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Diversity of long-chain toxins in *Tityus zulianus* and *Tityus discrepans* venoms (Scorpiones, Buthidae): Molecular, immunological, and mass spectral analyses ☆

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Abstract

In Venezuela, stings by *Tityus zulianus* scorpions produce cardiorespiratory arrest, whereas envenoming by *Tityus discrepans* involves gastrointestinal/pancreatic complications, suggesting structural and/or functional differences. We sought to compare their toxin repertoires through immunological, molecular, and mass spectral analyses. First, in vivo tests showed that neutralization of *T. zulianus* venom toxicity by the anti-*T. discrepans* antivenom was not complete. To compare *T. discrepans* and *T. zulianus* long-chain (sodium channel-active) toxins, their most toxic Sephadex® G-50 fractions, TdII and TzII, were subjected to acid–urea PAGE, which showed differences in composition. Amplification of toxin-encoding mRNAs using a leader peptide-based oligonucleotide rendered cDNAs representing twelve *T. discrepans* and two *T. zulianus* distinct toxin transcripts, including only one shared component, indicating divergence between *T. zulianus* and *T. discrepans* 5′ region-encoded, toxin signal peptides. A 3′-UTR polymorphism was also noticed among the transcripts encoding shared components Tz1 and Td4. MALDI–TOF MS profiling of TdII and TzII produced species-specific spectra, with seven of the individual masses matching those predicted by cDNA sequencing. Phylogenetic analysis showed that the unique *T. zulianus* transcript-encoded sequence, Tz2, is structurally related to *Tityus serrulatus* and *Centruroides* toxins. Together with previous reports, this work indicates that *T. zulianus* and *T. discrepans* toxin repertoires differ structurally and functionally.

Keywords: Antivenom; Mass spectrometry; Scorpion toxin; Scorpion venom; *Tityus zulianus*; *Tityus discrepans*; Toxin diversity; Venezuela; 3′-UTR

1. Introduction

The ability to detect biochemical and antigenic diversity in animal venoms has wide-ranging implications, particularly for antivenom production strategies and for the characterization of endemic areas. Inter- and intraspecific geographic variation in the concentration and/or function of toxic components has been extensively documented for snake venoms (for reviews, see Chippaux et al., 1991; Fry et al., 2003) but similar efforts to investigate diversity in arachnid venoms, including scorpions, are only beginning to emerge. *Tityus* is the most speciose of all scorpion genera, with almost 150 described species, distributed from Costa Rica to northern Argentina (Lourenço, 2002; González-Sponga, 2002). Originally reported from Brazil and Trinidad, sting by various *Tityus* species now represents a

Abbreviations: AU–PAGE, acidic, urea-containing polyacrylamide gel electrophoresis; MALDI–TOF MS, matrix-assisted laser desorption time-of-flight mass spectrometry; Na_v, voltage-gated sodium channel; RT–PCR, reverse transcriptase-coupled polymerase chain reaction; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; Td, *Tityus discrepans*; Tz, *Tityus zulianus*; 3′-UTR, 3′-untranslated region.

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pediatric emergency in northern South America, particularly in the mountainous ranges of Colombia (Otero et al., 2004) and Venezuela (Borges, 1996; De Sousa et al., 2000), where the genus reaches its highest species diversity. In Venezuela, the clinical outcome of *Tityus* poisoning depends on the species involved (Borges et al., 2004b). Sting by *Tityus zulianus* (Mérida and Táchira States, western Venezuela) often produces respiratory arrest and death by pulmonary oedema (Borges et al., 2002; Mazzei de Davila et al., 2002) whereas envenomation by *Tityus discrepans* in north-central Venezuela mainly causes pancreatic and gastrointestinal disorders (Mota et al., 1994). Venom-induced hyperamylasemia is significantly higher in the case of *T. zulianus*, and also the onset and evolution of the venom-induced pancreatic oedema differ between *T. discrepans* and *T. zulianus*, indicating the existence of structural and/or functional differences between their toxic components (Borges et al., 2004b). Additionally, clinical reports have documented the need to administer large doses of the anti-*Tityus discrepans* serum for the treatment of scorpion envenoming in *T. zulianus* endemic areas (Mazzei de Davila et al., 2002), suggesting that differences in antigenic reactivity exist between these venoms.

Given that differential venom toxicity and antigenicity might be related to species-specific venom proteomes, as reported in snakes (see for example Fry et al., 2002; Wickramaratna et al., 2003), the aim of this work was to investigate *T. zulianus* and *T. discrepans* venom diversity by comparing their toxin repertoires using immunological, molecular, and mass spectral techniques. First, in vivo tests were carried out to compare the neutralization of *T. zulianus* and *T. discrepans* venom lethality by the anti-*T. discrepans* antivenom. Then, a degenerate reverse transcriptase-coupled polymerase chain reaction (RT–PCR) was developed to amplify *T. zulianus* and *T. discrepans* transcripts coding for their long-chain (60- to 76-amino acids-long) toxins, whose abundance in whole scorpion venoms correlates with their lethality to vertebrates (Chávez-Olórtegui and Kalapothakis, 1997). These toxins, divided into the α- and β-subfamilies, alter the gating mechanism of voltage-gated sodium channels, producing sustained depolarization and massive release of neurotransmitters at peripheral and central nerve terminals (Martin-Eauclaire and Couraud, 1995; Possani et al., 1999) which, in turn, contribute to the cardiorespiratory complications seen in severe *Tityus* envenoming (Andrade et al., 2004; Benvenuti et al., 2002; Mazzei de Davila et al., 2002). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF MS) was used to compare the masses of individual *T. zulianus* and *T. discrepans* components in the long-chain toxin molecular weight range to assess whether the amplified transcripts were actually translated and present in the crude venom.

The data presented show that shared and species-specific long-chain toxins are produced by *T. zulianus* and *T. discrepans* scorpions. Also, common as well as different antigenic epitopes should exist between their toxic components to explain the partial in vivo neutralization of *T. zulianus* venom by the anti-*T. discrepans* serum.

2. Materials and methods

2.1. Scorpion venoms and antivenom

Adult *T. zulianus* scorpions were collected around the villages of El Bordo, Mesa Bolívar, and Santa Cruz de Mora, western Mérida State, Venezuela. Adult *T. discrepans* scorpions were collected near San Antonio de los Altos, Miranda State, central Venezuela. Venom pooled from 50 to 60 scorpions was obtained by manual stimulation of the telson according to Shulov and Zlotkin (1969) and lyophilized at −50 °C and 80 mbar of pressure. Prior to in vivo studies, lyophilized venom samples were suspended in NaCl 0.9% (w/v) and protein estimated according to Lowry et al. (1951). Anti-*T. discrepans* antivenom (Batch L031) was obtained from Centro de Biotecnología, Facultad de Farmacia, Universidad Central de Venezuela.

2.2. Gel filtration chromatography of T. zulianus and T. discrepans venoms

Crude venoms (5 mg of protein) were eluted through a Sephadex® G-50 column (0.9 × 50 cm) at a flow of 19 mL/h. Eluate absorbance was recorded at 280 nm and fractions pooled according to the chromatographic profile, then lyophilized; protein content in the pools was estimated as for the crude venom samples. Toxicity of chromatographic fractions was assessed by intraperitoneal injection into mice as described previously (Borges et al., 2004a).

2.3. Polyacrylamide gel electrophoresis

Electrophoresis of protein samples in acid–urea gels was carried out according to Wang et al. (1997) in 12.5% polyacrylamide gels, using 5% (v/v) acetic acid as running buffer and pyronine as tracking dye. Toxin Ts1 (a gift of Dr. Suely Vilela, University of São Paulo, Brasil) was used as an electrophoretic marker for toxin migration. Electrophoresis under denaturing conditions was carried out in the presence of sodium dodecyl sulfate according to Laemmli (1970).

2.4. RNA extraction

Total RNA was extracted from a pool of venom glands dissected from twenty adult *T. zulianus* and *T. discrepans* scorpions according to the procedure of Borges et al. (2004a). Integrity of RNA was confirmed by agarose gel electrophoresis in the presence of formaldehyde according to Sambrook et al. (1989).

2.5. Reverse transcriptase-coupled polymerase chain reaction (RT–PCR) from total T. zulianus and T. discrepans RNA

Complementary DNA (cDNA) was synthesized from 1 μg of total venom gland RNA of either *T. zulianus* or *T. discrepans* scorpions essentially as described by Borges et al. (2004a), using

2.5 μM of a modified oligo(dT) primer (5′–GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTT–3′). Appropriate amounts of cDNA were amplified by PCR using Advantage 2 polymerase mix (Clontech Labs., Palo Alto, CA, USA). Forward primer (2.5 μM) was a degenerate oligonucleotide (5′–GTTTATYWGCTGCTTITTKC–3′) designed to anneal to the 3′-end of the DNA region coding for the leader peptide of *Tityus* long-chain toxins sequenced thus far [Ts1, from *Tityus serrulatus* (Martin-Eauclaire et al., 1992); Ts3, also from *T. serrulatus* (Martin-Eauclaire et al., 1994); gamma-toxin like Tst1 and Tb1, from *Tityus stigmurus* and *Tityus bahiensis*, respectively (Becerril et al., 1996); TbTx5, from *T. bahiensis* (Kalapothakis et al., 2001), and Tz1, from *T. zulianus* (Borges et al., 2004a)]. Deoxyinosine (I) was included to reduce degeneracy at the 16th position (Kilpatrick et al., 1996). Reverse primer (0.5 μM) was 5′–GGCCACGCGTCGACTAGTAC–3′, which anneals to the 3′ end of oligo(dT) (modified)-primed cDNAs. The temperature profile of amplification consisted of one cycle at 94 °C (5 min), 30 cycles consisting of three steps at 94 °C (30 s), 40 °C (3 min), and 60 °C (3 min), respectively, and one last cycle at 72 °C (7 min). PCR fragments were ligated to the vector pCR2.1-TOPO (Invitrogen) and transformed into competent *Escherichia coli* DH5α cells which were then plated onto Luria-Bertani/agar plates containing 40 μg/mL of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside. Plasmids were isolated from recombinant colonies by the alkaline lysis method and sequenced using universal M13 primers in an automated ABI 373 DNA Sequencer at the Laboratorio de Secuenciación de Ácidos Nucléicos (CeSAAN, IVIC).

2.6. MALDI–TOF MS analyses

Mass spectra of positively charged ions from TdII and TzII gel filtration fractions (see Fig. 3) were analyzed by MALDI–TOF MS in a Biflex III MALDI–TOF MS (Bruker, FRG). Samples for analyses (200–500 μg) were lyophilized, resuspended in 100 μL of MilliQ water and diluted 10-fold with 0.1% (v/v) trifluoroacetic acid (TFA). A total of 1 μL of the diluted sample was mixed with 5 μL of matrix solution (10 mg/mL of 3,5-dimethoxy-4-hydroxycinnamic acid in a 1:1 mixture of acetonitrile and 0.1% TFA (v/v). Moreover, 1 μL from this mixture was spotted on the target plate. Mass spectra of positively charged ions were recorded on the Biflex III instrument operated in the linear mode. The total acceleration voltage and the detector voltage were 19 kV and 0.55 kV, respectively. A total of 100 to 150 single shots were accumulated for each sample. Masses were calculated from at least three independent analyses.

2.7. In vitro neutralization assays

To assess the capacity of the anti-*T. discrepans* antivenom to neutralize *T. discrepans* and *T. zulianus* venom lethality, a constant amount of venom ('challenge dose', $2DL_{50}$ of venom per mouse) was incubated, for 30 min at 37 °C, with various dilutions of antivenom, before intravenous injection into CD1 mice (19–21 g, Instituto Nacional de Higiene, Caracas). Five animals per dose tested were utilized, and survivors counted after 24 h (see Table 1 for details). LD_{50} values used in this study were as reported previously for *T. zulianus* (Borges et al., 2004a) and *T. discrepans* (Borges et al., 1990) venoms. As a control, venom samples ($2LD_{50}$ per mouse) were incubated in NaCl 0.9% under the same conditions as above and injected through the same route. The neutralizing capacity of the antivenom is expressed as effective dose 50% (ED_{50}), defined as the actual amount of antivenom (in mg of protein) which reduced by 50% the activity of the 'challenge dose' of venom. The ED_{50} values and the corresponding 95% confidence intervals were determined by the Spearman–Karber method (Finney, 1978).

Table 1
In vivo neutralization of *Tityus* scorpion venoms with anti-*Tityus discrepans* serum[a]

Venom	Serum injected per mouse (mg protein)	Surviving mice	Percentage (%)
T. discrepans	3.77	5/5	100
	2.89	4/5	80
	2.23	3/5	60
	1.71	0/5	0
	(Control)[b]	0/5	0
T. zulianus	6.97	5/5	100
	5.58	4/5	80
	4.57	3/5	60
	3.57	0/5	0
	(Control)[b]	0/5	0

These data are representative of two independent experiments.
[a] Mice were injected intravenously with $2LD_{50}$ of *T. discrepans* (100.4 μg) or *T. zulianus* (60.8 μg) venoms, in the presence of variable amounts of antivenom.
[b] Control mice were injected with $2DL_{50}$ of venom pre-incubated under the same conditions with NaCl 0.9%.

2.8. Phylogenetic analyses

Nucleotide sequences were curated and aligned in Seqapp (Gilbert, 1993). Sequences were aligned by eye. Phylogenetic analysis was performed with PAUP*4.b.10 (Swofford, 2002). A tree was constructed by distance-based optimality criteria (with a Neighbour-Joining phylogram). Clade support was inferred by bootstrapping.

3. Results

3.1. In vivo neutralization of T. zulianus and T. discrepans venoms by the anti-T. discrepans antivenom

Table 1 shows the results of the evaluation of the anti-*T. discrepans* antivenom neutralization efficiency. In the case of *T. zulianus* venom neutralization, ED_{50} (95% confidence limits are in parentheses) was 4.56 (3.89–5.35) mg of antivenom protein needed to reduce in 50% the lethality of the challenge dose ($2DL_{50}$ per mouse), twice the amount needed to achieve the same effect, 2.28 (1.89–2.75), for *T. discrepans* venom.

3.2. Composition of *T. zulianus* and *T. discrepans* venoms analyzed through SDS– and UA–PAGE of chromatographic fractions

Gel filtration chromatography of both *T. zulianus* and *T. discrepans* venoms followed at 280 nm produced three distinct absorbance peaks (Fig. 1A). The major peaks (pools TdII, from *T. discrepans*, and TzII, from *T. zulianus*) were the most toxic by intraperitoneal injection into CD1 mice, which showed signs of neurotoxicity that resembled those observed after injection with crude venoms (Borges et al., 2004a,b). SDS–PAGE confirmed that TdII and TzII contained the crude-venom lowest molecular mass proteins, that migrated as a single, diffuse band around 6.5 kDa (Fig. 1B). Due to the close proximity of molecular weights among Na_v channel-active scorpion toxins bearing different net charges, acid–urea (AU) PAGE is a more efficient tool to investigate the protein composition of TdII and TzII. Indeed, the AU–PAGE system described by Wang et al. (1997) for resolving cationic peptides of similar size, detected significant differences in the composition of both pools, as indicated by the presence of TdII- and TzII-specific cationic components (Fig. 1B, right side). Most abundant TdII and TzII components migrate close to Ts1, a well-characterized β-toxin from *T. serrulatus* venom, suggesting that they probably correspond to toxins of similar size/charge to Ts1. The most cationic components differ in migration between TdII and TzII; also differences in the migration of higher mol. weight components can be detected in *T. discrepans* and *T. zulianus* whole venoms (Fig. 1B).

3.3. Characterization of *T. discrepans* and *T. zulianus* long-chain toxin cDNAs

A total of 29 *E. coli* colonies harboring recombinant plasmids were processed for nucleotide sequencing from the *T. discrepans* cDNA library and 27 colonies from the *T. zulianus* library. Several clones from both libraries gave the same sequence, indicating that they were amplified from the same mRNA. All sequences have been deposited in GenBank and have the accession numbers DQ075226–DQ075243.

From the *T. discrepans* library, 12 distinct (309- to 314-bp long) nucleotide sequences were recovered, which were then translated to identify open reading frames. According to sequence homology to previously reported toxin precursor sequences from the genera *Tityus* and *Centruroides* (Fig. 2), *T. discrepans* clones Td1 to Td11 coded for 73-amino acid-long putative (voltage-gated sodium channel-active) toxin precursors, each comprising the last seven residues of the leader peptide and a 66-residue-long region containing the mature toxin and the C-terminal residues which are potentially removed post-translationally (Possani et al., 1999). Notably, the precursor identified as Td4 codes for a homologue of β-toxin Tz1, the most abundant *T. zulianus* toxic component (Borges et al., 2004a; GenBank AY874060). Nucleotide sequence identity of cDNAs encoding Td4 and Tz1 within the mature toxin coding region is 98%, with all replacements being third-position changes (Fig. 3). Clone Td1 was the most frequently isolated *T. discrepans* sequence (12 out of 29 cDNAs); its precursor structure is shown in

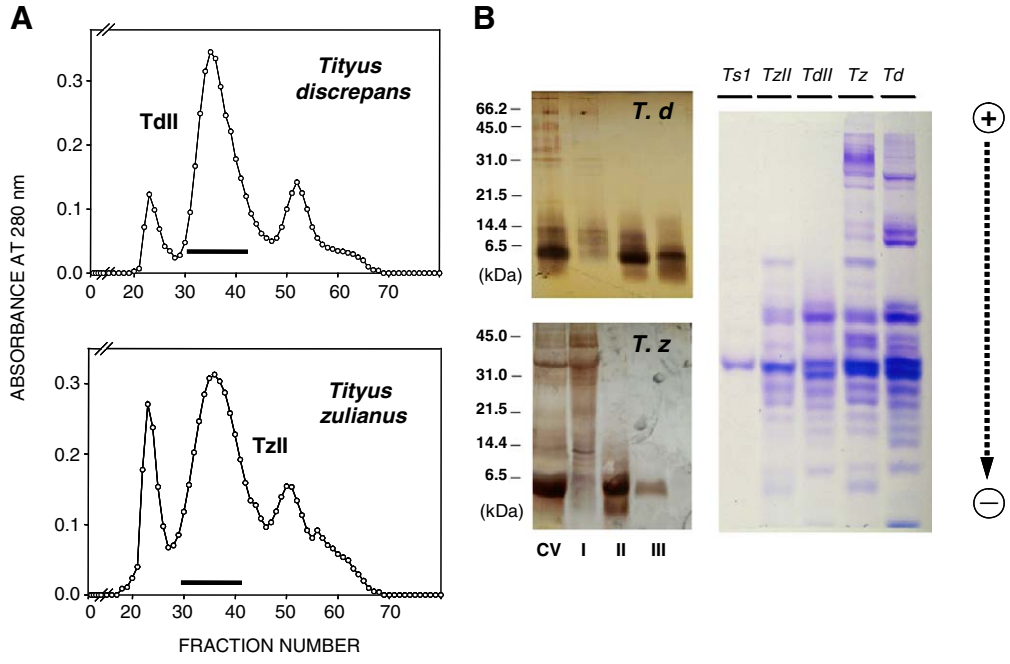

Fig. 1. Differences in protein composition between *Tityus discrepans* and *Tityus zulianus* venoms. (A) Chromatographic profiles obtained through Sephadex® G-50 (5 mg protein) of *T. discrepans* and *T. zulianus* venoms. Horizontal bars indicate fractions that were pooled into TdII and TzII. Both pools were lethal to BALB/c mice when injected intravenously with 50 μg of protein. (B) Left panels: SDS–PAGE (silver-stained 12.5% gel) of crude (CV) *Tityus discrepans* (T.d) and *Tityus zulianus* (T.z) venoms, and gel filtration pools (30 μg) TdI to TdIII and TzI to TdIII. Molecular weight markers are indicated on the left side. Right panel: AU–PAGE (12.5% gel) of pools TdII and TzII (30 μg), *T. zulianus* (Tz) and *T. discrepans* (Td) venoms (30 μg) and toxin Ts1 (0.5 μg). Electrophoretic polarity is shown on the right side of the gel.

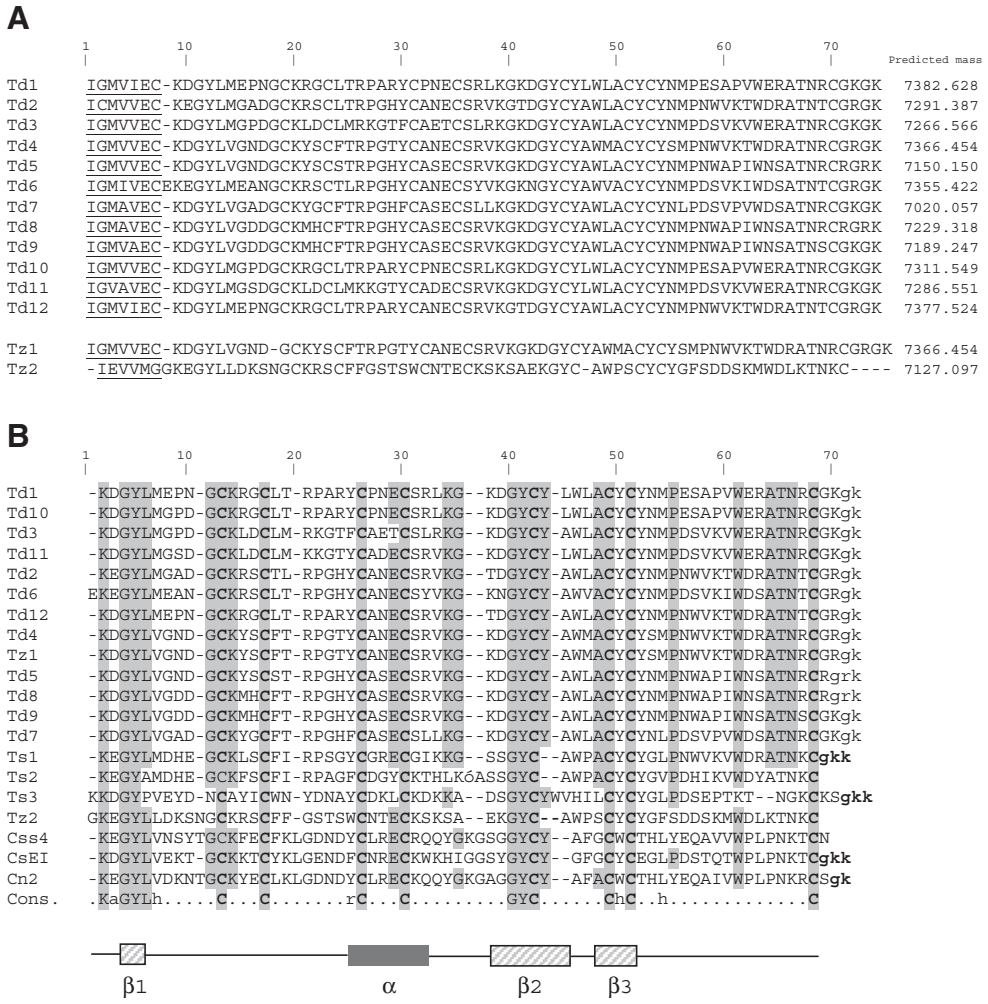

Fig. 2. (A) Amino acid sequences encoded by *Tityus discrepans* (Td, top alignment) and *Tityus zulianus* (Tz, bottom alignment) cDNA clones. Primary structure encoded by clone Tz1 is identical to that previously reported for beta-type toxin Tz1 from *T. zulianus* (AY874060; Borges et al., 2004a). Signal peptide sequences are singly underlined. Gaps (—) were introduced to improve sequence similarities. (B) Comparison of Td- and Tz-encoded primary structures with representative examples of Na_v channel-active *Tityus* and *Centruroides* toxins with known precursor sequences. Sequences were aligned according to the positions of the cysteine residues. *Centruroides* sequences are CsE1 (AF338460; Corona et al., 2001) and Cn2 (P01495; Vazquez et al., 1995); *Tityus* sequences are Ts1 (S21158; Martin-Eauclaire et al., 1992) and Ts3 (P45659; Martin-Eauclaire et al., 1994). Sequences of toxins Ts2 (P68411; Becerril et al., 1996) and Css4 (P60266; Possani et al., 1999), for which precursor sequences have not yet been isolated, are included for functional comparisons. A consensus sequence (Cons.) is written below the multiple sequence alignment with the corresponding amino acid when 100% identity is present and a lower-case letter when an acidic (a), aromatic (r), or hydrophobic (h) residue is conserved. The secondary structure elements identified in the Ts1 three-dimensional structure by Polikarpov et al. (1999) are shown below the alignment. Carboxyl-terminal basic residues, which are known to be removed post-translationally from CsE1, Cn2, Ts1 and Ts3, are in small case and boldface. Basic residues potentially removed by C-terminal processing from peptides encoded by Td clones are in small case. The predicted masses after C-terminal processing for the putative Td and Tz toxins are shown in panel A.

Fig. 4. Interestingly, the full amino acid sequences encoded by Td1 and Td8 precursors show similarity to the partial N-terminal sequences reported by D'Suze et al. (1996) for *T. discrepans* toxins TdII-4 and TdII-3, respectively (Fig. 3B). Differing from the other Td isolates, Td6 codes for a 74-residue-long toxin precursor containing a mature toxin region with 67 amino acids, assuming that Glu is at the N-terminus, similarly to *Centruroides sculpturatus* toxin precursors CsE9 and CsE9b (Corona et al., 2001) and toxin Cll9 from *Centruroides limpidus limpidus* (Corona et al., 2003).

Translation of the *T. zulianus* cDNA-encoded sequences rendered only two distinct precursors coding for putative long-chain toxins, one of them (20 out of 27 isolated cDNAs) corresponding to the already characterized Tz1. The second distinct, 304-bp-long *T. zulianus* isolated sequence (identified as Tz2) encoded a 69-residue-long toxin precursor. The nucleotide sequence and translated amino acid sequence of this putative long-chain toxin are shown in Fig. 5A. The precursor comprises the last five residues of the leader peptide and a 64-residue-long region encoding the mature toxin, with Gly as N-terminal residue based on the homology to toxins Tf4, from *Tityus fasciolatus* (Wagner et al., 2003), the non-toxic *T. serrulatus* protein, TsNTxP (Guatimosin et al., 1999) and Ts4, a weak toxin also from *T. serrulatus* (Marangoni et al., 1990; Sampaio et al., 1996) (see Fig. 5B). Identity to Tf4, TsNTxP and Ts4 were 60%, 55% and 54%, respectively.

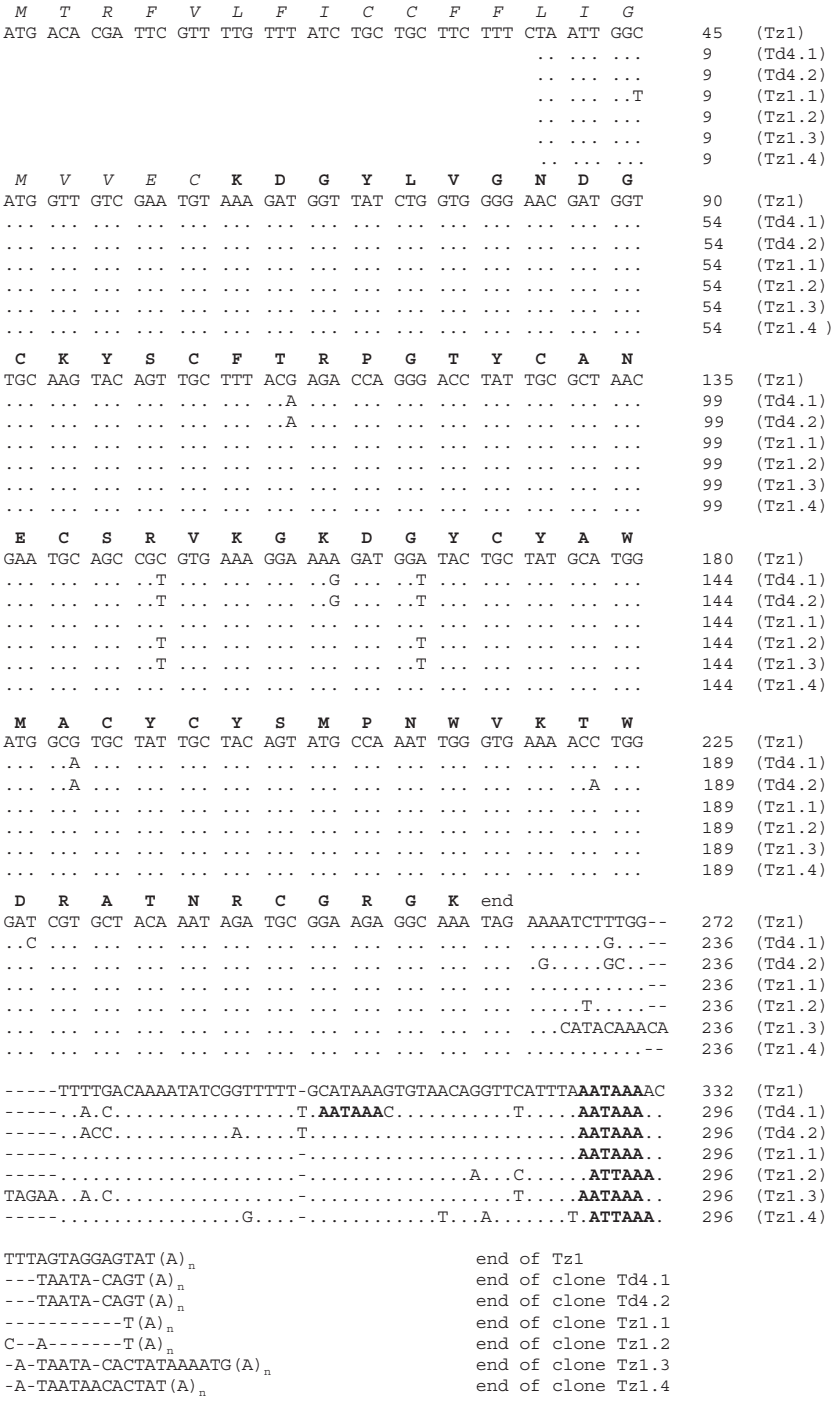

Fig. 3. Sequence variability in the mature toxin and 3′-UTRs of precursors coding for homologues of *T. zulianus* β-toxin Tz1. Nucleotide sequence of Tz1 mRNA (Borges et al., 2004a) is compared to that of clones Td4.1 and Td4.2 (*T. discrepans*) and clones Tz1.1 to Tz1.4 (*T. zulianus*). Amino acids encoded by Tz1 precursors are shown above the nucleotide sequence (signal peptide is italicized and the toxin sequence, including residues removed post-translationally, is in boldface). Nucleotides are numbered at the right side of the corresponding sequence. Dots indicate identity to the published Tz1 mRNA nucleotide sequence (Borges et al., 2004a); gaps (—) are introduced to maximize homology. Potential polyadenylation sites in the 3′-UTRs are in boldface.

3.4. Heterogeneity within the 3′-UTR of isolated T. zulianus Tz1 and T. discrepans Td4 precursors

Of all isolated *T. zulianus* cDNAs that coded for identical Tz1 mature coding regions (20), nine precursors shared a nucleotide sequence identical to the previously reported Tz1 mRNA (Borges et al., 2004a). The remaining Tz1 isolates presented sequence variations which can be grouped into four sequences, Tz1.1 to Tz1.4 (Fig. 3). Precursors Tz1.1, 1.2, and 1.3 contained third-position changes within the mature toxin region. All four Tz1.1 to 1.4 presented differences in the 3′-untranslated region, containing either the canonical AAUAAA

A

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 I   G   M   V   I   E   C   K   D   G   Y   L   M   E   P   N   G   C   +11
att ggc atg gtt atc gaa tgt aag gat ggt tat ctg atg gag ccc aat ggt tgc    54

 K   R   G   C   L   T   R   P   A   R   Y   C   P   N   E   C   S   R   +29
aaa agg ggt tgc ctt aca aga cca gca cgt tat tgc cct aac gaa tgc agc cgt   108

 L   K   G   K   D   G   Y   C   Y   L   W   L   A   C   Y   C   Y   N   +47
ttg aaa gga aaa gat ggt tac tgc tat tta tgg ttg gca tgc tat tgc tac aat   162

 M   P   E   S   A   P   V   W   E   R   A   T   N   R   C   G   K   g   +65
atg cca gaa tcg gct cca gtc tgg gaa cgt gct aca aat aga tgc gga aaa ggc   216

 k  End                                                                  +66
aaa tag aaaatctgtggttatcacaaaatatcggttttgcataaactgtaacaggttcatttaaataaaa 286

cttttaatagcagtgg(A)n
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B

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Td1          KDGYLMEPNGCKRGCLTRPARYCPNECSRLKGKDGYCYLWLACYCYNMPESAPVWERATNRCGKGK
                ||||||||||| ||||||| ||  |
TdII-4       KDGYLMEPNGCKLGCLTRPAKYCWXEE...

Td8          KDGYLVGADGCKYGCFTRPGHFCASECSLLKGKDGYCYAWLACYCYNLPDSVPVWDSATNRCGKGK
                ||||||| |||||||||||||||| |  |
TdII-3       KDGYLVGTDGCKYGCFTRPGHFCANEECL...
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Fig. 4. Nucleotide sequence of *Tityus discrepans* clone Td1 and translated amino acid sequence. (A) The predicted protein sequence is shown above the nucleotide sequence and is numbered starting from the toxin putative N-terminal residue, Lys. The signal peptide is italicized and the mature toxin region is in boldface; a potential polyadenylation site (AAUAAA; Wahle and Ruegsegger, 1999) is doubly underlined. Carboxyl-terminal residues, potentially removed post-translationally, are written in small case and boldface. (B) Comparison of the full sequences encoded by transcripts Td1 and Td8 and the partial N-terminal sequences of *T. discrepans* toxins TdII-3 and TdII-4 (D'Suze et al., 1996).

A

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 I   E   V   V   M   G   G   K   E   G   Y   L   L   D   K   S   N   G   +12
att gaa gtg gtg atg gga ggc aaa gaa ggt tac cta ttg gac aaa tcc aat gga    54

 C   K   R   S   C   F   F   G   S   T   S   W   C   N   T   E   C   K   +30
tgc aaa cgc agt tgt ttc ttc ggg tca aca tca tgg tgt aac acc gaa tgc aaa   108

 S   K   S   A   E   K   G   Y   C   A   W   P   S   C   Y   C   Y   G   +48
tcg aag tcg gca gaa aag ggt tac tgc gca tgg ccg tcg tgt tac tgc tac ggg   162

 F   S   D   D   S   K   M   W   D   L   K   T   N   K   C  End         +63
ttc agc gac gac tcg aaa atg tgg gat ttg aag acg aac aaa tgt tag aaataat   217

gcaatctgttaacactgaaaacactcttcaacatgaatttgtaacaagttattaaaatattaaagtctttcg 289

tccattgg(A)n
```

B

```
                1         10        20        30        40        50        60          %Identity
                |         |         |         |         |         |         |
Tz2     GKEGYLLDKSNGCKRSCFFGSTSWCNTECKSKSA-EKGYCAWP--SCYCYGFSDDSKMWDLKTNK--C     100
Tf4     GKEGYPADSK-GCKVTCFFTGVGYCDTECKLKKA-SSGYCAWP--ACYCYGLPDSASVWDSATNK--C      60
Ts1     -KEGYLMDHE-GCKLSCFIRPSGYCGRECGIKKG-SSGYCAWP--ACYCYGLPNWVKVWDRATNK--C      56
TsNTxP  GREGYPADSK-GCKITCFLTAAGYCNTECTLKKG-SSGYCAWP--ACYCYGLPDSVKIWTSETNK--Cgkk  55
TbIT-1  GKEGYPVDSR-GCKVTCFFTGAGYCDKECKLKKA-SSGYCAWP--ACYCYGLPDSVPVYDNASNK--CB    55
Ts2     -KEGYAMDHE-GCKFSCFIRPAGFCDGYCKTHLKASSGYCAWP--ACYCYGVPDHIKVWDYATNK--C      53
Ts4     GREGYPADSK-GCKITCFLTAAGYCNTECTLKKG-SSGYCAWP--ACYCYGLPESVKIWTSETNK--C      53
TsV     KKDGYPVEGD-NCAFACFGYDNAYCDKLCKDKKA-DDGYCVWSP-DCYCYGLPEHILKEPTKTSGR-C      43
Ts3     KKDGYPVEYD-NCAYICWNYDNAYCDKLCKDKKA-DSGYCYWVHILCYCYGLPDSEP---TKTNGK-CKS   40
Cons.   .baGY..a....C...Ch.....hC...C..K.....GYC.W....CYCYGh..........TN...C
```

Fig. 5. Structure of *T. zulianus* Tz2 precursor and sequence comparison. (A) Precursor structure. The predicted protein sequence is shown above the nucleotide sequence and is numbered starting from the putative N-terminal residue (+1) (Gly, by homology to proteins Tf4, Ts4 and TsNTxP, see Panel B); the signal peptide is italicized and the mature toxin region is in boldface; a potential polyadenylation site is doubly underlined. (B) Primary structure comparison of Tz2 with *T. fasciolatus* Tf4 (P83435; Wagner et al., 2003), *T. serrulatus* TsNTxP (077463; Guatimosin et al., 1999), *T. serrulatus* Ts4 (P45669; Marangoni et al., 1990), and *T. bahiensis* TbIT-1 (P60275). Sequences were aligned according to cysteine residues. In TbIT-1, B stands for Asx. In TsNTxP, the Carboxyl-terminal residue is not known; its precursor sequence is shown, indicating the putatively removed residues after processing (Guatimosin et al., 1999). A consensus sequence (Cons.) is written below the multiple sequence alignment with the corresponding amino acid when 100% identity is present and a lower-case letter when an acidic (a), basic (b), aromatic (r), or hydrophobic (h) residue is conserved.

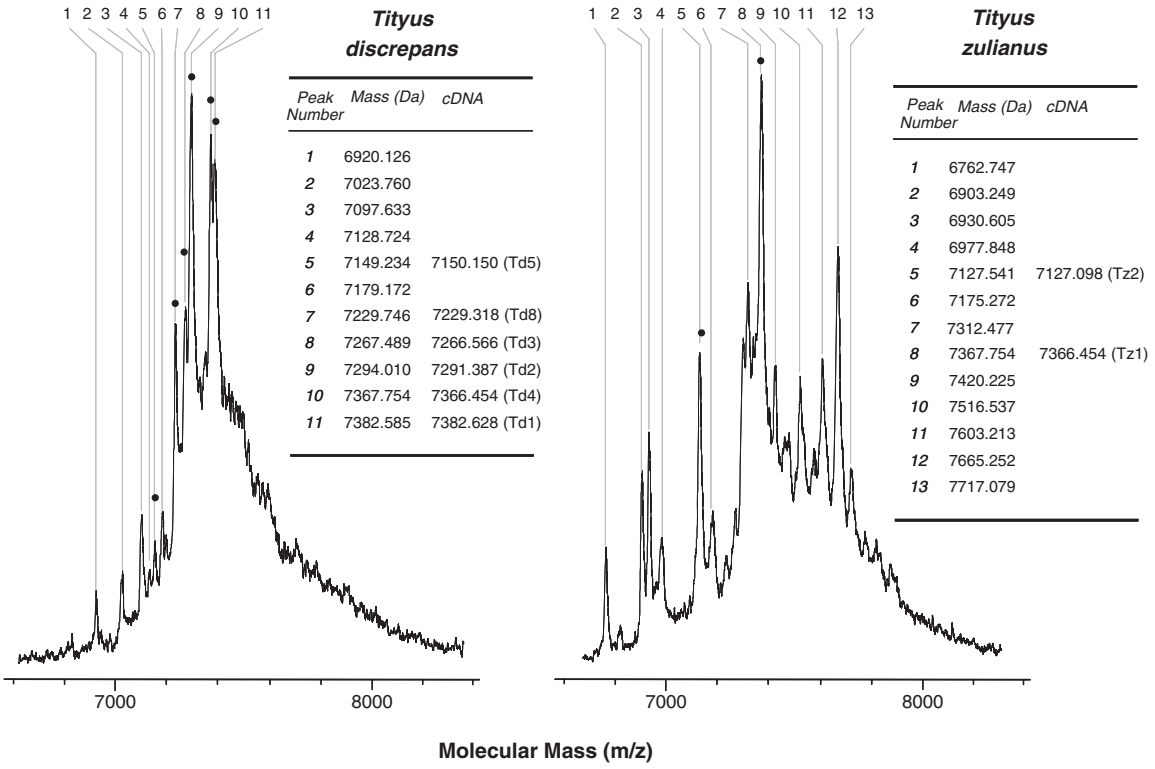

Fig. 6. MALDI–TOF mass spectrometry analysis of TzII and TdII gel filtration pools (see Fig. 1 for chromatographic profiles) in the 6000–8000 Da range. Spectra are representative of at least three analyses of TdII and TzII obtained by chromatography of independent venom samples. Filled circles indicate components with masses matching those calculated from transcripts isolated by RT–PCR from *T. discrepans* and *T. zulianus* PCR-based cDNA libraries. Masses of selected peaks are shown in the insets, together with the calculated masses of putative toxins, after removal of residues putatively processed post-translationally.

polyadenylation signal, found within the published Tz1 mRNA, or the site AUUAAA. Particularly, isolate Tz1.3 presented a 7-bp insertion at the beginning of its 3′-UTR, located within a region whose sequence diverges from the other isolates (Fig. 3). *T. discrepans* Tz1 homologue, Td4, also differ from the published Tz1 precursor and the other Tz1 isolates described in this work in their 3′-UTRs. In fact, two Td4 clones (Td4.1 and Td4.2) were isolated, which encoded identical mature toxin regions (containing only third-base changes) but different 3′-UTRs. Notably, a second canonical polyadenylation site (AAUAAA) is located in Td4.1 at 18–19 nucleotides upstream of the polyadenylation site found in the Tz1 clones.

3.5. MALDI–TOF MS analyses of *T. zulianus* and *T. discrepans* gel filtration fractions TzII and TdII

Mass spectral analyses via MALDI–TOF MS of TzII and TdII in the long-chain toxin mol. weight range (6–8 kDa) generated reproducible species-specific spectra (Fig. 6). The masses of 11 *T. discrepans* and 13 *T. zulianus* components were estimated and shown in the inset of Fig. 6.

The masses of six TdII and two TzII components matched closely (mass error from 0.04 to 2.62 Da) those predicted for *T. discrepans* (Td1, Td2, Td3, Td4, Td5, Td8) and *T. zulianus* (Tz1, Tz2) long-chain toxin precursors isolated in this work. To estimate the mass of each precursor (Peptide Mass Calculator v3.2, www.rna.rega.kuleuven.ac.be), it was assumed that all mature toxin regions encoded molecules with four intrachain disulfide bonds and that the rules for carboxy-terminal post-translational processing suggested by Possani et al. (1999) apply to *T. discrepans* and *T. zulianus* toxins, e.g. when a glycine residue precedes one (as in Td1, Td2, Td3, Td4, Td6, Td7, Td9, Td10, Td11, Td12, Td13 and Tz1) or two (as in Td5 and Td8) basic residues, the amino group of the glycine residue is used to amidate the residue preceding the glycine and both the Gly and basic residues are removed by a carboxyl-end peptidase (see Fig. 2).

3.6. Phylogenetic analysis of *T. discrepans* and *T. zulianus* putative long-chain toxins

A Neighbour-Joining phylogram was used to estimate the phylogenetic relationships among the amino acid sequences encoded by the isolated *T. discrepans* and *T. zulianus* cDNAs. *T. serrulatus* Ts1, Ts2 and Ts3, *Centruroides sculpturatus* CsE1 and *Centruroides noxius* Cn2 were used as outgroups (Fig. 7). Such outgroup selection is based on recent mitochondrial DNA data which suggest that Brazilian *Tityus* and Mexican and Central American *Centruroides* species are closely related to the Venezuelan *Tityus* species (A. Borges and E. Bermingham, unpublished results). All *T. discrepans* and *T. zulianus* Tz1 isolates are monophyletic. However, the newly isolated *T. zulianus* toxin cDNA, Tz2, is nested with the

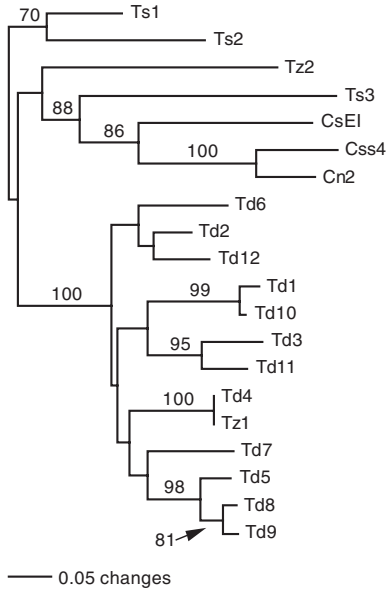

Fig. 7. Neighbour-Joining phylogram showing structural relationships of selected New World long-chain neurotoxins, including those deduced from the *T. discrepans* and *T. zulianus* precursors isolated in this work. The sequence data set corresponds to the alignment presented in Fig. 2, excluding residues putatively removed post-translationally. Sequences for *T. serrulatus* Ts1, Ts2 and Ts3, together with *C. sculpturatus* CsEI and *C. noxius* Cn2 were assigned as outgroups. Values on branching points are calculated bootstrap frequencies (%) of 1000 pseudoreplicates. Only nodes supported by >50% bootstrap frequencies are shown.

outgroup sequences *T. serrulatus* Ts3 and the *Centruroides* CsEI, Css4 and Cn2 (Fig. 7).

4. Discussion

4.1. Biochemical and immunological comparison of T. discrepans and T. zulianus venoms

This study provides evidence indicating that the long-chain toxin repertoires produced by two of the most toxic Venezuelan scorpion species, *Tityus discrepans* and *Tityus zulianus*, differ structurally and antigenically, a result which may have clinical and epidemiological implications. We postulated that the differences in quality and severity between the *T. discrepans* and *T. zulianus* envenoming syndromes, both in humans and mice (Mota et al., 1994; Mazzei de Davila et al., 2002; Borges et al., 2004b), could be related, at least in part, to structural/functional variations in their long-chain toxins, responsible for up to 90% of the lethality of crude scorpion venoms (Martin-Eauclaire and Couraud, 1995). To explore this prediction, isolation of the chromatographic pools containing the most toxic components (by i.p. injection into mice) from *T. discrepans* (TdII) and *T. zulianus* (TzII) allowed their direct comparison by electrophoretic and mass spectral analyses. AU–PAGE (Wang et al., 1997) provides good resolution of proteins and peptides sharing similar masses but differing in net charge, a case found in Na_v channel-active scorpion toxins. This technique revealed that cationic, low molecular mass components (ca. 6.5 kDa) with similar but also different net charges exist in TdII and TzII. MALDI–TOF MS analyses also demonstrated that (i) TdII and TzII contained components in the long-chain toxin mol. weight range (6–8 kDa) and (ii) that their spectra produced unique, species-specific mass fingerprints. Up to this stage, the evidence pointed out to the existence in both venoms of species-specific components.

Additionally, an in vivo neutralization test showed that the anti-*T. discrepans* antivenom is two-fold less effective in neutralizing the lethal effect of *T. zulianus* venom, in comparison with its high efficacy in the case of *T. discrepans* experimental envenoming. For instance, mice injected with 3.77 mg of antivenom per mouse resisted lethal challenge by an amount of *T. discrepans* venom equivalent to the LD_{100} while a similar dose (3.57 mg) provided no protection in the case of *T. zulianus*-envenomed animals (Table 1). These findings support the observations of local doctors from *T. zulianus* endemic areas, who have reported that elevated doses of the anti-*T. discrepans* serum are needed to treat envenomed children (Borges et al., 2002; Mazzei de Davila et al., 2002). Although some epitopes might be shared, as indicated by immunoblotting (Borges et al., 1999), such partial in vivo neutralization of *T. zulianus* venom suggests (i) the existence of non-neutralizable antigenic epitopes in those Na_v channel-active *T. zulianus* toxins that remain free upon antibody incubation, (ii) differences in activity of *T. zulianus* toxic components, and/or (iii) differences in bioavailability between their toxic components. The latter possibility is unlikely since the specific activity of hyaluronidase, an enzyme contributing to scorpion toxin diffusion (Pessini et al., 2001), does not differ significantly between *T. zulianus* and *T. discrepans* venoms (A. Borges, unpublished results). In contrast, in spite of a broadly similar three-dimensional structure, long-chain toxins display large sequence variations in the regions connecting the conserved secondary structure elements, producing lack of immunological cross-reactivity even if the toxins are found in the same venom (Granier et al., 1989; De Lima et al., 1993; Gazarian et al., 2005). Also, limited structural deviations in scorpion toxins can produce functional diversity. For instance, dramatic changes in toxin selectivity for the various Na_v channel subtypes can arise as a result of subtle variations at their functional (interacting) motifs (Gordon and Gurevitz, 2003).

4.2. Molecular investigation of T. discrepans and T. zulianus long-chain toxin diversity

To investigate the potential differences in structural and/or functional motifs between their long-chain toxins, inferred from our electrophoretic, spectral, and immunological findings, a molecular approach was then undertaken to isolate *T. discrepans* and *T. zulianus* toxin precursors and obtain insight from their encoded primary structures. High-fidelity RT–PCR amplifications from total venom gland RNA using a combination of a modified oligo(dT) primer and a degenerate, leader peptide-based primer rendered *T. discrepans* and *T. zulianus* precursor sequences clearly bearing the long-chain toxin structural fingerprint, including eight potential disulfide bridge-forming cysteines (Fig. 2). Among the set of isolated

T. discrepans precursors, two clones (Td4.1 and Td4.2), sharing the same encoded sequence as *T. zulianus* Tz1, were isolated, while no *T. zulianus* homologues of any of the remaining Td clones was found. Taking into consideration the obvious complexity of the *T. zulianus* toxin proteome (Borges et al., 2004a; see also mass profile of Fig. 6), the higher number of *T. discrepans* toxin mRNAs retrieved in this work, compared with only two *T. zulianus* sequences obtained under identical conditions, indicates divergence in the 5′ region-encoded signal peptide between those *T. zulianus* toxin mRNAs which remain to be characterized and their *T. discrepans* counterparts. In support of this view, recent isolation of a number of *T. zulianus* genomic clones encoding novel long-chain toxins have revealed signal peptides with variable nucleotide sequences around the degenerate primer binding site (data not shown).

4.3. T. discrepans toxin precursor sequences

Sequences encoded by *T. discrepans* precursors isolated in this work are more similar (identity in parentheses) to those of *T. zulianus* Tz1 (67–100%) and *T. serrulatus* Ts1 (55–66%) than to *T. serrulatus* TsV (P46115; Marangoni et al., 1995) (34–44%) and Ts3 (37–46%) (see Fig. 2), suggesting that these putative long-chain toxins belong to the scorpion β-toxin subfamily. Furthermore, a number of conserved residues, identified as functionally and structurally relevant in other β-toxins, are present in most *T. discrepans* encoded sequences (numbering of Fig. 2 alignment): (i) Glu-29, conserved in the alpha-helical regions of the β-toxins Ts1, CsEI, and Cn2, is only absent from isolate Td3; this residue is equivalent to Glu-28 in Css4, where forms a 'hot spot' that interacts electrostatically with a putative positive charge on the receptor site (Cohen et al., 2005); (ii) Trp-61 is equivalent to Trp-58 in Css4 and Trp-54 in Ts1, which chemical modification markedly reduced its toxicity (Hassani et al., 1999). In view of their similarity to Ts1, these putative *T. discrepans* peptides could be grouped structurally into the gamma-like long-chain toxin subclass according to the nomenclature of Becerril et al. (1997), who have classified *Tityus* toxins affecting Na_v channels in five subfamilies: 1 (gamma (Ts1)-like), 2 (III-8 (Ts2)-like), 3 (IV-5 (Ts3)-like), 4 (TsTx-VI (Ts4)-like) and 5 (TsTx-V (TsV)-like) toxins.

Overall, sequences encoded by *T. discrepans* precursors display a high degree of similarity among themselves, particularly in the last 20 carboxy-terminal most residues. Most non-conservative replacements among these precursors lie in the N-terminal region, which includes in *T. serrulatus* Ts1 the residues forming the alpha-helix and the loop connecting strand β1 to the alpha-helix (Polikarpov et al., 1999). It is noteworthy that these two regions encompass residues involved in channel recognition and subtype specificity in scorpion α- and β-toxins (Karbat et al., 2004; Cohen et al., 2005).

Some of the isolated *T. discrepans* precursors appear to code for actively translated peptides, judging from the close correspondence between their predicted masses and those determined by MALDI–TOF MS, assuming that the carboxyl-end processing rules of Possani et al. (1999) are valid in the case of these Venezuelan species. Furthermore, an additional indication that these transcripts encode active toxins comes from the similarity between the full sequences translated from precursors Td1 and Td8 and the N-terminal sequences of *T. discrepans* Na_v channel-active toxins TdII-3 (P60262) and TdII-4 (P60263) (D'Suze et al., 1996), respectively, although a proper comparison should await until these proteins are fully sequenced. Alternative carboxy-terminal processing mechanisms, as suggested for *T. serrulatus* Ts3 isoforms (Martin-Eauclaire et al., 1994), may also apply to these and the remaining toxin precursors whose predicted masses did not match the spectral data.

4.4. Tz2, a newly isolated T. zulianus precursor

This work also allowed isolation of a novel *T. zulianus* toxin precursor, Tz2, which codes for a putatively translated peptide (Fig. 5), since its predicted mass closely matches that of peak 5 in *T. zulianus* venom MALDI–TOF MS profile (Fig. 6). The codon corresponding to the last residue in Tz2 mRNA did not encode a Gly residue, which is known to allow post-translational amidation (Bougis et al., 1989), but was immediately followed by a stop codon. It is therefore impossible for this potential new protein to be amidated. Although by sequence comparisons, Tz2 is more related to β-toxins than to α-toxins (52–60% vs. 40–43% identity) (Fig. 5), it has 60% identity to the poorly toxic *T. fasciolatus* component Tf4, which belongs to subclass 4 of *Tityus* Na_v channel-active peptides (Wagner et al., 2003). Some Tz2 sequence features suggest that this peptide, when finally isolated, may belong to a new *Tityus* toxin class: (i) Position 10 is occupied by a conserved acidic residue in all *Tityus* toxins, except those in class 4, where it is a conserved basic (Pimenta et al., 2001); a Ser residue is found in Tz2 at this location; (ii) Tz2 contains conserved β-toxins residues Lys-14 and Trp-56, and also Glu-28; however, Lys-59 is in register with an equivalent residue in the α-toxins Ts3 and TsV, which is also present in this position in many toxins belonging to this class and proven to be crucial for bioactivity (Gordon et al., 1998). Whether this residue plays a similar role in Tz2 remains to be determined experimentally. Notably, the phylogenetic analysis (Fig. 7) grouped Tz2 together with *T. serrulatus* toxin Ts3 and *Centruroides* Cn2 and CsE1, suggesting structural relatedness with these proteins and making Tz2 clearly divergent from the *T. discrepans* toxin clade. Thus, in addition to the differences at the transcriptome level, *T. zulianus* scorpions are able to produce toxins structurally, and probably pharmacologically, unrelated to the *T. discrepans* toxin pool.

Given the limited number of isolated *T. zulianus* long-chain toxin sequences, we can only speculate at this point that the putatively non-neutralizable *T. zulianus* antigenic epitopes may contain amino acid replacements that abolish the binding of anti-*T. discrepans* toxin antibodies. For instance, Machado de Avila et al. (2004), and also Chávez-Olórtegui et al. (2002), have found that charged residues (i.e. Asp, Glu, and Lys) are crucial for antibody binding in Ts1, Ts2, Ts3, and TsNTxP, and

that these are usually replaced by Gly in the equivalent regions of those toxins lacking such antigenic epitopes.

4.5. 3′-UTR polymorphism in shared transcripts Tz1 and Td4

This work has uncovered a sequence polymorphism in the 3′-UTR among the various precursors that code for *T. zulianus* Tz1 and the *T. discrepans* Tz1 homologue, Td4. Of the twenty Tz1 clones isolated in this work (sharing an identical open reading frame), four sequence variants were found, of which Tz1.2 and Tz1.4 contain the polyadenylation signal AUUAAA instead of the canonical AAUAAA (Wahle and Ruegsegger, 1999), reported previously for Tz1 mRNA (Borges et al., 2004a) (Fig. 3). Such signal sequence has also been reported for other scorpion toxins such as the *Buthus occitanus* lipolysis factor (Soudani et al., 2004). The *T. discrepans* Tz1 homologues Td4.1 and Td4.2 also bear 3′-UTR sequences differing from the reported Tz1 and the other variants isolated here. Significantly, the Td4.1 sequence contains an additional polyadenylation signal (Fig. 3). Given that total RNA used for library construction was pooled from 20 individuals, these isoforms should not reflect individual variations in Tz1 precursors. The 3′-UTR determines the efficient termination and stability of the transcript and contains elements implicated in numerous processes such as mRNA turnover and translation (Proudfoot et al., 2002). Variation of toxin mRNA in the 3′-UTR could be an important mechanism in post-transcriptional regulation of gene expression, because modification within this region could contribute either to differences in mRNA stability and/or differential processing of primary transcripts, as seen in other systems (e.g. human HLA DQA1; Hoarau et al., 2004). Investigation of these possibilities is currently underway through the assessment of the half-lives and expression levels of these isoforms. It should be emphasized that the differences between the 3′-UTR sequences of *T. zulianus* Tz1 and *T. discrepans* Td4 mRNAs strongly suggest that the toxin transcriptomes of these two species differ in composition.

5. Concluding remarks

Regardless of the molecular basis for the above observations, the results presented clearly show species-specific differences in the composition of the venom gland proteome and transcriptome between *T. discrepans* and *T. zulianus*. Together with known dissimilarities in venom action between these species (see Borges et al., 1999, 2004b), this work raises the question of whether such underlying toxin diversity is to be encountered in the remaining Venezuelan species of this highly complex genus. A recent phylogeographic analysis performed on representative *Tityus* species have revealed that a geographical partition of mitochondrial DNA clades exist, with *T. discrepans* and *T. zulianus* belonging to quite separate groups, exhibiting >10% nucleotide divergence (A. Borges and E. Bermingham, unpublished results). Such analysis of genetic *Tityus* clades, together with the application of similar molecular/immunological approaches as the one presented herein, will set the basis for an investigation of venom diversity across *Tityus* species in northern South America.

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