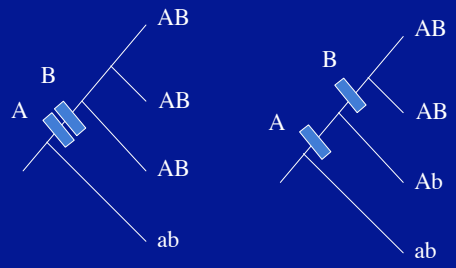
# Correlated evolution (not coevolution!)

Have two characters evolved together?

# Correlated character change

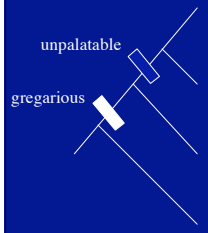


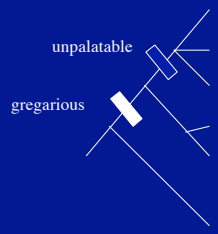

Does character A always arise with character B?
Does character A always precede character B?

# Gregariousness in aposematic butterfly larvae

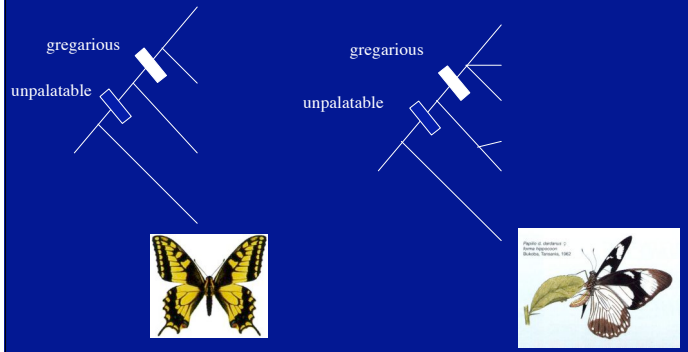
- Aposematic forms tend to be gregarious.
- R. A. Fisher suggested warning coloration evolved through kin selection.
- An individual may die during the "lesson" that teaches naïve predator not to eat brightly colored larvae, but if predator leaves kin alone, inclusive fitness of dead larvae is positive
- Laying eggs in clutches will result in kin groups on same plant
- **Prediction**: aggregation evolves before coloration

# Prediction based on kin selection

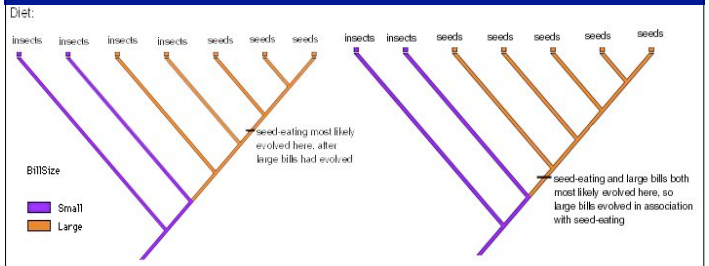



## Results: gregariousness evolves **after** unpalatability



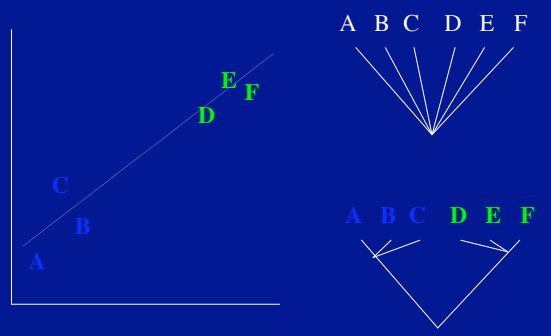

Determining the order of appearance for phylogenetically correlated characters is important for hypothesis testing




## Comparative method

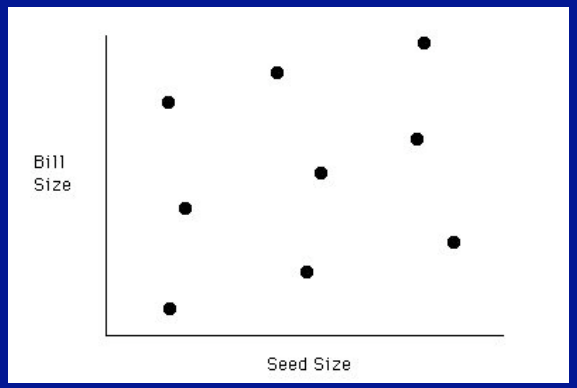
Phylogeny makes cross-species comparisons non independent

## Species aren't independent data points

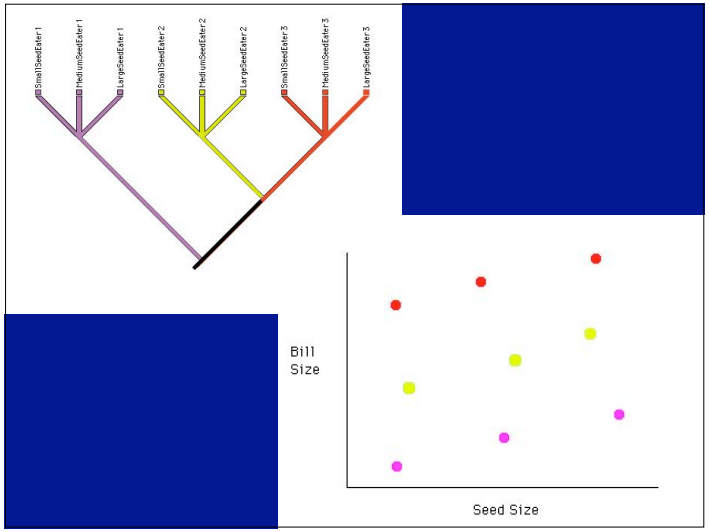


## Why Phylogeny (History) Matters

- **Overestimating the number of independent events in "natural experiments"**
  - Example: Six white species that live in the arctic.
  - What if the six species are fox, hare, ptarmigan, lynx, weasel, and fur seal?
  - What if the six species are all mice?




Bill size vs. favorite seed size in nine species of birds.
Is there a conspicuous pattern here?



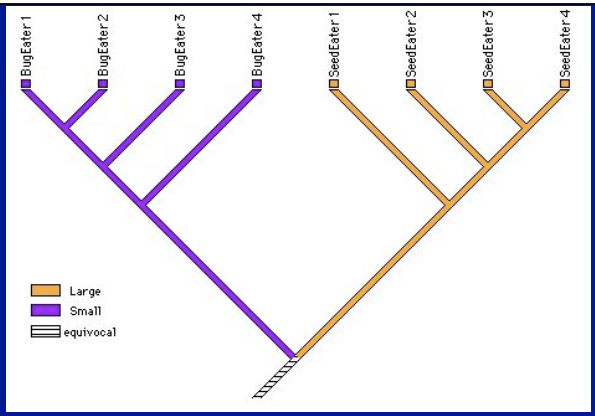

## How do we formally test adaptive hypotheses using a phylogeny?

**We need different methods for:**

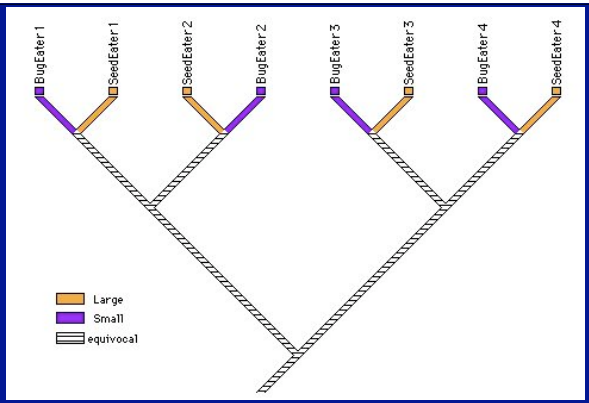
- **Discrete (qualitative) characters**: character traits that have qualitatively discrete states
  - E.g., Number of fingers, color, type of mating behavior
- **Continuous (quantitative) characters**: Characters that have continuous distributions
  - E.g., height, rate at which an enzyme functions, oil composition in a seed

## Discrete Character Traits

– If the **trait only evolved once, there is not strong evidence for adaptation**.
  - Could have just arisen that single time by chance
  - Thereafter maintained by descent




A pattern like this could be simply due to chance; bill size is inherited from ancestors, and not necessarily an adaptation for anything




A pattern like this, however, shows large bills evolving several times in association with seed eating. This kind of convergence is more consistent with true adaptation.

Comparative analyses that use phylogeny can also lead to the discovery of previously unknown adaptations

An example from a gene family in vertebrates, the voltage-gated sodium channels

Many deadly neurotoxins target sodium channels.

There should be selection for toxin resistance (I.e. decreased toxin binding), but how do we identify mutations that might lead to this?

Phylogeny can reveal patterns of parallel evolution.

- Pufferfish toxicology has been studied by the Japanese for over 100 years as a public health issue. The liver, gonads, and sometimes skin of at least 21 genera are known to contain tetrodotoxin and saxitoxin.

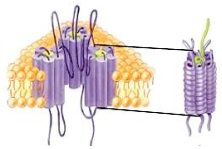
- For mammals (including humans), pufferfish poisoning results in paralysis, including paralysis of diaphragm muscle which can lead to respiratory failure.

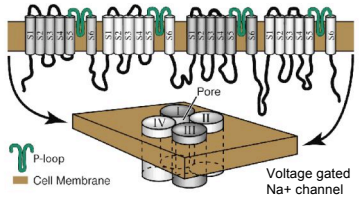
## Most neurotoxins affect the function of ion channels

- Voltage-dependent $K^+$ channels, $Ca^+$ channels, and $Na^+$ channels
- The voltage-gated $Na^+$ channel is targeted by diverse neurotoxins found widely in nature, of two types:

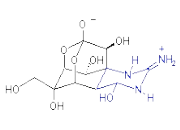
  Toxins that alter channel gating
  e.g. scorpion, anemone, and "arrow frog" toxins, also a wide variety of plant toxins

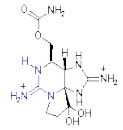
  Toxins that physically block the $Na^+$ ion pore
  e.g. the guanidinium toxins TTX and STX



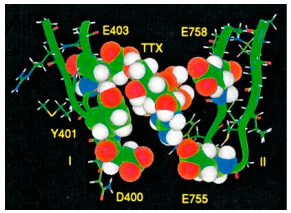


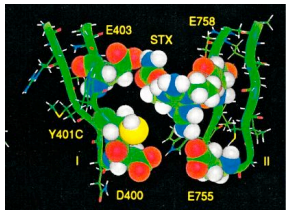

## The guanidinium toxins tetrodotoxin (TTX) and saxitoxin (STX)





- Each contains a positively charged guanidinium group (STX has two)
- Mode of toxicity in animals is by blocking voltage-gated $Na^+$ channels responsible for generating action potentials in nerve and muscle



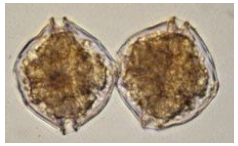




From Lipkind and Fozzard, 1994

## Origin and abundance of guanidinium toxins (1)

Paralytic Shellfish Poisoning (PSP) and some "red tides" are caused by saxitoxin, produced by explosive populations of marine dinoflagellates

A red tide bloom in Nova Scotia



*Alexandrium funyense*

## Origin and abundance of guanidinium toxins (2)

Tetrodotoxin has been detected in widely diverse organisms

- Marine Invertebrates:

Platyhelminthes (flatworms)
Chaetognaths (arrow worms)
Nemertines (ribbon worms)
Tunicates ("sea squirts" = primitive chordates)
Echinoderms (starfish and sea urchins)
Horseshoe crabs
The blue-ringed octopus (*Hapalochlaena* sp.)

- Marine and Terrestrial Vertebrates:

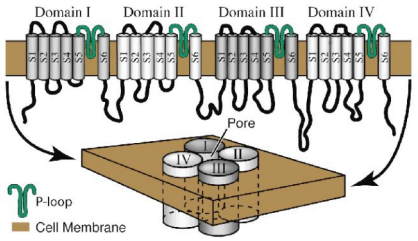
Several families of Tetraodontiform fish (pufferfish)
A few species of goby fish
Five genera of frogs
Seven genera of salamanders

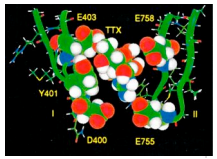
- Many strains of marine bacteria

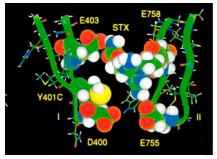
## TTX and STX bind to the $Na_v$ channel in a similar way

- TTX and STX competitively inhibit the binding of $Na^+$ ions, and of each other

- Target is residues surrounding the $Na^+$ ion **pore** formed by the four protein domains



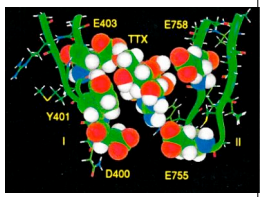



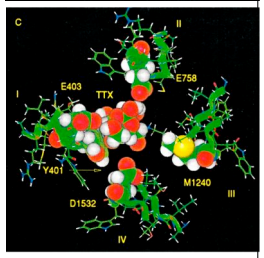

From Lipkind and Fozzard, 1994

## Models of STX and TTX binding involve pore region amino acids in all four $Na_v$ channel domains.

| Domain | Pore Sequence |
|---|---|
| I | D F/Y W E N |
| II | E W I E T |
| III | K G W M D |
| IV | A G W D G |

Inner ring  Outer ring




From Lipkind and Fozzard, 1994

Some mutations in the pore region are known to affect TTX/STX affinity

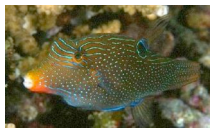
| Mutation | Known From | Toxin resistance (approximate) |
|---|---|---|
| Domain I D(F/Y)WEN aromatic-to-nonaromatic | Pufferfish[1], *Cynops* newts[2], three mammalian channels | ~ 190- to 2000-fold for TTX/STX |
| Domain II EWIET Glu-Asp | Softshell clam *Mya arenaria*[3] | STX: ~ 3000-fold |

| Domain | Pore Sequence |
|---|---|
| I | D F/Y W E N |
| II | E W I E T |
| III | K G W M D |
| IV | A G W D G |

*Takifugu* sp.

(1) Yotsu-Yamashita et al. 2000; (2) Kaneko et al. 1997; (3) Bricelj et al. 2005

Prediction:
Animals that are normally exposed to TTX and/or STX should possess $Na_v$ channel mutations that inhibit toxin binding


*Canthigaster solandri*


*Tetraodon sp.*


*Arothron nigropunctatus*


*Takifugu rubripes*


Both *Taricha* and *Thamnophis*

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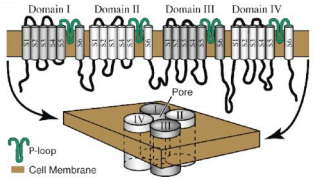
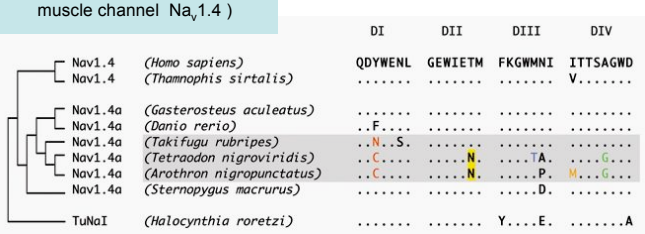
Protein sequence at the $Na^+$ ion pore is highly conserved among vertebrates, but more diverse in species of pufferfish (boxed in grey)

(Example from skeletal muscle channel $Na_v1.4$ )




Pufferfish boxed in grey

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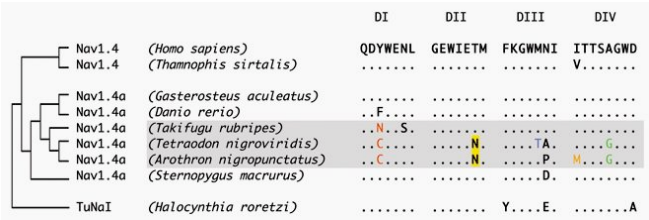
## Phylogenetic Inference Helps Identify Unique Mutation Events

For Domain I, this evolutionary tree of the skeletal muscle $Na_v$ channel $Na_v1.4$ shows two aromatic-to-nonaromatic amino acid replacement events (not three).

Shared replacements in Domain I (to C), Domain II (to N), and Domain IV (to G) were likely already present in the evolutionary ancestor of *Tetraodon* and *Arothron*.

In this example, *Tetraodon* has a unique replacement in Domain III (to T), and *Arothron* has a unique replacement in Domain IV (to M)




Pufferfish boxed in grey

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**Complication:** Vertebrate animals can have ten or more $Na_v$ channel genes in their genomes, each of which encodes a $Na_v$ channel isoform that has specialized expression in different excitable tissues.

| $Na_v1.1$ | $Na_v1.2$ | $Na_v1.3$ | $Na_v1.7$ | $Na_v1.4$ | $Na_v1.5$ | $Na_v1.8$ | $Na_v1.9$ | $Na_v1.6$ |
|---|---|---|---|---|---|---|---|---|
| CNS PNS | CNS | embryonic CNS | PNS | skeletal muscle | heart & skeletal muscle | PNS | PNS | CNS PNS |

Gene nomenclature and expression profile in mammals.
CNS=Central Nervous System. PNS=Peripheral Nervous System.
Chromosomal locations: Blue, HC2; Black, HC17; Orange, HC3; Green, HC12

**Therefore:** while invertebrates can acquire guanidinium toxin resistance via evolution of their single $Na_v$ channel, vertebrate animals must accumulate mutations in **all** their $Na_v$ channels in order to achieve whole-organism resistance. This implies **parallel evolution** of the isoforms.

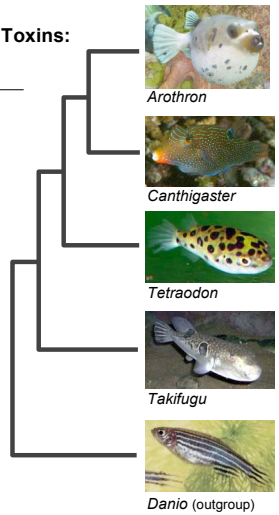
**Biodiversity of Resistance to Guanidinium Toxins: Adaptive Evolution of Animals and Genes**

In nature, how many different pore configurations have evolved in $Na_v$ channels that result in resistance to TTX/STX (reduced toxin binding)?

We can survey across genomes… and species.

Pufferfish are ideal organisms for studying the evolution of toxin resistance because:

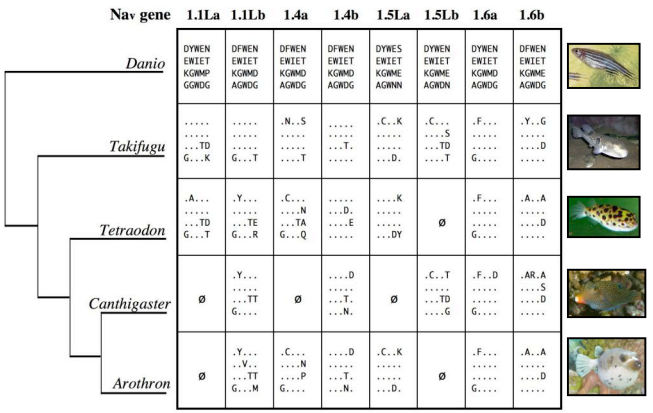
- The selective agent is known (TTX or STX)
- Critical binding region is known (channel ion pore)
- Functional effects of mutations can be measured empirically




## Methods

1. Survey the diversity of $Na_v$ channel pore configurations found in pufferfish using protein sequences for all $Na_v$ channel isoforms from four diverse species (up to eight $Na_v$ channel isoforms may exist in fish genomes). Compare with an outgroup control species (in this case, *Danio rerio*.)
2. Identify candidate mutations by comparing pore region sequence in pufferfish $Na_v$ channels with those in vertebrate animals that are not normally exposed to TTX/STX (e.g. *Danio rerio*, human, rat)
3. Use site-directed mutagenesis to introduce candidate mutations into an otherwise wild-type (toxin-sensitive) $Na_v$ channel
4. Express the mutant channel in a living cell (we use *Xenopus* oocytes)
5. Measure current across the cell membrane in the presence of varying concentrations of toxin (dose-response voltage clamp)

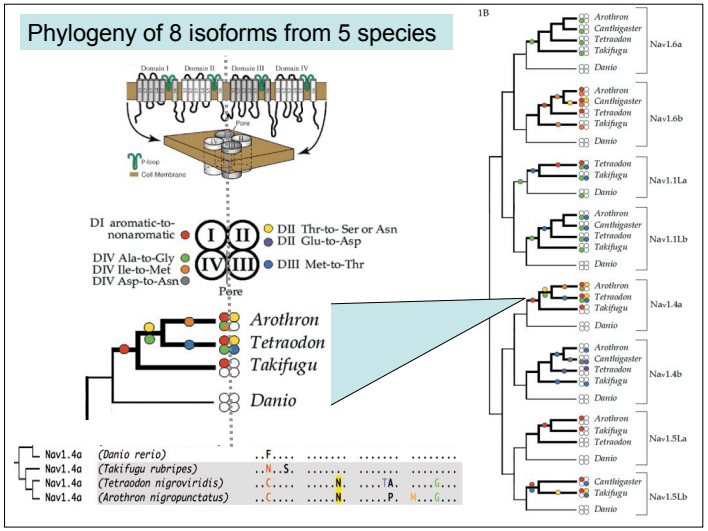
1.) Survey the diversity of $Na_v$ channel pore configurations found in four genera of pufferfish. Compare with an outgroup control species (*Danio rerio*.)




| $Na_V$ gene | 1.1La | 1.1Lb | 1.4a | 1.4b | 1.5La | 1.5Lb | 1.6a | 1.6b |
|---|---|---|---|---|---|---|---|---|
| *Danio* | DYWEN EWIET KGWMP GGWDG | DFWEN EWIET KGWMD AGWDG | DFWEN EWIET KGWMD AGWDG | DFWEN EWIET KGWMD AGWDG | DYWES EWIET KGWME AGWNN | DYWEN EWIET KGWME AGWDN | DYWEN EWIET KGWMD AGWDG | DFWEN EWIET KGWME AGWDG |
| *Takifugu* | ..... ..... ...TD G...K | ..... ..... ..... G...T | .N..S ..... ..... ....T | ..... ..... ...T. ..... | .C..K ..... ..... ...D. | .C... ....S ...TD ....T | .F... ..... ..... G.... | .Y..G ..... ..... ....D ..... |
| *Tetraodon* | .A... ..... ...TD G...T | .Y... ..... ...TE G...R | .C... ....N ...TA G...Q | ..... ...D. ....E ..... | ....K ..... ..... ...DY | Ø | .F... ..... ..... G.... | .A..A ..... ..... ....D ..... |
| *Canthigaster* | Ø | .Y... ..... ...TT G.... | Ø | ....D ..... ...T. ...N. | Ø | .C..T ..... ...TD ....G | .F..D ..... ..... G.... | .AR.A ....S ..... ....D ..... |
| *Arothron* | Ø | .Y... ..V.. ...TT G...M | .C... ....N ....P G.... | ....D ..... ...T. ...N. | .C..K ..... ..... ...D. | Ø | .F... ..... ..... G.... | .A..A ..... ..... ....D ..... |

Ø = gene not found, possibly lost from genome

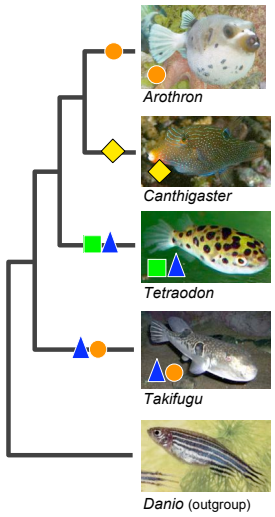
## Phylogeny of 8 isoforms from 5 species



## How Phylogeny Can Inform Us

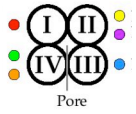
If toxin-resistant mutations appeared early in evolutionary history, modern descendants would have them in common. ***Homology.***

If toxin-resistant mutations appeared after the diversification (branching-off) of modern species, then modern species would show differences - possibly even unique solutions. ***Parallel Evolution.***

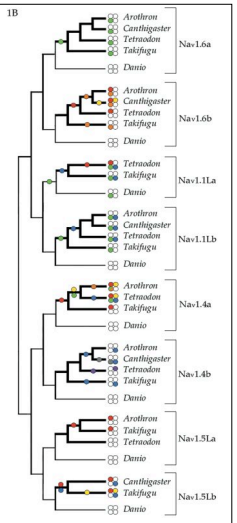



## Results: Massive Parallel Evolution.
Some solutions are identical, others are unique.

| Change | Minimum # of origins |
|---|---|
| DI aro./nonaro. | 5 |
| DII Glu-Asp | 1 |
| DII Thr-Ser/Asn | 3 |
| DIII Met-Thr | 6 |
| DIV Ala-Gly | 4 |
| DIV Ile-Met | 3 |




| | | | | | |
|---|---|---|---|---|---|
| I | D | F | W | E | N |
| II | E | W | I | E | T |
| III | K | G | W | M | D |
| IV | A | G | W | D | G |




3.) Use site-directed mutagenesis to introduce candidate mutations into an otherwise wild-type (toxin-sensitive) $Na_v$ channel

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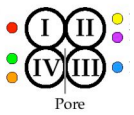
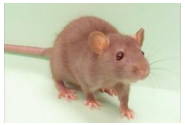
| DIV Ala-Gly | 4 |
| DIV Ile-Met | 3 |



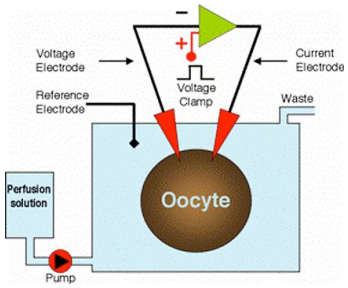

| | | | | | |
|---|---|---|---|---|---|
| I | D | F | W | E | N |
| II | E | W | I | E | T |
| III | K | G | W | M | D |
| IV | A | G | W | D | G |

We chose to study the Domain III M-T mutation, and the Domain IV A-G mutation, whose effects were unknown. Mutations were introduced into a $Na_v1.4$ (skeletal muscle) channel from rat, which is TTX- and STX-sensitive.

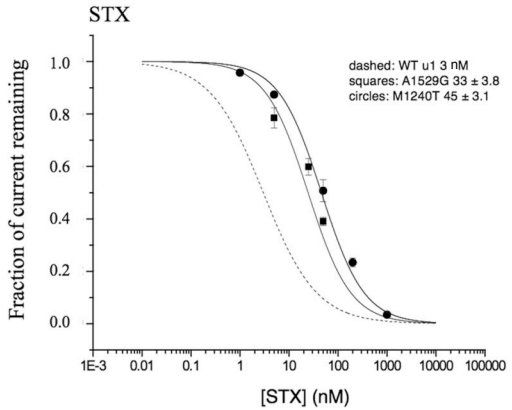


4.) Express the mutant channel in a living cell (*Xenopus laevis* oocyte)

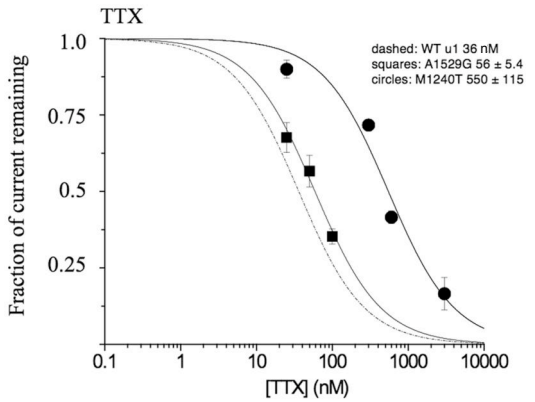
5.) Measure current across the cell membrane in the presence of varying concentrations of TTX and STX (dose-response voltage clamp)



**Dose/voltage response of expressed mutants in STX**
Voltage clamp in *Xenopus laevis* oocytes, Domain III M1240T, and Domain IV A1529G




**Dose/voltage response of expressed mutants in TTX**
Voltage clamp in *Xenopus laevis* oocytes, Domain III M1240T, and Domain IV A1529G




## A handful of solutions found in vastly different animals

| Mutation | Known From | Toxin resistance (approximate) |
|---|---|---|
| Domain I D(F/Y)WEN aromatic-to-nonaromatic | Pufferfish[1], *Cynops* newts[2], three mammalian channels | ~ 190- to 2000-fold for TTX/STX |
| Domain II EWIET Glu-Asp | *Tetraodon* $Na_v1.4b$ ; also softshell clam *Mya arenaria*[3] | STX: 3000-fold |
| Domain II EWIET Thr-Ser or -Asn | Pufferfish and mammal channels with Domain 1 nonaromatic | Unknown |
| Domain III KGWMD Met-Thr | Several pufferfish genes ; also flatworm *Bdelloura candida*[4] | TTX: 15.2-fold STX: 15-fold |
| Domain IV AGWDG Ala-Gly | Several pufferfish genes ; also $Na_v1.1La$ in *Danio rerio* | TTX: 1.5-fold STX: 11-fold |
| Domain IV (I1561) Ile-Met | Pufferfish $Na_v1.6b$ and $Na_v1.4a$; also *Thamnophis* garter snakes[5] | TTX: 2- to 5-fold |
| Domain IV AGWDG Asp-Asn | *Canthigaster* $Na_v1.4b$, also some *Thamnophis* garter snakes[5] | TTX: ~300-fold |

(1) Yotsu-Yamashita et al. 2000; (2) Kaneko et al. 1997; (3) Bricelj et al. 2005; (4) Blair & Anderson 1993;(5) Geffeney et al. 2005

Comparative analyses that use phylogeny can also lead to the discovery of previously unknown adaptations

There should be selection for toxin resistance (I.e. decreased toxin binding), but how do we identify mutations that might lead to this?

Phylogeny can reveal patterns of parallel evolution…

…and cause us to investigate those patterns even further.