The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey

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Where do the genetic variants underlying adaptive change come from? Are currently adaptive alleles recruited by selection from standing genetic variation within populations, moved through introgression from other populations, or do they arise as novel mutations? Here, we examine the molecular basis of repeated adaptation to the toxin of deadly prey in 3 species of garter snakes (*Thamnophis*) to determine whether adaptation has evolved through novel mutations, sieving of existing variation, or transmission of beneficial alleles across species. Functional amino acid substitutions in the skeletal muscle sodium channel ($Na_v1.4$) are largely responsible for the physiological resistance of garter snakes to tetrodotoxin found in their newt (*Taricha*) prey. Phylogenetic analyses reject the hypotheses that the unique resistance alleles observed in multiple *Thamnophis* species were present before the split of these lineages, or that alleles were shared among species through occasional hybridization events. Our results demonstrate that adaptive evolution has occurred independently multiple times in garter snakes via the de novo acquisition of beneficial mutations.

coevolution | genetic variation | sodium channel | tetrodotoxin | *Thamnophis*

The tempo and mode of adaptive evolution are driven, in large part, by the genetic basis of the traits targeted by selection (1, 2). The dynamics of adaptive evolution and the ability of populations to respond to environmental challenges are influenced by the number of genes underlying traits, the consequences of their interactions, and the magnitude of their effects (1, 2). Common across these architectural issues, however, is a more fundamental question—where does adaptive genetic variation come from? Three distinct pathways are possible but are rarely tested with respect to naturally arising adaptations. Populations are expected to maintain a degree of standing variation with low frequencies of neutral or mildly deleterious alleles at any locus (3). When environmental challenges arise, this standing variation may include alleles that suddenly have positive effects and which selection can recruit to generate adaptation (4–6). An alternative is that beneficial alleles may be present in related species or populations, and occasional hybridization introgresses those adaptive alleles into populations where selection favors their fixation (7–9). Finally, mutation may generate new alleles subsequent to a selective challenge (10, 11). Some of these novel variants may have positive fitness effects, and thereby spread through a population.

Populations are predicted to respond more quickly to selective challenges when adaptation capitalizes on standing variation, for 2 reasons. First, the requisite alleles are already present and available when a new challenge arises (4–6). Second, alleles present in standing variation have a head start because they are likely present in higher frequencies than de novo mutations (5, 6). For these reasons, we might expect adaptations to commonly arise through the sifting of existing variation (12, 13). The movement of beneficial alleles from other populations or species similarly introduces alleles that already have adaptive value (14, 15) and may circumvent the early disadvantage of negligibly low frequency. However, existing genetic variation may not include the large-effect alleles necessary to generate adaptive fitness consequences. In such cases, adaptation is expected to proceed more slowly, depending on the random generation of de novo variants and the ability of that variation to escape stochastic loss through drift (5, 6).

To test the alternative genetic origins of adaptation, we exploited a system displaying evolutionary convergence to a common selective pressure in phylogenetically independent lineages. Garter snakes (*Thamnophis*) appear to have independently evolved resistance to tetrodotoxin (TTX) possessed by their newt prey (*Taricha*). Newts of the genus *Taricha* possess the neurotoxin TTX (16, 17), which acts as a powerful chemical defense against virtually any predator (18). TTX binds to voltage-gated sodium channels in nerves and muscles, blocking the movement of sodium ions (Na^+) across the cell membrane and halting the propagation of action potentials that control nerve impulses (19, 20). By paralyzing nerves and excitable muscle cells, TTX causes immobilization, respiratory failure, and often death (18, 21). Despite the fact that TTX is one of the most potent neurotoxins known (22), garter snakes from a number of populations are able to prey on toxic *Taricha* (23–25). In fact, the levels of TTX resistance in garter snakes and concentrations of TTX in newts often covary over much of western North America, suggesting the two are engaged in a coevolutionary "arms race" characterized by adaptation and counteradaptation (17, 23, 26). The physiological and genetic mechanisms at least partially responsible for elevated TTX resistance have been described recently for 1 garter snake species (27, 28). Key amino acid changes in the skeletal muscle sodium channel ($Na_v1.4$) alter the molecular environment of the channel pore and dramatically alter TTX binding affinity to this protein (28).

Multiple species of garter snakes are known to engage in ecological interactions with toxic newts in western North America: *Thamnophis sirtalis* from coastal California (23), *Thamnophis couchii* from the southern Sierra Nevada mountain range of California (24), and *Thamnophis atratus* from coastal California (25) (Fig. 1). Resistance to TTX appears in both closely and distantly related garter snake taxa, suggesting independent evolution. However, the genetic basis of TTX resistance is known

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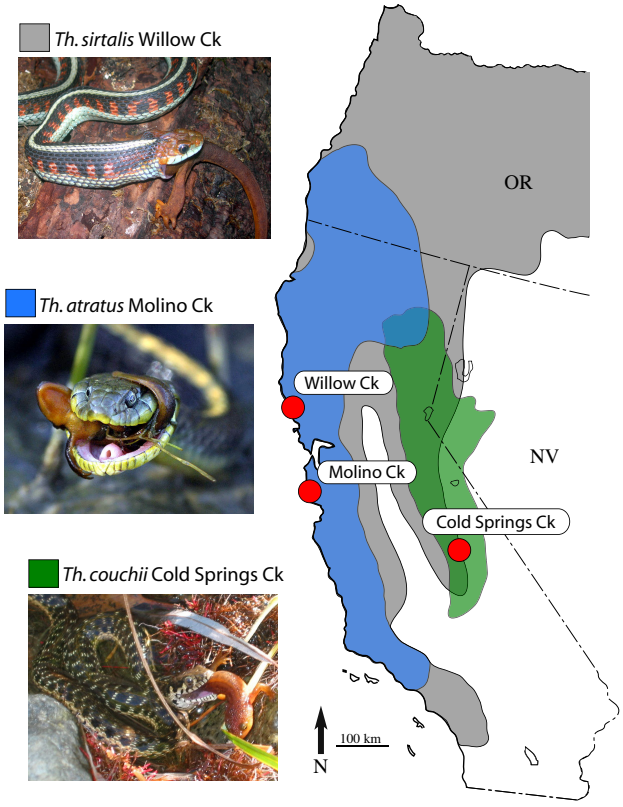

Fig. 1. Geographic distributions of focal *Thamnophis* species. In California and Oregon, the garter snakes *T. sirtalis* (gray), *T. atratus* (blue), and *T. couchii* (green) broadly overlap with newts of the genus *Taricha* that possess the lethal neurotoxin TTX. Despite the potent effects of TTX, some populations of garter snakes are known to prey newts (red).

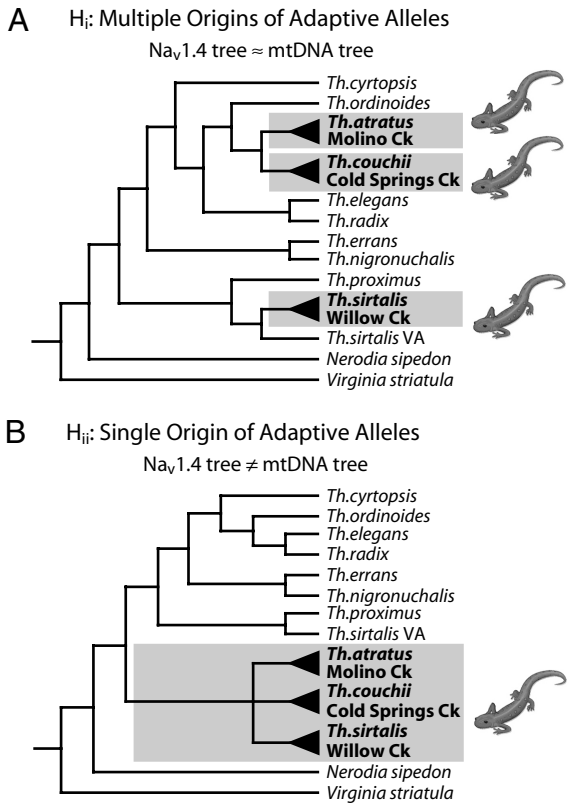

Fig. 2. Two alternative models of adaptive molecular evolution in garter snakes in response to a common selective pressure, TTX poisoning, imposed by their newt prey. (*A*) In the first hypothesis (*i*), each population of newt-consuming garter snakes (black cones) has independently evolved resistance to TTX through convergent changes in $Na_v1.4$, the locus targeted by TTX (gray boxes and newts). Thus, a phylogeny of $Na_v1.4$ alleles should closely resemble the established garter snake phylogeny based on mtDNA (33). (*B*) In the second scenario (*ii*), adaptive variation in $Na_v1.4$ has a single origin, and the occurrence of elevated TTX resistance in separate garter snake lineages is due to either the unique sorting (recruitment) or introgression of this adaptive variation. If this hypothesis is correct, then we expect TTX-resistant garter snakes to form a monophyletic clade in a $Na_v1.4$ phylogeny, in contrast to the *Thamnophis* phylogeny.

only for a single species, *T. sirtalis* (28). Furthermore, fossil and phylogeographic evidence indicates that newts have occupied western North America far longer than garter snakes (29–33), suggesting that exposure to newts as a prey source is ancient, certainly predating the split between *T. atratus* and *T. couchii*, and possibly the divergence of all western *Thamnophis* lineages. Because the ancestral condition for *Taricha* is possession of the neurotoxin TTX (26), garter snake lineages probably have a long history of selection from TTX. Given the significant difference in coalescence times for nuclear and mitochondrial genes (34), we cannot a priori rule out the retention of preexisting beneficial variation as the source of adaptive genetic variation underlying phenotypic convergence in TTX resistance. Furthermore, infrequent hybridization is known between garter snake species, even across diverse *Thamnophis* clades (35, 36). For alleles with a substantial fitness advantage, even low levels of introgression may be sufficient to allow the transfer of adaptive variation among species (7, 8).

We explored the origin of TTX resistance in the 3 species of garter snakes known to prey on toxic newts. To reconstruct the evolutionary sequence of elevated TTX resistance in *Thamnophis*, we collected TTX resistance data from garter snakes and several natricine relatives, collected DNA sequence data from the voltage-gated sodium channel $Na_v1.4$ of these snakes, and used gene trees to distinguish the signature of independent molecular evolution from that of incomplete lineage sorting or horizontal transfer. If elevated TTX resistance in *Thamnophis* has evolved through (*i*) independent changes in $Na_v1.4$, then a $Na_v1.4$ gene tree should roughly match the accepted garter snake phylogeny (33) (Fig. 2*A*). In contrast, if elevated TTX resistance in *Thamnophis* has occurred through (*ii*) the recruitment of adaptive variation that predates the splitting of these lineages, or horizontal transfer of beneficial alleles, then the 3 TTX-resistant taxa will form a clade in a phylogeny of $Na_v1.4$ alleles, contrary to the *Thamnophis* phylogeny (Fig. 2*B*). Thus, gene tree comparisons allow us to test whether phenotypic convergence is the result of novel mutations or evolution via existing genetic variation.

Results and Discussion

Elevated TTX Resistance in Thamnophis. Our phenotypic assay of TTX resistance demonstrates high levels of resistance in 3 species of *Thamnophis* (Fig. 3 and Table S1). *T. atratus* from the central California coast (Molino Creek, Santa Cruz County; and Pilar Point Harbor, San Mateo County) (Fig. 1) display high levels of resistance to TTX (Fig. 3 and Table S1). These *T. atratus* are among the most TTX-resistant snakes ever recorded; the amount of TTX required to slow the average *T. atratus* from the central coast to 50% of its normal crawl speed is >100 mass-adjusted mouse units (MAMUs), matched only by a few populations of *T. sirtalis* (23). In fact, the oral dose needed to reduce the crawl speed of a large *T. atratus* (200 g) from this population to 15% of its normal ability would roughly equal 900 human

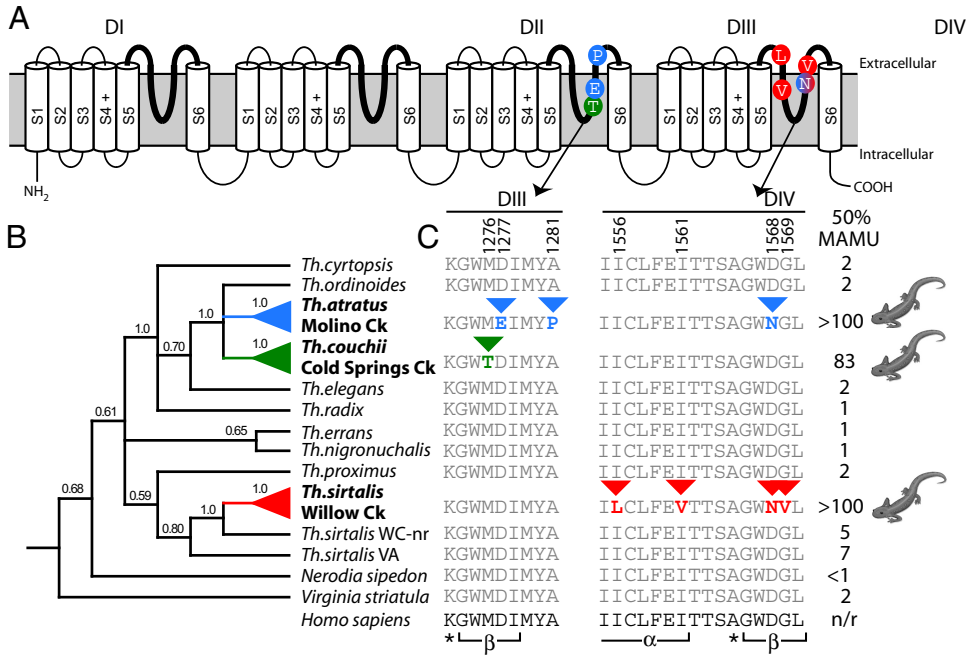

Fig. 3. Independent, adaptive molecular evolution in the skeletal muscle sodium channel ($Na_v1.4$). Amino acid replacements at sites important in TTX ligation in the P loops of $Na_v1.4$ are found only in TTX-resistant garter snakes and appear uniquely derived. (*A*) Structure of the α-subunit of $Na_v1.4$ showing the 4 domains (DI–DIV), their 6 transmembrane segments (S1–S6), and the linkers that connect segments. The 4 polypeptide chains that link S5 to S6 (bold) form the outer pore of the channel that allows selective permeation of Na^+ ions; however, a number of residues that form the outer pore bind strongly to TTX, which occludes the pore and halts Na^+ movement (see text for further discussion and citations). Approximate location of amino acid substitutions in DIII and DIV P loops (color-coded to species) discussed in text. (*B*) Phylogeny of $Na_v1.4$ alleles from *Thamnophis* and relatives based on 2.9 kb of $Na_v1.4$ sequence data (4.3-kb alignment), including the coding regions of all 4 P loops (1.0 kb) and portions of 3 linked introns (1.9 kb). The gene tree closely resembles independent estimates of garter snake phylogeny based on mtDNA (Fig. 2), and shows that elevated TTX resistance has evolved multiple times in garter snakes. Topology and nodal support values were estimated via Bayesian tree searches; some outgroups were pruned for simplicity; locality information is in Table S1. (*C*) Measures of whole-animal resistance to TTX (50% MAMU) alongside amino acid sequences of the DIII and DIV P loops (Table S1). Amino acid substitutions (arrows and replacements color-coded to species) occur at critical residues that change the structure and electrostatic environment of the pore and alter TTX binding affinity. Human sequence is given for comparison (M81758), but amino acid positions follow $Na_v1.4$ CDS from *T. sirtalis* AY851746; structures of the pore labeled below human sequence (*, selectivity filter; α, α-helix; β, β-strand).

lethal doses (21). Consequently, newts would have to be many times more toxic than any individual measured previously (17, 26) to impair these *T. atratus*. Similarly, multiple populations of *T. couchii* in the southern Sierra Nevada range (Tulare County, CA) possess elevated TTX resistance ($\bar{x}$ 50% dosage of 83 MAMUs). The population of *T. sirtalis* from Willow Creek (Sonoma County, CA) exhibits extensive phenotypic variation (Table S1) (23), with some individuals displaying extreme TTX resistance (50% dosage >100 MAMUs), and others (*T. sirtalis* WC-nr) showing only low levels of TTX resistance ($\bar{x}$ 50% dose of 5 MAMUs). Finally, our broad taxonomic survey of New World natricines confirms the hypothesis that elevated TTX resistance within *Thamnophis* is a derived trait (37); other species of *Thamnophis* and 4 natricine species display low levels of TTX resistance (<1–2 MAMUs).

Genetic Basis of TTX Resistance in *Thamnophis*. We examined the (partial) genetic underpinnings of TTX resistance by characterizing molecular changes in the skeletal muscle sodium channel gene $Na_v1.4$. This locus produces a channel-forming protein essential in muscle function that TTX selectively blocks (19, 20). A great deal of literature on the architecture of Na_v loci suggests that TTX fits into the outer pore of the channel (20, 38), and replacements at certain residues in the pore (P loop) dramatically alter TTX binding affinity (39–42), whereas substitutions elsewhere in the protein appear to have little effect (20, 40).

The entire α-subunit of $Na_v1.4$ in garter snakes encodes 1,875 residues (5,658 bp) and shows high structural and amino acid homology, as well as conservation of intron/exon boundaries with mammalian $Na_v1.4$ (*Homo* and *Rattus*) (Fig. S1). We found no difference between the genomic and transcribed sequences (cDNA) of $Na_v1.4$, suggesting that neither splice variation nor RNA editing plays a role in modulating TTX resistance in garter snakes (28). We detected variation in $Na_v1.4$ within and outside the P-loop regions. Changes outside the P loops ranged from 2 amino acid substitutions between *T. sirtalis* alleles to 17 between *T. couchii* and *T. sirtalis*. These substitutions are not expected to affect TTX binding, because they occur in the linker regions that connect transmembrane segments and have no contact with TTX (20, 40). Amino acid sequences within the P loop of $Na_v1.4$ are nearly invariant across garter snakes and relatives and are almost identical to mammalian sequences, suggesting the locus is under strong purifying selection because of its critical functional role. Amino acid substitutions in the pore-forming structures (pore α-helix, selectivity filter, β-strand; ref. 38) that interact with TTX are only found in TTX-resistant snakes (Fig. 3). We found replacements in the P loop of DIII in both *T. atratus* and *T. couchii*, in addition to changes in the DIV P loop of $Na_v1.4$ implicated previously in TTX resistance (28).

T. couchii have a single M1276T substitution [abbreviations follow standard notation: ancestral amino acid reported in front of residue location and followed by derived amino acid; positions follow $Na_v1.4$ complete coding sequence (CDS) from *T. sirtalis* (GenBank AY851746)] in the β-strand of DIII at a site important in TTX affinity to the outer pore (39, 41–43). An identical M→T replacement occurs in both $Na_v1.4a$ and $Na_v1.4b$ of

TTX-bearing pufferfish (44, 45) (teleost fish possess functional duplicates of most Na_v genes), and when this replacement was constructed in rat $Na_v1.4$ and functionally expressed, the amount of TTX required to block Na^+ current (IC_{50}) increased 15-fold (45).

T. atratus possess 3 amino acid changes in the P loops of $Na_v1.4$: 2 in DIII (D1277E and A1281P) and 1 in DIV (D1568N). The D1568N replacement in the β-strand of DIV, also seen in resistant *T. sirtalis* from Willow Creek (Fig. 3), occurs at a site that plays a major role in TTX ligation (39, 41–43, 46). Changing D→N at this position in rat $Na_v1.4$ (D1532) yields a 30- to 40-fold increase in TTX resistance (41, 42). The 2 DIII replacements, D1277E and A1281P, have not been functionally expressed; however, other replacements at D1277 do lead to minor changes in TTX-binding affinity (39, 42). P-loop replacements with only small effects on TTX ligation by themselves appear to have nearly ordinal effects on TTX sensitivity when combined with other resistant replacements (28, 47), so it is not surprising that both the D→E and A→P DIII replacements are also found in some pufferfish (44, 45).

These results do not exclude the possibility that mutations in other sodium channel paralogs contribute to adaptive variation in whole-animal resistance to TTX. On the contrary, diverse patterns of phenotypic variation in snake TTX resistance (23) and surveys of the sodium channels from pufferfish (45) suggest that variation in whole-animal performance is likely to involve multiple, perhaps convergent, adaptive changes across the entire gene family (45).

Evolution of TTX Resistance in *Thamnophis*. Current research is beginning to document the genetic basis of phenotypic adaptation (e.g., refs. 48 and 49), yet the origin of adaptive genetic variation is not generally known (4–6). Useful genetic variation fueling adaptive evolution may enter populations through a number of routes. Populations may acquire new beneficial mutations attendant to the selective challenge (10, 11). Alternatively, preexisting alleles may be recruited and subsequently fixed by selection (i.e., adaptation from standing variation; refs. 4–6, 12, 13). Similarly, adaptive variation may be introduced to populations or species through introgression (7–9, 14, 15). An ideal setting in which to test these alternative hypotheses for the origin of adaptive variation is an empirical system with a repeated pattern of convergent evolution in phylogenetically independent populations experiencing common selective pressures.

We established the evolutionary relationships of *Thamnophis* $Na_v1.4$ alleles to trace the origins of elevated TTX resistance in this system and to test alternative hypotheses for the origin of adaptive genetic variation. If elevated TTX resistance in *Thamnophis* has evolved through (*i*) independent changes in $Na_v1.4$, then a $Na_v1.4$ gene tree should roughly match the well-supported garter snake phylogeny (33). Alternatively, if elevated TTX resistance in *Thamnophis* has occurred through (*ii*) the recruitment of segregating adaptive variation or horizontal transfer of beneficial alleles, then the 3 TTX-resistant taxa will form a monophyletic grouping in a $Na_v1.4$ gene genealogy, contrary to the *Thamnophis* phylogeny. The 3 phylogenetic methods [maximum parsimony (MP), maximum likelihood (ML), and Bayesian (BI)] generally agree, and there are only a few areas of disagreement (only BI tree shown). Overall, phylogenetic relationships of *Thamnophis* $Na_v1.4$ alleles (Fig. 3) are largely concordant with independent estimates of garter snake relationships based on mitochondrial loci (33).

Assaying TTX resistance across garter snakes and relatives and mapping resistance data onto our $Na_v1.4$ phylogeny indicate that elevated TTX resistance is a derived trait, whereas the ancestral condition for garter snakes and relatives is low TTX resistance (37). The hypothesis of a single origin of adaptive $Na_v1.4$ variation linking TTX-resistant garter snakes through either incomplete lineage sorting or gene flow was rejected by statistical tests of hypothesis compatibility (MP, 2-tailed Wilcoxon signed-ranks test: L difference = 19, $z = -3.9618$, $P < 0.0001$; ML, 1-tailed SH test: $-$lnL difference = 54.6856, $P < 0.001$). Resistant forms of $Na_v1.4$ clearly arose subsequent to the common ancestor of *T. sirtalis* and other western *Thamnophis*. Thus, the topology of the $Na_v1.4$ gene tree, as well as the nature of the changes in the P loops, allow us to reject the hypotheses that elevated TTX resistance occurred through either the recruitment of ancient genetic variation in $Na_v1.4$ that predated the divergence of these lineages, or introgression of beneficial $Na_v1.4$ alleles across species. The recent common ancestry of *T. atratus* and *T. couchii* makes it difficult to determine whether the resistance alleles in those taxa existed before their divergence, although the unique functional mutations in the $Na_v1.4$ P loops in these 2 taxa suggest independent acquisition. Thus, we feel that our data are most consistent with the hypothesis that elevated TTX resistance has evolved 3 times independently within *Thamnophis* through convergent changes in $Na_v1.4$.

These results do not exclude the possibility that within each taxon (*T. atratus*, *T. couchii*, and *T. sirtalis*), the adaptive $Na_v1.4$ alleles were present as neutral or nearly neutral variants segregating at low frequency until promoted by selection. However, the striking conservation of $Na_v1.4$ P-loop residues across snakes (and even between snakes and mammals) hints that most variation in this region negatively affects sodium channel function. Biogeographic evidence further suggests that the ecological challenge of toxic newt prey predates the separation of these snake lineages (29–32), suggesting that resistance alleles arose after the initial selective context. An unequivocal answer to this problem will require more extensive sampling and analyses to fully trace the timing of origins of adaptive $Na_v1.4$ alleles within *T. atratus*, *T. couchii*, and *T. sirtalis* populations.

The results of this study provide a significant commentary on the convergent acquisition of adaptive alleles in natural populations faced with strong selective pressures. Adaptive changes in the TTX resistance phenotype have a simple genetic basis mediated by a few mutations of major effect. In addition, these amino acid changes occur in a critical locus with strong pleiotropic effects (39, 50) likely to result in significant molecular evolutionary constraints (51, 52). Taken together, these observations suggest that in situations where a few changes in a gene of major effect are involved, independent evolution may be a common motif (53). Adaptation via the recruitment of standing variation or hybridization may be more commonly observed in situations where polygenetic changes are required, or where adaptive alleles do not have deleterious pleiotropic effects on fitness and are essentially neutral in the absence of the selective pressure that renders them beneficial.

Methods

Bioassays. We collected TTX resistance data from 22 *T. atratus*, 84 *T. couchii*, and 22 *T. sirtalis* (Fig. 1 and Table S1). To provide a phylogenetic perspective on the evolution of elevated TTX resistance, we also collected resistance data from 228 specimens from 7 other garter snakes species representing the major *Thamnophis* clades (33) and from 34 snakes from 4 outgroup taxa representing pertinent New World natricine lineages (54) (Table S1). Some TTX resistance data came from our previous work (24, 37).

We measured TTX resistance by using a bioassay of whole-organism performance (23, 55). We first established an individual's ''baseline speed'' by racing it down a 4-m racetrack equipped with infrared sensors. We averaged the speed of 2 time trials to obtain an individual's baseline crawl speed. After 24 h of rest, we gave each snake an intraperitoneal injection of a known, mass-adjusted dose of TTX (Sigma). Thirty minutes after injection, we raced snakes on the track to determine ''postinjection speed.'' We repeated this process, resting snakes for 24 h and then increasing the dose of TTX, up to 5 total sequential TTX tests (0.5, 1, 2, 5, and 10 μg) per snake (48 h between trials). We scored ''resistance'' as the reduction of an individual's baseline sprint speed after an injection of TTX (postinjection speed/baseline speed). We

calculated a population (or species) dose–response curve from individual responses to the serial TTX injections by using a simple linear regression (56). From this regression model, we estimated the ''50% dose,'' (analogous to a 50% inhibition concentration), defined as the amount of TTX required to reduce the average snake to 50% of its baseline speed. Because TTX resistance is related to body size (55, 56), we transformed doses into MAMUs, the amount of TTX (in milligrams) required to kill a 20-g mouse in 10 min (see ref. 56). This correction allows us to directly compare TTX resistance between individuals, populations, or species. In some cases (because of extremely low or high resistance), we could not generate a sufficient range of phenotypic resistance to quantitatively estimate an accurate 50% dose; for these taxa, we report threshold values of <1 MAMU or >100 MAMUs without standard errors. Further details of the bioassay and information on captive care of snakes can be found elsewhere (23, 56); we followed institutional protocols for humane treatment and care of animals (Utah State University Institutional Animal Care and Use Committee no. 1008).

Sequence Data. We collected sequence data from $Na_v1.4$ from a subset of garter snakes and outgroup taxa assayed for TTX resistance (Table S1). Within garter snake populations, we intentionally sampled individuals with the most extreme (high and low) resistance phenotypes to maximize the potential to identify alternative alleles. The single α-subunit of Na_v loci forms a membrane-spanning channel that allows selective permeation of Na^+ ions (20, 50). This subunit consists of 4 domains (DI–DIV), each containing 6 transmembrane helices (S1–S6), with the polypeptide chains linking S5 and S6 creating the outer pore of the channel (Fig. 3) (20, 50). The 4 pore-forming segments (P loops) fold back into the membrane to create the outer pore, modeled as a cone and at the base of which lies a narrow selectivity filter (20, 38) that preferentially allows Na^+ ions to pass through the channel (the DEKA motif). The funnel shape of the outer pore is thought to come from 4 α-helix-turn-β-strand structures (one from each S5–S6 linker), with the last residue of each turn facing the pore to create the selectivity filter (38). These same structures that line the outer pore and permit selectivity and permeability of Na^+ through the channel bind strongly to TTX. TTX apparently fits into the vestibule through a combination of hydrogen and ionic bonds, steric attraction, and cation–π interaction (39–43, 46, 57), essentially docking in the outer pore and blocking Na^+ movement (20). Thus, we focused our investigation on amino acid variation in the 4 P loops of $Na_v1.4$, paying attention to the α-helix-turn-β-strand structures. We obtained the entire coding sequence (CDS) of $Na_v1.4$ from 7 garter snakes (4 species) to check for posttranscriptional modification (58).

We isolated and purified genomic DNA from muscle or liver tissue with the DNeasy Tissue Kit (Qiagen). We amplified the 4 regions of $Na_v1.4$ between the S5 and S6 transmembrane segments that form the P loop by using primers we designed specifically for snake $Na_v1.4$ (Table S2). Our amplicons included a linked intron in DI and portions of 2 introns in DIII (Fig. S1). We cleaned amplified products by using the ExcelaPure PCR Purification Kit (Edge Biosystems) and used purified template in cycle-sequencing reactions with Big Dye 3.1 (Applied Biosystems). After an isopropanol/ethanol precipitation, we ran cycle-sequenced products on an ABI 3130 automated sequencer (Applied Biosystems). We sequenced all samples in both directions.

We isolated and purified mRNA from fresh skeletal muscle with the RNeasy Mini Plus Kit (Qiagen). We reverse transcribed total mRNA to cDNA with the iScript Select cDNA Synthesis Kit (Bio-Rad) and oligo(dT) primer. We then amplified a series of overlapping pieces of $Na_v1.4$ to construct a complete contig of the locus by using primers we designed specifically for snake $Na_v1.4$ (Table S2). We cleaned and sequenced amplified products as above. We edited sequences by eye in Sequencher 4.2 (Gene Codes), aligned sequences with Clustal W 1.83 (59), and translated coding regions into amino acid sequences by using MacClade 4.08 (60). We deposited all sequences in GenBank (FJ570810–FJ571064, GQ154075–GQ154084).

Phylogenetic Analyses. We used MP, ML, and BI methods on the combined P-loop and flanking intron sequences to infer phylogenies of $Na_v1.4$ alleles. We excluded a region of intron 19 (1.3 kb flanking DIII P loop) where we could not confidently establish positional homology. The final dataset included more than 2.9 kb of $Na_v1.4$ sequence (4.3 kb aligned), 1.0 kb from the exons containing the P loops, and more than 1.9 kb from linked introns. We coded intron indels as an additional character. We pruned the dataset to include unique alleles only, and we polarized the dataset with the natricine *Virginia striatula*. We conducted MP reconstructions in PAUP* (61) with the branch-and-bound algorithm and assessed nodal support with 1,000 bootstrap pseudoreplicates. We executed ML analyses in PAUP* under the best-fit substitution model (HKY+I+Γ) with the heuristic search algorithm and estimated nodal support with 100 bootstrap pseudoreplicates. We performed mixed-model BI analyses in MrBayes 3.1.2 (62), dividing exons, introns, and indels into distinct partitions and conducting searches under the best-fit models (exons, HKY+I; introns, HKY+Γ) and the parsimony approximation model (63) for indels. We ran BI analyses for 10 million generations, sampling trees every 1,000 generations, and assessed nodal support by the frequency of recovered clades sampled after the stable equilibrium (62). Finally, we assessed the congruence between our $Na_v1.4$ gene trees and our expectations of allelic relationships under a model of a single origin of beneficial alleles with repeated recruitment or horizontal transfer of these alleles. We constrained the MP and ML searches in PAUP* to retain only those trees with a monophyletic TTX-resistant clade. We then compared the constrained and unconstrained MP and ML estimates of $Na_v1.4$ phylogeny in PAUP* by using a 2-tailed Wilcoxon signed-ranks test (64) and a 1-tailed multiple-comparisons likelihood ratio test (65) with 1,000 RELL bootstrap pseudoreplicates.

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Supporting Information

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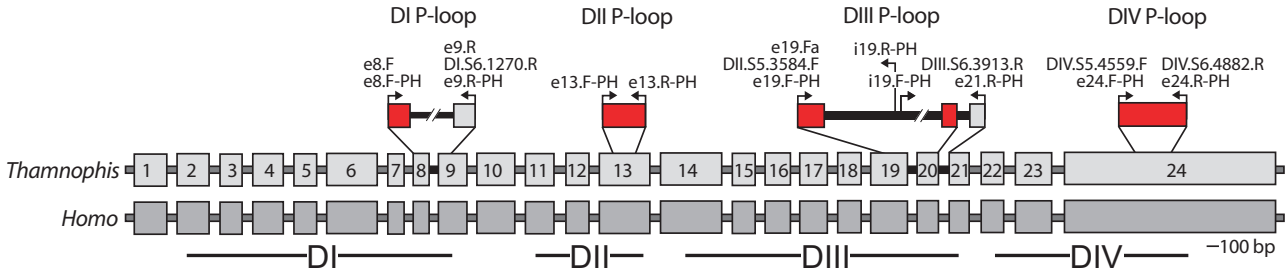

Fig. S1. Predicted exon/intron structure of *Thamnophis* $Na_v1.4$ based on comparison to *Homo* (M81758) and *Rattus* (M26643). The 24 exons (large boxes) are drawn roughly to scale, whereas untranslated regions (small boxes) are not; the 4 P loops (red boxes) and primers used to capture these regions are detailed above the locus; domain boundaries follow [Trimmer JS, et al. (1989) *Neuron* 3:33–49; George AL, Jr., Iyer GS, Kleinfield R, Kallen RG, Barchi RL (1993) *Genomics* 15:598–606]. We translated *Thamnophis* and mammal $Na_v1.4$ CDS in MacClade [Maddison DR, Maddison WP (2005) MacClade: Analysis of phylogeny and character evolution (Sinauer Associates, Sunderland, MA), Version 4.08], aligned these with ClustalW [Thompson JD, Higgins DG, Gibson TJ (1994) *Nucleic Acids Res* 22:4673–4680], and forced nucleotide sequences to follow resultant amino acid alignment in tranalign [Wu TD, Wantanabe CK (2005) *Bioinformatics* 21:1859–1875]. We then created an exon/intron map of mammal reference sequences by using GMAP 2 [Williams G (2002) tranalign (Genome Campus, Hinxton, UK)]. The introns sequenced here (introns 8, 19, and 20) match the assumed splicing sites exactly.

Table S1. Mean TTXresistance for *Thamnophis* species and related natricines, and samplessizes for TTX bioassay, individuals genotyped for the 4 $Na_v1.4$ Ploops, and samples sequenced for entire CDS of $Na_v1.4$

Taxon	Locality	x̄ 50% MAMUs*	SE†	Bioassay (adult/offspring), *n*‡	$Na_v1.4$ P-loop replacements	$Na_v1.4$ P-loop/CDS, *n*
T. atratus	Molino Creek, Santa Cruz County, CA; Pilar Point Harbor, San Mateo County, CA	>100	NA	22 (9/13)	DIII: D1277E DIII: A1281P DIV: D1568N	9/1
T. couchii	Cold Springs Creek, Tulare County, CA; Wishon, Tulare County, CA	83	0.41	84 (17/67)§	DIII: M1276T	8/1
T. cyrtopsis	Ladder Ranch, Sierra County, NM; Patagonia Lake, Santa Cruz County, AZ	2	0.65	38 (4/34)¶	–	1/–
T. elegans	San Simeon Creek Ranch, San Luis Obispo County, CA; Prewitt Creek, Monterey County, CA	2	0.63	24 (24/–)	–	9/1
T. errans	Mil Diez, Durango, Mexico	1	NA	1 (1/–)	–	1/–
T. nigronuchalis	Mil Diez, Durango, Mexico	1	NA	1 (1/–)	–	1/–
T. ordinoides	Adair, Benton County, OR	2	0.32	127 (–/127)‖	–	1/–
T. proximus	Krugerville, Denton County, TX; Splendora, Montgomery County, TX	2	0.51	37 (1/36)¶	–	1/–
T. radix	Corrumpa Creek, Union County, NM	1	NA	2 (2/–)	–	1/–
T. sirtalis	Willow Creek, Sonoma County, CA	>100	NA	10 (3/7)	DIV: I1556L DIV: I1561V DIV: D1568N DIV: G1569V	10/1**
T. sirtalis WC-nr	Willow Creek, Sonoma County, CA	5	NA	2 (2/–)	–	2/–
T. sirtalis VA	Mountain Lake Biological Station, Giles County , VA	7	2.25	10 (10/–)	–	4/–
Nerodia sipedon	Auburn, Lee County, AL	<1	NA	4 (4/–)	–	1/–
Storeria occipitomaculata	Cortland, Cortland County, NY	<1	NA	20 (8/12)	–	1/–
Tropidoclonion lineatum	Luray, Russell County, KS	<1	NA	5 (5/–)	–	1/–
Virginia striatula	North Houston, Harris County, TX	2	0.54	5 (5/–)	–	1/–

*Mass-adjusted measure of the amount of TTX required to slow a snake to 50% of its normal crawl speed (see *Methods*).
†Standard errors not estimable for all groups because of either extremely low or high levels of TTX resistance (see *Methods*) or small sample size.
‡Sample size reflects total number of snakes assayed for TTX resistance and includes wild-caught adults as well as neonates born to wild-caught females in the lab.
§From Brodie et al. [Brodie ED, III, et al. (2005) *J Chem Ecol* 31:343–356] but supplemented with additional samples.
¶From Motychak et al. [Motychak JE, Brodie ED, Jr., Brodie ED, III (1999) *Evolution* 53:1528–1535] but supplemented with additional samples.
‖From Motychak et al. [Motychak JE, Brodie ED, Jr., Brodie ED, III (1999) *Evolution* 53:1528–1535].
**T. sirtalis* complete coding sequence (CDS) from Geffeney et al. [Geffeney SL, Fujimoto E, Brodie ED, III, Brodie ED, Jr., Ruben PC (2005) *Nature* 434:759–763].

Table S2. Oligonucleotide primers used to amplify and sequence genomic DNA and messenger RNA (cDNA) in *Thamnophis* species and natricine relatives

Primer	Sequence (5′–3′)	Position*
5′UTR.F Nav1.4	GTTTTCCCAGTCACGACGTT	NA (5′ UTR)
SN46 1f †	GGGATATTGCTCATACCGTCA	NA (5′ UTR)
e1.Fa Nav1.4	GGGATATTGCTCATACCGTCA	10
e3.F Nav1.4	AGCCAACTGTGTCTTCATGACT	420
e4.F Nav1.4	AAGGTACTTGCTCGGGGATT	517
e4.R Nav1.4	ACATTCCTGCGAGATCCTTG	553
e5.Ra Nav1.4	CGTAGACTTGGGCAACGTCT	621
e5.F Nav1.4	GGGCAACGTCTCTGCTCTAC	630
e5.R Nav1.4	ACATTCCGTGTTCTTCGAGC	652
e6.F Nav1.4	TCTGTGAAGAAGCTCGCTGA	730
e6.Ra Nav1.4	ACTGGGACCTTTGACTGGC	804
e6.R Nav1.4	ACTGTTCATGGGGAACTTGC	967
e8.F-PH Nav1.4	GTGTCCAGAAGGATTTCTCTGC	1074
e8.F Nav1.4	CGAAACCCAAATTACGGCTA	1108
DI.S6.1270.R	GGCTCTTTCTATCTCATCAATTTAATCCTGGC	1270
e9.R-PH Nav1.4	TTTAATCCTGGCTGTGGTGG	1290
e9.R Nav1.4	GCAGAACAGAATGATGCCAC	1321
e11.Fa Nav1.4	TGTTGTCCAGTTTGGGTGAA	1789
e11.F Nav1.4	GTCCTGGACCCTTTTGTTGA	1834
e11.R Nav1.4	TGAATGTGGGCAATCTGGTA	1940
e12.F Nav1.4	TTCACAGCTGAGATGGTGCT	1972
e12.R Nav1.4	GGATGGAACATCTTTGACAGC	2032
e13.F-PH Nav1.4	GCATGCAGCTATTTGGGAAG	2264
e13.R-PH Nav1.4	CCCGATGACCATGACCATTA	2459
e14.R Nav1.4	TTAGCTCCTTCAGTGCCGAT	2516
e14.F Nav1.4	AGCTCCTTCAGTGCCGATAG	2518
e14.Ra Nav1.4	CACAGGCCAGGATTTCAAGT	2748
DIII.S5.3584.F	TCTTCTGGCTCATCTTCAGCATTATGGG	3584
e19.F-PH Nav1.4	CGCTGTGTCAATACCACCAC	3640
e19.Fa-PH Nav1.4	TGAAAATGCCACTGATGTCC	3723
i19.R-PH Nav1.4	GCACATCCAGATCAACATGC	NA (intron 19)
i19.F-PH Nav1.4	GGGGTTTTCAAAAAGCACTTC	NA (intron 19)
e21.R-PH Nav1.4	TATTGAGGCTGTTCCTCCTG	3862
DIII.S6.3913.R	GTGAAGAATGACCCAAAGATAATAAAGATGAC	3913
e24.F-PH Nav1.4	CCGAACTCTGCTCTTTGCTT	4491
DIV.S5.4559.F	TGGTTATGTTCATTTATTCCATTTTTGG	4559
e24.R-PH Nav1.4	ATCTGGACAACCCTGGCAGT	4754
DIV.S6.4882.R	GAGAATTTTAATGTAGCCACAGAGGAGAG	4882
e24.Fb Nav1.4	GGAGAAATTTATGGCTGCAA	5193
3′UTR.R Nav1.4	GTTGGTGTATGGTTCCAAATGA	NA (3′ UTR)

*Positions follow $Na_v1.4$ CDS from *T. sirtalis* AY851746.
†From Geffeney et al. [Geffeney SL, Fujimoto E, Brodie ED, III, Brodie ED, Jr., Ruben PC (2005) *Nature* 434:759–763]; all others designed herein.