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Snakes (Colubridae: *Erythrolamprus*) With a Complex Toxic Diet Show Convergent yet Highly Heterogeneous Voltage-gated Sodium Channel Evolution

Valeria Ramírez-Castañeda [1,2,*], Rebecca D. Tarvin [1], Roberto Márquez [3]

[1]Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, CA 94720, USA
[2]Department of Biological Sciences, Universidad de los Andes, Bogotá, AA 4976, Colombia
[3]Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061, USA

*Corresponding author: E-mail: esperando370@gmail.com.

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Abstract

Chemical defense has evolved convergently across multiple lineages and plays a crucial role in shaping ecological communities through selection for toxin resistance. Research on toxin resistance has been pivotal in understanding the genetic basis of trait evolution, as resistance can evolve through mutations in a few target genes, resulting in target-site resistance (TSR). However, in tropical ecosystems, multiple selective pressures from prey with different toxins create complex chemical scenarios for predators that require multiple resistance mechanisms. 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. In this study, we investigated the evolution of TSR in VGSC genes in relation to toxic frog predation in *Erythrolamprus* snakes, tracing the phylogenetic origin and geographic distribution of TSR-conferring genotypes across six species in this group. Our findings reveal convergent yet highly heterogeneous TSR evolution in at least two species that evolved to predate poisonous frogs, and possibly in a third one. Amino acid changes at nine resistance-related positions across eight VGSC genes were identified, suggesting a shared evolutionary path across this gene family. Four of these changes are known to provide tetrodotoxin resistance in other animals. We observed polymorphism in resistance-related sites across species and VGSC paralogs, hinting at a complex evolutionary history of alleles at these loci. These findings offer new insights into adaptive mechanisms in predators with complex toxic diets and introduce *Erythrolamprus* as a model to understand variation in toxin resistance mechanisms.

Key words: toxin resistance, poison frogs, tetrodotoxin, target-site resistance, convergent evolution.

Significance Statement

This study introduces a new evolutionary framework for predator–prey interactions involving multi-toxin prey. 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, populations, and species 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 a widespread antipredatory trait across the tree of life that plays a fundamental role in shaping ecological communities. Toxins have been described as "keystone molecules" because they can exert cascading effects throughout food webs (Ferrer and Zimmer 2012, 2013). For example, primary producers at the base of the food web (many plants) are infamous for having chemical defenses, and insects have adapted and widely diversified in response to plant toxins (Ehrlich and Raven 1964; Després et al. 2007). Along with chemical defenses evolves the ability to resist them, both in the organisms defending themselves (eg prey) and those that they target (eg predators). Three molecular resistance mechanisms have been proposed to counteract exposure to toxins: (i) detoxifying or clearing the toxin, (ii) expressing toxin-binding proteins or transporters that prevent the toxin from reaching its target site, and (iii) 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, 2023; Caty et al. 2019; Karageorgi et al. 2019; Abderemane-Ali et al. 2021; Alvarez-Buylla et al. 2022; Chen et al. 2022). 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) such as snakes, newts, and pufferfish, present a suite of convergent mutations in voltage-gated sodium channels (VGSCs) (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. 2012). 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 toxin resistance mechanisms in the presence of a highly diverse community. Interactions between diverse toxic amphibians and their predators impose complex selection pressures across multiple trophic levels, driving the evolution of toxin-resistance mechanisms across many genes and species within the trophic network (Daly et al. 1999; Asner et al. 2014; Salazar et al. 2018; González Montoya 2021). Among amphibian predators, the snake genus *Erythrolamprus* stands out for its ability to consume multiple poisonous frog species. A series of natural history observations suggest that these snakes are generalist predators that regularly include poisonous frogs in their diet (Santos et al. 2003; Acevedo et al. 2016; Pašukonis and Loretto 2020; Torres-Carvajal and Hinojosa 2020). Specifically, the Amazonian snake *Erythrolamprus reginae* and the Chocóan snake *Erythrolamprus epinephelus* 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; Instituto Humbolt 2022; Mosquera et al. 2022; Caicedo-Portilla et al. 2023). *Erythrolamprus* snakes also prey on toxic frogs of the family Dendrobatidae: *E. reginae* preys *Ameerega trivittata,* which secretes histrionicotoxin (HTX) and pumiliotoxin (PTX), among other alkaloids (Albarelli and Santos-Costa 2010; Santos et al. 2016; Pašukonis and Loretto 2020), and—notably—*E. epinephelus* is the only known predator of the highly toxic *Phyllobates terribilis,* which secretes batrachotoxin (BTX), one of the most potent natural toxins on earth (Myers et al. 1978). Populations of *E. epinephelus* from Costa Rica exhibit mutations in the voltage-gated sodium channel $Na_V1.4$ that may allow them to consume TTX-defended prey (Feldman et al. 2012; McGlothlin et al. 2016; Pearson and Tarvin 2022), but beyond this how *Erythrolamprus* are able to prey on toxic frogs remains unknown.

E. reginae and *E. epinephelus* both consume diverse neurotoxic prey, many of which produce multiple toxins. This ecological exposure likely imposed strong selective pressures on multiple molecular targets in these snakes. 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. 1999; Hille 2001; Santos et al. 2016). In non-resistant organisms, exposure to these toxins leads to respiratory and cardiac arrest, pain, and/or death (Daly et al. 1999; Santos et al. 2016). Therefore, TSR in *E. reginae* and *E. epinephelus* provides an ideal system to understand how

toxin resistance evolves from a gene family perspective, and more generally, how closely related genes with similar functions may respond together to adaptive challenges (McGlothlin et al. 2014, 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 underlied by mechanisms of varying similarity, ranging from phenotypes produced by largely distinct cellular and molecular pathways to those produced by identical molecular changes (Agrawal 2017). 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 (eg as in 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. Studying the evolution of TSRs across several species within a single genus that occupy different habitats and encounter distinct prey communities offers a unique opportunity to connect population-scale microevolutionary processes with macroevolutionary patterns underlying the same convergent trait.

Here, we explore the degree and evolutionary patterns of TSR across the VGSCs gene family within and among several *Erythrolamprus* species. We identify amino acid changes linked to TSR phenotypes and conduct ancestral sequence reconstructions across the VGSC gene family tree to test whether TSRs in both species evolved independently. We identify extensive allelic variability within and among species in this system, in contrast to other TSR models such as *Thamnophis* snakes or dendrobatid frogs. Our findings provide insight into resistance mechanisms in predators with chemically defended diets and highlight evolutionary and ecological processes that drive the shared evolutionary path of toxin resistance across multiple genes and species.

Results

VGSC Sequence Assembly and TSR Screening

We sequenced and assembled nine VGSC genes from eight *Erythrolamprus reginae* individuals and nine *E. epinephelus* individuals from three and seven different localities, respectively, using target enrichment (Fig. 1a & Table S2). In addition, we sequenced VGSCs for 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). In this study, we decided to 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. However, we sequenced an additional 74 genes related to neural communication, and potentially involved in toxin resistance. We make the reads containing data for all sequenced genes publicly available for future research on the molecular mechanisms underlying toxin resistance in snakes (Table S1).

We assembled complete coding sequences 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 neurotoxins (Fig. 2 & Fig. S2) (van Thiel et al. 2022). To screen for possible sites underlying convergent 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 (Supplementary Results and 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 to 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

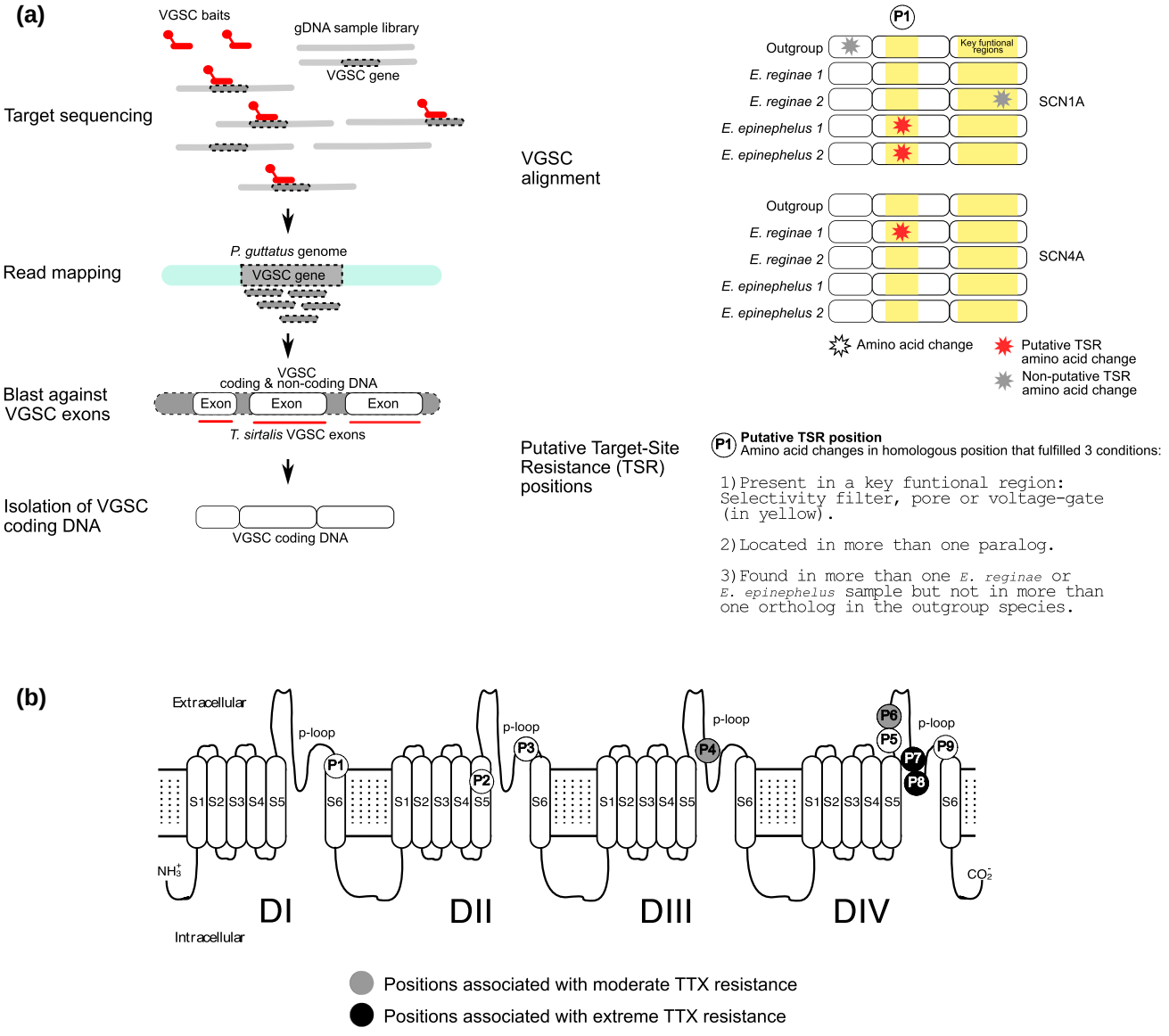

Fig. 1. Methodology to generate coding sequences of genes potentially involved in TSR and to identify putative TSR positions in *E. reginae* and *E. epinephelus*. a) Target sequencing was employed to enrich and sequence the VGSC genes (dark gray) and other nervous-system relevant genes from Colombian 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 (gray 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 (eg 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 (gray 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). Refer to Table S6 for detailed information and references.

(P7 & P8) TTX resistance based on computational models or electrophysiology experiments (Fig. 1b) (Geffeney et al. 2005; Wang et al. 2006; 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

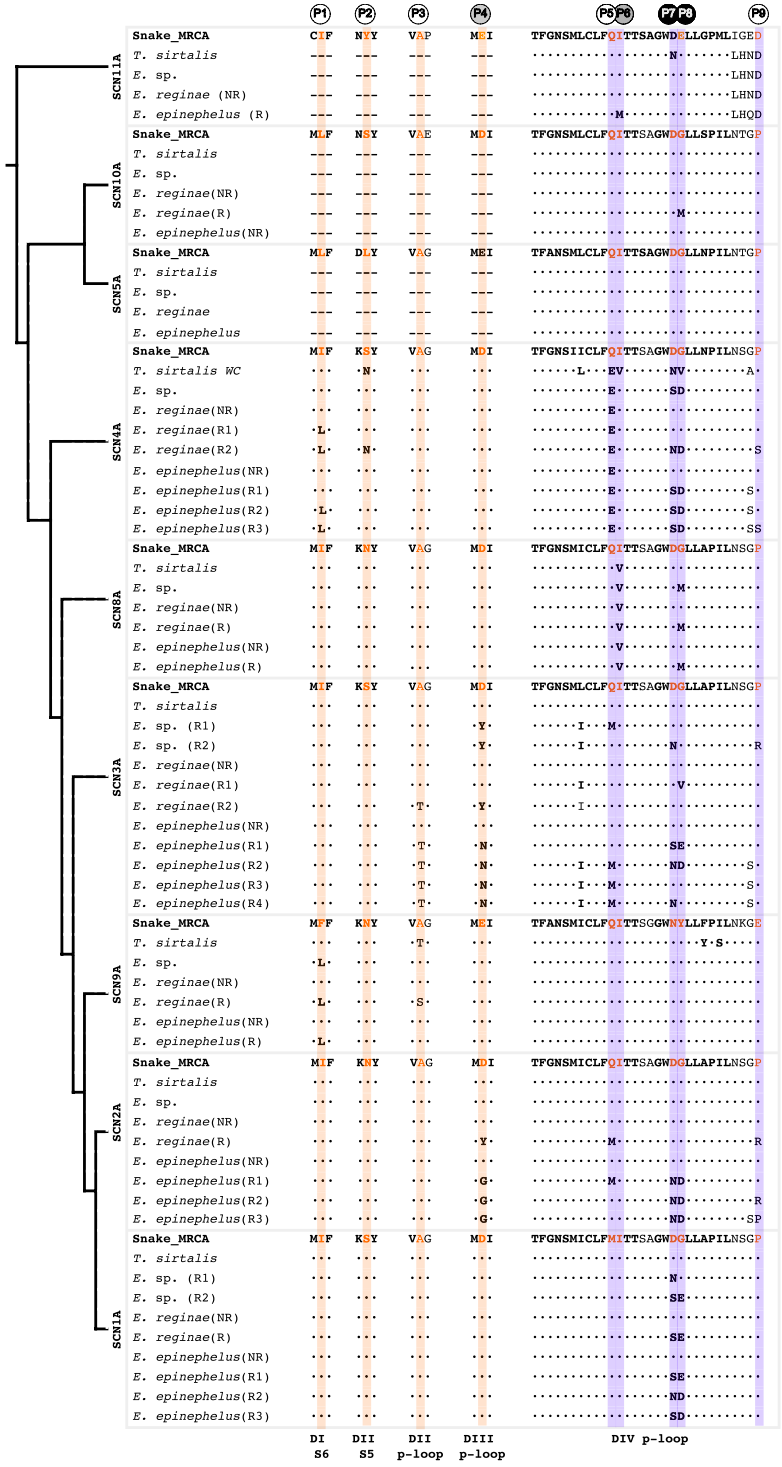

Fig. 2. A representative set of amino acid changes in nine homologous sites identified in nine VGSC genes in *Erythrolamprus reginae*, *E. epinephelus,* and *E.* sp. samples. Each focal species included individuals with haplotypes encoding a putatively non-resistant variant ("NR"), similar to the outgroup 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 or purple. Positions P5-P9, in purple, additionally showed evidence of positive, diversifying selection under MEME (likelihood-ratio test, $P < 0.05$). TTX-resistance-related positions are highlighted in grayscale by the level of resistance (see Fig. 1 for legend).

(Figs. 2 & 4). Five of the nine positions are located in DIV, where several TSR mutations have been identified for multiple neurotoxins across animals (eg Bricelj et al. 2005; Geffeney et al. 2005; Jost et al. 2008; Hanifin 2010; McGlothlin et al. 2016). 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 (Dataset S11), we showed that all the DIV focal sites were found to be under positive selection along several branches of the VGSC gene family tree (in purple, Fig. 2) (Kosakovsky Pond et al. 2020). Among those positions, only P6 became non-significant after applying a false discovery rate multiple testing correction (FDR) (Dataset S1a). All substitutions identified by MEME to be under positive selection are listed in Dataset S1-Sheet A. We note that, given the short length of our focal branches, parameters of the MEME model may not have been estimated accurately, so the statistical significance of these results should be interpreted with caution. This being said, considering multiple lines of evidence were used to select focal sites, uncertainty in MEME results does not affect our results in a significant way.

Additionally, we found other interesting substitutions located in important functional regions that only passed two TSR screening conditions. Although we do not 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 *Erythrolampus*, 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 and Supplementary Results). However, support for the position of *E.* sp. is low (bootstrap value ≈ 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 (Figs. 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. S4 and 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 alleles in different 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 (trans-species polymorphism), 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 (eg del Carlo et al. 2023). 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; most commonly, they were found at intermediate frequencies, or at high frequencies but not fixed species-wide (ie 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, Amazonas, where some TSR mutations are polymorphic within the population. If we only focus

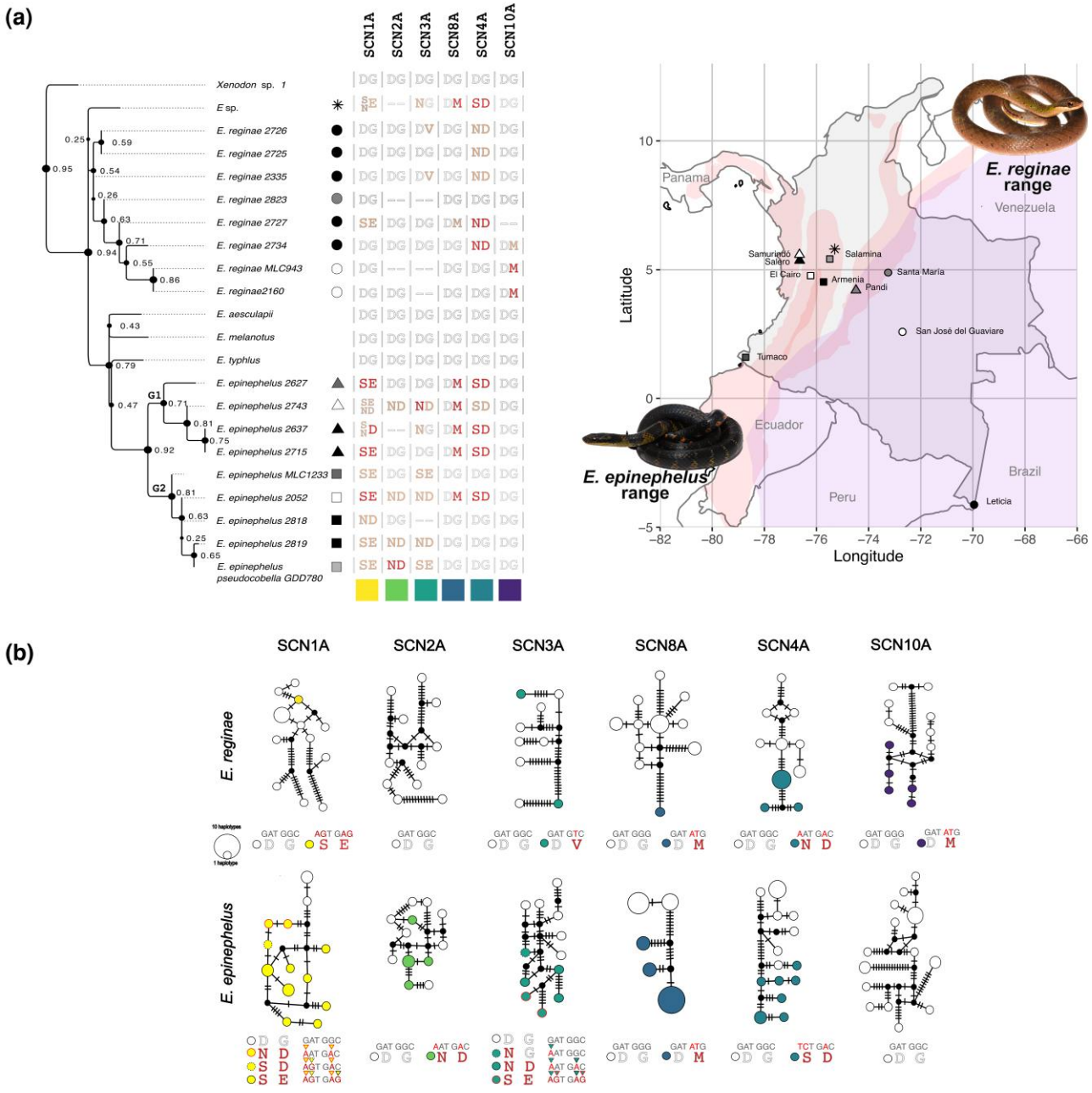

Fig. 3. Phylogenetic tree, geographic distribution and haplotype diversity of the *Erythrolamprus* samples used in this study. a) Phylogenetic tree based on two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*RAG2* and *c-mos*), alongside a visual representation of the P7 and P8 haplotypes 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 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, gray. 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) (Vázquez-Restrepo 2019). Neither of the photographs 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.

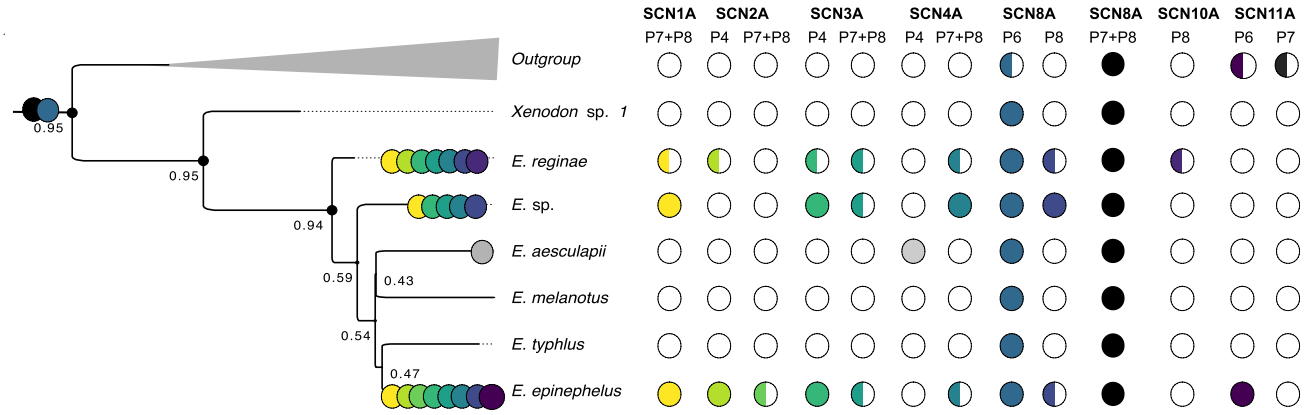

Fig. 4. Presence and distribution of the identified TTX-resistant changes from positions P4 (moderate TTX resistance), P6 (moderate TTX resistance), and P7 and P8 (both extreme TTX resistance) in the *Erythrolamprus* genus samples. Fully filled circles represent the presence of a particular substitution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

on the amino acid changes that have previously been associated with TTX resistance (P4, P6, P7, and 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 Fig. 5 and Fig. S2.

Fine-Scale Variation in and Linkage of Putative TSR Sites

The preceding evidence indicates that TSR evolved independently at nine homologous sites across eight VGSC genes 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 and 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 transition at each codon, then to "SD," which adds a second nucleotide transition that change the "N" to "S," and finally "SE," which requires a transversion or 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 explore 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 and samples 2726 and 2335 in Fig. S2), although we note that haplotype networks alone are not sufficient to recapitulate the origins of a particular

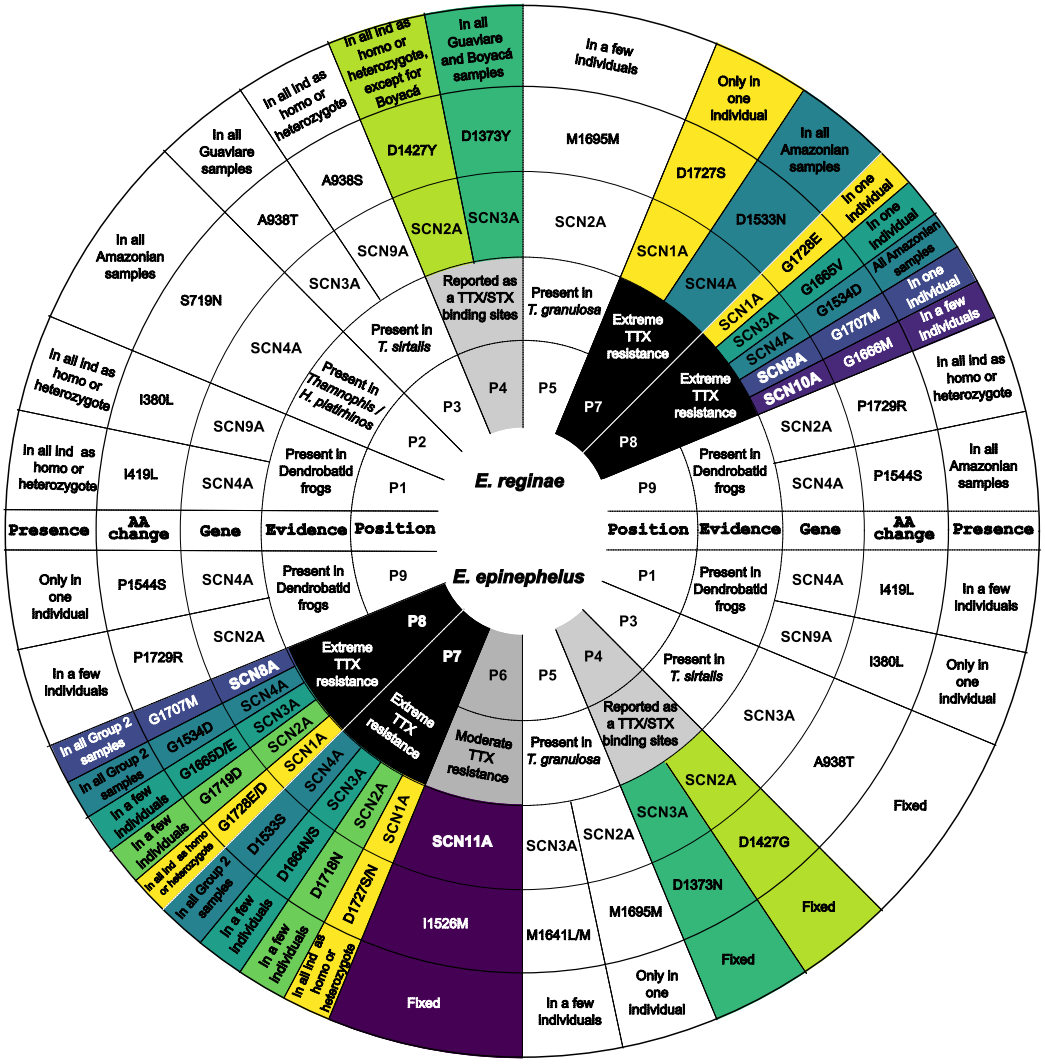

Fig. 5. Summary of the nine positions identified in this study as potential TSR-conferring sites for *Erythrolamprus reginae* and *E. epinephelus*. From the outer to the inner circle, we summarized for each position P1–P9 the presence of each amino acid change across the eight *E. reginae* individuals and nine *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 Figs. 3b and 5. According to the legend in Fig. 1, we highlighted in gray (P4 and P6) and black (P7 and P8) the TTX resistant positions. Ind., individual. Refer to Table S6 for detailed information and references.

mutation, as they do not explicitly account for recombination.

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 (Figs. 2 and 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, and P8 (Terlau et al. 1991; Bricelj et al. 2005; Geffeney et al. 2005; Tikhonov and Zhorov 2005, 2011; Hanifin and Gilly 2015; McGlothlin et al. 2016; Vaelli et al. 2020). For P4, electrophysiology and 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). The same asparagine ("N") that confers TTX resistance in *T. sirtalis* is present in the proteins encoded by the *SCN1A, SCN2A, SCN3A,* and *SCN4A* genes of *E. reginae*, *E. epinephelus*, and *E.* sp. (Figure 2 and see protein names in Table S2), suggesting these proteins also resist TTX. In addition, we confirmed that all the snake species sequenced in this study share the extreme TTX-resistant changes at P7 and P8—*SCN9A* and at P8—*SCN11A*, which 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 and 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 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 (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).

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 for reasons unrelated to toxin resistance. This being said, 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) (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*) (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 to 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) and 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. S3).

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,* and *SCN8A* 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). 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 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 (Stokes et al. 2014), which are common in Amazonian forests (V.R.C., pers. obvs). Identifying TTX in organisms requires specific extraction protocols and chemical analyses that are not often undertaken (Pearson and Tarvin 2022); thus, it is likely that more species may TTX than are currently recognized. 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 other toxins with similar targets. Two toxic molecules have been reported to bind at the same sites as TTX: STX and μ-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 μ-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 scorpion 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. 2006; Santos et al. 2016). In addition, amino acid changes in the DIII p-loop of rat sodium channels provide BTX resistance (Wang et al. 2006). Finally, although alternative functional explanations cannot be completely excluded, the high conservation of VGSCs, the deleterious effects of pore-region substitutions, and the repeated convergence across genes and species strongly support the interpretation that these substitutions contribute to toxin resistance.

Evolutionary Genetics of TSR Allele Polymorphisms

TSR as a phenotype appears to have evolved convergently in multiple species of *Erythrolamprus* snakes. At a population level, we detect finer molecular variation. 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 organs and individuals. There are at least three levels of molecular variation in our data that would contribute to organismal-level variation in resistance. First are different amino acid changes segregating at the same position (eg Figure 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 at 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 (Figs. 3a and 4). 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 differential presence of amino acid substitutions at TSR positions across the VGSC paralogs, as 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 or results in 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 (eg reduced speed and muscle force) (Feldman et al. 2016; Hague et al. 2018; del Carlo et al. 2023). The ecological relevance of this reduction of function has not been tested yet, but it seems likely that it could result in increased polymorphisms, 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. Furthermore, 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, which can also result in polymorphism within populations (Hermisson and Pennings, 2017). In view of the above, 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. 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 (eg [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/or balancing selection process could help maintain these polymorphisms across and within populations.

Concluding Remarks

Although the evolution of neurotoxin resistance has received considerable attention, the full complexity of this phenotype remains poorly understood, even when it is underlain by relatively simple mechanisms such as TSR. By analyzing the entire family of voltage-gated sodium channel proteins, we found considerable genetic variation at putative TSR sites across multiple paralogs, species, and populations, which points to complex ecological and physiological processes driving the evolution of this trait. Furthermore, we introduce a new model system well-suited to study such complex eco-evolutionary dynamics, especially in the context of a shared evolutionary path across gene families and convergent evolution between species. Finally, although this work focused on voltage-gated sodium channels, we make sequencing data for a wealth of additional ion transport proteins publicly available, which we hope will encourage future research on the complex molecular mechanisms and evolutionary dynamics involved in the evolution of neurotoxin 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 natural ecological communities 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 to 018 M). 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 at the Universidad Nacional de Colombia (Table S1). Our final dataset consisted of 9 *E. epinephelus* individuals corresponding to seven different localities (Fig. 3a), eight *E. reginae* individuals corresponding to three localities, and four *E. melanotus* individuals corresponding to three 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 and therefore cannot evaluate the impact of sex or sex-linked loci on allelic diversity (Gendreau et al. 2020).

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 reconcentrated some samples using a SpeedVac (Thermo Fisher Scientific) until they reached a minimum DNA concentration threshold of 40 ng/μL. We fragmented DNA to obtain 400-bp fragments by running three cycles of 30 s 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 *Protobothrops mucrosquamatus* 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 two to three 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 1.5 M (SD = 668 K) 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 and 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 2011133251460 081689024934. 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. 1999; Daly 2005), but less is known about binding sites or activities of the other toxins (Daly et al. 1999; 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 and S9) and two mitochondrial loci obtained through PCR and Sanger sequencing to reconstruct a phylogenetic tree for our samples and focal species (Dataset S6 and 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 two-stage bootstrap values developed in this program (Figs. 4 and 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. 2020), 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. 2020), 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 (ie hemiplasy), which could otherwise lead to incorrect inferences. The results of these ancestral reconstructions are included in Fig. 4, Fig. S4 and Dataset S4.

We screened VGSC sequences for signatures of positive, diversifying selection in a phylogenetic context using the MEME implemented in HyPhy, using default parameters (Kosakovsky Pond et al. 2020). MEME is a branch-site model that employs maximum likelihood to test for positive selection at each site, not assuming a ω single value across the tree (Murrell et al. 2012). Since we observed alleles with putatively different resistance phenotypes within some of our study species, we ran MEME tests with an alignment containing one sequence per species, in which resistant species were represented by a putative resistant variant. MEME was allowed to estimate a gene tree during the run (Dataset S11). 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. To correct for multiple testing, we conducted a FDR correction on the *P*-values generated by MEME using the p.adjust function in R (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 (π), 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.

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

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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).

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