, *E. epinephelus* individuals clustered into two highly supported clades: group 1 (G1 - triangles) constituted by samples from the inland Chocó rainforests and Cordillera Oriental, and group 2 (G2 - squares) from the Cordillera Central and Tumaco on the southern Pacific coast of Colombia (Fig 3A). These groupings fall in line with previous work proposing different subspecies, as well as cryptic diversity within *E. epinephelus* (Cope 1899; Torres-Carvajal and Hinojosa 2020).



We found high heterogeneity in the presence of putative TSR-conferring amino acid changes across individuals and populations of *E. reginae* and *E. epinephelus*. The VGSC gene tree for all samples used shows that resistant allele variants found within a species are more similar to the non-resistant variants within that species than to resistant alleles in the other species, suggesting independent and convergent origins of the resistant alleles (Fig. S2). Furthermore, ancestral sequence reconstructions support that all identified amino acid changes have independent origins within each species (Fig 4, Fig. S5 & Dataset S4). Together, this evidence strongly suggests that the putative TSR positions evolved convergently rather than in a common ancestor. We note, however, that when resistant species share the same TSR codon at a given site, the above result does not necessarily imply independent mutations. A single ancestral mutation could have persisted at low frequencies in all three species, with selection later increasing its frequency in resistant species while recombination with non-TSR alleles erased signs of shared ancestry. Given that these species shared a common ancestor ~10MY ago (Kumar et al. 2022), distinguishing between single or multiple mutational events is challenging without detailed knowledge of population genetic parameters, such as effective population sizes or the fitness tradeoffs of specific TSR mutations (e.g. (del Carlo et al. 2024)). In any case, our results support the independent rise in frequency of these genotypes within each species, regardless of their identity-by-descent status.

Most of the putative TSR amino acid changes are not fixed within species, or even within populations in Leticia, Amazonas; most commonly, they were found at intermediate frequencies, or at high frequencies but not fixed species-wide (i.e., some heterozygous individuals were found). We acknowledge that sampling limitations restrict our ability to interpret population-level variation, so we focus on polymorphisms within species rather than broader population structure, except in better-sampled localities like Leticia. If we only focus on the amino acid changes that have previously been associated with TTX resistance (P4, P6, P7 & P8), we find that none of them are fixed in *E. reginae*, but three are fixed in *E. epinephelus* (Fig 4). The TTX-resistant substitutions P7 & P8 - SCN9A are shared by all the suborder Serpentes, in concordance with

previous work (McGlothlin et al. 2016; Perry et al. 2018). Additionally, we did not find any clear correlation between the presence of amino acid changes from P1 to P9 and either geography or population structure, with two exceptions. Both of the extreme TTX-resistant substitutions in P7 & P8 of the *SCN4A* gene, the muscle-expressed VGSC, were only found in the Amazonian populations of *E. reginae* and in all of the G2 *E. epinephelus* individuals. To summarize the extreme variability found in the data, we illustrate the heterogeneity in genotypes at each of the putative TSR positions across the *E. reginae* and *E. epinephelus* samples, including any apparent geographic patterns, in Figs 5 & S2.

### **Fine-scale variation in and linkage of putative TSR sites**

The preceding evidence indicates that TSR evolved under similar molecular mechanisms in at least some of the nine positions highlighted, as substitutions at the same homologous sites of the VGSC genes appeared independently in three *Erythrolamprus* species. However, there is fine-scale variation in the (potentially) resistant phenotype at the molecular level. For example, if we focus on the sites where amino acid substitutions can provide extreme TTX resistance (P7 & P8), there is variation in the identity of the amino acid change and frequency of homozygosity across paralogs, species, and populations (Fig 3A). In *E. epinephelus*, at least four amino acid combinations were found at P7 and P8 in *SCN1A*: the ancestral “DG” combination, as well as the derived combinations “ND”, “SD”, and the most frequent “SE”. All *E. epinephelus* individuals carried at least one putatively toxin-resistant substitution at these two sites (Fig 3A). Amino acid changes at P7 and P8 in *SCN1A* show a progression in mutational distance from the ancestral genotype “DG” to “ND”, which is separated from “DG” by a single mutation at each codon, then to “SD” which adds a second nucleotide substitution from “N” to “S”, and finally “SE”, which requires an additional substitution from “D” to “E” (see *SCN1A* gene in Fig 3B). Interestingly, the two substitutions biochemically represent complementary polarity changes: at P7, from a negatively charged amino acid (“D”) to an uncharged one (“N” or “S”), and at P8, from an uncharged amino acid (“G”) to a negatively charged one (“D” or “E”).

Of the nine highlighted putative TSR positions, five are found in the DIV p-loop (P5-P9). Thus, we generated haplotype networks for DIV sequences within *E. epinephelus* and *E. reginae* to unveil the genealogical relationships between haplotypes with different amino acid identities at P7 and P8 (Fig 3B). Within each species, we found networks in most cases consistent with each putative TSR substitution arising once. However, in some instances, our results suggest that some variants may have evolved multiple times within a single species. For example, in the *SCN3A* gene in *E. reginae*, the two distant haplotypes carrying the “DV” variant could have originated independently (Fig 3B & samples 2726 and 2335 in Fig. S2), although we note that haplotype networks alone are not sufficient to recapitulate the origins of a particular mutation, as they don’t explicitly account for recombination.

Finally, to explore possible linked segregation between P7 and P8 across paralogs, we calculated pairwise linkage disequilibrium (LD) between alleles at these two specific positions. These positions were chosen because there is strong evidence supporting that they confer extreme resistance to TTX (Geffeney et al. 2005; McGlothlin et al. 2016; Vaelli et al. 2020). We found a relatively strong degree of linkage disequilibrium ( $R^2$  between 0.5 to 1) between P7 and P8 sites in the *SCN1A*, *SCN4A*, and *SCN8A* genes in both species (Fig S3). However, larger population sampling is necessary to make robust conclusions in this regard.

## **Discussion**

### **Putative phenotypic effects of the amino acid changes**

This study describes the independent evolution, and possibly origin, of putative toxin-resistance-conferring mutations in at least two species of the *Erythrolamprus* genus. We identified nine homologous positions across the VGSC family that exhibit amino acid substitutions in regions functionally important for neurotoxin resistance (Fig 2 & 5). These positions represent convergent changes in the same sequence location (homologous site) across genes, but not necessarily changes that encode the same amino acid, although we also observed identical substitutions in some cases. However, it is important to highlight that functional experiments are

necessary to confirm whether specific substitutions provide any TSR for biologically relevant toxins (Feldman et al. 2016; Reimche et al. 2022). With this caveat in mind, we hypothesize that it is likely that substitutions at the positions we identify affect *E. reginae* and *E. epinephelus* neurotoxin resistance phenotypes given their association with resistance to toxins like TTX, and because VGSC genes have been shown to be under strong purifying selection, so nearly-neutral amino-acid substitutions are unlikely, especially at critical parts of the protein like the p-loop, voltage gate, and channel pore (Zakon et al. 2008; Zakon 2012).

Of the nine putative TSR positions highlighted in this study, several amino acid changes at four of these positions (P4, P6, P7, and P8) have been shown to provide TTX resistance, and substitutions at the other five positions are shared with several other neurotoxin-resistant organisms (Fig 5). Electrophysiology experiments and computational models in different organisms provide evidence that TTX resistance is conferred by substitutions at P4, P6, P7 & P8 (Terlau et al. 1991; Bricelj et al. 2005; Geffeney et al. 2005; McGlothlin et al. 2016; Vaelli et al. 2020). For P4, computational models showed that replacing the wild-type aspartic acid (“D”) in the rat SCN2A with polar-charged amino acids provided moderate TTX and STX resistance (Terlau et al. 1991; Bricelj et al. 2005). For P6, electrophysiology experiments showed that amino acid changes provide moderate TTX resistance in *T. sirtalis* snakes and *Taricha* newts (Geffeney et al. 2005; Vaelli et al. 2020). For P7 and P8, electrophysiology experiments have shown that amino acid changes found in the *T. sirtalis* SCN4A channel cause extreme TTX resistance (McGlothlin et al. 2016; Perry et al. 2018). The same asparagine (“N”) that confers TTX resistance in *T. sirtalis* is present in the SCN1A, SCN2A, SCN3A, and SCN4A proteins of *E. reginae*, *E. epinephelus*, and *E. sp.* (Fig 2 & see protein names in Table S2), suggesting these proteins also resist TTX. In addition, we confirmed that all the snake species in this study, including all sampled *Erythrolamprus* individuals, share the extreme TTX-resistant changes at P7 & P8 - SCN9A and at P8 - SCN11A that were hypothesized to be ancestral to snakes, as well as changes in P6 that were reported in the SCN8A and SCN11A channels in some other species of the Colubridae family (Fig 2 & Fig S2), and are hypothesized to provide some level of ancestral

TTX resistance (McGlothlin et al. 2016; Perry et al. 2018). However, because we could not retrieve the DI, DII, and DIII from *SCN5A*, *SCN10A*, and *SCN11A*, we could not verify whether the sequenced individuals possessed additional TTX-resistant changes that have been hypothesized to be ancestral to snakes reported by (Perry et al. 2018).

Concordant with electrophysiological evidence, multiple TTX-resistant organisms exhibit changes at these positions (Table S6; see references in (van Thiel et al. 2022)). Based on the points above, the assumption that at least some of the identified mutations in *E. reginae*, *E. epinephelus*, and *E. sp.* are likely to be involved in TTX resistance seems reasonable (Fig 5). Although the phylogenetic position of *E. sp.* is uncertain, *E. sp.* possess different amino substitutions than the other two species (*E. reginae* and *E. epinephelus*) at some putative TSR positions, providing evidence for a possible third independent origin of neurotoxin resistance in the genus (Fig 2).

The previously discussed positions have been reported to cause a TTX-resistant phenotype. On the other hand, changes in P1, P2, P3, P5, and P9 are shared with different toxin-resistant vertebrates, but there is no available experimental or computational data regarding their effect on toxin resistance (Fig 2). Thus, it is important to note that some of these mutations may arise from mechanisms unrelated to toxin resistance. However, we hypothesize that some of these positions could contribute to the toxin-resistant phenotype (Fig 2). For example, amino acid changes in P1 and P9 are both shared with the *SCN4A* from dendrobatid frogs, but are not located directly in the DIV p-loop (Fig 2, Table S6) (Tarvin et al. 2016). Changes at P2 are shared with the TTX-resistant snakes from the *Thamnophis* genus and the hog-nosed snake *Heterodon platirhinos*, which preys on TTX-bearing newts (*Notophthalmus viridescens*; Table S6) (Brodie and Brodie 1990; Feldman et al. 2016).

### **The convergent evolution of the VGSCs between species and genes**

The presence of convergent or similar amino acid substitutions at the same homologous sites across genes in multiple species of the *Erythrolamprus* genus reveals that toxin resistance in

Royal Ground snakes has likely evolved – at least partly – through a shared molecular mechanism. Eight of nine VGSC genes contained mutations that encode amino acid changes at the putative TSR positions (Fig 2). Although we do not possess information about the tissue-specific expression of these channels in *Erythrolamprus* snakes, the VGSC gene family in amniotes contains 9-11 genes (nine in reptiles) that play different roles in the central and peripheral nervous system. In mammals, *SCN1A*, *SCN2A* and *SCN3A* are expressed in the brain, *SCN4A* is expressed in skeletal muscle, *SCN5A* in cardiac muscle, and *SCN8A* and *SCN9A* in the peripheral nervous system (Fux et al. 2018). The lizard *Anolis carolinensis* exhibits the same expression pattern (Fig S4). In *T. sirtalis*, *SCN10* and *SCN11A* are expressed in sensory neurons (Perry et al. 2018). The presence of heterogeneous amino acid changes in several VGSC paralogs within and among species makes clear that further research is necessary to understand how toxin sensitivity varies across tissues and populations in *E. reginae*, *E. epinephelus*, and *E. sp.*, and among different toxic molecules.

To our knowledge, we report the first potential evidence of TTX-resistance-conferring substitutions in reptiles at the *SCN1A*, *SCN2A*, and *SCN3A* genes, which are expressed exclusively in the brain in *Anolis carolinensis* (Fig S4) (Eckalbar et al. 2013), as well as in mammals (Fux et al. 2018). The fact that other TTX-resistant snakes, such as *Thamnophis* spp. do not exhibit TTX-resistant genotypes at these genes has been explained by the notion that TTX cannot cross the blood-brain barrier (McGlothlin et al. 2016). Our results open the possibility that, at least in *Erythrolamprus*, TTX may move across the blood-brain barrier, as occurs in pufferfish (Amano et al. 2022). Alternatively, these substitutions may be involved in resistance to other toxins that cross the blood-brain barrier. Finally, these proteins may be expressed in tissues other than the brain, such as the adrenal glands (Fig S4).

We found strong signatures of linkage disequilibrium between putative TSR mutations at different VGSC genes. Although some of these signatures could be caused by physical linkage or gene conversion, since some VGSC genes are clustered in tandem within chromosomes (Gendreau et al. 2020; Gendreau et al. 2021), we propose that selection for specific genotype combinations

across tissues may explain some of the observed linkage. For example, we show a correlation between positions P7 & P8 in *SCN1A*, *SCN4A*, and *SCN8A* in both *E. reginae* and *E. epinephelus* samples (Fig S3). These genes are located on three different chromosomes in *Crotalus viridis* (Gendreau et al. 2020). This evidence of linked segregation between genes expressed in different tissues suggests that the presence of differential TSRs between VGSC genes is necessary for the resistant phenotype. However, the available data are not sufficient to robustly support this hypothesis or clarify its potential causes.

### **Ecological context and relevance for VGSC evolution in *Erythrolamprus* snakes**

Our study has shown that resistance to TTX is highly plausible for *E. reginae*, *E. epinephelus*, and *E. sp* (Fig 4). Thus there are multiple ecological implications of the TTX-resistant phenotype according to the distribution and diet of each of these species. The Harlequin frogs in the genus *Atelopus* and the poison frog *Colostethus panamansis* are the only reported amphibians with TTX within the ranges of *E. reginae*, *E. epinephelus*, and *E. sp*. (Daly et al. 1994; Grant 2007; Pearson and Tarvin 2022). In Costa Rican populations of *E. epinephelus*, substitutions at P6, P7, and P8 in *SCN4A*, *SCN8A*, *SCN10A*, and *SCN11A* have been previously reported, indicating a broad geographic distribution of resistance alleles that could match with the presence of *Atelopus* frogs (Feldman et al. 2012; McGlothlin et al. 2016; Perry et al. 2018). These frogs have suffered a recent and drastic population decline, but in the past, they were abundant and sympatric with *Erythrolamprus* snakes across multiple biomes (La Marca et al. 2005; Lötters et al. 2023). In the *Colostethus* genus, the phylogenetic distribution of TTX secretion is still unclear. Although proposed for *C. ucumari* from visual observations, the presence of TTX was never chemically confirmed in this species, and recent studies have not found evidence of TTX in *C. imbricolus* or in the skin microbiome of *C. panamansis* (Grant 2007; Martin H. et al. 2020; González Montoya 2021). Furthermore, there are no known *Colostethus* species distributed in sympatry with *E. reginae*.

Additional conditions could explain the evolution of TTX-resistant substitutions. First, these snakes could prey on uncharacterized TTX-defended amphibians, or other tetrodotoxic animals, such as flatworms in the subfamily Geoplanidae, which are common in Amazonian forests (VRC, pers. obs). Our understanding of the presence of TTX in tropical organisms is limited, and further research is necessary to trace the presence of this molecule in the Andes, Amazon, and Chocó forests. Alternatively, the TTX-resistant substitutions exhibited by *Erythrolamprus* snakes could provide resistance to additional toxins with similar targets. Two toxic molecules have been reported to bind at the same sites as TTX: STX and  $\mu$ -conotoxin (Colquhoun et al. 1972; Lipkind and Fozzard 1994; Zhang et al. 2009; Stevens et al. 2011). These two toxins are found in freshwater organisms that could be part of the snakes' diet. However, there is a lack of surveys to trace the possible presence of STX and  $\mu$ -conotoxin in terrestrial organisms. Furthermore, some of the substitutions found in this study could provide resistance against other frog toxins. For example, PTX-B found in frogs from the *Oophaga* genus competes against *Scorpio* toxins and brevetoxins that bind to DI-S6 and DIV S3-S4 (Daly et al. 1990; Gusovsky et al. 1992; Stevens et al. 2011; Santos et al. 2016), and HTX found in *Ameerega* and other dendrobatid frogs inhibits the binding of [3H]BTX-B that affects the S6 channel pore (Lovenberg and Daly 1986; Wang et al. 2005; Santos et al. 2016). In addition, amino acid changes in the DIII p-loop of rat sodium channels provide BTX resistance (Wang et al. 2005). Finally, while we think it unlikely, it is possible that the positions identified in this study are not involved in toxin resistance at all; instead, they may be related to other physiological function and variation in these snake species.

### **Evolutionary genetics of TSR allele polymorphisms**

TSR is a molecular mechanism of toxin resistance that evolved convergently in multiple species of *Erythrolamprus* snakes. At a finer molecular level, we found that the identity and presence of different amino acid changes at P1–P9 are highly variable, suggesting that protein function and resistance varies across tissues and individuals. There are at least four levels of molecular variation in our data that would contribute to organismal-level variation in resistance. A first level



of variation is different amino acid changes segregating at the same position (e.g. Fig 3A). These amino acid changes could differentially impact protein function, producing multiple fitness peaks or exhibiting intermediate variants in the mutational path between non-resistant and resistant variants (for example; *SCN1A* and *SCN4A* in Fig 3B). Alternatively, different amino acid changes in the same position could produce the same toxin-resistant phenotype (Karageorgi et al. 2019; Mohammadi et al. 2022). For example, all observed substitutions at P7 and P8 exhibit biochemical convergence, maintaining consistent polarity and charge shifts. A second level of variation is the presence and absence of substitutions across the P1–P9 positions. In our data, some samples exhibit the complete array of putative TSR changes, while others have an “incomplete” version, even within populations (Fig 4 and Fig 3A). This pattern could emerge from balancing selection, as well as from incomplete or/and soft selective sweeps. Distinguishing between these evolutionary mechanisms remains a challenge and requires genome-wide data and larger sample sizes (Booker et al. 2017; Bitarello et al. 2023). A third level of variation is the presence and absence of amino acid changes with a potentially compensatory function. In this study, we defined compensatory amino acid changes as non-synonymous substitutions that evolved to maintain the protein function despite the adverse effects of TSR substitutions. We suggest that some of the putative TSR positions have a compensatory effect, for example, if the substitution is not directly located close to a toxin binding site (eg: P3 and P9 in Fig 2) (e.g. (Mohammadi et al. 2022)). Finally, a fourth level of variation is the differential presence of amino acid substitutions at TSR positions across the VGSC paralogs that were previously discussed (see ‘The convergent evolution of the VGSCs between species and genes’ discussion section).

The extensive molecular variation present at P1–P9 across the VGSC family is consistent with a selection regime that promotes polymorphism. Balancing selection, soft selective sweeps, and local adaptation could all produce the patterns that we encounter in our data. These mechanisms have been previously reported in predator-prey coevolutionary relationships, including in snake predators (Brodie et al. 2002; Schield et al. 2022). Some TSR substitutions, including at positions P6, P7, and P8, have been shown to impair sodium channel function, resulting in a trade-off

between toxin resistance and organism-level performance (i.e. reduced speed and muscle force) (Feldman et al. 2016; Hague et al. 2018; del Carlo et al. 2024). Alleles with weak tradeoffs can remain at low frequencies within a population for longer than those without tradeoffs, increasing the chances that they undergo soft selective sweeps (Hermisson and Pennings 2017). The ecological relevance of this reduction of function has not been tested yet, but it seems likely that it could result in balancing selection, especially considering that the impact of a trade-off between toxin resistance and locomotor performance/muscle force would depend on ecological aspects such as the density of toxic prey or the strength of predation pressures, which can vary over time and space within the range of a population.

In this same vein, the large geographic and ecological range of these species could promote local adaptation, with gene flow between locally adapted populations resulting in within-population polymorphisms. Local adaptation is a common mechanism for the evolution of VGSCs in *T. sirtalis* snakes, where TTX resistance varies widely across geography (Brodie et al. 2002; Feldman et al. 2009b), and may explain the diversity of VGSC alleles observed in this study (Fig 5). Prey availability, the specific toxic molecules, and the level of toxicity that *Erythrolamprus* snakes encounter are likely to be highly variable across species ranges. We propose that geographic heterogeneity of selection pressures contributes to and promotes a dynamic evolution of VGSCs, which can easily result in within-species polymorphism. In general, we do not find a clear or generalized geographical pattern that explains the presence of particular polymorphisms (Figs 3A & 5A). The Chocó and Amazon harbor a wider diversity of poison frogs than other biogeographic regions of Colombia where *E. reginae* and *E. epinephelus* occur (Lynch et al. 1997). The evolution of toxin resistant variants of the *SCN4A* channel is common in vertebrate predators and prey exposed to high levels of neurotoxins, such as TTX, that target VGSCs (Geffeney et al. 2005; Jost et al. 2008; Feldman et al. 2012; Hanifin and Gilly 2015). If the Amazon and Chocó rainforests harbor a higher abundance of tetrodotoxic amphibians, it is possible that populations in these regions are under stronger selective pressures to evolve toxin resistance. Concordantly, we found that the TSR mutations in the muscle-expressed *SCN4A*

channel were only present in individuals of the *E. reginae* Amazon population, and at much higher frequency in *E. epinephelus* clade 1, which occurs in the Chocó rainforest and the Eastern cordillera (allele freq. = 1), than in clade 2, which occurs in the Central Cordillera (allele freq = 0.25; Figs 3A & 5A). Nevertheless, broader geographic and within-population sampling, functional data on the physiological effects of the observed mutations, and further information on the distribution and toxicity of amphibian chemical defenses (e.g. (Pearson and Tarvin 2022)) are essential to understand the population dynamics of the resistant phenotype in these species, and to discriminate between selection regimes that influence them. For now, the data available suggest that local adaptation and balancing selection process could help maintain these polymorphisms across and within populations.

### **Concluding Remarks**

This work introduces a new model system for studying eco-evolutionary adaptations of toxin resistance in the highly chemically and biologically diverse forests of South America. This model provides an opportunity to investigate coordinated evolution across gene families in relation to toxin resistance, as well as convergent evolution between species. Although we focus on voltage-gated sodium channels, we provide extended sequencing data of other ion channels to facilitate future research on the molecular mechanism of toxin resistance. Urgent research on these topics is fundamental to understanding the impact of declining species and the resulting loss of complexity in the chemical composition of the forests and the maintenance of biodiversity.

### **Materials and Methods**

#### **Sample collection and target enrichment sequencing**

We collected tissue samples from 40 snakes from different locations across Colombia, spanning multiple ecosystems including grasslands, and lowland and montane wet forests, under permit

1177 (October 9, 2014, file No. IDB0359), granted by the Colombian Authority for Environmental Licenses (ANLA; Table S1). Collection protocols were approved by the Universidad de los Andes Bioethics Committee (protocol number C.FUA 14-018M). For each sample, we obtained mouth swabs, muscle tissue, or liver tissue, subsequently stored in 70% ethanol or RNAlater® (Life Technologies, Carlsbad, CA) at –80 °C. We also obtained tissue samples (tail clipping, muscle, or liver) through loans from the Instituto de Ciencias Naturales (ICN) at the Universidad Nacional de Colombia (Table S1). Our final dataset consisted of 9 *E. epinephelus* individuals corresponding to 7 different localities (Fig 3A), 8 *E. reginae* individuals corresponding to 3 localities, and 4 *E. melanotus* individuals corresponding to 3 localities, one Amazonian sample of *E. typhlus*, one Amazonian sample of *E. aesculapii*, as well as 17 individuals from 12 genera outside *Erythrolamprus*, which were used as outgroups (Table S1). Unfortunately, because whole animals were not collected or because of the current condition of the specimens, we do not have data on the sex of the individuals.

We used target enrichment and Illumina sequencing to obtain around 200 Kb that corresponded to the complete sequence of 74 genes from 14 gene families involved in neurological functions, and therefore putatively involved in neurotoxin resistance, and two nuclear genes commonly used in vertebrate phylogenetic reconstruction (Table S2). For this study, we focus on the 9 genes that make up the voltage-gated sodium channel alpha subunit family (VGSC family). To ensure transparency and accessibility, we have chosen to release the data of the 74 genes rather than keep them out of the public domain, despite the absence of accompanying analyses for other gene families. All generated data are publicly available on NCBI with BioProject ID PRJNA1055115. We extracted DNA using the Qiagen DNeasy kit and quantified it using a Qubit dsDNA HD Assay Kit (Thermo Fisher Scientific). We re-concentrated some samples using a SpeedVac (Thermo Fisher Scientific) until they reached a minimum DNA concentration threshold of 40 ng/ul. We fragmented DNA to obtain 400-bp fragments by running three cycles of 30 seconds using a Brandon 2800 ultrasonicator, and prepared 40 genomic DNA Illumina libraries using the Accel-NGS 1S Plus Swift bioscience™ library preparation kit, following the

recommendations of the manufacturer, except for the purification step when we used the AMPure XP beads (Beckman Coulter) for an average size selection of 400 bp. We indexed each sample using dual identifiers (Dual-indexing barcodes) from the Swift bioscience™ 1S Plus Dual Indexing kit. For a final quality check, we used Agilent Bioanalyzer High Sensitivity DNA® kit and the Qubit dsDNA HD Assay kit. On average we obtained libraries with 400-bp fragment sizes at 4 nM. Library preparations were performed at the Universidad de los Andes in Bogotá, Colombia.

We enriched the target genes using a custom set of MYbaits® in-solution (MYcroarray) enrichment probes, designed by the company using gene annotations extracted from the *Python bivittatus* (Genbank assembly ID: GCA\_000186305) and *Ophiophagus hanna* genomes (Genbank assembly ID: GCA\_000516915), or gene sequences from *Thamnophis sirtalis*, *Erythrolamprus reginae* and *Protophryne marmorata* generated in previous studies (Table S2). We chose the most relaxed design of the probes (Relaxed-4) to account for the wide phylogenetic breadth of our samples. We pooled 2 to 3 libraries for each capture, depending on the DNA concentration, to perform 16 enrichment reactions using the provider's protocol, and we purified the enriched libraries using Dynabeads® MyOne™ Streptavidin C1 (Life Technologies). Finally, we sequenced all libraries using one lane of the Illumina NextSeq500 platform (150-bp paired-end reads) at ACGT, Inc, Wheeling, IL. The mean number of reads obtained was 1.5M (SD = 668K) per sample (Table S1).

### **VGSC family sequence annotation and TSR screening**

Read quality was evaluated using FastQC v0.10.1 (Andrews 2010). We removed adapters and quality-trimmed reads using Trimmomatic v0.32 (Bolger et al. 2014). To generate nucleotide sequences for the VGSC family we used Bowtie2 v2.3.4.1 to map the cleaned reads against the *Pantherophis guttatus* genome (Genbank assembly ID: GCA\_029531705.1). We generated phased consensus sequences using Picard v2.9.0 (Anon 2018) and samtools v1.18 (Danecek et al. 2021). We used bcftools v1.12 to call genotypes (Danecek et al. 2021). The coordinates for

each gene in the *P. guttatus* genome were annotated manually using Hmmer v3.1b2 (Finn et al. 2011) against the bait sequences (Table S2), and sequences were extracted using bedtools/2.28.0 (Quinlan and Hall 2010). Finally, we used blast v2.7.1+ (Altschul et al. 1990) to annotate the exons against our sequences, and extracted and concatenated them to assemble the coding sequences for each gene (Dataset S3). The exons used as query in blast were *SCN1A*, *SCN2A*, *SCN3A*, *SCN4A*, *SCN8A*, and *SCN9A* from *T. sirtalis* exons, previously annotated by (McGlothlin et al. 2014). For the *SCN10A* and *SCN11A* genes, we used *P. guttatus* exon annotations, and for *SCN5A*, we used *Xenopus tropicalis* and *Gallus gallus* exons. Only the domain IV (DIV) of the latter three genes was recovered. Table S3 contains the annotated coordinates for each gene on the *P. guttatus* genome and GenBank ID of the query exon sequences.

The high conservation among VGSC genes poses challenges for gene annotation and increases the probability of assembling chimeric sequences. Thus, we generated a gene family tree using the newly generated consensus sequences and reference VGSC sequences from other vertebrates, and calcium channels as outgroups to verify the gene identity of each sequence (Fig S1, Table S4 & Dataset S2). We translated and aligned the sequences using Clustal Omega v1.2.3 as implemented in Geneious v2021.2.2 (<https://www.geneious.com>). Then, using the CIPRES gateway (Miller et al. 2010), we estimated a maximum likelihood VGSC protein family tree in IQtree v2.1.2 (Nguyen et al. 2015) under the LG model, and assessed support with ultrafast bootstraps using default parameters. We visualized the resulting tree in iTol v5 (Letunic and Bork 2021). The interactive tree can be displayed in the iTol portal using ID 2011133251460081689024934. Our reconstruction showed monophyletic groupings by gene, confirming our annotations and showing no evidence of chimeric sequences (Fig S1).

The lack of clear information regarding the diet of the *Erythrolamprus* snakes did not allow us to make comparisons among specific molecules that each species/population might encounter regularly or rarely. However, at least a few toxins that are found in the reported prey and in

sympatric poisonous frogs target the VGSC family (eg: TTX, HTX, BTX, PTX, etc) (Santos et al. 2016). The target sites of TTX and BTX have been characterized using X-ray crystallography (Garber and Miller 1987; Lipkind and Fozzard 1994; Daly et al. 1999a; Daly 2005), but less is known about binding sites or activities of the other toxins (Daly et al. 1999b; Daly 2005). Thus, to screen for substitutions potentially related to toxin insensitivity, we focused on sites previously linked to TTX resistance in *T. sirtalis*, as well as those that fulfilled the three requirements shown in Fig 1. Functional regions were annotated based on (Abderemane-Ali et al. 2021). Sequence residues are numbered based on *Mus musculus* protein sequences (See Uniprot number at Table S4).

### **Phylogenetic tree reconstruction**

To interpret the evolution of TSR across the VGSC gene family in the context of *Erythrolamprus* phylogenetic history, we used *c-mos* and *RAG2* sequences (from target sequencing Table S2, Dataset S8 & S9) and two mitochondrial loci obtained through PCR and Sanger sequencing to reconstruct a phylogenetic tree for our samples and focal species (Dataset S6 & S7). We amplified the *CO1* gene using primers dgLCO-1490 (5'-GGT CAA CAA ATC ATA AAG AYA TYG G-3') and dgHCO-2198 (5'-TAA ACT TCA GGG TGA CCA AAR AAY C-3') (Meyer 2003), and the *16S* gene using 16Sbr-H (5'-CCGGT CTGAA CTCAG ATCAC GT-3') and 16Sar-L (5'- CGCCT GTTTA TCAAA AACAT-3') (Palumbi et al. 2002), following the PCR conditions described in the respective papers. Sanger sequencing data are publicly available on NCBI (Accession number 16S gene: PV124458-PV124497 and CO1 gene: PV124506-PV124545; see table S1). After aligning each set of sequences with MAFFT using the *mafft-linsi* option (Yamada et al. 2016), we estimated maximum likelihood trees separately for each gene using IQ-TREE v2.1.2 (Nguyen et al. 2015), and assessed support using ultrafast bootstrapping with the default parameters in CIPRES gateway (Miller et al. 2010). We then concatenated the two mitochondrial genes and reran the analysis. Finally, we built a species tree using ASTRAL and estimated the multilocus

2-stage bootstrap values developed in this program (Figs 4 & individualizing each of the *E. reginae* and *E. epinephelus* samples Fig 3A) (Seo 2008; Zhang et al. 2018).

### **Ancestral reconstruction & positive selection tests**

To trace the origin of the focal ancestral state of amino acid changes, we used HyPhy (Kosakovsky Pond et al. 2019), to estimate joint maximum likelihood ancestral sequences at each node, using aligned codon sequences across the entire VGSC gene family tree. The FitMG94 model of codon evolution (Muse and Gaut 1994) was used for reconstructions. To generate a tree for ancestral reconstructions we selected one sequence per VGSC gene per outgroup species, and one sequence of every resistant and non-resistant variant of *E. reginae*, *E. epinephelus*, and *E. sp.*, as shown in Fig 2. We conducted a codon alignment using MAFFT and PAL2NAL (available as Dataset S5) (Suyama et al. 2006; Kosakovsky Pond et al. 2019), and built a gene tree of this alignment in IQtree using, the best codon-based substitution model determined with the flags -st CODON -m MFP+MERGE (Minh et al. 2020). We chose to use a whole gene family tree for ancestral sequence reconstruction because it better represents the evolutionary history of the specific genetic elements under study, accounting for possible discordance with the species tree (i.e. hemiplasy), which could otherwise lead to incorrect inferences. The results of these ancestral reconstructions are included in Fig 4, Fig S5 & Dataset S4.

We screened VGSC sequences for signatures of positive, diversifying selection in a phylogenetic context using the Mixed Effects Model of Evolution, MEME, implemented in HyPhy, using default parameters (Kosakovsky Pond et al. 2019). MEME is a branch-site model that employs maximum likelihood to test for positive selection at each site, not assuming a  $\omega$  single value across the tree (Murrell et al. 2012). We conducted a codon-based selection test for the same dataset used in the ancestral sequence reconstruction and we allowed the MEME program to estimate a gene tree (Dataset S5). This test was run on the DataMonkey server using default parameters (Weaver et al. 2018), the results were visualized on Hyphy Vision (Pond 2020) and are available at Fig 2 and Dataset S1A. It is important to emphasize that our intent in using MEME was to test whether the



nine positions previously identified under specific conditions as putatively toxin-resistance-related exhibited phylogenetic signatures of positive selection, rather than serving as the primary source of evidence for selection, or a site's involvement in TSR.

### **Intraspecific variation in VGSC genotypes**

Our data revealed that several mutations putatively involved in TSR were not fixed within *Erythrolamprus* species. Therefore, to further explore patterns of intraspecific variation we built minimum-spanning haplotype networks (epsilon = 0) in PopArt (Leigh and Bryant 2015) for domain DIV of all VGSC genes (Fig 3B), where the majority of putative TSR mutations were found. In addition, we calculated nucleotide diversity ( $\pi$ ), the number of segregating sites, the number of parsimony-informative sites, and Tajima's D, testing for  $D \neq 0$  with a permutation-based p-value, using PopArt (Table S5). Networks and statistics were calculated independently for *E. reginae* and *E. epinephelus* populations, as well as for the *E. melanotus* samples, which did not exhibit the potential TSR and were used for comparison. Finally, we assessed the co-segregation of SNPs related to toxin resistance by calculating the square correlation ( $r^2$ ) of a biallelic polymorphism at the domain IV of the VGSC. To obtain a pairwise statistic we used the *LDscan* function from the *pegas* R package (Paradis 2010). The results were plotted using ggplot2 and plotly packages in Rstudio (Wickham 2009; Sievert 2020) (Fig S3).

### **Data availability**

All target sequencing data is publicly available on NCBI with BioProject ID PRJNA1055115. Sanger sequencing data from the 16S gene are publicly available on NCBI under accession numbers PV124458–PV124497, and CO1 gene sequences are available under accession numbers PV124506–PV124545 (see Table S1).

### **List of abbreviations**

VGSC = Voltage-Gated Sodium Channel

TSR = Target-Site Resistance

D = Domain of the protein

S = Segment of the protein

TTX = Tetrodotoxin

BTX = Batrachotoxin  
HTX = Histronicotoxin  
PTX = Pumiliotoxin  
STX = Saxitoxin  
CTS = Cardiotonic steroids  
LRT = Likelihood Ratio Test  
P = Position  
LD = Linkage disequilibrium

### **Acknowledgments**

We thank the Program for Basic Science of Colciencias 712-2015 (No. 120471249694) and seed projects at the Universidad de Los Andes for funding this study. We also thank the Russell E. Train Education for Nature Program (EF14103) from the World Wildlife Fund (WWF) and the National Institutes of Health (NIGMS #R35GM150574 to RDT) that funded VRC expenses during the writing process. RM was partially supported by the Michigan Society of Fellows. Thanks to the Instituto de Ciencias Naturales at the Universidad Nacional, Fernando Vargas Salinas at the Universidad del Quindío, Jhon Tailor Rengifo at the Universidad Tecnológica del Chocó, Jhonatan David Echavarria, Nicomedes Asprilla and Elkin de Jesús Asprilla for loaning samples for this study. Thanks to Reserva Natural Taninboca in Leticia and the Reserves in Bajo Calima, Valle del Cauca, and Puerto Salgar, Cundinamarca for granting permission to work there. We are very grateful for all the local guides and workers who helped us and kindly shared time and knowledge with us. Also, for having the opportunity to observe and interact with snakes and frogs, observe them closely, and share a home with them, the forest, and the cities where they live. Special thanks to the great friends we made during the Master of Biological Sciences at the Universidad de Los Andes: Camilo Rodríguez, Pablo Palacios, Diana Motta, Iván Beltrán, Mabel González, Mileydi Betancourth-Cundar, Leonardo Moreno, Jorge Díaz Riaño, Leidy Barragán, Catalina Ramírez-Portilla, among others. They taught VRC how to work in the field, analyze data, understand science, and most importantly make those years, very happy years. We acknowledge that our fieldwork was conducted in territories where local communities continue to face exclusion from scientific endeavors and suffer from the imposition of capitalist and colonial activities.

Historically, science has contributed to these activities, and regrettably, there is no assurance that the findings from this or other biological research will not perpetuate these power structures in the future. Nevertheless, we affirm our commitment to conducting scientific work that actively opposes the imposition of patriarchy, capitalism, and colonialism on any body or territory. We hope that any use or application derived from this work will not be used to further these purposes. We thank the editor and the reviewers for your valuable comments. The Spanish translation was made using ChatGPT (Available on Dataset S10) (OpenAI 2023).

## References

- Abderemane-Ali F, Rossen ND, Kobiela ME, Craig RA, Garrison CE, Chen Z, Collieran CM, O'Connell LA, Bois JD, Dumbacher JP, et al. 2021. Evidence that toxin resistance in poison birds and frogs is not rooted in sodium channel mutations and may rely on “toxin sponge” proteins. *J. Gen. Physiol.* [Internet] 153. Available from: <https://doi.org/10.1085/jgp.202112872>
- Acevedo A, Martínez Cuesta M, Cabrera Pacheco J. 2016. Erythrolamprus epinephelus (Golden-bellied Snakelet) Diet. *Herpetol. Rev.* 47:310–311.
- Agrawal AA. 2017. Toward a Predictive Framework for Convergent Evolution: Integrating Natural History, Genetic Mechanisms, and Consequences for the Diversity of Life. *Am. Nat.* [Internet] 190:S1–S12. Available from: <https://www-journals-uchicago-edu.libproxy.berkeley.edu/doi/full/10.1086/692111>
- Albarelli LPP, Santos-Costa MC. 2010. Feeding ecology of Liophis reginae semilineatus (Serpentes: Colubridae: Xenodontinae) in eastern Amazon, Brazil. *Zool. Curitiba* [Internet] 27:87–91. Available from: <http://www.scielo.br/j/zool/a/JzrKsdmm53NMWPdFf4wJz7L/?lang=en>
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.
- Alvarez-Buylla A, Payne CY, Vidoudez C, Trauger SA, O'Connell LA. 2022. Molecular physiology of pumiliotoxin sequestration in a poison frog. *PLOS ONE* [Internet] 17:e0264540. Available from: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0264540>
- Amano M, Takatani T, Sakayauchi F, Oi R, Sakakura Y. 2022. The brain of the wild toxic marine pufferfishes accumulates tetrodotoxin. *Toxicon* [Internet] 218:1–7. Available from: <https://www.sciencedirect.com/science/article/pii/S0041010122002495>
- Andrews S. 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data. Available from: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
- Anon. 2018. Picard toolkit. *Broad Inst. GitHub Repos.* [Internet]. Available from: <http://broadinstitute.github.io/picard/>
- Asner GP, Martin RE, Tupayachi R, Anderson CB, Sinca F, Carranza-Jiménez L, Martinez P. 2014. Amazonian functional diversity from forest canopy chemical assembly. *Proc. Natl. Acad. Sci.* [Internet] 111:5604–5609. Available from: <https://www.pnas.org/doi/full/10.1073/pnas.1401181111>

- Bitarello BD, Brandt DY, Meyer D, Andrés AM. 2023. Inferring Balancing Selection From Genome-Scale Data. *Genome Biol. Evol.* [Internet] 15:evad032. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10063222/>
- Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* [Internet] 30:2114–2120. Available from: <https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btu170>
- Bolochio BE, Lescano JN, Cordier JM, Loyola R, Nori J. 2020. A functional perspective for global amphibian conservation. *Biol. Conserv.* [Internet] 245:108572. Available from: <https://www.sciencedirect.com/science/article/pii/S000632071932049X>
- Booker TR, Jackson BC, Keightley PD. 2017. Detecting positive selection in the genome. *BMC Biol.* [Internet] 15:98. Available from: <https://doi.org/10.1186/s12915-017-0434-y>
- Bricelj VM, Connell L, Konoki K, MacQuarrie SP, Scheuer T, Catterall WA, Trainer VL. 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. *Nature* [Internet] 434:763–767. Available from: <https://www-nature-com.libproxy.berkeley.edu/articles/nature03415>
- Brodie ED, Brodie ED. 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. *Evolution* [Internet] 44:651–659. Available from: <http://doi.wiley.com/10.1111/j.1558-5646.1990.tb05945.x>
- Brodie Edmund D., Ridenhour BJ, Brodie E. D. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. *Evolution* [Internet] 56:2067–2082. Available from: <http://doi.wiley.com/10.1111/j.0014-3820.2002.tb00132.x>
- del Carlo RE del, Reimche JS, Moniz HA, Hague MTJ, Agarwal SR, Iii EDB, Jr EDB, Leblanc N, Feldman CR. 2024. Coevolution with toxic prey produces functional trade-offs in sodium channels of predatory snakes. *eLife* [Internet] 13. Available from: <https://elifesciences.org/reviewed-preprints/94633>
- Caty SN, Alvarez-Buylla A, Byrd GD, Vidoudez C, Roland AB, Tapia EE, Budnik B, Trauger SA, Coloma LA, O'Connell LA. 2019. Molecular physiology of chemical defenses in a poison frog. *J. Exp. Biol.* 222.
- Chen Z, Zakrzewska S, Hajare HS, Alvarez-Buylla A, Abderemane-Ali F, Bogan M, Ramirez D, O'Connell LA, Du Bois J, Minor DL. 2022. Definition of a saxitoxin (STX) binding code enables discovery and characterization of the anuran saxiphilin family. *Proc. Natl. Acad. Sci.* [Internet] 119:e2210114119. Available from: <https://www.pnas.org/doi/10.1073/pnas.2210114119>
- Colquhoun D, Henderson R, Ritchie JM. 1972. The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol.* [Internet] 227:95–126. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1331265/>
- Cope ED. 1899. Contributions to the herpetology of New Granada and Argentina, with descriptions of new forms. Philadelphia: the Philadelphia Museums Available from: <https://www.biodiversitylibrary.org/bibliography/54674>
- Daly JW. 1995. The chemistry of poisons in amphibian skin. *Proc. Natl. Acad. Sci. U. S. A.* [Internet] 92:9–13. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/7816854>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC42808>
- Daly JW. 1998. Thirty Years of Discovering Arthropod Alkaloids in Amphibian Skin †. 3864:162–172.
- Daly JW. 2005. Nicotinic agonists, antagonists, and modulators from natural sources. *Cell. Mol.*

- Neurobiol.* [Internet] 25:513–552. Available from:  
<http://link.springer.com/10.1007/s10571-005-3968-4>
- Daly JW, Gusovsky F, McNeal ET, Secunda S, Bell M, Creveling CR, Nishizawa Y, Overman LE, Sharp MJ, Rossignol DP. 1990. Pumiliotoxin alkaloids: a new class of sodium channel agents. *Biochem. Pharmacol.* 40:315–326.
- Daly JW, Gusovsky F, Myers CW, Yotsu-Yamashita M, Yasumoto T. 1994. First occurrence of tetrodotoxin in a dendrobatid frog (*Colostethus inguinalis*), with further reports for the bufonid genus *Atelopus*. *Toxicon* [Internet] 32:279–285. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/8016850>
- Daly JW, Martin Garraffo H, Spande TF. 1999a. Alkaloids from Amphibian Skins. In: Alkaloids: Chemical and Biological Perspectives. Vol. 13. Elsevier. p. 1–161. Available from:  
<https://linkinghub.elsevier.com/retrieve/pii/S0735821099800247>
- Daly JW, Martin Garraffo H, Spande TF. 1999b. Alkaloids from Amphibian Skins. In: Alkaloids: Chemical and Biological Perspectives. Vol. 13. Elsevier. p. 1–161. Available from:  
<https://linkinghub.elsevier.com/retrieve/pii/S0735821099800247>
- Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, et al. 2021. Twelve years of SAMtools and BCFtools. *GigaScience* 10:giab008.
- Després L, David J-P, Gallet C. 2007. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol. Evol.* [Internet] 22:298–307. Available from:  
<https://www.sciencedirect.com/science/article/pii/S0169534707000651>
- Eckalbar WL, Hutchins ED, Markov GJ, Allen AN, Corneveaux JJ, Lindblad-Toh K, Di Palma F, Alföldi J, Huentelman MJ, Kusumi K. 2013. Genome reannotation of the lizard *Anolis carolinensis* based on 14 adult and embryonic deep transcriptomes. *BMC Genomics* [Internet] 14:49. Available from: <https://doi.org/10.1186/1471-2164-14-49>
- Entiauspe-Neto OM, Abegg AM, Koch C, Nunez LP, Azevedo WS, Moraes LJCL, Tiutenko A, Bialves TS, Loebmann D. 2021. A new species of *Erythrolamprus* (Serpentes: Dipsadidae: Xenodontini) from the savannas of northern South America. *Salamandra* [Internet] 57:196–218. Available from: <https://zenodo.org/records/4767059>
- Feldman CR, Brodie ED, Brodie ED, Pfrender ME. 2009a. The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. *Proc. Natl. Acad. Sci. U. S. A.* [Internet] 106:13415–13420. Available from:  
[www.pnas.org/cgi/doi/10.1073/pnas.0901224106](http://www.pnas.org/cgi/doi/10.1073/pnas.0901224106)
- Feldman CR, Brodie ED, Brodie ED, Pfrender ME. 2009b. The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. *Proc. Natl. Acad. Sci. U. S. A.* [Internet] 106:13415–13420. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/19666534>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2726340>
- Feldman CR, Brodie ED, Brodie ED, Pfrender ME. 2012. Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. *Proc. Natl. Acad. Sci. U. S. A.* [Internet] 109:4556–4561. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22392995>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3311348>
- Feldman CR, Durso AM, Hanifin CT, Pfrender ME, Ducey PK, Stokes AN, Barnett KE, Brodie ED, Brodie ED. 2016. Is there more than one way to skin a newt? Convergent toxin resistance in snakes is not due to a common genetic mechanism. *Heredity* 116:84–91.
- Finn RD, Clements J, Eddy SR. 2011. HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res.* [Internet] 39:W29–W37. Available from:

- <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3125773/>
- Fritz SA, Rahbek C. 2012. Global patterns of amphibian phylogenetic diversity. *J. Biogeogr.* [Internet] 39:1373–1382. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2699.2012.02757.x>
- Fux JE, Mehta A, Moffat J, Spafford JD. 2018. Eukaryotic Voltage-Gated Sodium Channels: On Their Origins, Asymmetries, Losses, Diversification and Adaptations. *Front. Physiol.* [Internet] 9. Available from: <https://www.frontiersin.org/article/10.3389/fphys.2018.01406>
- Gallardo AO, Carrillo-Chica E, Lorenzo JEV. 2022. ANFIBIOS Y REPTILES del Aeropuerto Alfredo Vásquez Cobo.
- Garber SS, Miller C. 1987. Single Na<sup>+</sup> channels activated by veratridine and batrachotoxin. *J. Gen. Physiol.* [Internet] 89:459–480. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/2435846>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2215904>
- Geffeney SL, Fujimoto E, Brodie ED, Brodie ED, Ruben PC. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. *Nature* [Internet] 434:759–763. Available from: [www.nature.com/nature](http://www.nature.com/nature).
- Gendreau KL, Hague MTJ, Feldman CR, Brodie ED, Brodie ED, McGlothlin JW. 2020. Sex linkage of the skeletal muscle sodium channel gene (SCN4A) explains apparent deviations from Hardy–Weinberg equilibrium of tetrodotoxin-resistance alleles in garter snakes (*Thamnophis sirtalis*). *Heredity* [Internet] 124:647–657. Available from: <https://www.nature.com/articles/s41437-020-0300-5>
- Gendreau KL, Hornsby AD, Hague MTJ, McGlothlin JW. 2021. Gene Conversion Facilitates the Adaptive Evolution of Self-Resistance in Highly Toxic Newts. *Mol. Biol. Evol.* [Internet] 38:4077–4094. Available from: <https://doi.org/10.1093/molbev/msab182>
- González Montoya MC. 2021. Eco-metabolomics approach for understanding the chemodiversity of cocktails found on poison frogs. Available from: <https://repositorio.uniandes.edu.co/handle/1992/55119>
- Grant T. 2007. A new, toxic species of *Colostethus* (Anura: Dendrobatidae: Colostethinae) from the Cordillera Central of Colombia. *Zootaxa* [Internet] 1555:39–51. Available from: <https://biotaxa.org/Zootaxa/article/view/zootaxa.1555.1.3>
- Gusovsky F, Padgett WL, Creveling CR, Daly JW. 1992. Interaction of pumiliotoxin B with an “alkaloid-binding domain”; on the voltage-dependent sodium channel. *Mol. Pharmacol.* [Internet] 42:1104–1108. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/1336116>
- Hague MTJ, Toledo G, Geffeney SL, Hanifin CT, Brodie ED, Brodie ED. 2018. Large-effect mutations generate trade-off between predatory and locomotor ability during arms race coevolution with deadly prey. *Evol. Lett.* [Internet] 2:406–416. Available from: <http://doi.wiley.com/10.1002/evl3.76>
- Hanifin CT. 2010. The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. *Mar. Drugs* [Internet] 8:577–593. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20411116>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2857372>
- Hanifin CT, Gilly WF. 2015. Evolutionary history of a complex adaptation: Tetrodotoxin resistance in salamanders. *Evolution* 69:232–244.
- Hermisson J, Pennings PS. 2017. Soft sweeps and beyond: understanding the patterns and probabilities of selection footprints under rapid adaptation. *Methods Ecol. Evol.*

- [Internet] 8:700–716. Available from:  
<https://onlinelibrary.wiley.com/doi/abs/10.1111/2041-210X.12808>
- Hille B. 2001. Ion channels of excitable membranes. Sinauer
- Hurtado-Gomes JP. 2016. Systematics of the genus *Erythrolamprus* Boie 1826 (Serpentes: Dipsadidae) based on morphological and molecular evidence.
- Instituto Humbolt. 2022. Herpetofauna de ocho Biodiversidades de Colombia - Bioblitz 2022. Available from: [http://i2d.humboldt.org.co/resource?r=herp\\_bioblitz\\_2023](http://i2d.humboldt.org.co/resource?r=herp_bioblitz_2023)
- Jiménez-Ortega A, Renjifo J, García Y. 2004. Una aproximación a la herpetofauna (anfibios y reptiles) del municipio de Novita departamento del Chocó – Colombia. :39–44.
- Jost MC, Hillis DM, Lu Y, Kyle JW, Fozzard HA, Zakon HH. 2008. Toxin-resistant sodium channels: Parallel adaptive evolution across a complete gene family. *Mol. Biol. Evol.* 25:1016–1024.
- Karageorgi M, Groen SC, Sumbul F, Pelaez JN, Verster KI, Aguilar JM, Hastings AP, Bernstein SL, Matsunaga T, Astourian M, et al. 2019. Genome editing retraces the evolution of toxin resistance in the monarch butterfly. *Nature* 574:409–412.
- Kosakovsky Pond SL, Poon AFY, Velazquez R, Weaver S, Hepler NL, Murrell B, Shank SD, Magalis BR, Bouvier D, Nekrutenko A, et al. 2019. HyPhy 2.5—A Customizable Platform for Evolutionary Hypothesis Testing Using Phylogenies. *Mol. Biol. Evol.* [Internet] 37:295–299. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8204705/>
- Kumar S, Suleski M, Craig JM, Kasprowitz AE, Sanderford M, Li M, Stecher G, Hedges SB. 2022. TimeTree 5: An Expanded Resource for Species Divergence Times. *Mol. Biol. Evol.* [Internet] 39:msac174. Available from: <https://doi.org/10.1093/molbev/msac174>
- La Marca E, Lips KR, Lötters S, Puschendorf R, Ibáñez R, Rueda-Almonacid JV, Schulte R, Marty C, Castro F, Manzanilla-Puppo J, et al. 2005. Catastrophic Population Declines and Extinctions in Neotropical Harlequin Frogs (Bufonidae: Atelopus)1. *Biotropica* [Internet] 37:190–201. Available from:  
<https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1744-7429.2005.00026.x>
- Leigh JW, Bryant D. 2015. popart: full-feature software for haplotype network construction. *Methods Ecol. Evol.* [Internet] 6:1110–1116. Available from:  
<https://onlinelibrary.wiley.com/doi/abs/10.1111/2041-210X.12410>
- Letunic I, Bork P. 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* [Internet] 49:W293–W296. Available from:  
<https://doi.org/10.1093/nar/gkab301>
- Lipkind GM, Fozzard HA. 1994. A structural model of the tetrodotoxin and saxitoxin binding site of the Na<sup>+</sup> channel. *Biophys. J.* 66:1–13.
- Lötters S, Plewnia A, Catenazzi A, Neam K, Acosta-Galvis AR, Alarcon Vela Y, Allen JP, Alfaro Segundo JO, de Lourdes Almendáriz Cabezas A, Alvarado Barboza G, et al. 2023. Ongoing harlequin toad declines suggest the amphibian extinction crisis is still an emergency. *Commun. Earth Environ.* [Internet] 4:1–8. Available from:  
<https://www.nature.com/articles/s43247-023-01069-w>
- Lovenberg T, Daly JW. 1986. Histronicotoxins: effects on binding of radioligands for sodium, potassium, and calcium channels in brain membranes. *Neurochem. Res.* 11:1609–1621.
- Lynch JD. 2005. Discovery of the richest frog fauna in the world-an exploration of the forests to the north of Leticia. *Rev. Acad. Colomb. Cienc. Exactas Físicas Nat.* [Internet] 29:581–588. Available from:  
<https://biblat.unam.mx/es/revista/revista-de-la-academia-colombiana-de-ciencias-exactas-fisicas-y-naturales/articulo/discovery-of-the-richest-frog-fauna-in-the-world-an-exploration-of-the-forests-to-the-north-of-leticia>

- Lynch JD, Ruiz-Carranza PM, Ardila-Robayo MC. 1997. Biogeographic patterns of Colombian frogs and toads (Patrones biogeográficos de las ranas y los sapos de Colombia). *Rev. Acad. Colomb. Cienc. Exactas Físicas Nat.* 21:237–248.
- Martin H. C, Ibáñez R, Nothias L-F, Caraballo-Rodríguez AM, Dorrestein PC, Gutiérrez M. 2020. Metabolites from Microbes Isolated from the Skin of the Panamanian Rocket Frog *Colostethus panamansis* (Anura: Dendrobatidae). *Metabolites* [Internet] 10:406. Available from: <https://www.mdpi.com/2218-1989/10/10/406>
- McGlothlin JW, Chuckalovcak JP, Janes DE, Edwards SV, Feldman CR, Brodie ED, Pfrender ME. 2014. Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake *Thamnophis sirtalis*. *Mol. Biol. Evol.* 31:2836–2846.
- McGlothlin JW, Kobiela ME, Feldman CR, Castoe TA, Geffeney SL, Hanifin CT, Toledo G, Vonk FJ, Richardson MK, Brodie ED, et al. 2016. Historical Contingency in a Multigene Family Facilitates Adaptive Evolution of Toxin Resistance. *Curr. Biol.* 26:1616–1621.
- Meyer CP. 2003. Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. *Biol. J. Linn. Soc.* [Internet] 79:401–459. Available from: <https://academic.oup.com/biolinnean/article-lookup/doi/10.1046/j.1095-8312.2003.00197.x>
- Michaud EJ, Dixon JR. 1989. Prey items of 20 species of the Neotropical colubrid snake genus *Liophis*. *Herpetol. Rev.* 20:39–41.
- Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: 2010 Gateway Computing Environments Workshop (GCE). New Orleans, LA, USA: IEEE. p. 1–8. Available from: <http://ieeexplore.ieee.org/document/5676129/>
- Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R. 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol. Biol. Evol.* [Internet] 37:1530–1534. Available from: <https://doi.org/10.1093/molbev/msaa015>
- Mohammadi S, Herrera-Álvarez S, Yang L, Rodríguez-Ordoñez M del P, Zhang K, Storz JF, Dobler S, Crawford AJ, Andolfatto P. 2022. Constraints on the evolution of toxin-resistant Na,K-ATPases have limited dependence on sequence divergence. *PLOS Genet.* [Internet] 18:e1010323. Available from: <https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1010323>
- Mohammadi S, Yang L, Harpak A, Herrera-Álvarez S, Rodríguez-Ordoñez M del P, Peng J, Zhang K, Storz JF, Dobler S, Crawford AJ, et al. 2021. Concerted evolution reveals co-adapted amino acid substitutions in Na+K+-ATPase of frogs that prey on toxic toads. *Curr. Biol.* [Internet] 31:2530–2538.e10. Available from: [https://www.cell.com/current-biology/abstract/S0960-9822\(21\)00462-0](https://www.cell.com/current-biology/abstract/S0960-9822(21)00462-0)
- Mosquera JTR, Palacios MYR, Albornoz LMR. 2022. Colección Científica de Referencia Zoológica del Chocó- Herpetología. Available from: <https://www.gbif.org/es/dataset/2de2f65e-7031-4844-a461-687b8c197689>
- Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Pond SLK. 2012. Detecting Individual Sites Subject to Episodic Diversifying Selection. *PLOS Genet.* [Internet] 8:e1002764. Available from: <https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1002764>
- Muse SV, Gaut BS. 1994. A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. *Mol. Biol. Evol.* 11:715–724.



- Myers CW, Daly JW, 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 AMNH* ; v. 161, article 2. Available from: <http://digitallibrary.amnh.org/handle/2246/1286>
- Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* [Internet] 32:268–274. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4271533/>
- Nori J, Villalobos F, Loyola R. 2018. Global priority areas for amphibian research. *J. Biogeogr.* [Internet] 45:2588–2594. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/jbi.13435>
- OpenAI. 2023. ChatGPT. Available from: <https://chat.openai.com/>
- Palumbi S, Martin A, Romano S, Grabowski G. 2002. Simple Fool's Guide THE SIMPLE FOOL'S GUIDE TO PCR.
- Paradis E. 2010. pegas: an R package for population genetics with an integrated–modular approach. *Bioinformatics* [Internet] 26:419–420. Available from: <https://doi.org/10.1093/bioinformatics/btp696>
- Pašukonis A, Loretto M-C. 2020. Predation on the Three-striped poison frog, *Ameerega trivittata* (Boulenger 1884; Anura: Dendrobatidae), by *Erythrolamprus reginae* (Linnaeus 1758; Squamata: Collubridae). Available from: <https://www.biotaxa.org/hn/article/view/60062>
- Pearson KC, Tarvin RD. 2022. A review of chemical defense in harlequin toads (Bufonidae: *Atelopus*). *Toxicon X* [Internet] 13:100092. Available from: <https://www.sciencedirect.com/science/article/pii/S2590171022000029>
- Perry BW, Card DC, McGlothlin JW, Pasquesi GIM, Adams RH, Schield DR, Hales NR, Corbin AB, Demuth JP, Hoffmann FG, et al. 2018. Molecular Adaptations for Sensing and Securing Prey and Insight into Amniote Genome Diversity from the Garter Snake Genome. *Genome Biol. Evol.* [Internet] 10:2110–2129. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6110522/>
- Pinto-Erazo MA, Calderón Espinosa ML, Medina Rangel GF, Méndez Galeano MÁ. 2020. Herpetofauna de dos municipios del suroeste de Colombia. *Biota Colomb.* [Internet] 21:41–57. Available from: [http://www.scielo.org.co/scielo.php?script=sci\\_abstract&pid=S0124-53762020000100041&lng=en&nrm=iso&tlng=en](http://www.scielo.org.co/scielo.php?script=sci_abstract&pid=S0124-53762020000100041&lng=en&nrm=iso&tlng=en)
- Pond S. 2020. MEME analysis result visualization. *Observable* [Internet]. Available from: <https://observablehq.com/@spond/meme>
- Quinlan AR, Hall IM. 2010. BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* [Internet] 26:841–842. Available from: <http://code.google.com/p/bedtools>
- Reimche JS, del Carlo RE, Brodie Jr ED, McGlothlin JW, Schlauch K, Pfrender ME, Brodie III ED, Leblanc N, Feldman CR. 2022. The road not taken: Evolution of tetrodotoxin resistance in the Sierra garter snake (*Thamnophis couchii*) by a path less travelled. *Mol. Ecol.* [Internet] 31:3827–3843. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/mec.16538>
- Salazar D, Lokvam J, Mesones I, Vásquez Pilco M, Ayarza Zuñiga JM, de Valpine P, Fine PVA. 2018. Origin and maintenance of chemical diversity in a species-rich tropical tree lineage. *Nat. Ecol. Evol.* [Internet] 2:983–990. Available from: <https://www.nature.com/articles/s41559-018-0552-0>
- Santos JC, Coloma L a, Cannatella DC. 2003. Multiple, recurring origins of aposematism and diet

- specialization in poison frogs. *Proc. Natl. Acad. Sci. U. S. A.* [Internet] 100:12792–12797. Available from:  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=240697&tool=pmcentrez&rendertype=abstract>
- Santos JC, Tarvin RD, O'Connell LA. 2016. A Review of Chemical Defense in Poison Frogs (Dendrobatidae): Ecology, Pharmacokinetics, and Autoresistance. In: Schulte BA, Goodwin TE, Ferkin MH, editors. *Chemical Signals in Vertebrates 13*. Cham: Springer International Publishing. p. 305–337.
- Saporito RA, Donnelly MA, Spande TF, Garraffo HM. 2011. A review of chemical ecology in poison frogs. *Chemoecology* [Internet] 22:159–168. Available from:  
<http://link.springer.com/10.1007/s00049-011-0088-0>
- Schild DR, Perry BW, Adams RH, Holding ML, Nikolakis ZL, Gopalan SS, Smith CF, Parker JM, Meik JM, DeGiorgio M, et al. 2022. The roles of balancing selection and recombination in the evolution of rattlesnake venom. *Nat. Ecol. Evol.* [Internet] 6:1367–1380. Available from: <https://www.nature.com/articles/s41559-022-01829-5>
- Seo T-K. 2008. Calculating Bootstrap Probabilities of Phylogeny Using Multilocus Sequence Data. *Mol. Biol. Evol.* [Internet] 25:960–971. Available from:  
<https://doi.org/10.1093/molbev/msn043>
- Sievert C. 2020. Interactive Web-Based Data Visualization with R, plotly, and shiny. Chapman and Hall/CRC Available from: <https://plotly-r.com>
- Stevens M, Peigneur S, Tytgat J. 2011. Neurotoxins and Their Binding Areas on Voltage-Gated Sodium Channels. *Front. Pharmacol.* [Internet] 2:71. Available from:  
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3210964/>
- Suyama M, Torrents D, Bork P. 2006. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Res.* 34:W609–612.
- Tarvin RD, Borghese CM, Sachs W, Santos JC, Lu Y, O'Connell LA, Cannatella DC, Harris RA, Zakon HH. 2017. Interacting amino acid replacements allow poison frogs to evolve epibatidine resistance. *Science* 357:1261–1266.
- Tarvin RD, Pearson KC, Douglas TE, Ramírez-Castañeda V, Navarrete MJ. 2023. The Diverse Mechanisms that Animals Use to Resist Toxins. *Annu. Rev. Ecol. Evol. Syst.* [Internet] 54:null. Available from: <https://doi.org/10.1146/annurev-ecolsys-102320-102117>
- Tarvin RD, Santos JC, O'Connell LA, Zakon HH, Cannatella DC. 2016. Convergent Substitutions in a Sodium Channel Suggest Multiple Origins of Toxin Resistance in Poison Frogs. *Mol. Biol. Evol.* [Internet] 33:1068–1080. Available from: <https://doi.org/10.1093/molbev/msv350>
- Terlau H, Heinemann SH, Stühmer W, Pusch M, Conti F, Imoto K, Numa S. 1991. Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. *FEBS Lett.* [Internet] 293:93–96. Available from:  
<https://www.sciencedirect.com/science/article/pii/0014579391811596>
- van Thiel J, Khan MA, Wouters RM, Harris RJ, Casewell NR, Fry BG, Kini RM, Mackessy SP, Vonk FJ, Wüster W, et al. 2022. Convergent evolution of toxin resistance in animals. *Biol. Rev.* [Internet] 97:1823–1843. Available from:  
<https://onlinelibrary.wiley.com/doi/abs/10.1111/brv.12865>
- Torres-Carvajal O, Hinojosa KC. 2020. Hidden diversity in two widespread snake species (Serpentes: Xenodontini: Erythrolamprus) from South America. *Mol. Phylogenet. Evol.* [Internet] 146:106772. Available from:  
<https://www.sciencedirect.com/science/article/pii/S1055790320300440>
- Vaelli PM, Theis KR, Williams JE, O'Connell LA, Foster JA, Eisthen HL. 2020. The skin microbiome

- facilitates adaptive tetrodotoxin production in poisonous newts. Baldwin IT, Robert CA, Robert CA, González Montoya MC, editors. *eLife* [Internet] 9:e53898. Available from: <https://doi.org/10.7554/eLife.53898>
- Vandendriessche T, Abdel-Mottaleb Y, Maertens C, Cuypers E, Sudau A, Nubbemeyer U, Mebs D, Tytgat J. 2008. Modulation of voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels by pumiliotoxin 251D: A “joint venture” alkaloid from arthropods and amphibians. *Toxicon* 51:334–344.
- Vázquez-Restrepo D. 2019. CalPhotos: *Erythrolamprus epinephalus*. Available from: [https://calphotos.berkeley.edu/cgi/img\\_query?enlarge=0000+0000+0519+1034](https://calphotos.berkeley.edu/cgi/img_query?enlarge=0000+0000+0519+1034)
- Wang S-Y, Mitchell J, Tikhonov DB, Zhorov BS, Wang GK. 2005. How batrachotoxin modifies the sodium channel permeation pathway: computer modeling and site-directed mutagenesis. *Mol. Pharmacol.* [Internet] 69:788–795. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16354762>  
<http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.105.018200>
- Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL. 2018. Datamonkey 2.0: A Modern Web Application for Characterizing Selective and Other Evolutionary Processes. *Mol. Biol. Evol.* [Internet] 35:773–777. Available from: <https://doi.org/10.1093/molbev/msx335>
- Wickham H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer International Publishing
- Yamada KD, Tomii K, Katoh K. 2016. Application of the MAFFT sequence alignment program to large data—reexamination of the usefulness of chained guide trees. *Bioinformatics* [Internet] 32:3246–3251. Available from: <https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btw412>
- Zakon HH. 2012. Adaptive evolution of voltage-gated sodium channels: The first 800 million years. *Proc. Natl. Acad. Sci.* [Internet] 109:10619–10625. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22723361>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3386883>  
<http://www.pnas.org/cgi/doi/10.1073/pnas.1201884109>
- Zakon HH, Zwickl DJ, Lu Y, Hillis DM. 2008. Molecular evolution of communication signals in electric fish. *J. Exp. Biol.* [Internet] 211:1814–1818. Available from: <https://doi.org/10.1242/jeb.015982>
- Zhang C, Rabiee M, Sayyari E, Mirarab S. 2018. ASTRAL-III: polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinformatics* [Internet] 19:153. Available from: <https://doi.org/10.1186/s12859-018-2129-y>
- Zhang M-M, McArthur JR, Azam L, Bulaj G, Olivera BM, French RJ, Yoshikami D. 2009. Synergistic and Antagonistic Interactions between Tetrodotoxin and  $\mu$ -Conotoxin in Blocking Voltage-gated Sodium Channels. *Channels Austin Tex* [Internet] 3:32–38. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2878737/>

## Figures

**Figure 1.** Methodology to generate coding sequences of genes potentially involved in target-site resistance (TSR) and to identify putative TSR positions in *E. reginae* and *E. epinephelus*. A) Target sequencing was employed to enrich and sequence the voltage-gated sodium channel (VGSC) genes (dark grey) and other nervous-system relevant genes from Colombian snake species. The resulting reads were mapped to VGSCs in the *Pantherophis guttatus* genome (light blue) and phased consensus sequences were generated. These sequences were then BLASTed against *Thamnophis sirtalis* VGSC exons (red lines), enabling us to assemble partial or complete VGSCs for each sample. Sequences from all VGSC genes and samples were aligned. Using this VGSC family alignment, we applied three specific conditions to identify putative TSR positions (see Putative TSR positions step). If a position met all three conditions, the amino acid change (star) was characterized as a putative TSR position (red star and circle; position 1, "P1"); if not, it was not selected (grey star). B) Unfolded two-dimensional VGSC structural representation. Six transmembrane segments (S1 – S6), the pore (S5 – S6) and one extracellular p-loop are found in each of four domains (DI – DIV). The nine amino acid positions of interest are highlighted in circles with the corresponding position number (e.g. P1 in DI-S6). Positions known to provide TTX resistance are highlighted by the level of toxicity: P4 and P6 exhibit moderate TTX and STX resistance (grey circles) (Terlau et al. 1991; Geffeney et al. 2005; Vaelli et al. 2020). P7 and P8 confer extreme TTX resistance (black circles) (Geffeney et al. 2005).

**Figure 2.** A representative set of amino acid changes in nine homologous sites identified in eight VGSC genes in *Erythrolamprus reginae*, *E. epinephelus*, and *E. sp.* samples. Each focal species included individuals with haplotypes encoding a putatively non-resistant variant ("NR"), similar to the outgroup sequence, as well as one or more haplotypes with substitutions categorized as possible "resistant" variants ("R"). Additional singleton changes that we identified in these regions are detailed in Dataset S1B. For comparison, we include haplotypes from *Thamnophis sirtalis*, a snake with known TTX resistance (Genbank numbers in Table S4). The reference sequence corresponds to the reconstruction of each gene's sequence in the Most Recent Common Ancestor of snakes (snake MRCA). The positions of interest (P1-P9) are highlighted in orange.

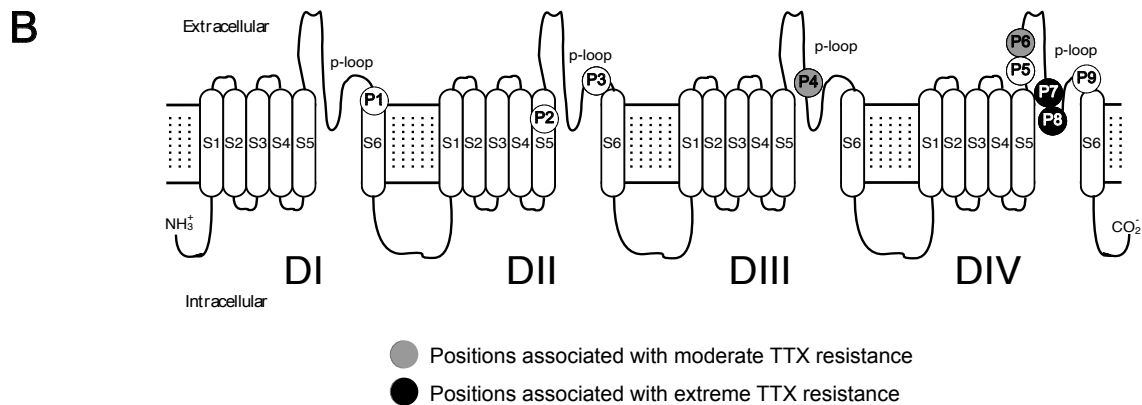
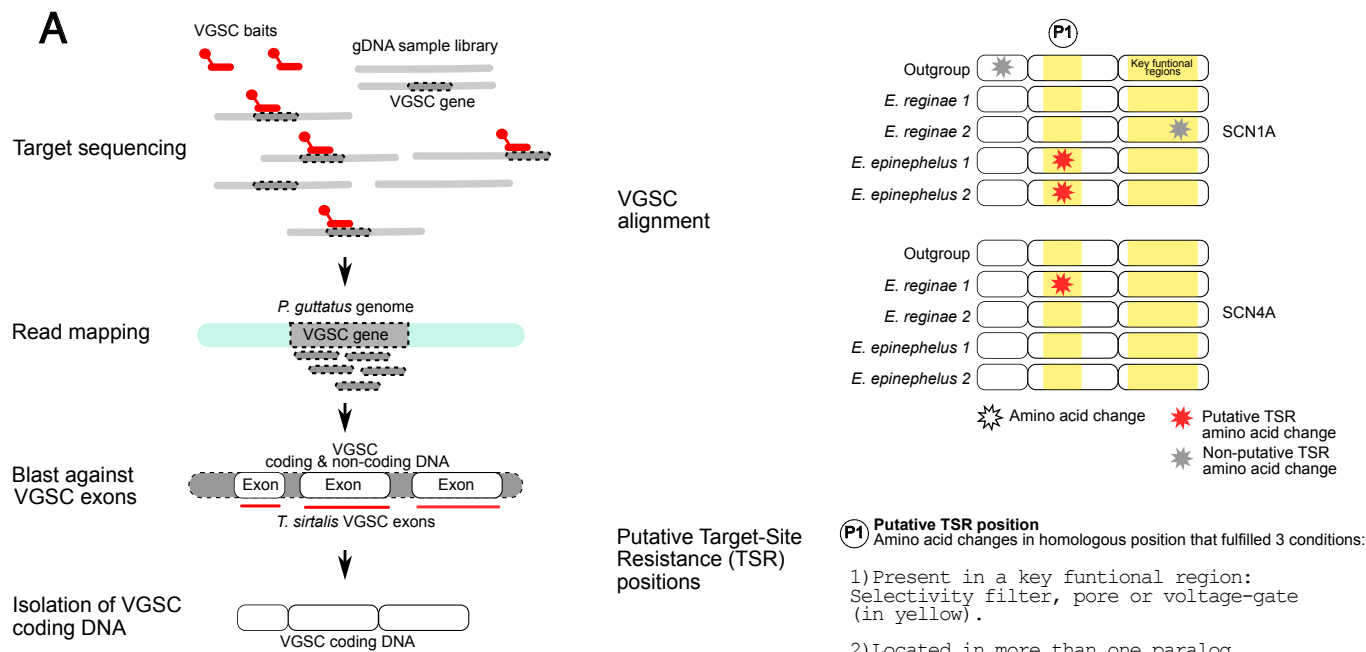
Substitutions that additionally showed evidence of positive, diversifying selection under the Mixed Effects Model of Evolution, MEME, (likelihood-ratio test (LRT)  $p < 0.05$ ) are highlighted in purple. TTX-resistance-related positions are highlighted in greyscale by the level of toxicity (see Fig. 1 for legend).

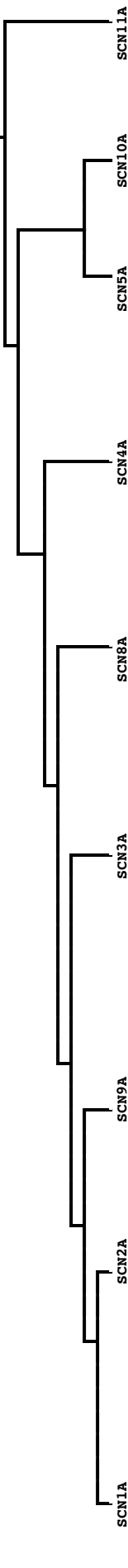
**Figure 3.** Phylogenetic tree, geographic distribution and haplotype diversity the *Erythrolamprus* samples used in this study. A) Phylogenetic tree based on two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*RAG2* and *c-mos*), alongside a visual representation of the P7 and P8 haplotypes for the VGSC genes and map of the *Erythrolamprus* samples. Node labels represent bootstrap support. In the visual representation the amino acid “DG” (Asp-Gly) is considered a non-resistant genotype in these groups (white transparent amino acid codes). Resistant haplotypes found in homozygosis are shown in red and resistant haplotypes that occur in heterozygosis with the non-resistant haplotype are shown in orange. The map shows the distribution and sampling localities for each species. . *E. reginae* samples are represented as circles: Amazonia, black; Guaviare, white; Boyacá, grey. Given that our samples of *E. epinephelus* formed two groups, we divided samples into two groups: Group 1 represented as triangles (G1), and group 2 as squares (G2). The distributional range of *E. reginae* is shown in purple and *E. epinephelus* in red. Photo credits: *E. reginae* from Leticia, Amazonas, courtesy of Dario Alarcón Naforo; *E. epinephelus* from Caldas, Antioquia, courtesy of Daniel Vásquez-Restrepo via CalPhotos (CC BY-NC-SA 3.0 © 2019) (Vásquez-Restrepo 2019). Neither of the photos corresponds to samples used in this study. B) Haplotype networks of the region containing positions P7 and P8 in domain IV (DIV) of each VGSC gene for *E. reginae* and *E. epinephelus*. Codon mutations leading to resistant genotypes, and the color-coding for indicating the haplotypes containing them are illustrated under each haplotype network.

**Figure 4.** Presence and distribution of the identified TTX-resistant changes from positions P4 (moderate TTX resistance), P6 (moderate TTX resistance), and P7 and P8 (both extreme TTX resistance) in the *Erythrolamprus* genus samples. Fully filled circles represent the presence of a

particular substitution in all individuals of that species. Half-filled circles represent sites where the amino acid change is not present across all individuals of that species (this only applies to species with more than one sample: *E. melanotus*, *E. reginae* and *E. epinephelus*). On the phylogeny we indicate the inferred origin of amino acid changes at the above positions based on HyPhy ancestral sequence reconstructions. The phylogeny corresponds to the species tree inferred by ASTRAL.

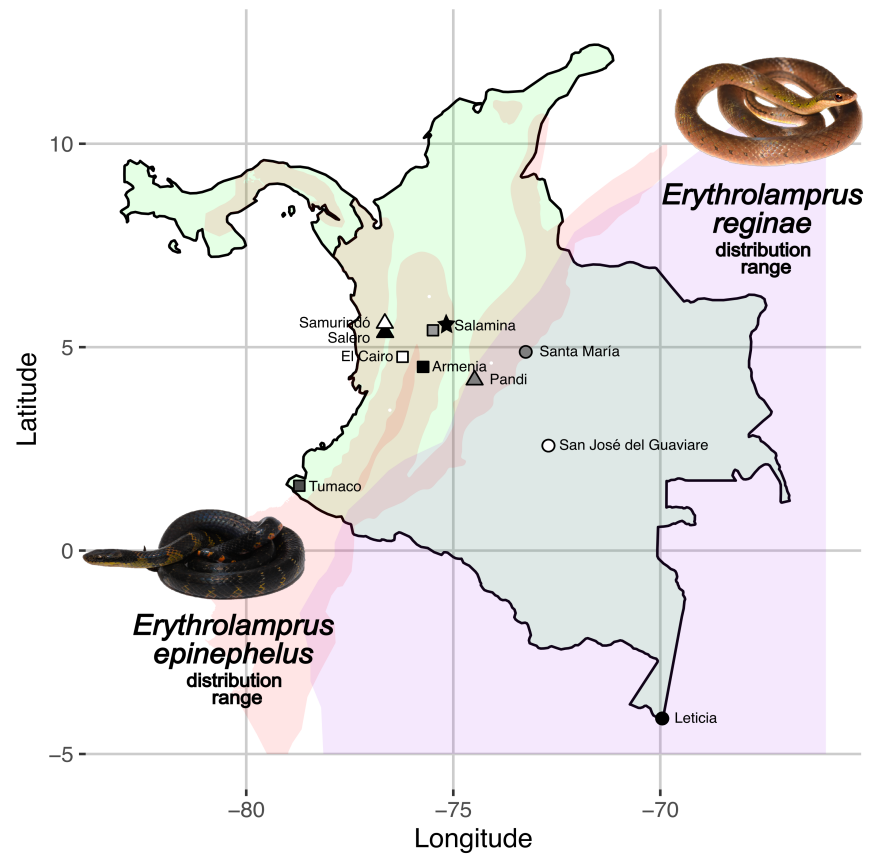
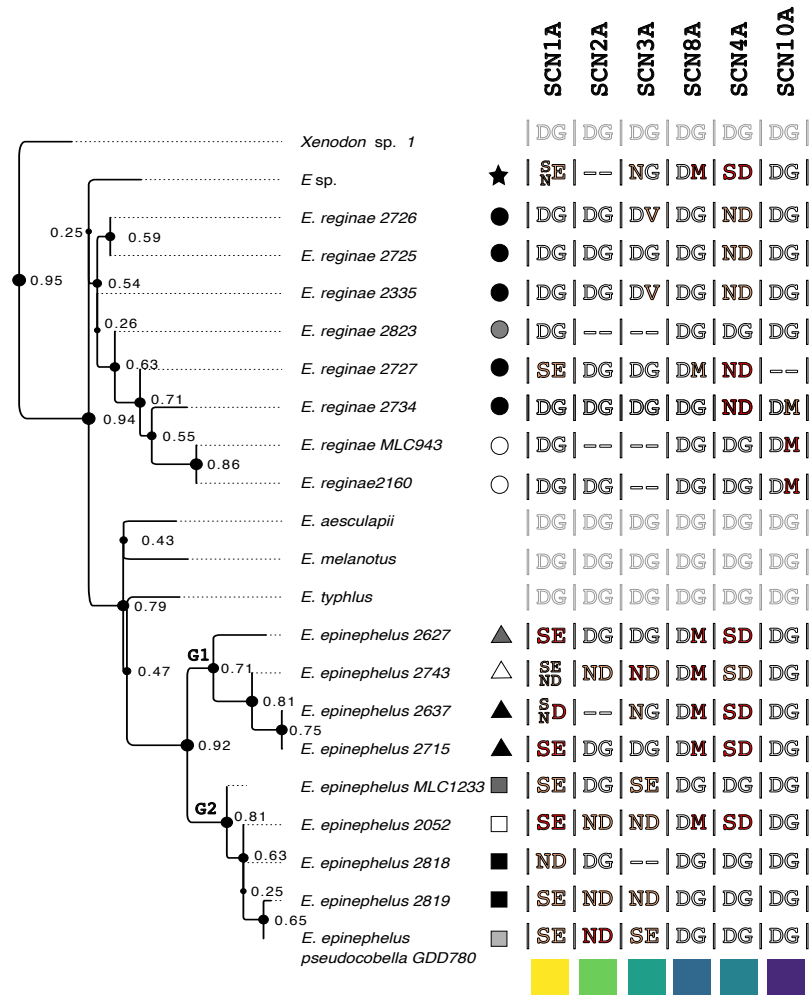
**Figure 5.** Summary of the nine positions identified in this study as potential TSR-conferring sites for *Erythrolamprus reginae* and *E. epinephelus*. From the outer to the inner circle, we summarized for each position P1-P9 the presence of each amino acid change across the 8 *E. reginae* individuals and 9 *E. epinephelus* individuals (Presence), the amino acid change and position with *Mus musculus* gene annotation (AA change), the gene(s) where this change was found (Gene), and the evidence for toxin resistance in previous electrophysiological or computational studies or presence in other toxin-resistant organisms (Evidence). Genes, changes, and presence of alleles are color-coded using the same color scheme as in Fig. 3B and Fig. 5. According to the legend in Fig. 1, we highlighted in grey (P4 and P6) and black (P7 and P8) the TTX resistant positions. Ind., individual.



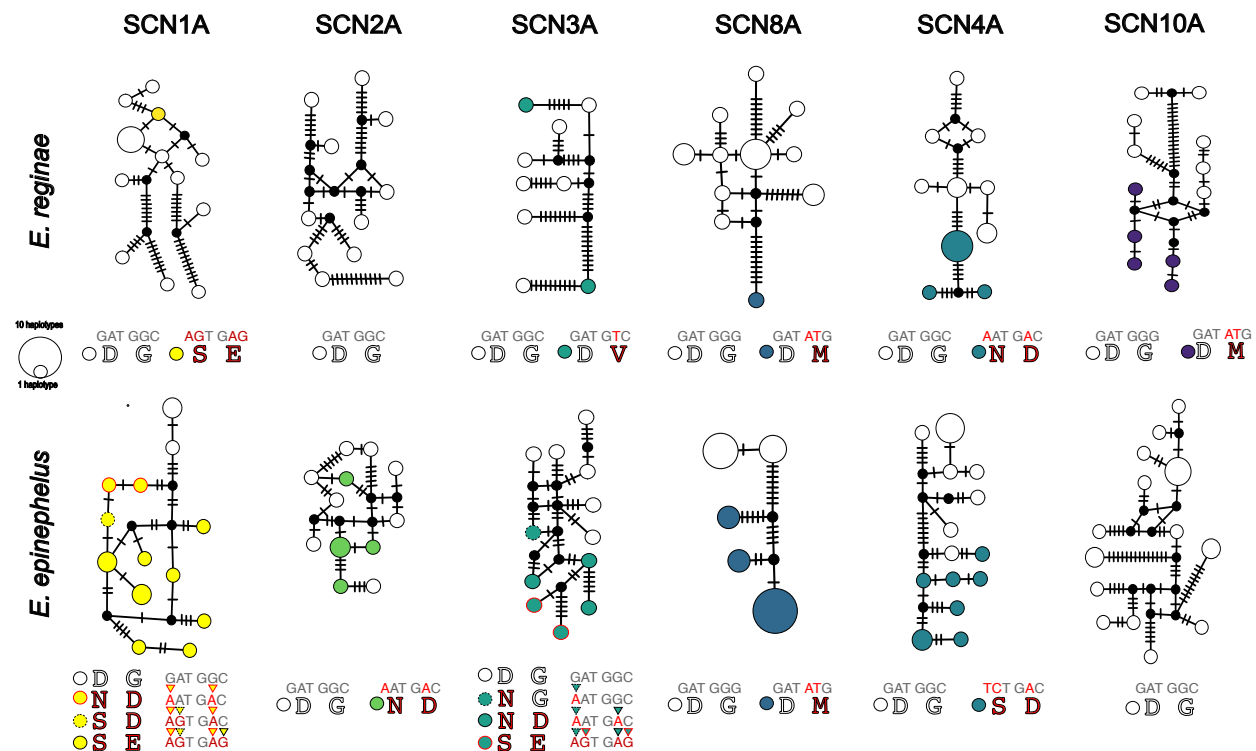
			(P1)	(P2)	(P3)	(P4)	(P5)	(P6)	(P7)	(P8)	(P9)
	SCN11A	Snake_MRCA	CIF	NYV	VAP	MEI	TFGNSMLCLF	QITTSAGWDELLG	PMLIGED		
		T. sirtalis	---	---	---	---	.....	.....	N.....	.....	LHND
		E. sp.	---	---	---	---	.....	.....	.....	.....	LHND
		E. reginae (NR)	---	---	---	---	.....	.....	.....	.....	LHND
		E. epinephelus (R)	---	---	---	---	.....	.....	M.....	.....	LHQD
	SCN10A	Snake_MRCA	MLF	NSY	VAE	MDI	TFGNSMLCLF	QITTSAGWDG	LLSPILNTG	P	
		T. sirtalis	---	---	---	---	.....	.....	.....	.....	
		E. sp.	---	---	---	---	.....	.....	.....	.....	
		E. reginae(NR)	---	---	---	---	.....	.....	.....	.....	
		E. reginae(R)	---	---	---	---	.....	.....	M.....	.....	
	SCN5A	Snake_MRCA	MLF	DLY	VAG	MEI	TFANSMLCLF	QITTSAGWDG	LLNPILNTG	P	
		T. sirtalis	---	---	---	---	.....	.....	.....	.....	
		E. sp.	---	---	---	---	.....	.....	.....	.....	
		E. reginae	---	---	---	---	.....	.....	.....	.....	
		E. epinephelus	---	---	---	---	.....	.....	.....	.....	
	SCN4A	Snake_MRCA	MIF	KSY	VAG	MDI	TFGNSIICLF	QITTSAGWDG	LLNPILNSG	P	
		T. sirtalis WC	... .	N.	... .	... .	.....	L.....	EV.....	NV.....	A.
		E. sp.	... .	... .	... .	... .	.....	.....	E.....	SD.....	
		E. reginae(NR)	... .	... .	... .	... .	.....	.....	E.....	.....	
		E. reginae(R1)	...L.	... .	... .	... .	.....	.....	E.....	.....	
E. reginae(R2)		...L.	N.	... .	... .	.....	.....	E.....	ND.....	S	
E. epinephelus(NR)		... .	... .	... .	... .	.....	.....	E.....	.....		
E. epinephelus(R1)		... .	... .	... .	... .	.....	.....	E.....	SD.....	S.	
E. epinephelus(R2)		...L.	... .	... .	... .	.....	.....	E.....	SD.....	S.	
SCN8A	Snake_MRCA	MIF	KNY	VAG	MDI	TFGNSMICLF	QITTSAGWDG	LLAPILNSG	P		
	T. sirtalis	... .	... .	... .	... .	.....	.....	V.....	.....		
	E. sp.	... .	... .	... .	... .	.....	.....	V.....	M.....		
	E. reginae(NR)	... .	... .	... .	... .	.....	.....	V.....	.....		
	E. reginae(R)	... .	... .	... .	... .	.....	.....	V.....	M.....		
	E. epinephelus(NR)	... .	... .	... .	... .	.....	.....	V.....	.....		
	E. epinephelus(R)	... .	... .	... .	... .	.....	.....	V.....	M.....		
SCN3A	Snake_MRCA	MIF	KSY	VAG	MDI	TFGNSMLCLF	QITTSAGWDG	LLAPILNSG	P		
	T. sirtalis	... .	... .	... .	... .	.....	.....	.....	.....		
	E. sp. (R1)	... .	... .	... .	Y.	.....	I.....	M.....	.....		
	E. sp. (R2)	... .	... .	... .	Y.	.....	I.....	.....	N.....	R	
	E. reginae(NR)	... .	... .	... .	... .	.....	.....	.....	.....		
	E. reginae(R1)	... .	... .	... .	... .	.....	I.....	.....	V.....		
	E. reginae(R2)	... .	... .	T.	Y.	.....	I.....	.....	.....		
	E. epinephelus(NR)	... .	... .	... .	... .	.....	.....	.....	.....		
	E. epinephelus(R1)	... .	... .	T.	N.	.....	.....	.....	SE.....		
	E. epinephelus(R2)	... .	... .	T.	N.	.....	I.....	M.....	ND.....	S.	
	E. epinephelus(R3)	... .	... .	T.	N.	.....	I.....	M.....	.....	S.	
	E. epinephelus(R4)	... .	... .	T.	N.	.....	I.....	M.....	N.....	S.	
SCN9A	Snake_MRCA	MFF	KNY	VAG	MEI	TFANSMICLF	QITTSGGWNY	LLFPILNKGE			
	T. sirtalis	... .	... .	T.	...	.....	.....	.....	Y.S.....		
	E. sp.	...L.	...	...	...	.....	.....	.....	.....		
	E. reginae(NR)	... .	...	...	...	.....	.....	.....	.....		
	E. reginae(R)	...L.	...	S.	...	.....	.....	.....	.....		
	E. epinephelus(NR)	... .	...	...	...	.....	.....	.....	.....		
SCN2A	Snake_MRCA	MIF	KNY	VAG	MDI	TFGNSMICLF	QITTSAGWDG	LLAPILNSG	P		
	T. sirtalis	... .	...	...	...	.....	.....	.....	.....		
	E. sp.	... .	...	...	...	.....	.....	.....	.....		
	E. reginae(NR)	... .	...	...	...	.....	.....	.....	.....		
	E. reginae(R)	... .	...	...	Y.	.....	.....	M.....	.....	R	
	E. epinephelus(NR)	... .	...	...	...	.....	.....	.....	.....		
	E. epinephelus(R1)	... .	...	...	G.	.....	.....	M.....	ND.....		
SCN1A	Snake_MRCA	MIF	KSY	VAG	MDI	TFGNSMICLF	MITTSAGWDG	LLAPILNSG	P		
	T. sirtalis	... .	...	...	...	.....	.....	.....	N.....		
	E. sp. (R1)	... .	...	...	...	.....	.....	.....	SE.....		
	E. sp. (R2)	... .	...	...	...	.....	.....	.....	.....		
	E. reginae(NR)	... .	...	...	...	.....	.....	.....	.....		
	E. reginae(R)	... .	...	...	...	.....	.....	.....	SE.....		
	E. epinephelus(NR)	... .	...	...	...	.....	.....	.....	.....		
	E. epinephelus(R1)	... .	...	...	...	.....	.....	.....	SE.....		
E. epinephelus(R2)	... .	...	...	...	.....	.....	.....	ND.....			
E. epinephelus(R3)	... .	...	...	...	.....	.....	.....	SD.....			
			DI S6	DII S5	DII p-loop	DIII p-loop	DIV p-loop				

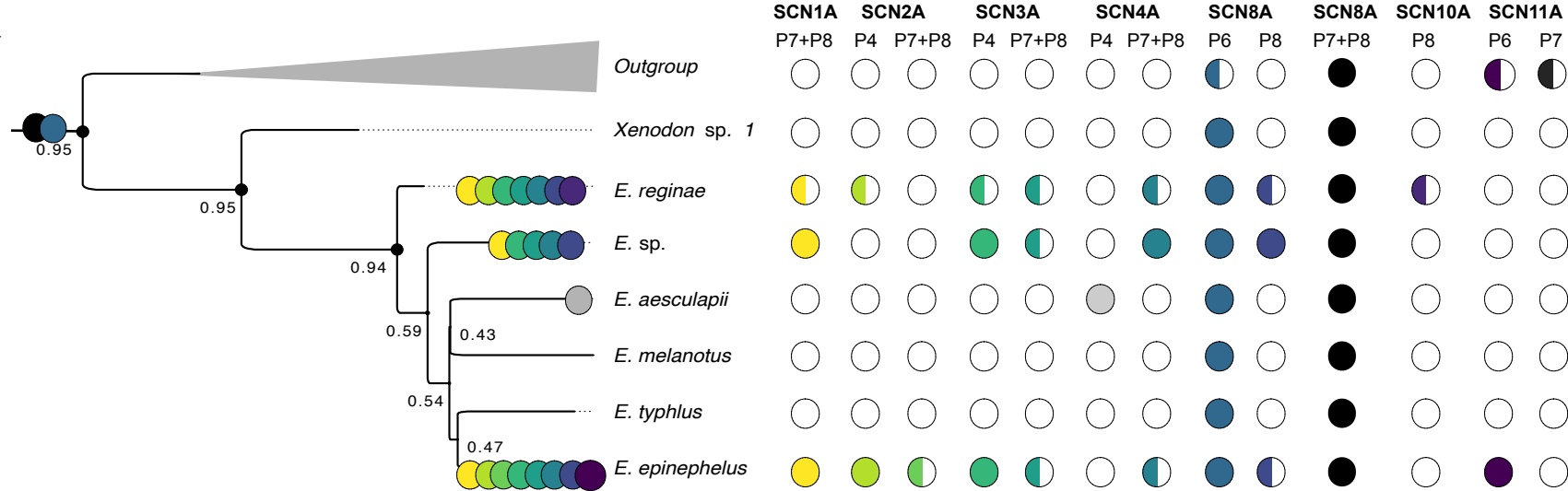


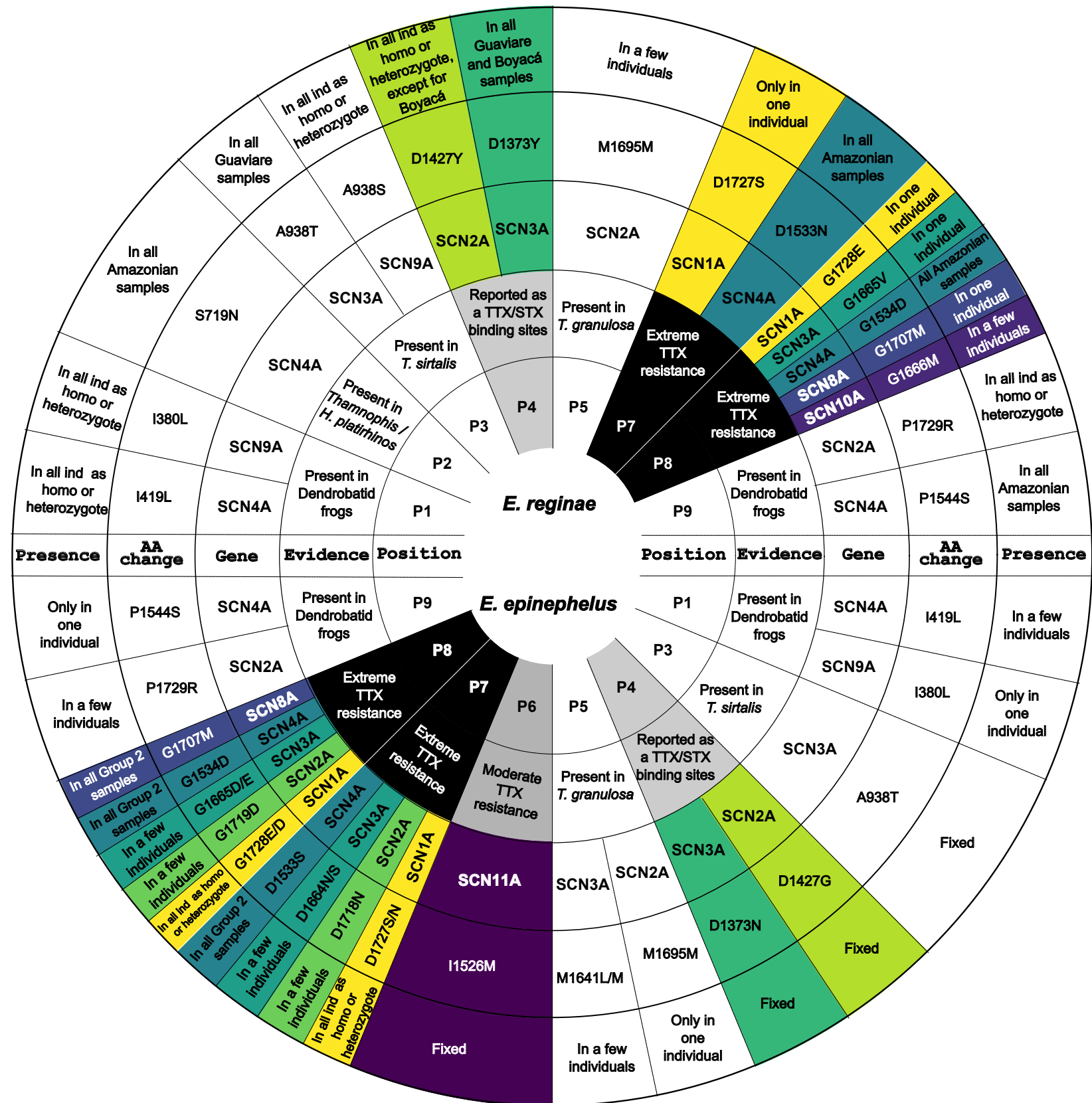
A



B







# **Supporting Information for Snakes (*Erythrolamprus* spp.) with a complex toxic diet show convergent yet highly heterogeneous voltage-gated sodium channel evolution**

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## **This PDF file includes:**

Supporting text  
Figures S1 to S5  
Tables S1 to S6  
SI References

## **Other supporting materials for this manuscript include the following:**

Datasets S1 to S10

## **Supporting Text**

## **Supplementary Results**

### **VGSC Sequence Assembly and Phylogenetic Tree Reconstruction**

We sequenced the VGSC family of 8 *Erythrolamprus reginae* samples from different 3 localities and 9 *E. epinephelus* samples from 7 localities. We analyzed 40 samples of 21 snake species that coexist in the same habitats, including 25 individuals corresponding to six species of the *Erythrolamprus* genus: *E. aesculapii*, *E. typhlus*, *E. melanotus*, *E. reginae*, *E. epinephelus*, and one individual designated as *Erythrolamprus* sp. that was previously identified as *Erythrolamprus epinephelus* but according to our phylogeny corresponds to a different species that lives in sympatry with *E. epinephelus* (Fig 3A & Table S1). The phylogeny was reconstructed using two mitochondrial (COI, 16S) and two nuclear (c-mos, RAG2) gene sequences. This tree shows high

support for nodes among the families and subfamilies, as well as between *Xenodon* and the *Erythrolamprus* genera (bootstrap value > 0.9) (Fig 3A).

On average, 64% (SD = 13.9%) of the reads from each library mapped to the *P. guttatus* genome, as expected considering the phylogenetic distance between our focal species and *P. guttatus*. However, the successfully aligned reads resulted in an average sequencing coverage of 51.24x (SD = 23.03x) (Table S1), which allowed us to obtain phased haplotype sequences for the VGSC covered regions accurately. We were able to reconstruct the complete or near-complete coding sequence for six of the nine VGSC genes found in snakes (*SCN1A*, *SCN2A*, *SCN3A*, *SCN4A*, *SCN8A*, *SCN9A*) in all targeted species, as well as Domain IV sequences for the remaining three (*SCN5A*, *SCN10A*, *SCN11A*). In the gene tree, our sequences clustered unambiguously with orthologous sequences and they matched the relationships between paralogs reconstructed in prior studies confirming their correct assembly and annotation (Fig S1) (Liebeskind et al. 2015; Rogers et al. 2018; Gendreau et al. 2021).

## Supplementary Methods

**Fig. S4. Methods.** Protein match results from *Anolis carolinensis* transcriptome. These results were downloaded from supplementary information in Eckalbar et al., 2013 (Eckalbar et al. 2013). Nine organ transcriptomes (skin, brain, skeletal muscle, heart, adrenal gland, embryo, lung, ovary, and liver) were used to search for voltage-gated sodium channels using a text finder by typing each of the gene names. Then the number of matches per tissue was plotted using the ggplot package from R (Wickham 2009).

**Fig. S1.** Voltage-gated Sodium Channel (VGSC) protein family tree. This tree was reconstructed using amino acid sequences in this study and sequences from model vertebrate and reptile species available on GenBank (Table S4). Each color represents one monophyletic group corresponding to a single VGSC protein. From this study, only domain IV (DIV) was retrieved from *SCN5A*, *SCN10A* and *SCN11A*. As reported in the literature, the genes *SCN1A*, *SCN2A*,

and SCN3A in *Xenopus tropicalis* are not homologous to the reptiles and mammals genes and form a monophyletic group (Zakon et al. 2011; Rogers et al. 2018). Additionally, the SCN2A gene was paraphyletic between mammals and reptiles (Liebeskind et al. 2015). The alignment used to build this tree is found in database S2.

**Fig. S2.** Extract from Fig. S1. VGSC gene tree to show nine positions of interest. Alignment of the nine homologous amino acid changes in the VGSC family found as putative TSR sites for *Erythrolamprus reginae*, *Erythrolamprus epinephelus*, and *Erythrolamprus* sp. species. Dots represent the same amino acid change as the outgroup highlighted in red for each protein. Below is a consensus sequence showing amino acids >50% conserved in the alignment. Each color represents a monophyletic VGSC protein. Node size depends on the bootstrap values shown in the legend.

**Fig. S3.** Pairwise Linkage Disequilibrium (LD) for the TTX extreme resistance-conferring substitutions found in P7 and P8. A) LD for *E. epinephelus* substitutions. Each gene and corresponding amino acid change is exhibited for each axis. The values correspond to an  $r^2$  correlation obtained using the *LDscan* function from the *pegas* R package. A higher  $r^2$  is consistent with a higher linkage between the two sites. B) A) LD for *E. reginae* substitutions. Each gene and corresponding amino acid change is exhibited for each axis. The values correspond to an  $r^2$  correlation obtained using the *LDscan* function from the *pegas* R package. A higher  $r^2$  is consistent with a higher linkage between the two sites.

**Fig. S4.** Number of positive matches for each of the VGSC genes in the expression profile of different *Anolis carolinensis* organs (Eckalbar et al. 2013). Each VGSC gene is shown in the legend. Data was obtained from published literature (see Supplementary methods).

**Fig S5.** Gene tree used to run an ancestral reconstruction and a MEME selection test in Hyphy. Alignment of the nine homologous amino acid changes in the VGSC family found as putative TSR sites for *Erythrolamprus reginae*, *Erythrolamprus epinephelus*, and *Erythrolamprus* sp. species. The ancestral position of these positions is represented in the gene tree. Dots represent the same

amino acid change as the outgroup highlighted in red for each protein. Below is a consensus sequence showing amino acids >50% conserved in the alignment.

**Table S1.** Information from samples used in this study. Species name, ID, locality, coordinates, collected tissue, previous toxic prey reports, number of reads, number of mapped reads, coverage, and alignment rate are included.

**Table S2.** The list of genes used to design the baits for target sequencing. The name of the gene and protein, organism, length of the sequence, and GenBank reference are included.

**Table S3.** VGSC gene location in the *Pantherophis guttatus* genome after annotation. In the table, we provide information for Ensembl gene ID, gene ID, gene name in *P. guttatus* GFF file, region name in *P. guttatus* genome, scaffold in *P. guttatus* genome, position in the scaffold, and strand direction. To eliminate the introns from the sequences we used published exon regions and reported in the table the source organism, the number of exons, and the GenBank ID. Finally, we described the exon location in the *P. guttatus* genome exons and the final number of exons obtained in this study.

**Table S4.** VGSC protein sequence GenBank ID for model vertebrates and reptiles used in the protein tree. Species names and common names are provided. *CACNA1A* was used as an outgroup.

**Table S5.** List of statistics obtained from haplotype networks for the domain IV of VGSC genes in *E. reginae*, *E. epinephelus*, and *E. melanotus* samples using PopArt (Leigh and Bryant 2015). Tajima D statistic (TajID) and the permutation-based p-value for Tajima D (P\_tajID). In addition, the number of segregating sites (segre\_sites),  $\pi$  (pi), and the number of parsimonious sites (pars\_i\_sites) is provided.

**Table S6.** Summary of the nine reported homologous positions and additional sites that are shared with other toxin-resistant organisms. We provided the functional region where the site is located (Region), the number assigned in this study for the position (Position), gene, amino acid



change, presence in samples from this study, organisms with substitutions at this site, and the reference for that report (Reference). From the table, <sup>a</sup>Amphibian's *SCN1A*, *SCN2A*, and *SCN3A* are not orthologs for *SCN1A*, *SCN2A*, and *SCN3A* in mammals and reptiles. <sup>b</sup>*SCN2A* is paraphyletic between reptiles and mammals. Amino acid notations are based on the *Mus musculus* *SCN2A*, however, this is not homologous to the reptile *SCN2A* ortholog, P4\* Reported as a TTX and STX target site from structural models (Terlau et al. 1991), P6\*\* Site reported to confer moderate TTX resistance (Geffeney et al. 2005; Vaelli et al. 2020). P7 & P8 \*\*\*Site reported to confer extreme TTX resistance (Geffeney et al. 2005), and \*\*\*\*Site reported to confer *SCN4A* BTX resistance (Wang and Wang 1998).

**Dataset S1 (separate file).** Excel sheet including all interesting sites found and MEME test results. A) Sheet A, includes all the interesting sites found in segment 4 (S4), S5, S5, and p-loop of the *Erythrolamprus* genus samples. It includes an assigned number for the substitution (Mutation number), the gene name (Gene), the protein name (Protein), the mutation site in the *Mus musculus* protein, if the change was present in another ortholog (shared change in ortholog), orthologs mutation numbers assign for this study, observations. In addition, we included if the change is only found in the *Erythrolamprus* genus, if it is shown in Fig 2, and if it has a P-value <0.07 from the MEME test. Finally, we include information on whether it fulfilled the requirements to be included as a potential TSR in this study. B) Sheet B provides all the MEME matches with p-values <0.1. We included gene, region, amino acid change in *Mus musculus* protein, orthologs that share this change (Shared in other orthologs), observations if this change is showed in Dataset S1 – Sheet A, if showed in Fig 2, and the p-value obtained from the MEME test.

**Dataset S2 (separate file).** The amino acid alignment from the VGSC used to reconstruct the protein tree in fasta format (Fig S1 & Table S4).

**Dataset S3 (separate file).** Phased VGSC gene sequences annotated for this study in fasta format. We only obtained DIV for *SCN5A*, *SCN10A*, and *SCN11A*.

**Dataset S4 (separate file).** Hyphy ancestral sequence reconstruction results in json format.

**Dataset S5 (separate file).** Codon alignment from the VGSC used for the Hyphy ancestral reconstruction and the MEME selection test in fasta format (Fig S5, DatasetS1A & Dataset S4).

**Dataset S6 (separate file).** CO1 gene sequences used to estimate the species tree.

**Dataset S7 (separate file).** 16S gene sequences used to estimate the species tree.

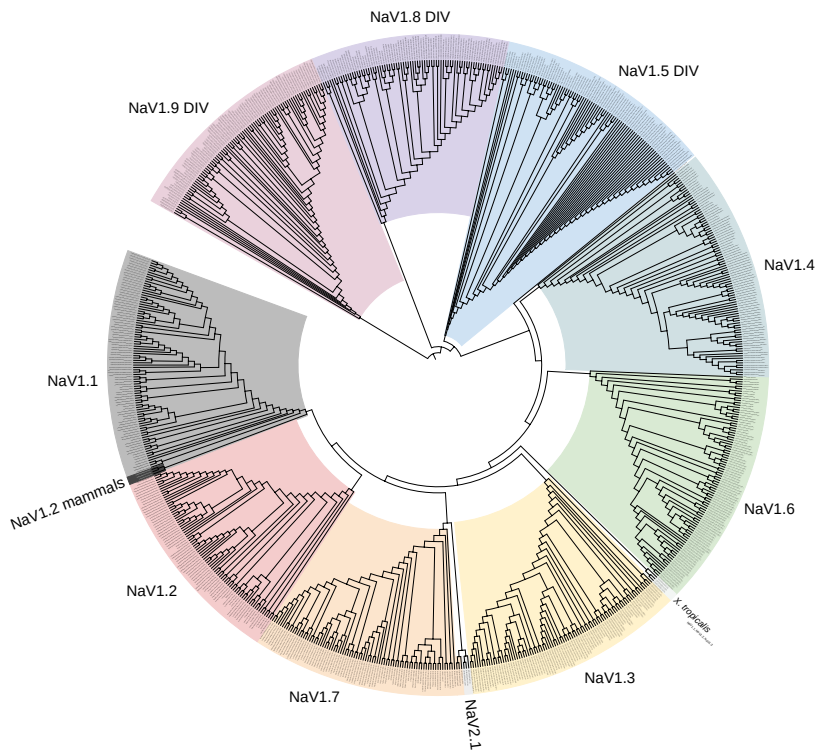
**Dataset S8 (separate file).** RAG2 gene sequences used to estimate the species tree.

**Dataset S9 (separate file).** c-mos gene sequences used to estimate the species tree.

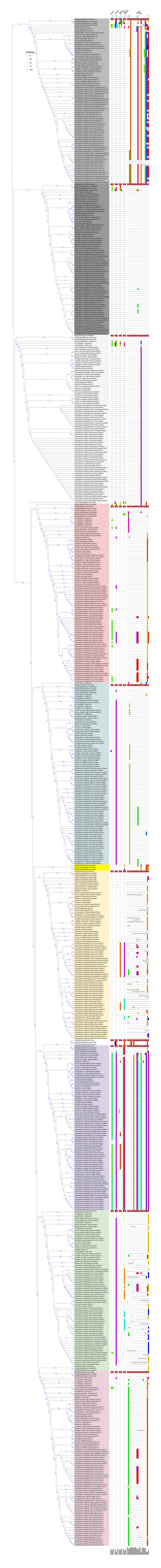
**Dataset S10 (separate file).** Spanish translation of the complete manuscript. The Spanish translation was made using ChatGPT (OpenAI 2023). raducción en español del manuscrito completo. La traducción fue hecha usando ChatGPT (OpenAI 2023).

## SI References

- Eckalbar WL, Hutchins ED, Markov GJ, Allen AN, Corneveaux JJ, Lindblad-Toh K, Di Palma F, Alföldi J, Huentelman MJ, Kusumi K. 2013. Genome reannotation of the lizard *Anolis carolinensis* based on 14 adult and embryonic deep transcriptomes. *BMC Genomics* [Internet] 14:49. Available from: <https://doi.org/10.1186/1471-2164-14-49>
- Geffeney SL, Fujimoto E, Brodie ED, Brodie ED, Ruben PC. 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. *Nature* [Internet] 434:759–763. Available from: [www.nature.com/nature](http://www.nature.com/nature).
- Gendreau KL, Hornsby AD, Hague MTJ, McGlothlin JW. 2021. Gene Conversion Facilitates the Adaptive Evolution of Self-Resistance in Highly Toxic Newts. *Mol. Biol. Evol.* [Internet] 38:4077–4094. Available from: <https://doi.org/10.1093/molbev/msab182>
- Leigh JW, Bryant D. 2015. popart: full-feature software for haplotype network construction. *Methods Ecol. Evol.* [Internet] 6:1110–1116. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/2041-210X.12410>
- Liebeskind BJ, Hillis DM, Zakon HH. 2015. Convergence of ion channel genome content in early animal evolution. *Proc. Natl. Acad. Sci. U. S. A.* [Internet] 112:846–851. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25675537>  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4345596>
- OpenAI. 2023. ChatGPT. Available from: <https://chat.openai.com/>
- Rogers RL, Zhou L, Chu C, Márquez R, Corl A, Linderroth T, Freeborn L, MacManes MD, Xiong Z, Zheng J, et al. 2018. Genomic Takeover by Transposable Elements in the Strawberry Poison Frog. *Mol. Biol. Evol.* [Internet] 35:2913–2927. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6278860/>
- Terlau H, Heinemann SH, Stühmer W, Pusch M, Conti F, Imoto K, Numa S. 1991. Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. *FEBS Lett.* [Internet] 293:93–96. Available from: <https://www.sciencedirect.com/science/article/pii/0014579391811596>
- Vaelli PM, Theis KR, Williams JE, O'Connell LA, Foster JA, Eisthen HL. 2020. The skin microbiome facilitates adaptive tetrodotoxin production in poisonous newts. Baldwin IT, Robert CA, Robert CA, González Montoya MC, editors. *eLife* [Internet] 9:e53898. Available from: <https://doi.org/10.7554/eLife.53898>
- Wang S-Y, Wang GK. 1998. Point mutations in segment I-S6 render voltage-gated Na<sup>+</sup> channels resistant to batrachotoxin. *Proc. Natl. Acad. Sci.* [Internet] 95:2653–2658. Available from: <https://www.pnas.org/doi/10.1073/pnas.95.5.2653>
- Wickham H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer International Publishing
- Zakon HH, Jost MC, Lu Y. 2011. Expansion of Voltage-dependent Na<sup>+</sup> Channel Gene Family in Early Tetrapods Coincided with the Emergence of Terrestriality and Increased Brain Complexity. *Mol. Biol. Evol.* [Internet] 28:1415–1424. Available from: <https://doi.org/10.1093/molbev/msq325>

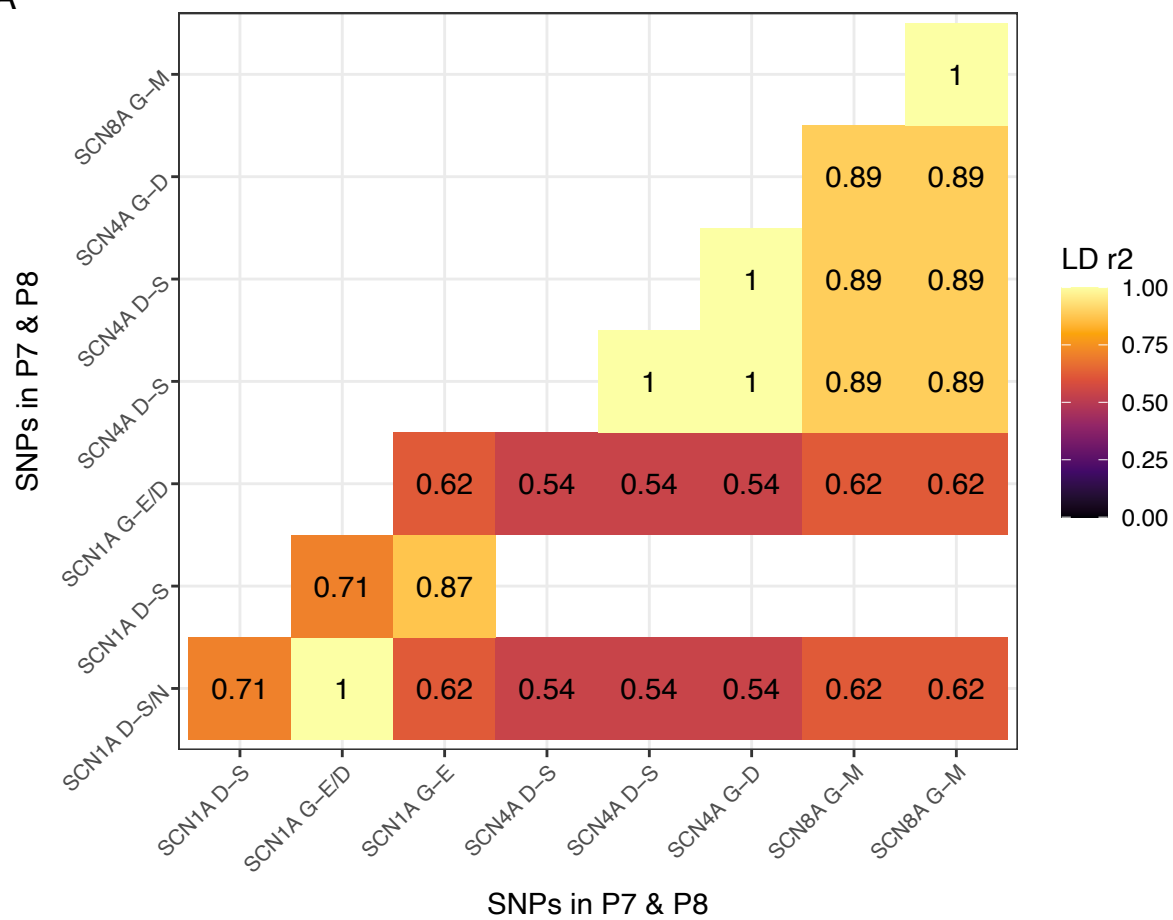




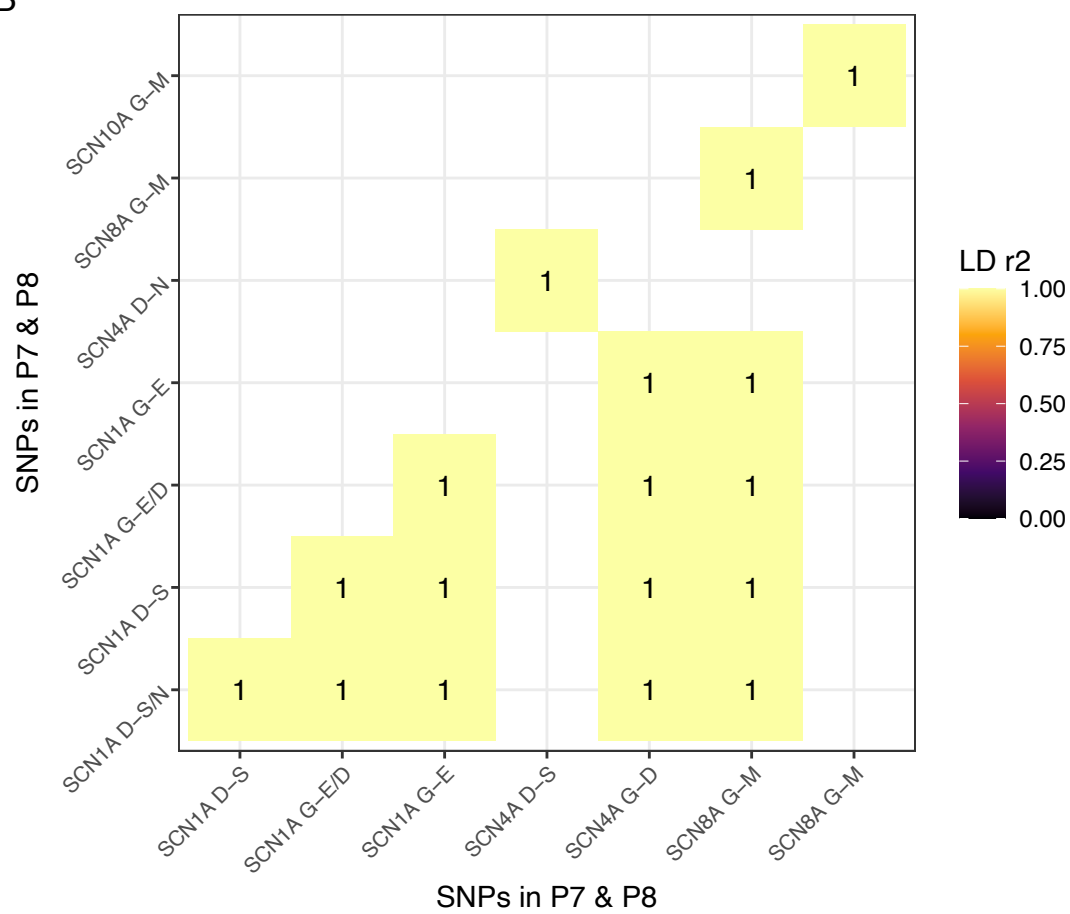




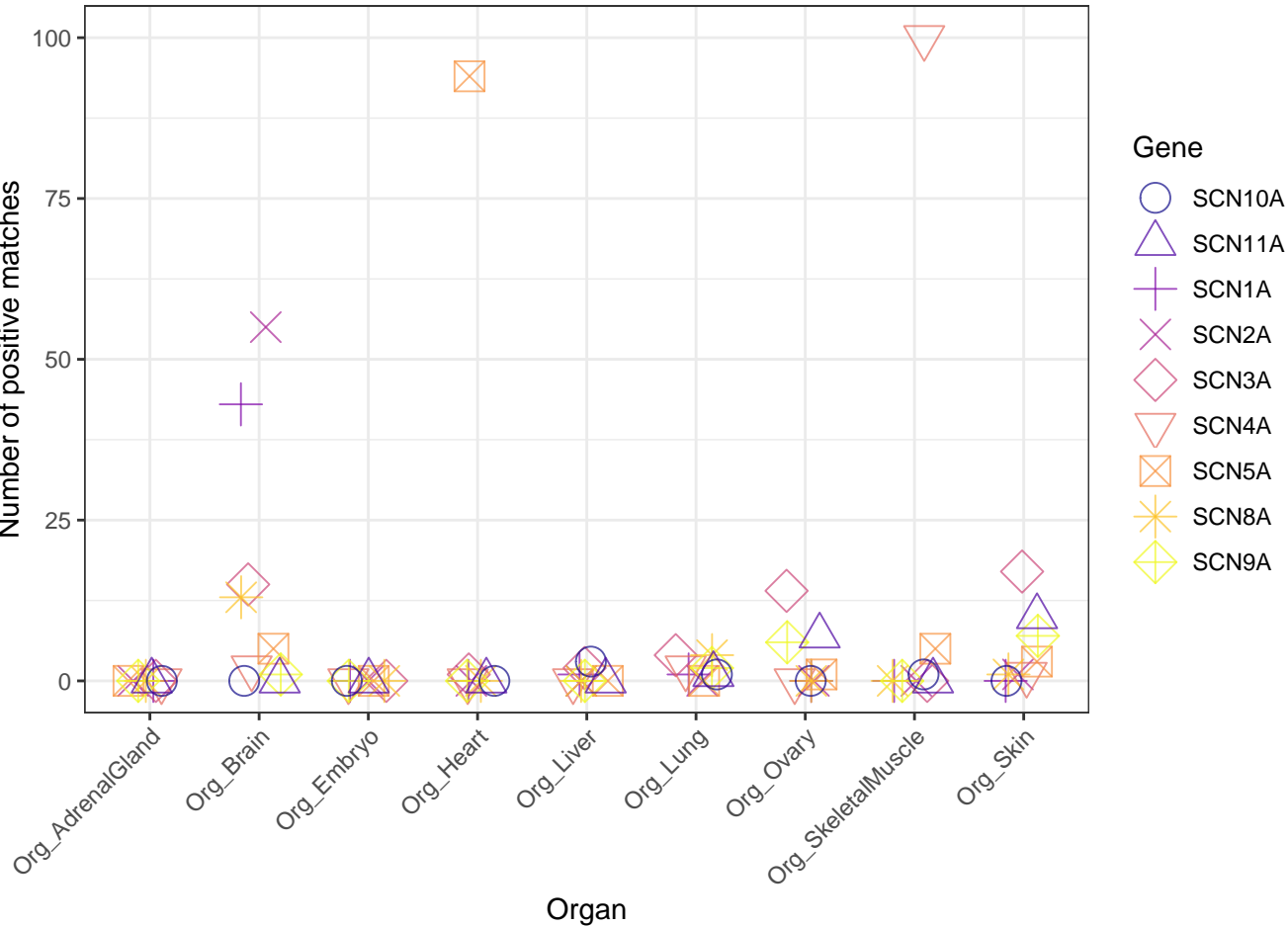
A



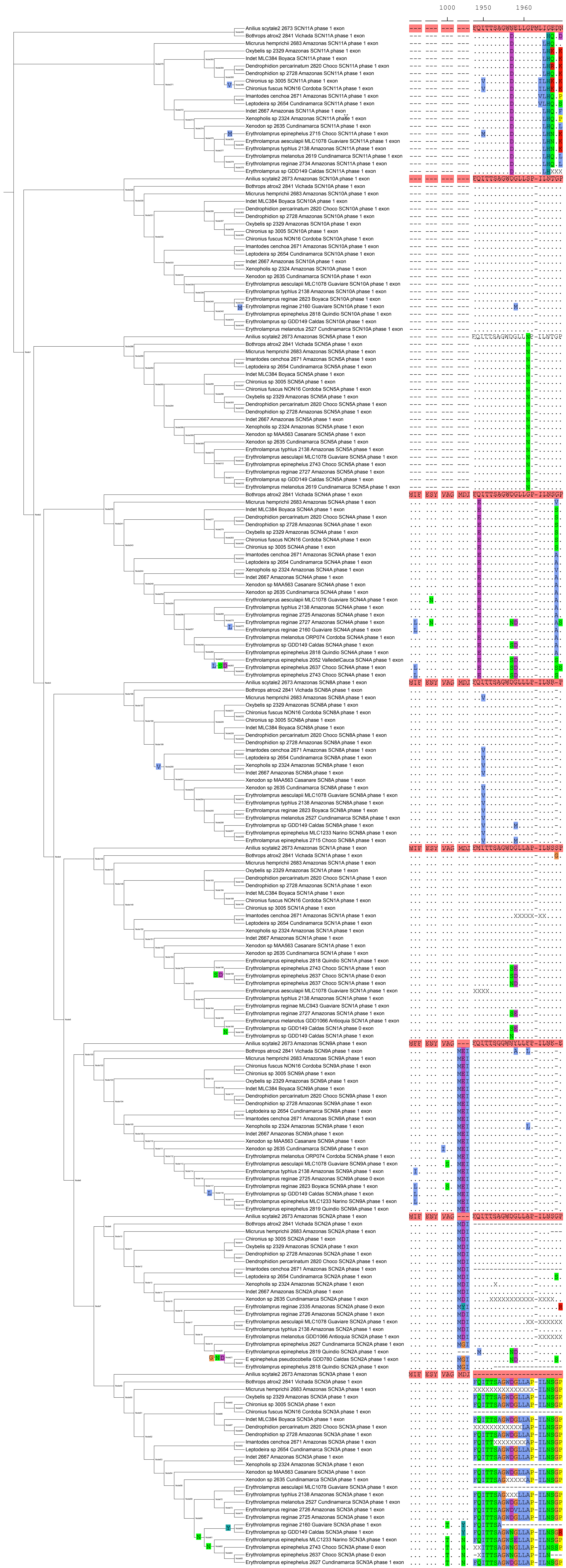
B



Number of positive matches









Genus	species	Sample number	Tissue	Location	Latitude	Longitude	Loan from the Instituto de Ciencias Naturales ICN	Collection date	Collected by	Toxic prey reported for the species	Reported by	Sequence name	Total number of reads	Total of mapped reads	Santools depth for the VGSC genes	SRA accession number	NCBI 16S gene sequence accession number	NCBI CO1 gene sequence accession number
<i>Anillius</i>	<i>scytale</i>	2673	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	NA	NA	Anillius_scytale2_2673_Amazonas_	1534248	938718	50.99	SRR27351694	PV124461	PV124506
<i>Bothrops</i>	<i>atrox</i>	2841	Muscle	Bojonawi, Vichada	4.422513	-69.293534	No	5 December 2015		Viperid snake	NA	Bothrops_atrox2_2841_Vichada_	1191006	770283	14.85	SRR27351693	PV124460	PV124517
<i>Chironius</i>	<i>fuscus</i>	NON16	Liver	Cordoba	8.315783	-75.763029	Yes	Unknown		<i>Dendrobates</i> sp.	Martins & Oliveira, 1999	Chironius_fuscus_NON16_Cordoba_	819994	761840	31.49	SRR27351674	PV124496	PV124510
<i>Chironius</i>	sp.	3005	Liver	Unknown	NA	NA	No	March 2015	Carlos Eduardo Burbano	NA	NA	Chironius_sp_3005_	1260596	1148728	72.65	SRR27351655	PV124497	PV124511
<i>Dendrophidion</i>	<i>percarinatum</i>	2820	Swab	Victoria, Chocó	5.510271	-76.870179	No	12 November 2015	Pablo Palacios	NA	NA	Dendrophidion_per carinatum_2820_C hoco_	1620156	1447769	47.66	SRR27351668	PV124493	PV124515
<i>Dendrophidion</i>	sp.	2728	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	Leptodactylidae	da Costa et al., 2007	Dendrophidion_sp_2728_Amazonas_	2626194	2312102	47.41	SRR27351667	PV124494	PV124516
<i>Erythrolamprus</i>	<i>epinephelus</i>	GDD780	Liver	Salamina, Caldas	5.414167	-75.49086	Yes	Unknown		<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2019	E_ epinephelus_pseud ocobella_GDD780_Caldas_	2934160	1757575	136.44	SRR27351688	PV124466	PV124522
<i>Erythrolamprus</i>	<i>aesculapii</i>	MLC1078	Liver	San José del Guaviare, Guaviare	2.579343	-72.701531	Yes	Unknown	Martha Calderón	NA	NA	Erythrolamprus_ae sculapii_MLC1078_Guaviare_		718798	33.12	SRR27351666	PV124479	PV124545
													1341098					
<i>Erythrolamprus</i>	<i>epinephelus</i>	2052	Swab	El Cairo, Valle del Cauca	4.761248	-76.225751	No	January 2013	Fernando Vargas	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2012	Erythrolamprus_epi nephelus_2052_Val ledelCauca_	1256588	776051	38.27	SRR27351665	PV124482	PV124543
<i>Erythrolamprus</i>	<i>epinephelus</i>	2627	Muscle	Pandi, Cundinamarca	4.193432	-74.485421	No	September 2014	Diego Gómez	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2013	Erythrolamprus_epi nephelus_2627_Cu ndinamarca_	1403810	800360	59	SRR27351664	PV124465	PV124528
<i>Erythrolamprus</i>	<i>epinephelus</i>	2637	Tail	Salero, Chocó	5.358701	-76.646274	No	November, 2014	Jonar David Echavarría	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2014	Erythrolamprus_epi nephelus_2637_Ch oco_	781256	503536	40.37	SRR27351663	PV124476	PV124540
<i>Erythrolamprus</i>	<i>epinephelus</i>	2715	Muscle	Salero, Chocó	5.358701	-76.646274	No	February, 2015	Jonar David Echavarría	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2015	Erythrolamprus_epi nephelus_2715_Ch oco_	1974580	1099925	40.16	SRR27351692	PV124477	PV124541
<i>Erythrolamprus</i>	<i>epinephelus</i>	2743	Muscle	Samurindó, Chocó	5.586063	-76.65271	No	16 June 2015	Pablo Palacios	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2016	Erythrolamprus_epi nephelus_2743_Ch oco_	1407946	780031	34.05	SRR27351691	PV124475	PV124539
<i>Erythrolamprus</i>	<i>epinephelus</i>	2818	Tail	Armenia, Quindío	4.514719	-75.728273	No	9 September 2015	Fernando Vargas	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2017	Erythrolamprus_epi nephelus_2818_Qu indio_	2415800	1297113	31.13	SRR27351690	PV124480	PV124508
<i>Erythrolamprus</i>	<i>epinephelus</i>	2819	Tail	Armenia, Quindío	4.514719	-75.728273	No	9 September 2015	Fernando Vargas	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2018	Erythrolamprus_epi nephelus_2819_Qu indio_	2031030	1260041	56.41	SRR27351689	PV124481	PV124544
<i>Erythrolamprus</i>	<i>epinephelus</i>	MLC1233	Liver	Tumaco, Nariño	1.59251	-78.71841	Yes	Unknown	Martha Calderón	<i>Phyllobates</i> <i>terribilis</i> , <i>Oophaga</i> <i>pumilio</i> y <i>Atelopus</i> sp.	Myers et al., 1978; Saporito et al., 2007; Feldman et al., 2020	Erythrolamprus_epi nephelus_MLC1233_Narino_	839978	556962	37.72	SRR27351687	PV124478	PV124542
<i>Erythrolamprus</i>	<i>melanotus</i>	2527	Swab	Vereda Las Brisas, Puerto Salgar, Cundinamarca	5.495372	-74.645447	No	July 2014	Javier Mendéz	<i>Dendrobates</i> <i>truncatus</i>	NA	Erythrolamprus_me lanotus_2527_Cun dinamarca_	3194044	1918246	66.77	SRR27351677	PV124487	PV124526
<i>Erythrolamprus</i>	<i>melanotus</i>	2619	Tail	Vereda Las Brisas, Puerto Salgar, Cundinamarca	5.495372	-74.645447	No	July 2014	Javier Mendéz	<i>Dendrobates</i> <i>truncatus</i>	NA	Erythrolamprus_me lanotus_2619_Cun dinamarca_	1358548	754555	35.6	SRR27351676	PV124489	PV124525
<i>Erythrolamprus</i>	<i>melanotus</i>	2824	Tail	Puerto Boyacá, Boyacá	5.967765	-74.594422	No	1 November 2015	Alvaro	<i>Dendrobates</i> <i>truncatus</i>	NA	Erythrolamprus_me lanotus_2824_Boya ca_	1151054	696894	36.84	SRR27351675	PV124488	PV124527
<i>Erythrolamprus</i>	<i>melanotus</i>	GDD1066	Liver	Caucasia, Antioquia	7.983238	-75.216681	Yes	Unknown		NA	NA	Erythrolamprus_me lanotus_GDD1066_Antioquia_	1298420	834553	40.33	SRR27351678	PV124486	PV124523
<i>Erythrolamprus</i>	<i>melanotus</i>	ORP074	Liver	Cordoba	8.315783	-75.763029	Yes	Unknown		<i>Dendrobates</i> <i>truncatus</i>	NA	Erythrolamprus_me lanotus_ORP074_C ordoba_	2767184	1753635	53.87	SRR27351673	PV124484	PV124524
<i>Erythrolamprus</i>	<i>reginae</i>	2160	Liver	San José del Guaviare, Guaviare	2.579343	-72.701531	No	4 January 2013	Martha Calderón	<i>Ameerega</i> <i>trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2020	Erythrolamprus_re ginae_2160_Guavi are_	1687730	1007857	46.46	SRR27351672	PV124474	PV124533
<i>Erythrolamprus</i>	<i>reginae</i>	2335	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	2014		<i>Ameerega</i> <i>trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2021	Erythrolamprus_re ginae_2335_Amaz onas_	2925784	1886358	83.85	SRR27351671	PV124470	PV124537
<i>Erythrolamprus</i>	<i>reginae</i>	2725	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	<i>Ameerega</i> <i>trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2021	Erythrolamprus_re ginae_2725_Amaz onas_	929410	571811	31.71	SRR27351662	PV124469	PV124536
<i>Erythrolamprus</i>	<i>reginae</i>	2726	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	<i>Ameerega</i> <i>trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2022	Erythrolamprus_re ginae_2726_Amaz onas_	1549044	984442	71.67	SRR27351661	PV124468	PV124535
<i>Erythrolamprus</i>	<i>reginae</i>	2727	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	<i>Ameerega</i> <i>trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2023	Erythrolamprus_re ginae_2727_Amaz onas_	889910	554819	49.64	SRR27351660	PV124473	PV124534

Genus	species	Sample number	Tissue	Location	Latitude	Longitude	Loan from the Instituto de Ciencias Naturales ICN	Collection date	Collected by	Toxic prey reported for the species	Reported by	Sequence name	Total number of reads	Total of mapped reads	Samtools depth for the VGSC genes	SRA accession number	NCBI 16S gene sequence accession number	NCBI CO1 gene sequence accession number
<i>Erythrolamprus</i>	<i>reginae</i>	2734	Tail	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	23 February 2015	Camilo Rodríguez	<i>Ameerega trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2024	Erythrolamprus_reginae_2734_Amazonas_	871918	540503	41.72	SRR27351659	PV124471	PV124531
<i>Erythrolamprus</i>	<i>reginae</i>	2823	Tail	Santa María, Boyacá	4.883333	-73.25	No	1 November 2015	Sebastián di Domenico	<i>Ameerega trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2021	Erythrolamprus_reginae_2823_Boyaca_	1762468	929956	39.76	SRR27351658	PV124483	PV124530
<i>Erythrolamprus</i>	<i>reginae</i>	MLC943	Liver	San José del Guaviare, Guaviare	2.579343	-72.701531	Yes	Unknown	Martha Calderón	<i>Ameerega trivittata</i> y <i>Allobates</i> sp.	Martins & Oliveira, 1999; Pašukonis & Loretto, 2021	Erythrolamprus_reginae_MLC943_Guaviare_	1529356	961647	60.52	SRR27351657	PV124472	PV124532
<i>Erythrolamprus</i>	sp.	GDD149	Liver	Salamina, Caldas	5.414167	-75.49086	Yes	Unknown		NA	NA	Erythrolamprus_sp._GDD149_Caldas_	1952682	1233236	101.58	SRR27351656	PV124485	PV124538
<i>Erythrolamprus</i>	<i>typhlus</i>	2138	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	June 2013	Camilo Rodríguez	NA	NA	Erythrolamprus_typhlus_2138_Amazonas_	1811940	1138727	45.38	SRR27351686	PV124467	PV124529
<i>Imantodes</i>	<i>cenchoa</i>	2671	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	NA	NA	Imantodes_cenchoa_2671_Amazonas_	1474608	302982	42.38	SRR27351685	PV124491	PV124512
<i>Indet</i>		2667	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	19 April 2015	Camilo Rodríguez	NA	NA	Indet_2667_Amazonas_	981758	653147	40.08	SRR27351684	PV124458	PV124519
<i>Indet</i>		MLC384	Liver	Puerto Boyacá, Boyacá	5.967765	-74.594422	Yes	Unknown	Martha Calderón	NA	NA	Indet_MLC384_Boyaca_	1741704	1591697	52.09	SRR27351683	PV124459	PV124509
<i>Leptodeira</i>	sp.	2654	Muscle	El Nilo, Cundinamarca	4.306568	-74.631007	No	November 2014	Sebastián di Domenico	<i>Buconidae</i> y <i>Leptodactylus</i> sp.	Moore et al., 2009	Leptodeira_sp._2654_Cundinamarca_	1087086	768121	38.26	SRR27351682	PV124495	PV124514
<i>Micrurus</i>	<i>hemprichii</i>	2683	Muscle	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	December 2015	Camilo Rodríguez	Elapid snake	NA	Micrurus_hemprichii_2683_Amazonas_	253494	105081	29.56	SRR27351681	PV124490	PV124513
<i>Oxybelis</i>	sp.	2329	Tail	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	2014		NA	NA	Oxybelis_sp._2329_Amazonas_	2248838	2079244	94.04	SRR27351680	PV124492	PV124507
<i>Xenodon</i>	sp.	2635	Muscle	El Nilo, Cundinamarca	4.306568	-74.631007	No	November 2014	Sebastián di Domenico	NA	NA	Xenodon_sp._2635_Cundinamarca_	886950	586506	32.2	SRR27351679	PV124463	PV124521
<i>Xenodon</i>	sp.	MAA563	Liver	Orocue, Casanare	4.910406	-71.434187	Yes	Unknown		NA	NA	Xenodon_sp._MAA563_Casanare_	1651884	1040052	76.74	SRR27351670	PV124464	PV124520
<i>Xenopholis</i>	sp.	2324	Swab	Tanimboca, Leticia, Amazonas	-4.127576	-69.953655	No	2014		<i>Allobates femoralis</i>	Ringler et al., 2010	Xenopholis_sp._2324_Amazonas_	1836124	1231851	66.73	SRR27351669	PV124462	PV124518
												Mean	1582009.45	1026393.8	51.2375			
												SD	668034.3888	498224.9183	23.02558908			

Family name	Protein	Gene	Organism used for design	Target sequence length (bp)	Genbank number
Voltage-gated sodium channel (alpha subunit)	NaV1.1	SCN1A	<i>Thamnophis sirtalis</i>	5542	BK008860.1
	NaV1.2	SCN2A	<i>Thamnophis sirtalis</i>	5747	BK008861.1
	NaV1.3	SCN3A	<i>Thamnophis sirtalis</i>	6012	BK008862.1
	NaV1.4	SCN4A	<i>Thamnophis sirtalis</i>	5628	BK008863.1
	NaV1.5	SCN5A	<i>Python bivittatus</i>	2706	AEQU02000001.1
	NaV1.6	SCN8A	<i>Thamnophis sirtalis</i>	5964	BK008864.1
	NaV1.7	SCN9A	<i>Thamnophis sirtalis</i>	5895	BK008865.1
	NaV1.8	SCN10A	<i>Python bivittatus</i>	1883	AEQU02000001.1
	NaV1.9	SCN11A	<i>Python bivittatus</i>	2003	AEQU02000001.1
Nicotinic receptor	Alpha1	CHRNA1	<i>Ophiophagus hannah</i>	1530	PRJNA201683
	Alpha2	CHRNA2	<i>Python bivittatus</i>	1545	AEQU02000001.1
	Alpha3	CHRNA3	<i>Python bivittatus</i>	1899	AEQU02000001.1
	Alpha4	CHRNA4	<i>Python bivittatus</i>	1272	AEQU02000001.1
	Alpha5	CHRNA5	<i>Python bivittatus</i>	1485	AEQU02000001.1
	Alpha6	CHRNA6	<i>Python bivittatus</i>	1188	AEQU02000001.1
	Alpha7	CHRNA7	<i>Ophiophagus hannah</i>	1464	PRJNA201683
	Alpha9	CHRNA9	<i>Python bivittatus</i>	1524	AEQU02000001.1
	Alpha10	CHRNA10	<i>Python bivittatus</i>	1089	AEQU02000001.1
	Beta1	CHRNA1	<i>Ophiophagus hannah</i>	1518	PRJNA201683
	Beta2	CHRNA2	<i>Python bivittatus</i>	1374	AEQU02000001.1
	Beta3	CHRNA3	<i>Python bivittatus</i>	1383	AEQU02000001.1
	Beta4	CHRNA4	<i>Python bivittatus</i>	1566	AEQU02000001.1
	Delta	CHRNA5	<i>Python bivittatus</i>	1608	AEQU02000001.1
	Epsilon	CHRNA6	<i>Python bivittatus</i>	1619	AEQU02000001.1
	Gamma	CHRNA7	<i>Python bivittatus</i>	2313	AEQU02000001.1
Canal de calcio voltaje dependiente (Subunidad alfa)	CaV1.1	CACNA1S	<i>Ophiophagus hannah</i>	2808	PRJNA201683
	CaV1.2	CACNA1C	<i>Python bivittatus</i>	1239	AEQU02000001.1
	CaV1.3	CACNA1D	<i>Python bivittatus</i>	5715	AEQU02000001.1
	CaV1.4	CACNA1F	<i>Python bivittatus</i>	6375	AEQU02000001.1
	CaV2.1	CACNA1A	<i>Python bivittatus</i>	6909	AEQU02000001.1
	CaV2.2	CACNA1B	<i>Python bivittatus</i>	1574	AEQU02000001.1
	CaV2.3	CACNA1E	<i>Python bivittatus</i>	6870	AEQU02000001.1
	CaV3.1	CACNA1G	<i>Python bivittatus</i>	5931	AEQU02000001.1
	CaV3.2	CACNA1H	<i>Python bivittatus</i>	3855	AEQU02000001.1
	CaV3.3	CACNA1I	<i>Python bivittatus</i>	6510	AEQU02000001.1
Voltage-gated calcium channel (alpha subunit)	Kv1.1	KCNA1	<i>Python bivittatus</i>	1485	AEQU02000001.1
	Kv1.2	KCNA2	<i>Python bivittatus</i>	1500	AEQU02000001.1
	Kv1.3	KCNA3	<i>Ophiophagus hannah</i>	1467	PRJNA201683
	Kv1.4	KCNA4	<i>Ophiophagus hannah</i>	1347	PRJNA201683
	Kv1.5	KCNA5	<i>Python bivittatus</i>	1683	PRJNA201683
	Kv1.7	KCNA7	<i>Ophiophagus hannah</i>	1542	PRJNA201683
	Kv1.8	KCNA10	<i>Ophiophagus hannah</i>	2016	PRJNA201683
Voltage-gated	Kv2.1	KCNB1	<i>Ophiophagus hannah</i>	588	PRJNA201683
	Kv2.2	KCNB2	<i>Python bivittatus</i>	2724	AEQU02000001.1
	Kv3.1	KCNC1	<i>Python bivittatus</i>	1863	AEQU02000001.1

potassium channel (Shaw - subfamily C)	Kv3.2	KCNC2	<i>Python bivittatus</i>	1137	AEQU02000001.1
	Kv3.4	KCNC4	<i>Python bivittatus</i>	1884	AEQU02000001.1
Voltage-gated potassium channel (Shal - subfamily D)	Kv4.1	KCND1	<i>Python bivittatus</i>	1706	AEQU02000001.1
	Kv4.2	KCND2	<i>Python bivittatus</i>	1896	AEQU02000001.1
	Kv4.3	KCND3	<i>Ophiophagus hannah</i>	1110	PRJNA201683
Voltage-gated potassium channel (subfamily F)	Kv5.1	KCNF1	<i>Python bivittatus</i>	702	AEQU02000001.1
Voltage-gated potassium channel (subfamily G)	Kv6.1	KCNG1	<i>Ophiophagus hannah</i>	1464	PRJNA201683
	Kv6.2	KCNG2	<i>Python bivittatus</i>	1533	AEQU02000001.1
	Kv6.3	KCNG3	<i>Ophiophagus hannah</i>	1575	PRJNA201683
	Kv6.4	KCNG4	<i>Python bivittatus</i>	1332	AEQU02000001.1
Voltage-gated potassium channel (eag - subfamily H)	Kv10.1	KCNH1	<i>Python bivittatus</i>	1527	AEQU02000001.1
	Kv11.1	KCNH2	<i>Python bivittatus</i>	987	AEQU02000001.1
	Kv12.2	KCNH3	<i>Python bivittatus</i>	1737	AEQU02000001.1
	Kv12.3	KCNH4	<i>Python bivittatus</i>	2880	AEQU02000001.1
	Kv10.2	KCNH5	<i>Python bivittatus</i>	3126	AEQU02000001.1
	Kv11.2	KCNH6	<i>Python bivittatus</i>	2943	AEQU02000001.1
	Kv11.3	KCNH7	<i>Python bivittatus</i>	3168	AEQU02000001.1
	Kv12.1	KCNH8	<i>Python bivittatus</i>	3510	AEQU02000001.1
Voltage-gated potassium channel (KGT subfamily Q)	Kv7.1	KCNQ1	<i>Python bivittatus</i>	3324	AEQU02000001.1
	Kv7.2	KCNQ2	<i>Python bivittatus</i>	1977	AEQU02000001.1
	Kv7.3	KCNQ3	<i>Python bivittatus</i>	1228	AEQU02000001.1
	Kv7.4	KCNQ4	<i>Python bivittatus</i>	2121	AEQU02000001.1
	Kv7.5	KCNQ5	<i>Python bivittatus</i>	1893	AEQU02000001.1
Voltage-gated potassium channel (subfamily S)	Kv9.1	KCNS1	<i>Python bivittatus</i>	2652	AEQU02000001.1
	Kv9.2	KCNS2	<i>Python bivittatus</i>	1401	AEQU02000001.1
	Kv9.3	KCNS3	<i>Python bivittatus</i>	1434	AEQU02000001.1
Voltage-gated potassium channel (subfamily V)	Kv8.1	KCNV1	<i>Python bivittatus</i>	1512	AEQU02000001.1
	Kv8.2	KCNV2	<i>Python bivittatus</i>	1275	AEQU02000001.1
Recombination activating gene 1	RAG-1	RAG1	<i>Thamnophis sirtalis</i>	1578	XM_014075888.1
Proto-oncogen	c-mos	MOS	<i>Erythrolamprus reginae</i>	600	GQ895819.1
Na+/K+ pump Sodium-potassium ATPase pump	ATP1A1	ATP1A1	<i>Protobothrops mucrosquamatus</i>	3560	XM_015818080.2
Total	76 genes			188530	

Ensembl gene ID	Gene ID	Gene name	Gene name in <i>P. guttatus</i> genome	Region name in <i>P. guttatus</i> genome	Scaffold in <i>P. guttatus</i> genome	Start position	End position	Strand	Blast exons sequence organism	Number of exons	<i>T. sirtalis</i> GenBank ID	<i>P. guttatus</i> genome exons location	Final number of exons obtained in consensus sequences
ENSG00000185313	6336	SCN10A	SCN10A	LOC117654779	NW_023010900.1	4655826	4745976	positive	<i>Pantherophis guttatus</i>	Unknown	NA	>NW_023010900.1:4745742-4745976 >NW_023010900.1:4733142-4733270 >NW_023010900.1:4732282-4732393 >NW_023010900.1:4729093-4729222 >NW_023010900.1:4723382-4723474 >NW_023010900.1:4721962-4722172 >NW_023010900.1:4720944-4721008 >NW_023010900.1:4717495-4717637 >NW_023010900.1:4705653-4705851 >NW_023010900.1:4704506-4704662 >NW_023010900.1:4701801-4702200 >NW_023010900.1:4697639-4697766 >NW_023010900.1:4696160-4696399 >NW_023010900.1:4693380-4693593 >NW_023010900.1:4692383-4692737 >NW_023010900.1:4691710-4692130 >NW_023010900.1:4690255-4690375 >NW_023010900.1:4683255-4683379 >NW_023010900.1:4680302-4680457 >NW_023010900.1:4677148-4677322 >NW_023010900.1:4675396-4675519 >NW_023010900.1:4663461-4663601 >NW_023010900.1:4662734-4662840 >NW_023010900.1:4660239-4660510 >NW_023010900.1:4655825-4658206	1 (only NW_023010900.1:4655825-4658206)
ENSG00000168356	11280	SCN11A	SCN11A	LOC117654780	NW_023010900.1	4780111	4814439	positive	<i>Pantherophis guttatus</i>	Unknown	NA	>NW_023010900.1:4814022-4814439 >NW_023010900.1:4810452-4810866 >NW_023010900.1:4807935-4808046 >NW_023010900.1:4804784-4804893 >NW_023010900.1:4801088-4801243 >NW_023010900.1:4800193-4800367 >NW_023010900.1:4797970-4798099 >NW_023010900.1:4796334-4796544 >NW_023010900.1:4794216-4794273 >NW_023010900.1:4791630-4791772 >NW_023010900.1:4790079-4790180 >NW_023010900.1:4784648-4784919 >NW_023010900.1:4780110-4781267	1 (only NW_023010900.1:4780110-4781267)
ENSG00000183873	6331	SCN5A	LOC117654807	LOC117654807	NW_023010900.1	4267418	4641777	positive	<i>Pantherophis guttatus</i>	Unknown	NA	Artificial exons created by mapping <i>X. tropicalis</i> , <i>G. gallus</i> exons againsts <i>P. guttatus</i> genome (ENSXETG00000004251, ENSGALG00000006112)	1 (only artificial exon 26 NW_023010900.1:4240810-4242090, see Supp fasta sequence PanGut_SCN5A_exon
ENSG00000007314	6329	SCN4A	SCN4A	LOC117664772	NW_023010717.1	15870385	16001819	positive	<i>Thamnophis sirtalis</i>	26 (10a-b)	KJ9088920, KJ908899, KJ908873, KJ908907	NA. Domain 4 DG variant 15998696-701	26
ENSG00000153253	6328	SCN3A	LOC117670973	LOC117670973	NW_023010694.1	29423555	29504825	negative	<i>Thamnophis sirtalis</i>	26 (5a-b)	KJ908864, KJ908874, KJ908876, KJ908896, KJ908912, KJ908917	NA	26
ENSG00000136531	6326	SCN2A	SCN2A	LOC117670994	NW_023010694.1	29523345	29636194	positive	<i>Thamnophis sirtalis</i>	26 (5a-b)	KJ908862, KJ908876, KJ908914, KJ908934, KJ908936	NA	26

ENSG00000144285	6323	SCN1A	SCN1A	LOC117671051	NW_023010694.1	29853090	29956985	negative	Thamnophis sirtalis	26 (5a-b)	KJ908875, KJ908900, KJ908903, KJ908904, KJ908930	NA	26
ENSG00000169432	6335	SCN9A	SCN9A	LOC117671080	NW_023010694.1	29980741	30103981	negative	Thamnophis sirtalis	26 (5a-b)	KJ908861, KJ908865, KJ90879, KJ908889, KJ908913, KJ908918, KJ908929, KJ908931, KJ908932, KM066119	NA	26
ENSG00000196876	6334	SCN8A	SCN8A	SCN8A	NW_023010713.1	7660736	7777256	negative	Thamnophis sirtalis	26 (5a-b)	KJ908863, KJ908869, KJ908872, KJ908891, KJ908892, KJ908908, KJ908922, KJ908928, KJ908933, KJ908935, KJ908937	NA	26

Species	Common name	SCN1A	SCN2A	SCN3A	SCN4A	SCN5A	SCN7A	SCN8A	SCN9A	SCN10A	SCN11A	CACNA1A
<i>Homo sapiens</i>	Human	ENSG00000144285	ENSG00000136531	ENSG00000153253	ENSG00000007314	ENSG00000183873	ENSG00000136546	ENSG00000196876	ENSG00000169432	ENSG00000185313	ENSG00000168356	ENSG00000141837
<i>Mus musculus</i>	Mouse	ENSMUSG00000064329	ENSMUSG00000075318	ENSMUSG00000057182	ENSMUSG00000001027	ENSMUSG00000032511	ENSMUSG00000034810	ENSMUSG00000023033	ENSMUSG00000075316	ENSMUSG00000034533	ENSMUSG00000034115	ENSMUSG00000034656
<i>Rattus norvegicus</i>	Brown rat	ENSRNOG00000053122	ENSRNOG00000005018	ENSRNOG00000005007	ENSRNOG00000012134	ENSRNOG00000015049	ENSRNOG00000029342	ENSRNOG00000005309	ENSRNOG00000006639	ENSRNOG00000032473	ENSRNOG00000032884	ENSRNOG00000052707
<i>Pelodiscus sinensis</i>	Chineese softshell turtle	NA	XP_006137387.1	ENSPSIG00000002683	ENSPSIG00000007410		NA	ENSPSIG00000016907	NA	NA	NA	NA
<i>Xenopus (Silurana) tropicalis</i>	Tropical clawed frog	ENSXETG00000020846	ENSXETG00000021004	ENSXETG00000008965	ENSXETG00000014235	ENSXETG00000004251	NA	XP_031751845.1	NA	NA	NA	ENSXETG00000005585
<i>Gallus gallus</i>	Chicken	NA	ENSGALG00000011009	ENSGALG00000011040	ENSGALG00000034427	ENSGALG00000006112	NA	ENSGALG000000043728	ENSGALG000000027793	NA	ENSGALG000000027702	NA
<i>Anolis carolinensis</i>	Green anole	XP_016850756.1	XP_008113359.1	ENSACAG00000001038	ENSACAG00000012739	XP_016853093.1	NA	XP_003216967.1	NA	ENSACAG00000009381	DAA34938.1	XP_016851635.1
<i>Crocodylus porosus</i>	Australian saltwater crocodile	XP_019409121.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
<i>Crotalus tigris</i>	Tiger rattlesnake	NA	XP_039208732.1	NA	NA	NA	NA	XP_039225747.1	NA	NA	NA	XP_039198770.1
<i>Gekko japonicus</i>		XP_015271637.1	NA	NA	XP_015273726.1	NA	NA	NA	NA	NA	NA	XP_015274358.1
<i>Lacerta agilis</i>		NA	NA	NA	NA	XP_033020790.1	NA	NA	NA	NA	NA	XP_032995621.1
<i>Notechis scutatus</i>	mainland tiger snake	NA	NA	NA	NA	NA	NA	XP_026537110.1	NA	NA	NA	NA
<i>Pantherophis guttatus</i>		NA	XP_034282445.1	NA	NA	NA	NA	XP_034268352.1	NA	NA	NA	XP_034262813.1
<i>Podarcis muralis</i>	Common wall lizard	NA	XP_028603348.1	NA	XP_028558585.1	NA	NA	XP_028566865.1	NA	NA	NA	XP_028573171.1
<i>Pogona vitticeps</i>	central bearded dragon	XP_020645073.1	NA	NA	XP_020653693.1	NA	NA	NA	NA	NA	NA	XP_020656028.1
<i>Protobothrops mucrosquamatus</i>		XP_015679123.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	XP_029139859.1
<i>Pseudonaja textilis</i>		NA	XP_026552908.1	NA	NA	NA	NA	NA	NA	NA	NA	XP_026551939.1
<i>Python bivittatus</i>	Burmese python	XP_025024892.1	NA	NA	XP_025020302.1	NA	NA	NA	NA	XM_025170661.1	NA	XP_025024299.1
<i>Thamnophis elegans</i>	Western terrestrial garter snake	NA	XP_032088260.1	NA	NA	NA	NA	XP_032066422.1	NA	NA	NA	XP_032094925.1
<i>Thamnophis sirtalis</i>	Garter snake	DAA64620.1	BK008861	DAA64622.1	AAW68223.1	NA	NA	XP_013919059.1	DAA64625.1	NA	NA	XP_013919992.1
<i>Zootoca vivipara</i>	common lizard	NA	NA	NA	XP_034990651.1	NA	NA	XP_034959711.1	NA	NA	NA	XP_034960755.1

Gene	Statistic	<i>E. melanotus</i> populations	<i>E. epinephelus</i> populations	<i>E. reginae</i> populations
SCN4A	pi	0.00110134	0.00623501	0.00481437
SCN4A	segre_sites	4	27	18
SCN4A	parsi_sites	3	18	16
SCN4A	TajID	-0.0982008	0.0969218	0.429003
SCN4A	P_tajID	0.51931	0.451365	0.337249
SCN1A	pi	0.00381741	0.00851769	0.0147774
SCN1A	segre_sites	7	21	36
SCN1A	parsi_sites	4	18	21
SCN1A	TajID	0.0615766	-0.168225	-0.403858
SCN1A	P_tajID	0.460798	0.548656	0.632769
SCN8A	pi	0.00103455	0.00684603	0.00578833
SCN8A	segre_sites	4	21	31
SCN8A	parsi_sites	2	21	16
SCN8A	TajID	-0.82229	0.924114	-1.3866
SCN8A	P_tajID	0.76534	0.189834	0.916945
SCN5A	pi	0.0013435	0.00644039	0.00661084
SCN5A	segre_sites	4	33	25
SCN5A	parsi_sites	3	20	22
SCN5A	TajID	0.625888	-0.658467	0.302653
SCN5A	P_tajID	0.281088	0.723216	0.379728
SCN9A	pi	0.00097629	0.0064706	0.00299235
SCN9A	segre_sites	7	22	14
SCN9A	parsi_sites	0	19	13
SCN9A	TajID	-1.83913	1.8508	0.0745492
SCN9A	P_tajID	0.985512	0.0362667	0.46006
SCN10A	pi	0.00284818	0.0114683	0.010454
SCN10A	segre_sites	8	53	36
SCN10A	parsi_sites	5	37	31
SCN10A	TajID	0.146031	-0.750273	-0.096777
SCN10A	P_tajID	0.433441	0.753235	0.520843
SCN11A	pi	0.00456442	0.0149666	0.0101428
SCN11A	segre_sites	17	58	29
SCN11A	parsi_sites	2	37	25
SCN11A	TajID	-1.64072	-1.09902	-0.046264
SCN11A	P_tajID	0.961922	0.853184	0.503815



Region	Position in this study	Gene	Amino acid change	Presence in samples from this study	Organisms with substitutions at this site	Reference
DII S1	NA	SCN4A	N1025N	All snakes	All snakes, mice, change to D in birds. N in Pith	Bodawatta et al., 2022
DII S5	In Fig 2 position 2	SCN4A	S719N	In <i>E. reginae</i> and <i>E. aesculapii</i>	<i>Thamnophis</i> spp. and <i>Heterodon platirhinos</i>	Genbank ref: FJ570810, FJ570811, FJ570812, BK008863 and KT277703
DI p-loop	In Fig 2 position 1	SCN4A	I419L	In <i>E. reginae</i> and <i>E. aesculapii</i>	Dendrobatidae frogs	Genbank ref: KT989187
		SCN9A	I380L	<i>E. reginae</i> , <i>E. sp.</i> , <i>E. epinephelus</i> , <i>E. typhlus</i>	Dendrobatidae frogs in SCN4A	NA for this ortholog
	NA	SCN8A	F371Y	Fixed in <i>E. epinephelus</i>	Tetrodotoxic newts	Vaelli et al. 2020; Gendreau et al., 2021
	NA	SCN4A	V748I	<i>Bothrops atrox</i> , <i>E. sp.</i> and <i>Xenopholis</i> sp.	Dendrobatidae frogs	Márquez et al., 2018
DIII p-loop	In Fig 2 position 4*	SCN2A <sup>a-b</sup>	D1427G/Y	<i>E. epinephelus</i> , <i>E. reginae</i>	In SCN4A in <i>A. pryeri</i> and <i>T. atractus</i>	Feldman et al. 2012; McGlothlin et al. 2016
		SCN3A <sup>a</sup>	D1373N/Y	Fixed in <i>E. epinephelus</i> and <i>E. reginae</i> . Present in <i>E. sp.</i>		
		SCN9A	D1408E	Present in all snakes		
DIV p-loop	In Fig 2 position 5	SCN1A <sup>a</sup>	Q1719M	Present in all snakes and <i>A. carolinensis</i>	<i>T. granulosa</i>	Vaeilli et al. 2020
		SCN2A <sup>a-b</sup>	Q1710M	<i>E. reginae</i> , <i>E. epinephelus</i> and <i>Imantodes cenchoa</i>	In SCN1A and SCN3A in <i>T. granulosa</i>	
		SCN3A <sup>a</sup>	Q1656M/L	<i>E. reginae</i> and <i>E. sp.</i>	<i>T. granulosa</i>	
	In Fig 2 position 6**	SCN8A	I1699V	<i>Erythrolamprus</i> , <i>Leptodeira</i> , <i>Imantodes</i> , <i>Xenopholis</i> and <i>Micrurus</i>	Present in several colubrid genera. <i>T. granulosa</i> (SCN4A and SCN8A)	McGlothlin et al. 2016; Perry et al., 2018; Gendreau et al., 2021
		SCN11A	I1526M/V	Fixed in <i>E. epinephelus</i> . <i>Chironius fuscus</i> and <i>Chironius</i> sp.	<i>T. sirtalis</i>	
	In Fig 2 position 7***	SCN1A <sup>a</sup>	D1727S/N	<i>E. epinephelus</i> , <i>E. sp.</i> and <i>E. reginae</i>	In TTX resistant organism in other orthologs	None for SCN1A, SCN2A and SCN3A orthologs. For SCN4A Josh et al. 2008; Feldman et al. 2012; Hanifin & Gilly, 2015
		SCN2A <sup>a-b</sup>	D1718N	<i>E. epinephelus</i>		
		SCN3A <sup>a</sup>	D1664N/S	<i>E. epinephelus</i> and <i>E. sp.</i>		
		SCN4A	D1533S/N	<i>E. epinephelus</i> , <i>E. sp.</i> and <i>E. reginae</i>	In pufferfish, tetrodotoxic newts, and <i>Thamnophis</i> snakes	McGlothlin et al. 2016
		SCN9A	D1699N	Present in all snakes	Across all snakes	
		SCN11A	D1533N	<i>Anilius scytale</i>	<i>T. sirtalis</i>	
	In Fig 2 position 8***	SCN1A <sup>a</sup>	G1728S/E	<i>E. epinephelus</i> , <i>E. sp.</i> and <i>E. reginae</i>	Tetrodotoxic newts and pufferfish	Josh et al. 2008; Vaeilli et al. 2020; Gendreau et al. 2021
		SCN2A <sup>a-b</sup>	G1719D	<i>E. epinephelus</i>	Tetrodotoxic newts	Vaelli et al. 2020; Gendreau et al. 2021
		SCN3A <sup>a</sup>	G1665D/V/E	<i>E. epinephelus</i> , <i>E. sp.</i> and <i>E. reginae</i>	In TTX resistant organism in other orthologs	NA for this ortholog
		SCN4A	G1533D	<i>E. epinephelus</i> , <i>E. sp.</i> and <i>E. reginae</i>	In pufferfish, tetrodotoxic newts, and <i>Thamnophis</i> snakes	Josh et al. 2008; Feldman et al. 2012; Hanifin & Gilly, 2015
		SCN8A	G1707M	<i>E. epinephelus</i> , <i>E. sp.</i> and <i>E. reginae</i>	In <i>E. epinephelus</i>	McGlothlin et al. 2016
		SCN9A	G1700Y/S/A	Present in all snakes	Across reptiles	McGlothlin et al. 2014; McGlothlin et al. 2016; Perry et al. 2018
		SCN10A	G1666M	<i>E. reginae</i>		
		SCN11A	A1534E	Present in all snakes		
	In Fig 2 position 9	SCN2A <sup>a-b</sup>	P1729R	<i>E. epinephelus</i> , <i>E. reginae</i> , <i>E. melanotus</i> and <i>E. typhlus</i>	Dendrobatidae frogs in SCN4A	NA for this ortholog
		SCN3A <sup>a</sup>	P1675R	<i>E. melanotus</i> and <i>E. sp.</i>		
		SCN4A	P1544S	<i>E. reginae</i> and <i>E. epinephelus</i>	Dendrobatidae frogs	KT989154, KT989158
DI S6	NA	SCN11A	I394V****	Present in several snakes genera. Including <i>Erythrolamprus</i>	Dendrobatidae frogs in SCN4A	Tarvin et al. 2016; Márquez et al., 2018
	A443D-SCN4A	SCN11A	E406D	Present in all snakes	Mantella and Dendrobati frogs in SCN4A	Tarvin et al. 2016; Márquez et al., 2018
	E406D-SCN11A	SCN4A	A443D	<i>Erythrolamprus</i> genus		

DII S6	NA	SCN4A	T774L/V	Leptodeira, <i>Dendriphidion</i> , <i>Oxybelis</i> , <i>Chironius</i> and <i>E.</i> <i>epinephelus</i>	Dendrobatidae frogs	Márquez et al., 2018
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<sup>a</sup>Amphibian's SCN1A, SCN2A, and SCN3A are not orthologs for SCN1A, SCN2A and SCN3A in mammals and reptiles.

<sup>b</sup>SCN2A is paraphyletic between reptiles and mammals. Amino acid notations are based on the *Mus musculus* SCN2A, however, this is not a reptile SCN2A ortholog.

\*Reported as a TTX and STX target site from structural models (Terlau et al. 1991)

\*\*Site reported to confer moderate TTX resistance (Geffeney et al. 2005; Vaelli et al. 2016)

\*\*\*Site reported to confer extreme TTX resistance (Geffeney et al. 2005)

\*\*\*\*Site reported to confer SCN4A BTX resistance (Wang & Wang, 1998)