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Research Article

Adaptive evolution of the vertebrate skeletal muscle sodium channel

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Abstract

Tetrodotoxin (TTX) is a highly potent neurotoxin that blocks the action potential by selectively binding to voltage-gated sodium channels (Na_v). The skeletal muscle Na_v ($Na_v1.4$) channels in most pufferfish species and certain North American garter snakes are resistant to TTX, whereas in most mammals they are TTX-sensitive. It still remains unclear as to whether the difference in this sensitivity among the various vertebrate species can be associated with adaptive evolution. In this study, we investigated the adaptive evolution of the vertebrate $Na_v1.4$ channels. By means of the CODEML program of the PAML 4.3 package, the lineages of both garter snakes and pufferfishes were denoted to be under positive selection. The positively selected sites identified in the p-loop regions indicated their involvement in $Na_v1.4$ channel sensitivity to TTX. Most of these sites were located in the intracellular regions of the $Na_v1.4$ channel, thereby implying the possible association of these regions with the regulation of voltage-sensor movement.

Key words: skeletal muscle voltage-gated Na ($Na_v1.4$) channel, tetrodotoxin (TTX), positive selection, pufferfish, garter snake.

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Introduction

Tetrodotoxin (TTX) is a highly potent neurotoxin, first isolated from toxic pufferfishes in 1950 (Yokoo, 1950). For many years, these animals were considered to be the only source of this toxin. However, in 1964, it was also detected in California newts (Mosher *et al.*, 1964), and later, in a large variety of animal species, including goby fishes, starfishes and frogs (Miyazawa and Noguchi, 2001). It was further demonstrated that the accumulation in pufferfishes and other animals could be attributed to their food chain starting from TTX-producing marine bacteria, rather than from *de novo* synthesis (Miyazawa and Noguchi, 2001).

Voltage-gated Na^+ channels play an important role in regulating the generation and propagation of action potentials, in response to electrical excitability throughout nerves, muscles and the heart (Marban *et al.*, 1998). They contain four homologous domains (DI – DIV), each of which with six transmembrane segments (S1-S6), as well as a re-entrant (P-loop) between S5 and S6 (Stuhmer *et al.*, 1989). TTX is able to block the current of sodium ions, especially by binding to voltage-gated Na^+ channels, thereby resulting in animal-death (Narahashi *et al.*, 1967). The P-loop regions of the Na^+ channels are responsible for the selectivity of Na^+ ions. Residue mutations in these regions are able to affect TTX binding to Na^+ channels (Lipkind and Fozzard, 2000). Therefore, the P-loop regions are considered to be crucial in avoiding TTX mediated animal death.

Pufferfishes can accumulate extremely high concentrations of TTX without any adverse effect. This resistance is attributed to TTX-resistant skeletal muscle Na^+ (Na^+ 1.4) channels. Pufferfish normally eat a TTX-rich diet, thus sustaining a strong, long-term natural-selection pressure to drive the evolution of TTX resistance in Na^+ 1.4 channels, also a beneficial gain in their defense against natural enemies (*i.e.* predators) (Venkatesh *et al.*, 2005). On the other hand, by preying on TTX-bearing newts, some North American garter snakes (*Thamnophis sirtalis*) might have been compelled to independently evolve resistance to the neurotoxin itself (Geffeney *et al.*, 2002, 2005). In contrast, this did not occur in most mammals and other organisms, possibly due to the absence of the toxin in their diet. However, it remains unclear whether the difference in $Na_v1.4$ channel sensitivity to TTX in non-mammalian vertebrates (*e.g.* pufferfish) and mammals is associated with adaptive evolution or not. In the present study, we used a robust codon-substitution model in PAML package (Yang, 2007) to investigate the adaptive evolution of $Na_v1.4$ channels in certain vertebrate species. Branch-site tests revealed that the $Na_v1.4$ channels in garter snakes and pufferfish were under positive selection. Eight and five positively selected sites were identified in garter snake and pufferfish lineages, respectively. It is worthy of note that, regardless of the presence in either garter snakes or pufferfishes, most of the positively selected sites were located in the intracellular regions of the $Na_v1.4$ channel, thereby implying that these re-

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gions play a crucial role in the adaptive evolution of the channel itself.

Methods

Sequence analysis

In order to investigate the adaptive evolution of skeletal muscle $Na_v1.4$ channels, 19 complete gene sequences of these same channels from TTX-sensitive mammals, electric fishes and zebrafish, as well as TTX-resistant garter snakes and pufferfish, were retrieved from GenBank and Swiss-port. The sequence selection comprised three pufferfish sequences: *Takifugu pardalis (Tp)*(accession number: AB030482), *Takifugu rubripes (Tr)* (DQ221249), and *Tetraodon nigroviridis (Tn)* (DQ221251), four garter snakes: (AY851743 to AY851746) from Bear Lake (BL), Warrenton (War), Benton (Ben) and Willow Creek (WC), nine mammalians: *Canis familiaris (cf)* (XM_848303), *Macaca mulatta (macaca)* (XM_001116451), *Mus musculus (mus)* (NM_133199), *Rattus norvegicus (Rn and RnSkM1)* (NM_013178 and Y17153), *Homo sapiens (Hs and HsSkM1)* (NM_000334 and AY212253), *Bos taurus (Bos)* (XR_028741), and *Equus caballus* (*Ec*) (NM_001081761), one zebrafish (NM_001039825), and two electric fish *Sternopygus macrurus (Sm)* (AF378144) and *Electrophorus electricus (Ee)* (X01119). Protein-coding sequences were aligned based on translated protein sequences using the Clustal W program implemented in MEGA 4 (Thompson *et al.*, 1994). In order to gain an insight into evolutionary relationships, a phylogenetic tree based on the amino acid alignments was constructed by using the NJ (neighbor-joining) method implemented in MEGA 4.0 (Tamura *et al.*, 2007), and the reliability of the tree was estimated using bootstrap method with 1000 replications (Felsenstein, 1985).

Adaptive evolutionary analysis

Maximum likelihood analysis was employed to detect adaptive evolution in $Na_v1.4$ channels, using the CODEML program in the PAML 4.3 package, which has been proven to be a powerful tool for inferring positively selected sites (Zhang *et al.*, 2005). Positive selection is generally measured by the rate-ratio of non-synonymous substitutions per non-synonymous site (dN) to that of synonymous substitutions per synonymous site (dS) (ω = dN/dS). The ω values of > 1, 1 and < 1 indicate positive (diversifying) selection, random drift and negative (purifying) selection, respectively. Branch-site tests were employed to detect positive selection along various evolutionary lineages. In model A, three ω ratios ($0 < \omega_0 < 1$, $\omega_1 = 1$, $\omega_2 > 1$) and 2 ω ratios ($0 < \omega_0 < 1$, $\omega_1 = 1$) were assigned to the foreground and background branches, respectively. The null model (model A') was the same as model A, but with a fixed $\omega_2 = 1$. To detect whether positive selection affects a small number of sites along the pufferfish lineage (branch a in Figure 1), the pufferfish lineage was set to be the foreground branch, and others to be the background branch in model A. The same process was then used to detect positive selection acting on the lineages of garter snakes and mammals, by assigning each of the two lineages in turn as the foreground branch.

Results and Discussion

Evolution of $Na_v1.4$ channel genes

Invertebrate species only possess one or, at the most, two Na^+ channel genes, compared to the 9 in non-mammalian vertebrates and 10 in mammals (Lopreato *et al.*, 2001). The greater number of these genes in the case of vertebrates, as a whole, came about by multiple gene duplication (Goldin, 2002; Novak *et al.*, 2006). Within its respective gene family, the $Na_v1.4$ channel has been well-studied. Mainly expressed in skeletal muscles, its chromosomal localization and evolutionary relationships are distant from the rest (Yu and Catterall, 2003). These skeletal-muscle $Na_v1.4$ channels present species-specific sensitivity to TTX. In pufferfishes, they have evolved resistance to TTX, besides also serving, in certain species, as TTX-specific chemoreceptors when the toxin is imposed as a female pheromone (Venkatesh *et al.*, 2005). Certain other non-mammalian vertebrates, such as North American garter snakes, have also developed $Na_v1.4$ channel resistance to TTX throughout the long-term evolutionary process (Geffeney *et al.*, 2005). Notwithstanding, in mammals and three other fishes, these channels have not developed this resistance, thus remaining very highly TTX sensitive.

A phylogenetic tree was constructed with MEGA 4.0 (Tamura *et al.*, 2007), in order to investigate the evolutionary relationships of $Na_v1.4$ channels among vertebrates. Four groups of sequences from pufferfish, three other fishes, garter snakes and mammals formed four well-sup-

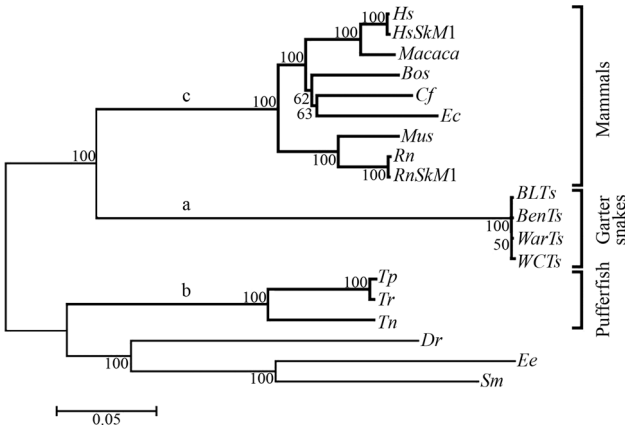

Figure 1 - Phylogenetic tree of $Na_v1.4$ channel genes in vertebrates. The phylogenetic tree was constructed with MEGA 4.0 using the Neighbor-Joining method. The reliability of the tree was evaluated by the bootstrap approach with 1000 replications.

ported clades in the tree (with bootstrap values of 100%) (Figure 1). The $Na_v1.4$ channels in the mammalian and the three fish branches were sensitive to TTX, in contrast to the pufferfish and garter snake, which were resistant, thereby indicating that the tree topology was consistent with both the functional and species divergence therein. The difference in sensitivity to TTX implied the existence of certain crucial residual sites contributing to functional divergence among different vertebrates. Therefore, it was inferred that the gain in resistance to TTX in the $Na_v1.4$ channels of pufferfishes and garter snakes was due to adaptation to a new environment, thus bettering the chances of preying on TTX-bearing organisms.

Test of positive selection and identification of positively selected sites

Darwinian natural selection foments protein evolution by accumulating advantageous mutations for adaptation to a changing environment. During vertebrate evolution, the $Na_v1.4$ channels in pufferfishes and garter snakes developed a resistance to TTX (Geffeney *et al.*, 2002, 2005; Venkatesh *et al.*, 2005). To address whether this specific gain could be associated with positive selection, the maximum likelihood model in the CODEML program of the PAML 4.3 package was used to detect positive selection acting on the $Na_v1.4$ channel (Yang, 2007). The branch-site model was applied to estimating variation in the pattern of substitution across sites along pufferfish (branch a), garter snake (branch b), and mammalian (branch c) lineages (Figure 1). The results are shown in Table 1.

The results revealed that signs of positive selection were detected in groups of garter snakes, pufferfishes and mammals ($p < 0.001$). In the garter snake lineage, 3.5% of the $Na_v1.4$ channel sites were identified as having undergone strong positive selection, with $\omega = 504.78$. At the level of Bayes empirical Bayes (BEB) posterior probability = 0.95, 8 positively selected sites were identified (Table 1). For the pufferfish lineage, 2% of the $Na_v1.4$ channel sites were identified, with $\omega = 131.46$. Five specific sites were identified in this branch at $p > 0.95$ (Table 1). As regards mammalian lineage, 2.2% of the $Na_v1.4$ channel sites were identified, with $\omega = 46.97$. From these results, it can be inferred that positive selection has played a crucial role in the evolution of skeletal muscle $Na_v1.4$ channels.

Location of positively selected sites

The sodium channel is composed of four domains (I-IV), each consisting of six transmembrane segments (S1-S6) (Figure 2). The S4 transmembrane α-helical, possessing many positively charged residues, is the voltage

Table 1 - Maximum likelihood (ML) estimates with the branch-site model for the $Na_v1.4$ channel gene.

Models	df	Parameters under null model	Parameters under alternative model	lnL_0 (lnL_1)	$2\Delta l$	p-value	Positively selected sites[a]	BEB prob. of sites
Branch-site model A								
Pufferfish group as foreground MA' *vs.* MA	1	MA' (fix $\omega_2 = 1$) $p_0 = 0.837$, $\omega_0 = 0.062$ $p_1 = 0.137$ ($p_{2a}+p_{2b} = 0.026$)	MA $p_0 = 0.844$, $\omega_0 = 0.063$ $\omega_2 = 131.46$ $p_1 = 0.136$ ($p_{2a}+p_{2b} = 0.020$)	-33818.74 (-33808.45)	20.58	< 0.001	1207	0.956
							1425	0.958
							1638	0.974
							1654	0.979
							1771	0.988
Garter snake group as foreground MA' *vs.* MA	1	MA' (fix $\omega_2 = 1$) $p_0 = 0.840$, $\omega_0 = 0.062$ $p_1 = 0.133$ ($p_{2a}+p_{2b} = 0.027$)	MA $p_0 = 0.840$, $\omega_0 = 0.063$ $\omega_2 = 504.78$ $p_1 = 0.125$ ($p_{2a}+p_{2b} = 0.035$)	-33814.66 (-33790.68)	47.96	< 0.001	60	0.992
							928	0.953
							933	0.961
							951	0.989
							1005	0.975
							1336	0.951
							1805	0.966
							1818	0.997
Mammalian group as foreground MA' *vs.* MA	1	MA' (fix $\omega_2 = 1$) $p_0 = 0.845$, $\omega_0 = 0.063$ $p_1 = 0.137$ ($p_{2a}+p_{2b} = 0.028$)	MA $p_0 = 0.846$, $\omega_0 = 0.063$ $\omega_2 = 46.97$ $p_1 = 0.132$ ($p_{2a}+p_{2b} = 0.022$)	-33820.17 (-33806.53)	27.28	< 0.001	358	0.973
							485	0.968
							486	0.985
							1201	0.986

[a]The numbering of amino acids is according to the rat $Na_v1.4$ protein sequence (AAA41682).

sensor. Four extracellular loops (the p-loop between the S5 and S6 segments of four domains) dip down into the membrane to form the mouth of the pore by facing each other. The p-loops are responsible for ion selectivity of the channel (*i.e.* preference for Na^+ ions). The S4 segment can initiate conformational changes, thereby leading to the movement of S5 and S6 segments, both of which control the opening and closing of the channel (Marban *et al.*, 1998). TTX binds to the outer vestibule of the pore, which is composed of amino acid residues in re-entrant P-loops (Figure 2). TTX binding occludes the pore, thereby preventing the extracellular entry of Na^+ ions. The TTX-binding region covers the amino acid residues located in and between two rings of the P-loops. The outer ring is formed by residues E403 in domain I, E758 in domain II, M1240 in domain III and D1532 in domain IV, while the inner ring includes residues D400 in domain I, E755 in domain II, K1237 in domain III and A1529 in domain IV (rat Nav1.4, AAA41682) (Soong and Venkatesh, 2006). The amino acid changes occurring in the TTX-binding region, especially in sites at 401 and 758, have been demonstrated to play crucial roles in sensitivity to TTX.

Positive selection generally represents a functional adaptation. To investigate the potential relationship of positive selection to gained $Na_v1.4$ channel resistance to TTX in pufferfish and garter snakes, the positively selected sites identified in rat $Na_v1.4$ channels were mapped (Figure S1). All the 8 positively selected sites identified in the garter snake lineage were located in the intracellular regions of the $Na_v1.4$ channel. Of the 5 sites in the pufferfish lineage, three (60%) were located in intracellular regions, one in the P-loop of domain III, and one in the transmembrane region. Two positive sites in mammalian lineage were located in the P-loops of domain I and IV, and another two in the intracellular regions. Worthy of note, the positively selected sites in the p-loops were located outside the two rings of p-loops.

Despite the importance of the two rings of amino acid residues in the TTX-binding region in TTX sensitivity, they are not the sole determinant of the resistance to TTX. A compelling example is the mammalian cardiac $Na_v1.5$ channel, which has the same two rings of amino acid residues in this specific binding region as do mammalian skeletal muscle $Na_v1.4$ and other Na_v channels. The cardiac channel appears to be TTX-resistant, whereas the remainder are sensitive to much lower amounts of TTX (Soong and Venkatesh, 2006). Therefore, the location of positively selected site (1207) in the p-loops of the $Na_v1.4$ channel gives to understand a potential association with gaining TTX resistance in the pufferfish lineage, in spite of it not covering the TTX-binding region itself.

Environmental change, especially in diet, is one of the major driving forces in organismic evolution. Where the diet often includes poison (*e.g.* TTX), strong survival stress compels animals to develop the adequate resistance. In the case of pufferfishes, this occurred as regards TTX in response to their diet generally including TTX-bearing organisms, such as starfish, gastropods and shrimps (Miyazawa and Noguchi, 2001). In certain populations of North American garter snakes that feed on tetrodotoxic newts, strong survival pressure compelled them to evolve resistance of the $Na_v1.4$ channel to TTX as a means of adaptation to a toxin bearing diet (Geffeney *et al.*, 2002). The higher ω values in pufferfishes and gar-

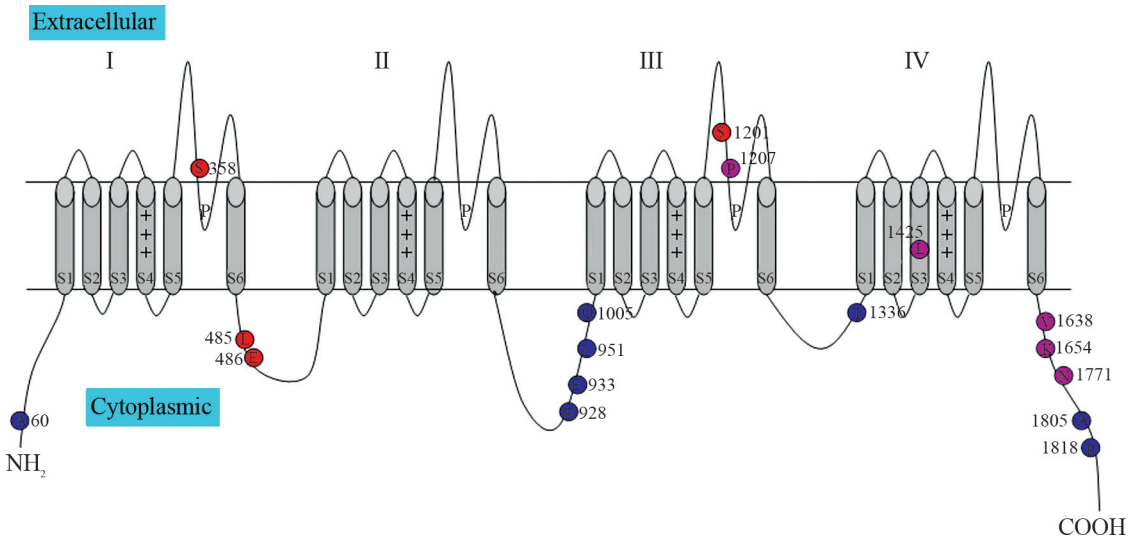

Figure 2 - Two-dimensional structure of the rat $Na_v1.4$ channel. A sodium channel is composed of four domains (I – IV), each consisting of six transmembrane segments (S1-S6). The α-helical S4 segment, which possesses many positive charges, is the voltage sensor. The P-loops between the S5 and S6 segments of each domain face each other, thus forming a pore that is the outer mouth of the channel. The loop between domains III and IV is the inner mouth of the channel. Cylinders indicate transmembrane α-helices, whereas lines represent the hydrophilic portions of the channel. Pink, blue and red amino acids represent positively selected sites identified in the pufferfish, garter snake and mammalian lineages, respectively (for detail see Figure 1). Numbering of the amino acids is according to the rat $Na_v1.4$ protein sequence (AAA41682).

ter snakes than in mammals, clearly indicate that certain beneficial nonsynonymous mutations became fixed (accumulated) through Darwinian natural selection, for them to so possess this specific resistance. In most mammals, their natural diets do not include TTX-bearing organisms, whereby their $Na_v1.4$ channels became functionally more constrained.

In pufferfish, one site in the P-loop region might be associated with the gain of TTX-resistance by $Na_v1.4$ channels. Three sites, located in cytoplasmic regions, could possibly be associated with Na^+ channel activation, by influencing internal conformation (Figure 2) (Marban *et al.*, 1998; Yu and Catterall, 2003). However, the site at 1425 is located within the S3 transmembrane α-helical of domain IV, and thus probably not associated with TTX-binding (Figure 2). In the garter-snake lineage, all positively selected sites of the $Na_v1.4$ channel were detected in cytoplasmic regions, especially in the linker regions between domains II and III. Previous studies showed that the linker between domains III and IV mediated the quick inactivation of Na_v channels (Rohl *et al.*, 1999). The bias of positively selected sites occurring in the intracellular regions implied an association with TTX-blocking action. Therefore, the large amino-terminal and carboxy-terminal tails, as well as II-III linker may contribute to activating Na^+ channels by influencing internal conformation (Marban *et al.*, 1998; Yu and Catterall, 2003). More positively selected sites occurring in these inner linkers possibly implied activity as gating inner controllers of voltage-sensor movement, thereby contributing to the activation of Na^+ channels. In the mammalian lineage, four sites were detected as having undergone positive selection. Due to the absence of TTX in the mammalian diet, positive selection acting on the mammalian $Na_v1.4$ channel might infer a novel functional divergence.

In summary, the adaptive evolution of the $Na_v1.4$ channel in vertebrates was investigated. Phylogenetic analyses showed these channels to be well divided into four large clades, pufferfishes, other fishes, garter snakes and mammals. The lineages of both garter snakes and pufferfishes were detected to have gone through stronger positive selection. Eight and five positively selected sites were identified in the garter snake and pufferfish lineages, respectively. The location of these sites in the $Na_v1.4$ channels implied that some were associated with the gain of $Na_v1.4$ channel resistance to TTX, as well as potential adaptation to a TTX-containing environment. Furthermore, it was noted that most of the positively selected sites, regardless of being in garter snakes or pufferfish, were located in the intracellular regions of the $Na_v1.4$ channel, making an inference of potential roles in voltage-sensor movement.

Acknowledgments

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Supplementary Material

The following online material is available for this article:

Figure S1 - Alignment of the complete sequence of Nav1.4 channels in pufferfishes, garter snakes and mammals.

This material is available as part of the online article from http//scielo.br/gmb.

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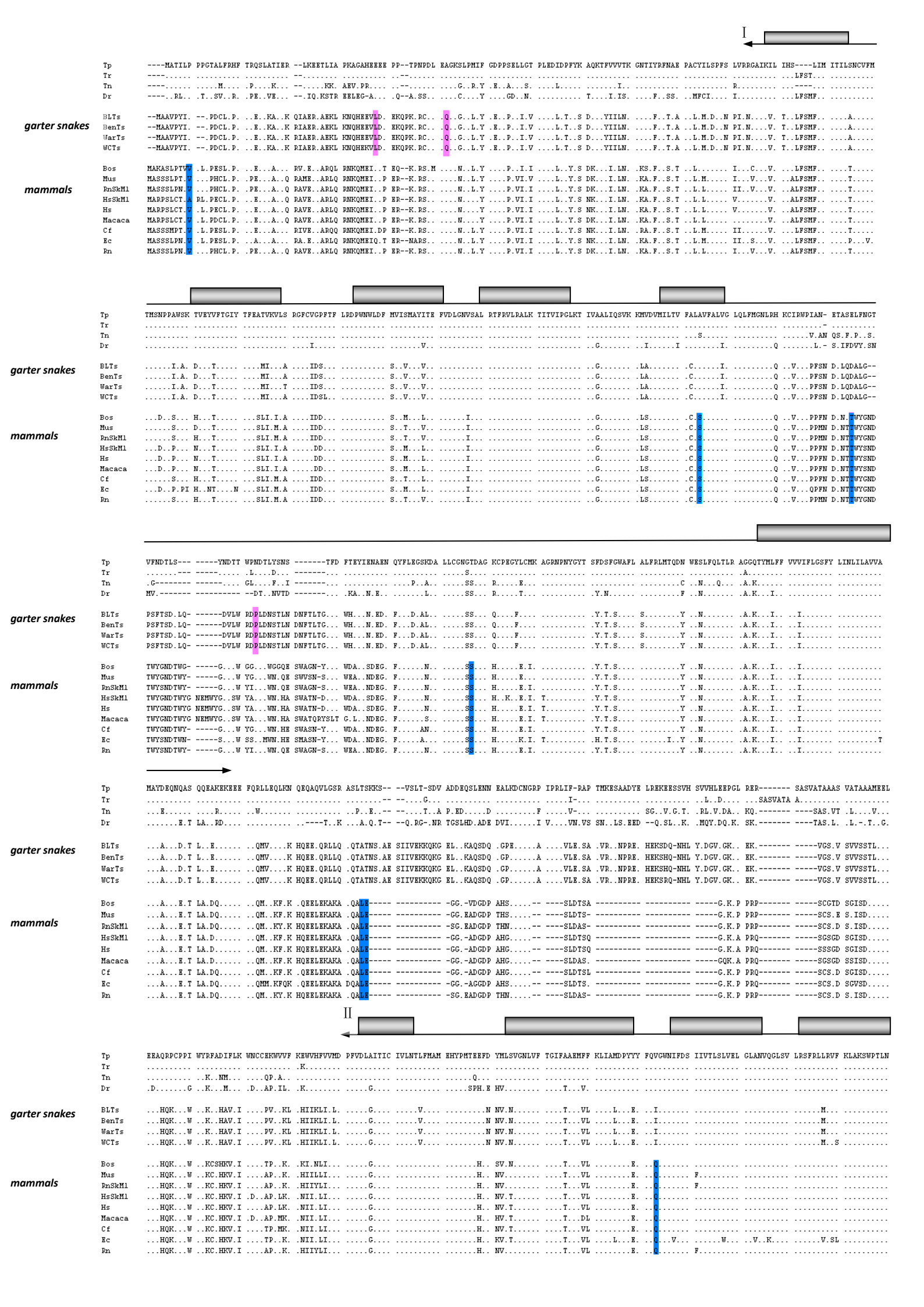
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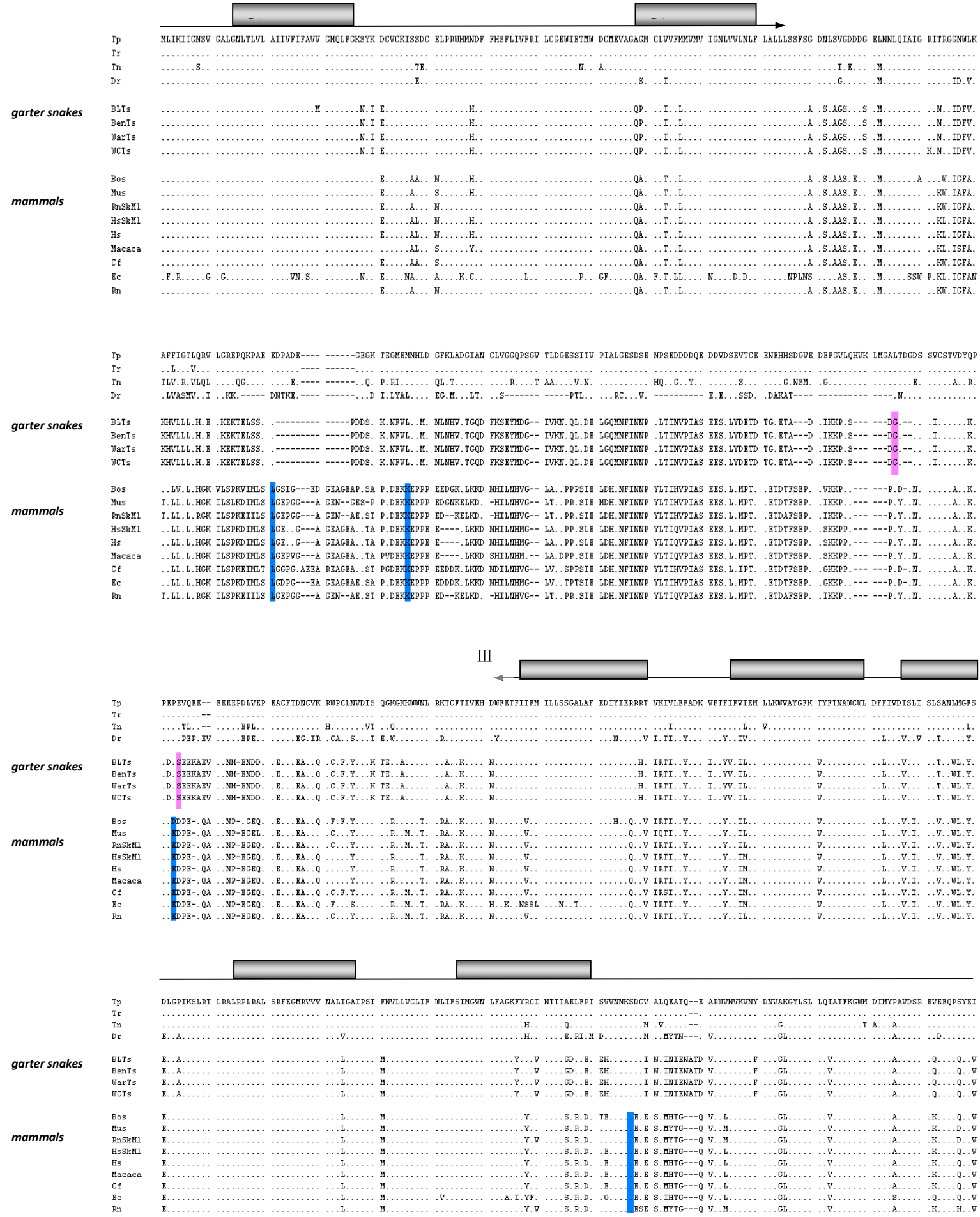
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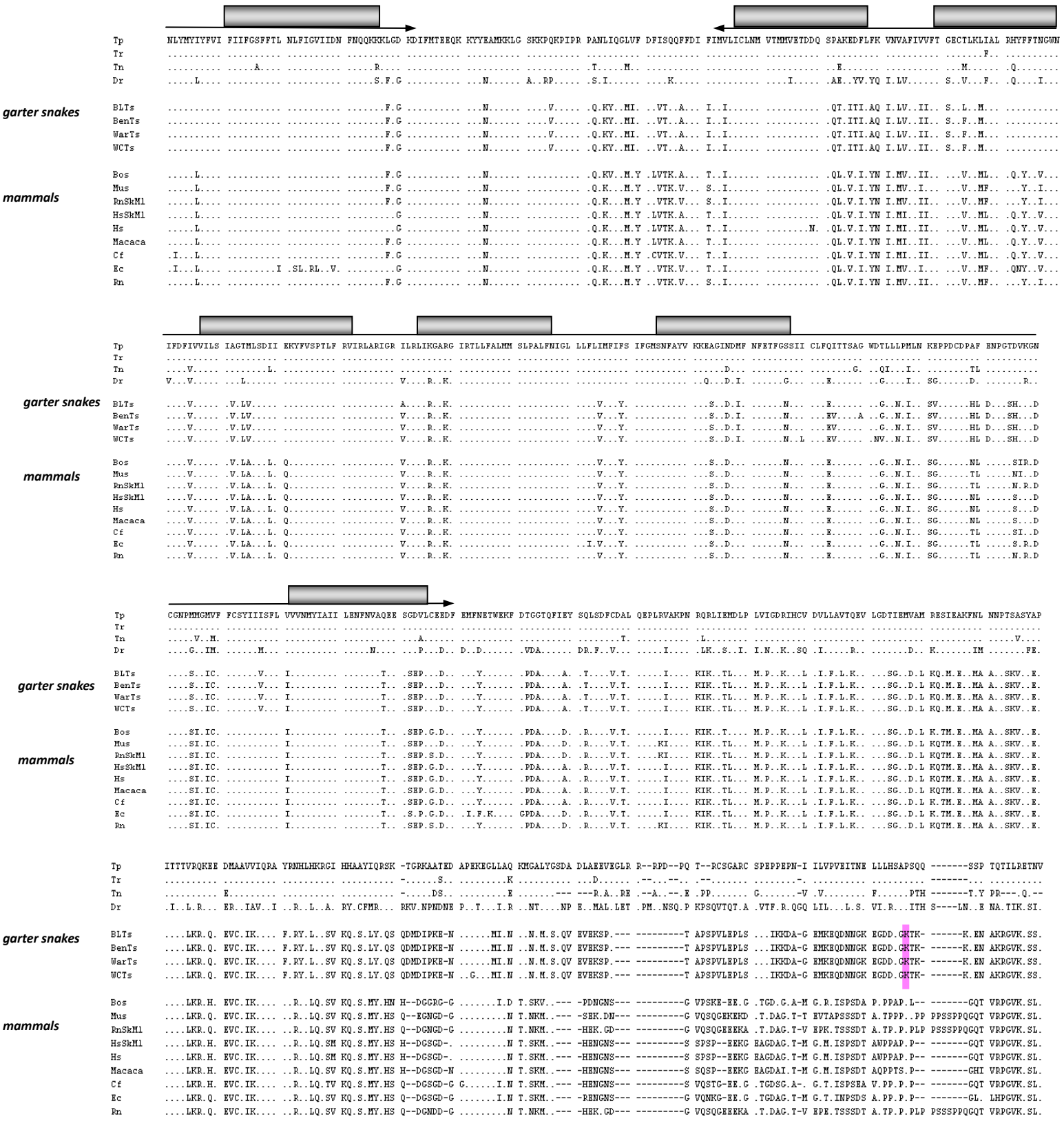

Figure S1 Alignment of the complete sequence of $Na_v1.4$ channels from pufferfish, garter snakes and mammals.

The alignment was constructed with Clustal W implemented in MEGA 4.0. Predicted four domains and the secondary structure of each domain are indicated at the top of the sequences. Deduced amino acid sequence of the rat sodium channel that shows the putative six transmembrane segments of each domain is marked as rectangle. Sites under positive selection are highlighted in purple and blue.