Short Communication

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Scorpions from the primeval subgenus *Archaeotityus* produce putative homologs of *Tityus serrulatus* toxins active on voltage-gated sodium channels

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Abstract: It has been proposed that the subgenus *Archaeotityus* comprises the most ancient species group within the medically important scorpion genus *Tityus*. cDNA encoding sodium-channel active toxins from the type species of this subgenus, *Tityus clathratus* (central Venezuela), have been isolated and sequenced. Two cDNAs were retrieved that encoded 61 amino acid-long putative neurotoxins named Tcl1 and Tcl2. Sequence identity was highest (87%) when both were compared with β-toxin Ts1 from the Brazilian scorpion *Tityus serrulatus* and its homologs from *T. bahiensis*, *T. stigmurus*, and *T. costatus*. A Bayesian analysis indicated statistical support for the grouping of *T. clathratus* Tcl1 and Tcl2 with Brazilian gamma-like β-toxins, reinforcing previous phylogenetic studies which suggested an evolutionary relationship between the subgenus *Archaeotityus* and scorpion species inhabiting southeast South America belonging to the subgenus *Tityus*.

Key words: *Archaeotityus*, scorpions, scorpion toxins, *Tityus*, *Tityus clathratus*, *Tityus serrulatus*.

Tityus is unquestionably the most complex genus of scorpions from a taxonomical standpoint (1-4). It is annually responsible for numerous casualties in several endemic areas of Latin America and the Caribbean (1-7). With over 190 described species, there is toxinological and clinical evidence indicating diversity in venom action and/or composition across *Tityus* distribution range. For example, envenoming by *T. obscurus* (formerly *T. cambridgei*) in northeastern Brazil typically presents with central neurotoxicity as opposed to the mainly autonomic manifestations associated with envenoming by *T. serrulatus* in the southeast (8). In Venezuela, envenoming by *T. zulianus* (Andean range) often produces respiratory arrest and death by pulmonary edema, whereas *T. discrepans* (north-central range) sting causes mainly pancreatic and gastrointestinal disorders (6, 9, 10). Various degrees of toxicity have been associated with different *Tityus* venoms depending on the species, with medium lethal doses (in mice) ranging from 0.773 (*T. stigmurus*) to 12.136 mg/kg (*T. obscurus*) (11 ,12). Notably, the smallest *Tityus* species, now belonging to the subgenus *Archaeotityus*, are only mildly toxic to humans (e.g. *T. uruguayensis*, *T. pussilus*, and *T. silvestris*) (2, 13-15). Toxicity of *Tityus* venoms is mostly due to the action of peptides targeting voltage-gated sodium channels (Na_v), which have been classified as α- and β-toxins depending on whether they alter the kinetics of Na_v inactivation or activation respectively (16).

Medically important *Tityus* species belong to the subgenera *Atreus* and *Tityus*, proposed by Lourenço (2), together with *Archaeotityus*, *Brazilotityus*, and *Caribetityus*, in order to organize the morphological groups already described within the genus. *Archaeotityus*

species (n = 24) comprise small (18-40 mm) and highly pigmented scorpions that are distributed throughout South America and the Caribbean and also Panamá and Costa Rica (2, 3, 17, 18). According to Mello-Leitão (19) and Lourenço (20), *Archaeotityus* occupies a plesiomorphic position among *Tityus* morphological groups/subgenera. Thus, its variegated pigmentation and the stronger distal tooth of the dorsal median carinae are considered primitive characters because they are only found in the juvenile stages of all remaining *Tityus* species (19).

While most toxins in the genus have been isolated and characterized from species in subgenera *Atreus* and *Tityus*, only scarce information is available on the venoms and toxins produced by *Archaeotityus*. Envenomation by *Archaeotityus* sp. is of poor medical relevance, probably due to the low amount of venom injected by scorpions in this subgenus (21). Primary structure determination of *Archaeotityus* toxins should throw light on their evolutionary relationship with other *Tityus* toxins that target ion channels considering the primitive status of this subgenus as suggested previously (19, 20).

We undertook a molecular approach to recover amino acid sequences encoding Na_v-active toxins from *Tityus clathratus*, the type species of *Archaeotityus*. This approach has identified protein sequences with molecular masses that correspond to *bona fide* toxins from other *Tityus* species confirmed by mass spectrometry (10). Adult scorpions (n = 12) were collected in Sanare, Lara State, western Venezuela (09°45´N, 69°20´W), and classified according to the taxonomic keys provided by González-Sponga (22). Total RNA was obtained from venom glands as described by Borges *et al*. (10). Animals were subjected to manual venom milking 48 hours before the dissection to increase production of toxin-encoding mRNAs (23). Scorpions subjected to dissection were deposited at the Scorpion Collection (CELT), Research Group on Applied Toxinology and Venomous Animals, Scholl of Health Sciences, University of Oriente, where they were given the catalog numbers CELT-1130 to CELT-1141.

Complementary DNA (cDNA) was synthesized from 1 µg of total venom gland RNA using the modified oligo(dT) primer 5´-GGCCACGCGTCGACTAGTAC TTTTTTTTTTTTTTT-3´ and subsequently amplified via the polymerase chain reaction using the primer 5´-GGCCACGCGTCGACTAGTAC-3´ and the degenerate oligonucleotide 5´-GTTTATYWGCTGCTTITTKC-3´. The latter primer was designed to anchor at the 3´-end of the DNA region coding for the leader peptide of *Tityus* long-chain toxins under the amplification conditions described by Borges *et al*. (10).

PCR fragments were ligated to the vector pCR2.1-TOPO® (Invitrogen, USA) and transformed into competent *Escherichia coli* DH5α cells which were then plated onto Luria-Bertani/agar plates containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal, 40 µg/mL). Plasmids were isolated from recombinant colonies by the alkaline lysis method and sequenced using universal M13 primers in an automated ABI 3130XL DNA Sequencer (Applied Biosystems, USA) at the Nucleic Acid Sequencing Laboratory, Venezuelan Institute for Scientific Research (24).

Thirty seven clones were recovered from X-Gal-containing agar plates and subjected to DNA sequencing. Seven colonies encoded transcript Tcl1 and 30 encoded transcript Tcl2. Nucleotide sequences (312 and 314 bp respectively) are presented in Figure 1. GenBank accession numbers are HQ632799 (Tcl1) and HQ632800 (Tcl2). Both transcripts encode 71 amino acid-long proteins with the C-terminal-most 64 residues bearing high similarity to the mature peptide region of scorpion toxins targeting Na_v channels based on comparisons using the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and including the eight cysteines involved in formation of disulphide bridges.

The amino acid sequences of mature Tcl1 and Tcl2 are 79% identical to each other. Main differences occur at the C-terminus where Asp55, Arg56 and Arg60 (in Tcl1) are replaced by Ser55, Tyr56 and Thr60 in Tcl2. The deduced molecular masses of the processed 61-residue-long *T. clathratus* putative neurotoxins are (in Da) 6966 (for Tcl1) and 6913 (for Tcl2) assuming that the two C-terminal-most lysine residues of both proteins (Figure 1) are removed post-translationally upon amidation via the amino group of Gly62, as is the case for other *Tityus* toxins (25).

Figure 2 shows the alignment of putative neurotoxins Tcl1 and Tcl2 with *Tityus* toxin

Tcl1

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ATC GGC GTG GTG GTG GAA AGT AAA GAA GGT TAT CTC ATG GAT CAC GAA GGC TGC  54
I   G   V   V   V   E   S   K   E   G   Y   L   M   D   H   E   G   C  +11

AAA CTC AGT TGT TTC ATC AGA CCG GCG GGA TAC TGT GGT AGA GAA TGT TCG ATA 108
K   L   S   C   F   I   R   P   A   G   Y   C   G   R   E   C   S   I  +29

AAG AAA GGG AAA AAT GGC TAC TGC CGC TGG CCA GCG TGT TAC TGC TAC GAT CTA 162
K   K   G   K   N   G   Y   C   A   W   P   A   C   Y   C   Y   D   L  +47

CCC GGC TGG GCC AAA GTT TGG GAC AGA GCG ACG AAC AGA TGT GGG AAA AAA TGA 216
P   G   W   A   K   V   W   D   R   A   T   N   R   C   G   K   K   *  +64

ATTTCTCACCAGTGAAATTCTCTTCACAATGGAATTGTAACAATTAATGGAAATAGATTAAAATGTCTTGCA

TAATAAAAAAAAAAAAAAAAAAA
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Tcl2

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ATC GGC GTG GTG GTG GAA AGT AAA GAA GGT TAT CTC ATG GAT CGC GAA GGC TGC  54
I   G   V   V   V   E   S   K   E   G   Y   L   M   D   R   E   G   C  +11

AAA CTC AGT TGT TTC ATC AGA CCG TCG GGA TAC TGT GGT AGA GAA TGT GAA ATA 108
K   L   S   C   F   I   R   P   S   G   Y   C   G   R   E   C   E   I  +29

AAG AAA GGG TCC TCG GGC TAC TGC CGC TGG CTA GCG TGT TAC TGC TAC GGA CTA 162
K   K   G   S   S   G   Y   C   A   W   L   A   C   Y   C   Y   G   L  +47

CCC GAT AGG GTG AAA GTT TGG AGC TAC GCA ACA AAC ACG TGC GGG AAA AAA TGA 216
P   D   R   V   K   V   W   S   Y   A   T   N   T   C   G   K   K   *  +64

ATCATTCACCACCGAAATCTGTAAAAATGAATTGTAACAAGTTTGGAAAGAAATAAAAAGTCTTGTATTAAAA

AAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide sequences of *Tityus clathratus* clones Tcl1 and Tcl2 and translated amino acid sequences. The predicted protein sequence is shown below the nucleotide sequence and is numbered starting from the putative N-terminal residue, Lys. The signal peptide is underlined in the amino acid sequences; potential polyadenylation sites (AAUAAA and AAUUAA) are underlined in the nucleotide sequences (26).

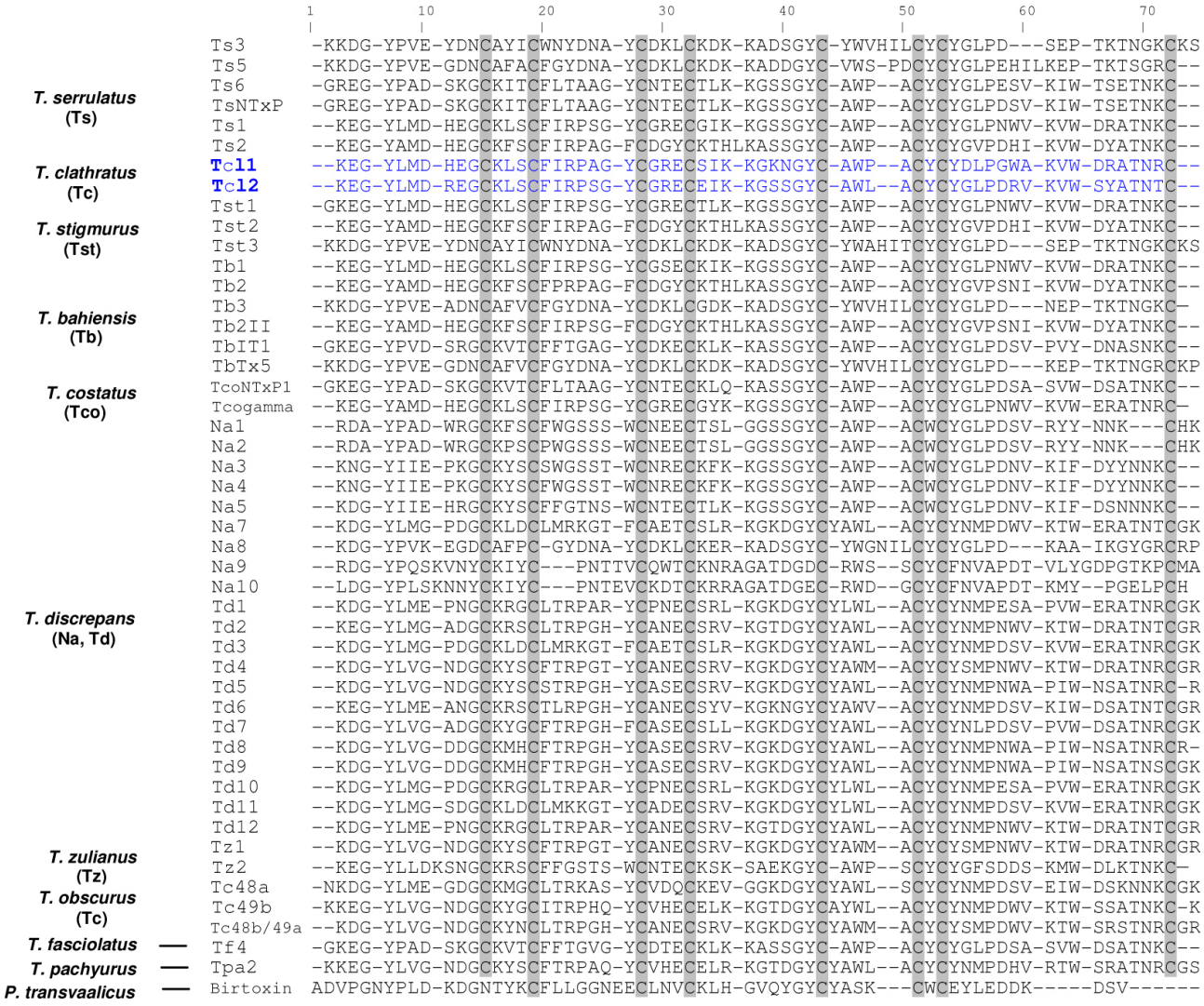

Figure 2. Alignment of amino acid sequences encoded by *T. clathratus* transcripts Tcl1 and Tcl2 (in blue) with Na_v-active scorpion toxins from other *Tityus* species and birtoxin from *Parabuthus transvaalicus*. Sequences were aligned according to the positions of the cysteine residues (in gray). Residues potentially removed posttranslationally were excluded. Accession numbers (per scorpion species) for the listed sequences are: *P. transvaalicus*: birtoxin (P58752) (27); *T. bahiensis* (Tb): Tb1 (AAB36318), Tb2 (P56609), Tb3 (P56608), Tb2II (P60276), TbIT-1 (P60275) (28); TbTx5 (P0C5K8) (29); *T. cambridgei* (Tc): Tc48b/49a (P60213) (30), Tc48a (P60212) (31), Tc49b (P60214) (30); *T. costatus* (Tco): Tcogamma (clone 1) (AY740683), TcoNTxP1 (Q5G8A8) (32); *T. discrepans* (Td): Td1 (DQ075226), Td2 (FN392273), Td3 (DQ075229), Td4 (DQ075232), Td5 (DQ075237), Td6 (DQ075239), Td7 (DQ075242), Td8 (FN392275), Td9 (DQ075228), Td10 (DQ075230), Td11 (DQ075233), Td12 (DQ075234) (10), Na1 (FN392277), Na2 (FN392278), Na3 (FN392279), Na4 (FN392280), Na5 (FN392281), Na6 (FN392282), Na7 (FN392283), Na8 (FN392284), Na9 (FN392285), Na10 (FN392286) (33); *T. fasciolatus* (Tf): Tf4 (P83435) (12); *T. pachyurus* (Tpa): Tpa2 (P84631) (34); *T. serrulatus* (Ts): Ts1 (P15226), Ts2 (P68410), Ts3 (P01496), Ts5 (P46115), Ts6 (P45669) (35), TsNTxP (AAC25688) (36); *T. stigmurus* (Tst): Tst1 (AAB36321), Tst2 (AAB36322), Tst3 (P0C8X5) (35); *T. zulianus* (Tz): Tz1 (AY874060) (37), Tz2 (DQ075241) (37).

sequences available from GenBank, which are from various origins in South America including Venezuela (*T. discrepans* and *T. zulianus*), the Brazilian Amazon/French Guiana region (*T. obscurus*), the Brazilian southeast (*T. serrulatus, T. bahiensis, T. costatus,* and *T. stigmurus*), the Brazilian central region (*T. fasciolatus*), and Colombia (*T. pachyurus*). Sequences were aligned using the program Seaview v.4.2.11 (38). Of all aligned toxins, Tcl1 and Tcl2 show remarkable sequence resemblance (87% identity) to *T. serrulatus* Ts1 (also known as gamma toxin) and also to gamma-like toxins Tb1 (from *T. bahiensis*), Tst1 (from *T. stigmurus*) (35) and the peptide encoded by *T. costatus* clone 1 (Tcogamma) (32). Identity to β-toxin Ts2 from *T. serrulatus* is lower (68 and 70% identity for Tcl1 and Tcl2 respectively). Toxins from the scorpions *T. discrepans* and *T. zulianus* (which distribution ranges overlap with *T. clathratus* in Venezuela) and also those from *T. obscurus* and *T. pachyurus* display lower (50-67%) identities to Tcl1 and Tcl2 compared with the Brazilian Ts1 and gamma-like toxins.

It is clear that Tcl1 and Tcl2 belong to the β-toxin family, bearing the same secondary structure elements found by Polikarpov *et al*. (39) in Ts1, the most abundant *T. serrulatus* β-toxin (40). Since *Tityus* venoms also contain α-toxins (16), the fact that we did not retrieve cDNAs coding for putative *T. clathratus* α-toxins could be either due to their absence in *T. clathratus* venom or that their cDNAs were not amplified under our conditions due to variations in the leader-peptide nucleotide sequence of *T. clathratus* α-toxin genes, which may prevent the degenerate primer from anchoring at the selected site, identified as conserved amongst genes encoding other *Tityus* toxins (10). A thorough venom proteomic approach should help clarify whether this toxin group is produced by *Archaeotityus* species.

Both Tcl1 and Tcl2 share with Ts1 critical residues involved in the β-toxin pharmacophore (Glu26, Tyr22, Ile29) (41). Also, residue Gly24, which participates in antigenic recognition in Ts1, is conserved in both *T. clathratus* putative homologs (42). Gly28, also a residue of antigenic importance in Ts1, is not conserved in Tcl1 and Tcl2, but this position is also variable in other gamma-like toxins (42). Significantly, Gly24 and the pharmacophore residues lie within the α-helical region, which is highly conserved in Ts1, gamma-like toxins (Tb1, Tst1, Tcogmamma) and *T. clathratus* homologs (see alignment in Figure 2) (39, 42). The discontinuous antigenic epitope encompassing the amino- (^{1}KEGY^{5}) and carboxy- (^{46}GLPXXVKV^{53}) terminal regions of *T. serrulatus* toxins is conserved in Tcl2, but the critical Gly46 is replaced in Tcl1 by Asp (43). At the gene level, both *T. clathratus* precursors exhibit 82% identity with respect to Ts1, with changes in Tcl1 and Tcl2 cDNAs mostly comprising third-position replacements in the region encoding the central domain (residues 27-33).

A Bayesian analysis was performed to investigate the phylogenetic relationships of *T. clathratus* Tcl1 and Tcl2 with *Tityus* toxins retrieved from GenBank. Figure 3 shows a consensus tree obtained after Bayesian reconstruction using the WAG model of protein evolution selected in ProtTest (44). Birtoxin, a three disulfide-bridge β-like toxin from the South African scorpion *Parabuthus transvaalicus* was chosen as outgroup since the probable ancestor of North and South American α- and β-toxins was a three-disulfide bridge toxin related to birtoxin (27, 45, 46).

There is reasonable (Bayesian posterior probability, BPP = 0.61) support for a monophyletic clade comprising toxins structurally and/or functionally related to the β-group which consists of two subclades. The first subclade (BPP = 0.98) contains *T. serrulatus* Ts2 and Ts2-like toxins. The second subclade (BPP = 0.87) comprises Ts1 and gamma-like toxins from *T. bahiensis* (Tb1), *T. costatus* (Tcogamma) and *T. stigmurus* (Tst1) with *T. clathratus* Tcl2 as a sister sequence. A subclade (BPP = 0.85) internal to the gamma-like group includes toxins from Venezuela (Tz1, Td1-Td12, Na7), Colombia (Tpa2) and the Brazilian Amazon/French Guiana region (Tc49b, Tc48b, Tc49a) with Tcl1 as a distantly related (*p*-uncorrected distance: 40%) sister sequence. Both Tcl1 and Tcl2 are equally distant to the subclade comprising Ts1 and related proteins (*p*-uncorrected distance: 20%), in agreement with their sequence homology to gamma-like toxins. The topology of the tree in the remaining branches cannot be assessed with certainty given the lower node support (BPP < 0.50) for the groupings, with the exception of α-toxins from *T. serrulatus* (Ts3, Ts5), *T. bahiensis* (Tb3), *T. stigmurus* (Tst3), and *T. discrepans* (Na8) which form a monophyletic group (BPP = 1.0) lying outside the β-toxin clade as reported before (45, 46).

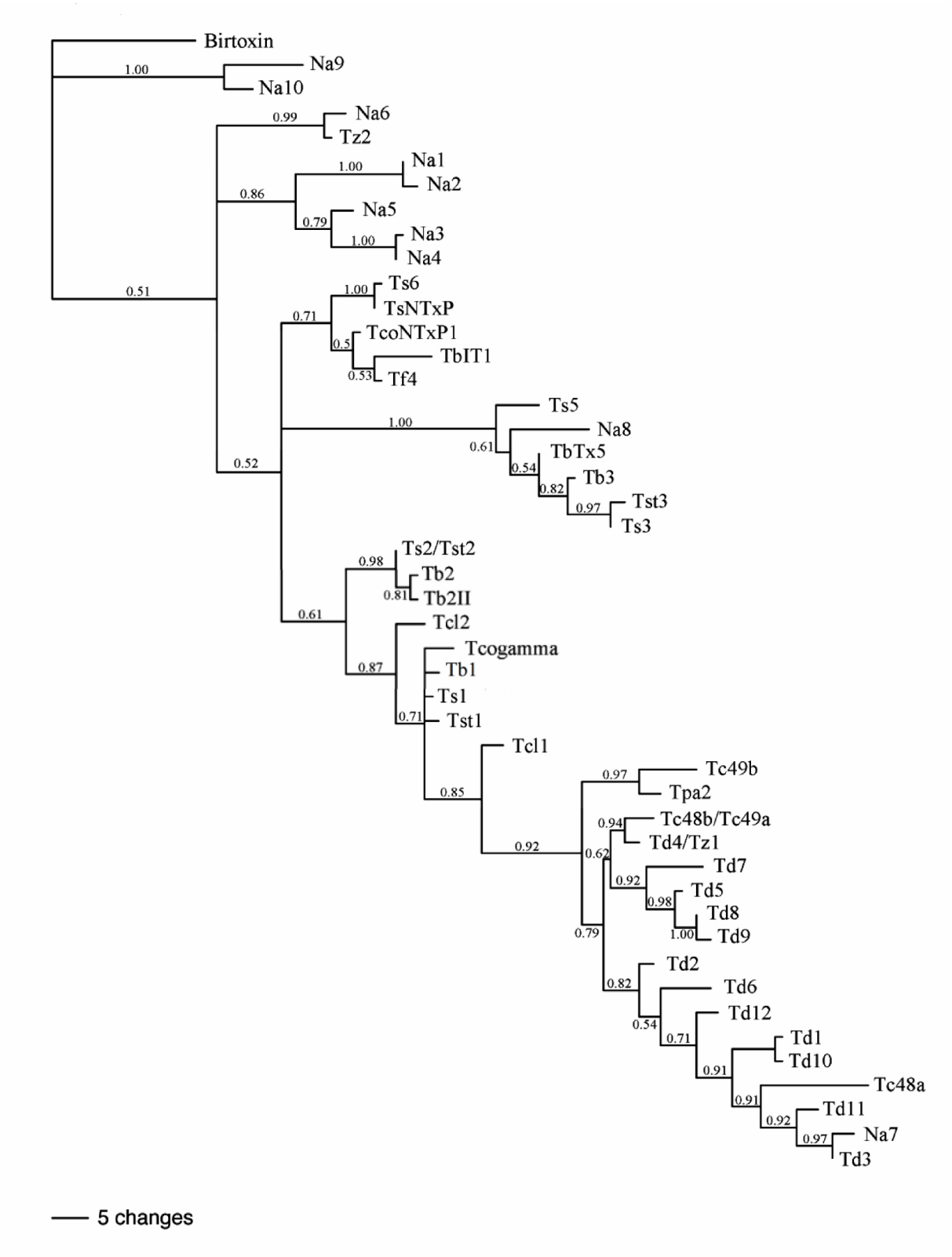

Figure 3. Bayesian maximum likelihood consensus phylogram depicting phylogenetic relationships of *Tityus* Na_v-active neurotoxins available from GenBank and *T. clathratus* Tcl1 and Tcl2 precursors isolated in this work. The sequence data set corresponds to the alignment presented in Figure 1. *P. transvaalicus* birtoxin was assigned as outgroup. Phylogenetic analysis was performed using the WAG model of protein evolution. Two Monte Carlo Markov chain were run 2,000,000 generations (burnin = 200,000). Clade support was inferred by bootstrapping. Bayesian posterior probability (BPP) was calculated for branching points. Only nodes supported by BPP > 0.5 are shown.

The fact that the *T. clathratus* genome encodes Na_v toxins with a very close structural fingerprint to Ts1 and its homologs indicates a toxinological affinity not anticipated in previous studies, especially considering that *T. clathratus* inhabits an area about 4000 km north of its south eastern Brazilian congeners and that it is morphologically unrelated to them. Homologs of *T. serrulatus* Ts1 had only been found so far in other Brazilian *Tityus* such as *T. bahiensis*, *T. stigmurus* and *T. costatus*, species belonging to the subgenus *Tityus* (32, 35). Significantly, *T. discrepans* and *T. zulianus*, two species sympatric with *T. clathratus* in Venezuela, do not produce such homologs but Na_v toxins with divergent N- and C-termini with respect to Ts1 (10, 33). In fact, in a recent phylogenetic analysis of *Tityus* scorpion Na_v toxins, Guerrero-Vargas et al. (47) revealed a strong toxinological divergence among *T. pachyurus, T. obscurus, T. discrepans* and *T. zulianus* (in the subgenus *Atreus*) from the northern part of the Amazon Basin, and *T. serrulatus, T. bahiensis, T. stigmurus, T. costatus* and *T. fasciolatus* (in the subgenus *Tityus*), which live south of the Amazon.

Thus far *T. clathratus* is the only species from the north of the Amazon Basin producing putative toxin homologs of southern Brazilian *Tityus*, which provides further support for the evolutionary relationship between subgenera *Archaeotityus* (which has a trans-Amazonian distribution) and *Tityus* and its separation from the subgenus *Atreus* as suggested previously by Borges *et al*. (4). The latter authors, in their molecular phylogenetic analysis of 21 *Tityus* species using two mitochondrial DNA markers (cytochrome oxidase subunit 1 and ribosomal 16S rRNA), found that the *Archaeotityus* type species, *T. clathratus*, groups with the Brazilian *T. serrulatus* into a single clade which strongly diverges (53-57% nucleotide divergence) with respect to its other congeners in Venezuela, Trinidad and Panama in the subgenus *Atreus*.

No hypothesis for relationships amongst *Tityus* subgenera had been put forward prior to the present findings or those of Borges *et al*. (4). Only Lourenço (20) has suggested that the primitive *Archaeotityus* is closely related to *Caribetityus*, a subgenus endemic to the Caribbean island of Hispaniola (currently Dominican Republic and Haiti) and that the Cuban genera *Alayotityus* and *Tityopsis*, together with *Caribetityus* and *Archaeotityus*, are the possible proto-elements of continental *Tityus*, all sharing a common ancestor in South America.

Probably our results implicate that such common ancestor, evolutionary and toxinologically shared by subgenera *Archaeotityus* and *Tityus*, was distributed in South America before formation of the Amazon Basin and therefore prior to cladogenesis of the *Atreus* group, mainly restricted to the northern part of the subcontinent and whose species evolved different toxin repertoires (47). It is clear that the molecular analysis of more *Archaeotityus* species is needed to evaluate whether they produce Ts1 homologs as *T. clathratus*, and also to determine their phylogenetic affinity with species in the subgenus *Tityus*.

All in all, this research constitutes an initial point to study the evolution of *Tityus* venoms, the most speciose of all scorpion genera, comparing the putative toxins from an ancient species group with toxins from other species within the genus that have been well characterized.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee of the Institute of Experimental Medicine, Central University of Venezuela.

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