RESEARCH ARTICLE

Genetic and toxinological divergence among populations of *Tityus trivittatus* Kraepelin, 1898 (Scorpiones: Buthidae) inhabiting Paraguay and Argentina

Adolfo Borges1,2*, Antonieta Rojas de Arias1, Sabrina de Almeida Lima3, Bruno Lomonte4, Cecilia Díaz4, Carlos Chávez-Olórguei5, Matthew R. Graham5, Evanguedes Kalapothakis6, Cathia Coronel1, Adolfo R. de Roodt7

1 Centro para el Desarrollo de la Investigación Científica (CEDIC), Asunción, Paraguay, 2 Laboratorio de Biología Molecular de Toxinas y Receptores, Instituto de Medicina Experimental, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela, 3 Laboratorio de Inmunología, Departamento de Bioquímica e Imunología, Instituto de Ciencias Biológicas, Universidad Federal de Minas Gerais, Belo Horizonte, Brasil, 4 Instituto Clodomiro Picado, Universidad de Costa Rica, San José, Costa Rica, 5 Departamento de Biología, Eastern Connecticut State University, Willimantic, Connecticut, United States of America, 6 Departamento de Biología General, Instituto Clodomiro Picado, Universidad de Costa Rica, San José, Costa Rica, 7 Instituto Nacional de Producción de Biológicos “Carlos G. Malbrán”, Buenos Aires, Argentina

* borges.adolfo@gmail.com

Abstract

Envenomation by scorpions in genus *Tityus* is a public health problem in Tropical America. One of the most medically significant species is *Tityus trivittatus*, which is known to occur from southwest Brazil to central-northern and eastern Argentina. In this work, we studied the lethality, composition, antigenicity, and enzymatic activity of venom from a *T. trivittatus* population found further north in urban areas of eastern Paraguay, where it has caused serious envenomation of children. Our results indicate that the population is of medical importance as it produces a potently toxic venom with an LD50 around 1.19 mg/kg. Venom neutralization in preliminary mouse bioassays was complete when using Brazilian anti-*T. serrulatus* antivenom but only partial when using Argentinean anti-*T. trivittatus* antivenom. Venom competitive solid-phase enzyme immunoassays and immunoblotting from Argentinean and Paraguayan *T. trivittatus* populations indicated that antigenic differences exist across the species range. SDS-PAGE showed variations in type and relative amounts of venom proteins between *T. trivittatus* samples from Argentina and Paraguay. MALDI-TOF mass spectrometry indicated that while some sodium channel toxins are shared, including β-toxin Tt1g, others are population-specific. Proteolytic activity by zymography and peptide identification through nESI-MS/MS also point out that population-specific proteases may exist in *T. trivittatus*, which are postulated to be involved in the envenoming process. A time-calibrated molecular phylogeny of mitochondrial COI sequences revealed a significant (8.14%) genetic differentiation between the Argentinean and Paraguayan populations, which appeared to have diverged between the mid Miocene and early Pliocene. Altogether, toxinological and genetic evidence indicate that *T. trivittatus* populations from Paraguay and...
Argentina correspond to distinct, unique cryptic species, and suggest that further venom and taxonomic diversity exists in synanthropic southern South American *Tityus* than previously thought.

**Author summary**

Scorpionism (the medical consequence of scorpion stings in humans) is a neglected health problem in tropical and subtropical areas associated with poverty. This study is the first to compare venoms among core (Argentinean) and peripheral (Paraguayan) populations of the noxious *Tityus trivittatus*, the most medically important scorpion in the southernmost section of South America. The work demonstrated the lethality of the venom of urban populations of *T.* *trivittatus* in Paraguay, where it has caused severe cases in children. We obtained data indicating that there are significant differences in venom composition and function, and also recognition by therapeutic antivenoms available in the region, among these Argentinean and Paraguayan scorpion populations which historically have been assigned to the same species. Our genetic study revealed that in fact these two populations diverged between ~15–5 Million years ago, indicating they are distinct species. These results indicate that southern South American scorpions in the genus *Tityus* which co-distribute with humans are more diverse in terms of their venoms and species composition than previously thought, and that further studies are warranted to design more effective therapeutic tools against scorpionism in the region to tackle such diversity.

**Introduction**

Envenoming by scorpions belonging to the genus *Tityus* is a public health problem in southern South America, which has been classified as a hyperendemic area of scorpionism [1,2]. In southeast Brazil the most problematic scorpion is *T. serrulatus* Lutz & Mello, a parthenogenetic species currently expanding its range and responsible for most severe envenomations in the area [3]. The second most medically important scorpion in the region is *T. trivittatus* Kraepelin, a species responsible for the majority of severe scorpion envenomations in Argentina, mostly in children [4,5]. The species’ range extends from central-northern and eastern Argentina to eastern Paraguay and southeast Brazil, and has been predicted to increase in response to ongoing global climate change [6].

Venoms from *T. serrulatus* and *T. trivittatus* contain low molecular mass toxins that affect the gating mechanism of various voltage-sensitive ion channels [7]. The main lethal toxins in *Tityus* venoms are sodium channel (Nav)-active toxins (NaTxs), which affect either the activation or inactivation components of sodium channel currents in excitable cells, producing sustained depolarization and massive discharge of neurotransmitters [8]. Rapid tissue distribution of these toxins has resulted in high mortality rates in children under 10 years of age, so severe stings require prompt treatment with specific antivenoms and intensive cardiorespiratory support [9].

The medical significance of *T. trivittatus* was unknown outside Argentina until recently, when severely envenomed children were reported from eastern Paraguay. These cases presented with psychomotor agitation, profuse sweating, serum hypokalemia, and altered cardiac frequency as a consequence of left ventricular dysfunction [10]. Unlike Argentinean populations of *T. trivittatus*, which are parthenogenetic [11], those that are common in urban areas of eastern Paraguay, including the capital city of Asunción exhibit sexual dimorphism [10,11].
Venom from Argentinean populations has been thoroughly studied from clinical, immunological, biochemical, pathological and toxicological perspectives [12,7,4]. However, considering the potential medical importance of *T. trivittatus* outside Argentina, its predicted changing distribution due to global warming, and the reported divergence in venom composition and action even among closely related *Tityus* species [1], further study is urgently needed. In this contribution, we studied the lethality, neutralization by available antivenoms, proteolytic activity, and molecular mass fingerprinting of venom from an urban *T. trivittatus* population from Paraguay. Our study revealed significant toxinological and genetic divergence between the Paraguayan samples and *T. trivittatus* from Argentina, indicating that they probably comprise unique cryptic species.

### Methods

#### Ethical statement

The Animal Research Ethics Committee of the Centro para el Desarrollo de la Investigación Científica reviewed the study protocol involving mice for toxicity and neutralization assays on 02/04/2019 and approved the research (approval code: 01/2019). The institutional Animal Research Ethics Committee follows the guidelines for animal research established by the United States National Research Council (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf).

#### Scorpion venoms

Scorpions from Paraguayan population of *T. trivittatus* (Fig 1) were collected from crevices and pipelines at human dwellings within the urban area of Asunción, Paraguay. Live specimens were transferred to the lab where they were housed with water *ad libitum* and fed with crickets (*Acheta domesticus*). Venom was extracted from male and female scorpions by electrical stimulation of the telson following procedures in [13] and lyophilized at -50 °C and 80 mBar of pressure. Prior to *in vivo* or *in vitro* studies, lyophilized samples, containing an equal proportion of venom from male and female specimens, were dissolved in either phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄) or doubly distilled water, respectively. Venom from the Argentinean population of *T. trivittatus* was from specimens collected in Paraná, Entre Ríos. Venom from *T. serrulatus* was also obtained by electrical stimulation of the telson of specimens collected in Belo Horizonte, Minas Gerais, Brasil. Venom from *T. discrepans* was obtained electrically from specimens collected in San Antonio de los Altos, Miranda, Venezuela. Venom protein content was estimated by the Lowry method [14].

#### Venom Lethality (LD₅₀)

The toxicity of crude venom from *T. trivittatus* (Paraguay) was assessed using NIH Swiss mice weighing 20–22 g obtained from the Instituto de Investigaciones en Ciencias de la Salud, Asunción, Paraguay. Mice (four animals per dose) were injected intraperitoneally (i.p.) with the following doses (in venom mg/kg body weight): 2.48, 1.66, 1.10, 0.74, and 0.49. A control group received the same injection (0.2 mL) of PBS. Mice were observed for 48 h after injection for symptoms of intoxication and death. Median lethal dose (LD₅₀) values (in mg/kg) and corresponding 95% confidence intervals (CI) were calculated using Probit analysis according to the Spearman–Karber method [15]. Animal manipulations were performed according to the regulations of the Centro para el Desarrollo de la Investigación Científica (CEDIC), Asunción, Paraguay (section 2.13).
In vivo neutralization tests

A preliminary in vivo assessment of the neutralization capacity of scorpion therapeutic anti-venoms (AVs) was conducted using a single dose of AV sufficient to neutralize 3–5 LD$_{50}$ of the corresponding control venoms as indicated by the manufacturers [16,17]. Venom samples of T. trivittatus (Paraguay) containing 3×LD$_{50}$ (LD$_{50}$ = 23.8 μg/20 g mouse) were incubated for 1 h at 37˚C with 100 μl of AVs (see below) mixed with 100 μl of PBS as suggested in standardized procedures [18]. After incubation, samples were injected (i.p.) into NIH Swiss mice (four animals per antivenom group) for the neutralization assay. Three control groups (four mice each) were used: a first group received a mixture of snake antivenom (Anti- (Bothrops jararaca + Crotalus durissus terrificus), Fundação Ezequiel Dias, Belo Horizonte, Brazil) and 3×LD$_{50}$ of T. trivittatus (Paraguay) venom per mouse and incubated as above. A second group received PBS (200 μl) and 3×LD$_{50}$ venom per mouse, incubated and injected as described above. A third group only received PBS. Two scorpion AVs were assayed: anti-T. serrulatus (Instituto Vital Brazil, Niterói, Brasil) (Batch 186001, expiration date 10/31/2021), and anti-T. trivittatus (Instituto Nacional de Productos Biológicos “Carlos G. Malbrán”, Buenos Aires, Argentina) (Batch L930, expiration date 07/31/2020). The procedure was repeated twice. Surviving mice were counted at 48 h. Protein concentration in AVs was measured by the Biuret method [19], using a Proti 2 protein determination kit (Wiener, Rosario, Argentina). All experiments were conducted before the expiration dates of the antivenoms.

Cross-recognition by western blot

Electrophoresis of venoms in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels was carried out with 20% gels using Tris-Glycine as running buffer and stained with either Coomassie Brilliant Blue R-250 or silver staining, as outlined in [20]. For immunoblotting, venom samples (typically 10–15 μg protein) were solubilized in reducing sample buffer (BioRad),
separated by SDS-PAGE, and subsequently transferred to nitrocellulose paper. Membranes were blocked with blocking buffer [1% (w/v) skimmed non-fat milk, 0.3% (v/v) Tween 20/PBS] for 1 h and then incubated with therapeutic sera from immunized horses, diluted in the same blocking buffer (1/1,000), for 1.5 h. Membranes were washed with 0.05% (v/v) Tween 20/PBS and incubated with goat anti-horse horseradish peroxidase-conjugated secondary antibody (Sigma) (diluted 1/50,000) in blocking buffer for 1 h. Membranes were washed once again and blots were developed using Luminata Forte Western HRP Substrate (Millipore).

**Solid-phase enzyme immunoassay (ELISA)**

One hundred nanograms of venom protein each from Argentinean and Paraguayan *T. trivittatus* was adsorbed to the surface of separate sets of wells of MaxiSorp flat bottom microtitration plates (Nunc) at 4°C for 12–14 h, and blocked with 3% (w/v) bovine serum albumin containing 0.05% (v/v) Tween-20 for 3 h at room temperature. Plates were washed with saline containing 0.05% Tween-20 and then 100 μl of different AV (anti-*T. trivittatus*, Argentina) dilutions (1:200 to 1:51,200) were added to wells and incubated for 45 min at room temperature. Plates were washed again and 100 μl of horseradish peroxidase (HRP)-conjugated anti-horse immunoglobulin (Sigma) diluted 1:50,000 was added to each well and incubated as described. Wells were washed a third time and 100 μl of o-phenylenediamine (Sigma) (1 mg/ml) plus 4 μl of 30% (v/v) hydrogen peroxide were added. Spectrophotometric determination of color change was recorded at 495 nm (A495nm) in a Multi-Mode Microplate Reader (BioTek). Anti-(Bothrops jararaca + Crotalus durissus terrificus) snake AV (FUNED, Belo Horizonte, Brasil) was included as a negative control. Data were fitted by five-parameter logistic regression implemented in GraphPad Prism 7 (GraphPad Software, San Diego, CA). AV dilutions corresponding to half-maximal A495nm values were calculated based on this software.

**Competitive solid-phase enzyme immunoassay**

Inhibition of binding of anti-*T. trivittatus* (INPB, Argentina) antivenom to solid-phase-bound *T. trivittatus* venom from Argentina by competing with venoms from *T. trivittatus* (Paraguay) and *T. trivittatus* (Argentina) in solution was carried out according to the method of King et al. [21]. Briefly, antivenom samples, at a dilution corresponding to half the maximal binding to solid-phase antigens, were pre-incubated for 1 hour at room temperature with serially diluted venoms starting at 100 ng/ml. Then, 100 μl of the mixtures was allowed to bind to solid-phase venoms for 1 hour and the bound horse F(ab)\(_2\)s were detected as described in section 2.5. Values represent the highest inhibition of antibody binding to solid-phase venoms when high concentrations of competitor venoms in the liquid phase were used.

**Mass spectrometry of crude venoms**

Mass spectra of positively charged ions from scorpion venoms were analyzed by MALDI–TOF MS in a Biflex III MALDI–TOF MS (Bruker, FRG). Samples for analyses (200–500 μg) were lyophilized, dissolved in 100 μL of ultra-pure 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)]. One μL from this mixture was spotted on the target plate. Mass spectra of positively charged ions were recorded on a Autoflex 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.
Mass spectrometry identification of electrophoretic bands

Selected Coomassie-stained protein bands of the SDS-PAGE electrophoresed venoms were excised and in-gel digested overnight with sequencing-grade trypsin (Sigma), after reduction of disulfide bonds with DTT and alkylation with iodoacetamide, in an automated workstation (Intavis). The resulting peptides were submitted to nESIMS/MS on a Q-Exactive Plus mass spectrometer (Thermo). Twelve μL of each tryptic digest were loaded on a 2 cm×75 μm trap column, washed, and separated at 200 nL/min on a C18 Easy-spray analytical column (15 cm×75 μm, 3 μm particle) using a nano-Easy 1200 chromatograph. A gradient from 0.1% formic acid (solvent A) to 80% acetonitrile with 0.1% formic acid (solvent B) was developed as follows: 1–5% B in 1 min, 5–26% B in 25 min, 26–79% B in 4 min, 79–99% B in 1 min, and 99% B in 4 min, for a total of 35 min. MS spectra were acquired in positive mode at 2.0 kV, with a capillary temperature of 200˚C, using 1 μscan at 400–1600 m/z, maximum injection time of 50 msec, AGC range of 1×10⁶, and resolution of 70,000. The top 10 ions with 2–5 positive charges were fragmented with an AGC target of 3×10⁶, minimum AGC 2×10⁵, maximum injection time 110 ms, dynamic exclusion time 5 s, and resolution 17,500. MS/MS spectra were searched for matches against protein sequences contained in the UniProt/SwissProt database (Arachnida, January 2020) using Peaks X software. Cysteine carbamidomethylation was set as a fixed modification, while deamidation of asparagine or glutamine and methionine oxidation were set as variable modifications, allowing up to 3 missed cleavages by trypsin. Parameters for match acceptance were set to FDR < 0.1%, -10lgP protein score ≥70, with at least 1 unique peptide.

Determination of Proteolytic and Hyaluronidase activity by Substrate Zymography

To determine proteolytic activity and the molecular weight of proteases present in the venoms, gelatin zymography was performed according to [22]. Briefly, venom proteins (20 μg) were separated by SDS-PAGE in 20% gels containing type-A gelatin (Sigma) at a concentration of 0.25 mg/mL under non-reducing conditions. After washing for 1 h with 1% (v/v) Triton X-100 to remove SDS, gels were incubated at 37˚C for 24 h in 50 mM Tris-HCl, pH 8.0, containing 5 mM CaCl₂, and stained with Coomassie Blue R-250. Hyaluronidase activity present in the venoms (20 μg) was determined by the method reported by [23] based on SDS-PAGE in a 12% gel containing 0.5 mg/mL hyaluronic acid from rooster comb (Sigma). Incubation buffer (0.1 M NaCl, 0.1 M sodium phosphate) was adjusted to pH 6.6. Gels were stained with Alcian Blue 8GX (Sigma).

DNA extraction and PCR amplification

Total DNA was extracted according to [24] from pedipalp muscle of two T. trivittatus specimens collected inside homes in Asunción, Paraguay (specimen 1 from Ciudad Nueva, 25.293631 S, 57.615761 W; specimen 2 from Barrio Jara, 25.274722 S, 57.603333 W). Scorpions were identified by David J. Guerrero, Natural History Museum, Asunción, based on [25,11]. Amplification and sequencing of the nucleotide sequence encoding the N-terminal portion of cytochrome oxidase subunit I (COI hereafter) was performed according to [26] using primers LCO1490: 5’-GGTCAACAAATCATATAAGATATGG-3’ [27], and HCOEXTERNB: 5’- CCTATTGAWARAACATARTGAAAAATG-3’ [28]. Amplified fragments were bidirectionally sequenced using an Applied Biosystems 3130 Genetic Analyzer DNA sequencer as previously described [24]. Sequences generated for this study were deposited at GenBank under the accession numbers MT800756 and MT808337.
Phylogenetic analyses

A phylogeny and divergence dates among individuals were simultaneously estimated using Bayesian inference (BI) in BEAST 1.8.0 [29]. Consensus sequences were aligned in Geneious v. 7.1.7 (Biomatters Ltd., Auckland, New Zealand) using MUSCLE [30], checked for accuracy by eye, and trimmed to minimize missing characters. We determined the best-fit model of nucleotide substitution with MEGAX [31] using the Bayesian Information Criterion. We generated an.xml file in BEAUTi (BEAST package) using the best-fit substitution model (HKY+G), the uncorrelated lognormal clock model, and the Yule tree prior. Preliminary BEAST runs using the uncorrelated lognormal clock model revealed a low uclld.stdev value (<1.0), so we used a strict clock model for final runs (as suggested in the BEAST manual). To calibrate the BEAST analyses, we used normal clock rate priors with a mean rate (uclld.mean) of 0.007 substitutions per site per million years, as previously estimated for other buthid scorpions [32]. Following [33], we adjusted the standard deviation so 95% of the normal distribution included minimum and maximum rates estimated for COI in other studies of scorpions (SD: 0.00270). We conducted two independent MCMC runs for 20 million generations each and sampled every 10,000 generations. TRACER 1.6 was used to confirm adequate effective sample sizes and that Markov chains reached stationarity and convergence. The runs were combined to produce a maximum clade credibility tree using TEEANNOTATOR (BEAST package) and visualized in FIGTREE 1.4.0 (http://tree.bio.ed.ac.uk/software/).

Statistical analyses

The Spearman-Karber method [15] was used to determine venom lethality. ELISA curves were analyzed by non-linear regression and half-maximal A490nm values (including their 95% confidence intervals) calculated using the software Prism7.0 (GraphPad Inc., CA). The significance of statistical differences between half-maximal values corresponding to AV recognition of *T. trivittatus* venoms from Argentina and Paraguay was evaluated using the Extra sum-of-squares F test implemented in Prism7.0 (p < 0.05).

Results

Lethality of *T. trivittatus* venom from Paraguay

Medium lethal dose in NIH Swiss mice intraperitoneally injected with *T. trivittatus* venom (a pool from female and male specimens) was estimated as 1.19 mg/kg (95% CI: 0.89–1.71). Mice injected with doses as low as 0.74 mg/kg presented with signs of acute toxicity such as profuse salivation, piloerection, urination, voiding of feces, extension rigidity of the hindlimbs, and dyspnea starting 10 minutes after venom administration. At lower doses (0.74 and 1.10 mg/kg) manifestations subsided after 45 minutes. When doses were lethal, death was usually recorded 45–60 min post-injection. In some mice injected with lethal doses (1.66 and 2.48 mg/kg) mouth bleeding was observed.

*In vivo* neutralization of *T. trivittatus* venom from Paraguay by therapeutic scorpion antivenoms

To test the neutralizing capacity of therapeutic anti-*Tityus* antivenoms towards venom from the Paraguayan population of *T. trivittatus*, Swiss mice were injected i.p. with 0.2 mL amounting to 3×LD₅₀ pre-incubated with 100 μL of antivenoms produced in Brazil, and Argentina. Table 1 summarizes representative survival data from two independent experiments, including a negative control, using snake AV (anti-*B. jararaca* + *C. durissus terrificus*), and a positive control, comprising mice injected with 3×LD₅₀ in the presence of PBS. The Brazilian
Instituto Vital Brazil) anti-\textit{T. serrulatus} showed the highest protection (100% survival), followed by the Argentinean anti-\textit{T. trivittatus} (INPB thereinafter) AV (50% survival). Mice injected with mixtures of venom and Brazilian AV showed no symptoms of toxicity. Mice injected with mixtures of venom and Argentinean AV presented with toxicity signs such as dyspnea, salivation and piloerection after 10 minutes, which subsided 1 hour post-injection in the case of surviving animals. Protein concentrations of tested AVs were $28.1 \pm 0.9 \text{ mg/mL}$ (Brazilian AV) and $33.1 \pm 2.8 \text{ mg/mL}$ (INPB AV).

### SDS-PAGE and cross-recognition by immunoblotting of \textit{Tityus} spp. venoms using therapeutic antivenoms

Fig 2 shows the result of immunoblottings performed to identify \textit{T. serrulatus} and \textit{T. trivittatus} (Argentina and Paraguay) venom protein components recognized by Brazilian and Argentinean scorpion AVs. Venom from Venezuelan \textit{T. discrepans} was included as an additional control considering its reported low recognition by the Brazilian AV and its phylogenetic separation from southern South American \textit{Tityus} spp. including \textit{T. serrulatus} and \textit{T. trivittatus} [1]. Blots developed with the Brazilian AV showed a greater number of recognized components in \textit{T. serrulatus} and \textit{T. trivittatus} (both populations) compared to the INPB AV, particularly the low molecular mass fraction ($< 10 \text{ kDa}$) which corresponds to scorpion toxins targeting ion channels [34]. This fraction was only weakly detected using Argentinean

| Treatment | $\text{LD}_{50}$ | Surviving mice /total mice | Survival percentage |
|-----------|-----------------|---------------------------|-------------------|
| Phosphate buffer saline | 0 | 4/4 | 100 |
| Phosphate buffer saline | 3 | 0/4 | 0 |
| Snake AV (Funed, Brazil) | 3 | 0/4 | 0 |
| Anti-\textit{T. serrulatus} AV (Instituto Vital Brazil, Brazil) | 3 | 4/4 | 100 |
| Anti-\textit{T. trivittatus} AV (INPB, Argentina) | 3 | 2/4 | 50 |

\textit{a} Data are representative of two experiments conducted independently.

https://doi.org/10.1371/journal.pntd.0008899.t001

Fig 2. Cross-recognition by immunoblotting of \textit{Tityus} spp. venoms using therapeutic AVs. (Left panel) Silver-stained SDS-PAGE (20% gel) under reducing conditions of \textit{Tityus} spp. venoms from Paraguay (PAR, \textit{T. trivittatus}), Argentina (ARG, \textit{T. trivittatus}), Venezuela (VEN, \textit{T. discrepans}), and Brazil (BRA, \textit{T. serrulatus}). (Right panels) Blots developed with therapeutic horse sera from Brazil and Argentina. Arrows on the left indicate migration of \textit{T. trivittatus} population-specific components. Arrow on the right indicates migration of low molecular mass scorpion toxins. MW, molecular mass markers.

https://doi.org/10.1371/journal.pntd.0008899.g002
antibodies. Most intensely recognized protein bands corresponded to high molecular mass components in both AVs tested (17–60 kDa). The INPB AV recognized a band of 30 kDa and a faint signal corresponding to the low mass neurotoxic fraction in Paraguayan samples, whereas the 30 kDa component was more intensely detected and an additional band of 20 kDa was recognized in the case of the Argentinean population. *T. discrepans* venom components were only weakly recognized by both AVs. Banding patterns in silver-stained SDS-PAGE gels differed between *T. trivittatus* venoms from Paraguay and Argentina, notably with proteins of 17, 19, 25, and 40 kDa being only present in the Argentinean venom, together with an intense 30-kDa component, and a fraction migrating around 10 kDa only found in the Paraguayan population (Fig 2, left panel).

**T. trivittatus** venom antigenicity evaluated by ELISA

Considering the differences in electrophoretic composition and *in vivo* and *in vitro* reactivity towards the INPB AV between venoms from Paraguayan and Argentinean *T. trivittatus* populations, ELISA tests were performed to compare their antigenicity. Particularly, we wanted to investigate quantitatively antigenic differences between these venoms as indicated by immunoblotting. Fig 3 shows titration of AV reactivity towards venoms of both populations (Panel A) (Data available in S1 Data). To estimate the statistical significance of differences between recognition of both venoms by the INPB AV, half-maximal AV dilution values were compared by the Extra Sum-of-Squares F-test implemented in GraphPad Prism4. A comparison of these values (Paraguay: 4.16 ± 0.15, 95%CI: 3.95–5.01; Argentina: 3.97 ± 0.06, 95%CI: 3.86–4.16) rendered the difference nonsignificant (F = 1.71, p = 0.198), implying that *T. trivittatus* venoms from Paraguay and Argentina were similarly recognized by the anti-*T. trivittatus* (INPB) AV. Panel B shows the results of a competitive ELISA assay for testing the inhibition capacity of *T. trivittatus* venoms from Paraguay and Argentina on the binding of INPB horse antibodies to immobilized *T. trivittatus* (Argentina) venom (Data available in S2 Data). Whereas venom from Argentina produced 18.1 ± 3.3% free antibodies at the maximal venom dose tested (10 μg/mL), incubation with venom from Paraguay produced 77.2 ± 4.2% free antibodies at the same concentration.

![Fig 3](https://doi.org/10.1371/journal.pntd.0008899.g003)
Enzyme activity comparison between *T. trivittatus* venoms from Paraguay and Argentina by Zymography

Fig 4 shows the results of the evaluation of in-gel enzyme activity by substrate zymography in *T. trivittatus* venoms. In the presence of hyaluronic acid as a substrate, we detected hyaluronidase activity at 40–50 kDa in both venoms. In the presence of gelatin, several bands with proteolytic activity were identified, which were distinct between *T. trivittatus* populations. The main proteolytic component unique to the Argentinean population venom migrated at 37 kDa, whereas the main component of the Paraguayan population was close to 110 kDa. Other population-specific, higher molecular mass minor components were also evident in both zymograms.

MALDI-TOF mass spectrometry assessment of *T. trivittatus* (Paraguay and Argentina) venom composition

Fig 5 shows spectra obtained through MALDI-TOF to compare protein mass distributions in venoms from the two *T. trivittatus* populations. Table 2 presents a list of the main ions observed in both venoms by MALDI-TOF. In the NaTx mass range (6–8 kDa), peptides unique to the Paraguayan population were components with m/z 6726.6, 6916.5, and 7263.5 Da. Whereas peptides unique to the Argentinean population were 6630.0, 6754.5, 6787.5,
Proteomic identification of proteins in 30-kDa SDS PAGE bands from *T. trivittatus* venoms

A major component of venoms from both *T. trivittatus* populations is a protein of molecular mass ca. 30 kDa, which appears at a higher abundance in the Argentinean samples (Figs 2 and 4). Therefore, we digested bands excised from SDS-PAGE gels (Fig 4) with trypsin and analyzed the resulting peptides by nanoelectrospray ionization tandem MS/MS (nESIMS/MS). Tables 3 and 4 summarize the identified venom peptides. Significant sequence matches were found for peptides from both populations with putative venom metalloproteinases from *T. bahiensis* (identified through transcriptomics [35]), metalloserrulases from *T. serrulatus* (which are metalloproteases identified both at the molecular and functional levels [36,37]), and a hyaluronidase from *T. bahiensis* (UniProtKB A0A0C9RFM5) (identified at the transcript level [35]). Both *T. trivittatus* venoms shared all listed *T. bahiensis* putative proteases and *T. serrulatus* metalloserrulases 18 (UniProtKB A0A1S5QN52) and 20 (UniProtKB A0A1S5QN67). Peptides matching *T. serrulatus* metalloserrulases 1 (UniProtKB A0A076L876) and 16 (UniProtKB A0A1S5QN57) (from *T. serrulatus*) were only found in the venom from Paraguay (Table 3).
Evolutionary distance between \textit{T. trivittatus} populations from Paraguay and Argentina

Considering the variations in venom composition and antigenicity between venoms from conspecific populations of \textit{T. trivittatus}, we asked whether significant genetic differences occurred among populations as well. Mitochondrial DNA COI data, which is used for DNA barcoding \cite{38}, revealed 6 amino acid replacements between Argentinean and Paraguayan \textit{T. trivittatus} populations, whereas the closely related \textit{T. confluens} exhibited 5 replacements in the same region (Fig 6). The replacements all occur in highly polymorphic COI areas, particularly transmembrane (M), extracellular (E), and intracellular (I) segments M3, E2 and I2 \cite{39}. In the time-calibrated phylogeny generated with BEAST \cite{40} (Fig 7), the Paraguayan \textit{T. trivittatus} is 8.14% divergent (at the nucleotide level) from \textit{T. trivittatus} from Argentina, and 8.97% from \textit{T. confluens}. \textit{T. trivittatus} from Argentina and \textit{T. confluens} are 8.47% divergent. Divergence time estimates (using calibration data from \cite{41}) indicate that the \textit{T. trivittatus} populations from northern Argentina and Paraguay diverged between the middle Miocene and early Pliocene (~15–5 Million years ago (Ma)).

Discussion

This study is the first to compare venoms among core (Argentinean) and peripheral (Paraguayan) populations of the noxious scorpion \textit{Tityus trivittatus}. Our results indicate that the population inhabiting urban areas of eastern Paraguay is of potential medical importance, as its LD$_{50}$ value is within the range of other congeneric species associated with lethal envenomations. Table 5 shows a comparison of the calculated LD$_{50}$ with venom lethality values obtained for other \textit{Tityus} spp. in South America in mouse bioassays using the same injection route.

Particularly, venom lethal potency for the Paraguayan population is comparable to those reported from the Argentinean provinces of Entre Ríos, Santa Fé, Córdoba, La Rioja, and
Table 3. Protein matches obtained by nESI-MS/MS of tryptic peptides from the SDS-PAGE 30 kDa band of *Tityus trivittatus* (Paraguay) venom\(^{a,b}\). Accession codes in bold correspond to protein matches unique to the Paraguayan *T. trivittatus* population.

| Accession | -10lgP | % Cov | #Pept | #Unique | Avg. mass | m/z | z | Matching peptide sequences\(^a\) | Description |
|-----------|--------|-------|-------|---------|-----------|-----|---|--------------------------------|-------------|
| A0A0C9QKU3 | 221.51 | 32 | 24 | 12 | 44081 | 484.2586 | 3 | K.YVHSDIIYKANK.Y | Putative metalloproteinase, TSA: *Tityus bahiensis* Tbah00944 mRNA sequence (Fragment) |
| | | | | | | 665.6638 | 3 | K.EVDQNGKYHSDIIYK.A | |
| | | | | | | 577.7932 | 2 | K.EVDQNGKYHSDIIYKANK.Y | |
| | | | | | | 569.2990 | 2 | K.YVHSDIIYK.A | |
| | | | | | | 473.7247 | 2 | K.ANKYYC(+57.02)K.N | |
| | | | | | | 487.7671 | 2 | Y.VHSIIYK.A | |
| | | | | | | 499.7458 | 2 | K.EVDQNGKYHSDIIYK.A | |
| | | | | | | 479.9159 | 2 | Q.N(+-.98)GKYVHSDIIYK.A | |
| | | | | | | 681.8162 | 2 | K.EVDQNGKYHSDIIYK.D | |
| | | | | | | 593.6390 | 2 | S.DVQNGKYHSDIIYK.A | |
| | | | | | | 526.9367 | 2 | S.DIYKANKYYC(+57.02)K.N | |
| | | | | | | 571.7819 | 2 | K.ENEPYIKESDQNGKYVH.D | |
| | | | | | | 412.2187 | 2 | Y.DTM(+15.99)NLDIKIR.L | |
| A0A0C9S3A4 | 185.28 | 23 | 15 | 14 | 43397 | 734.3187 | 2 | K.VGQA(Q57.02)DDSDDYNER.V | Putative metalloproteinase, TSA: *Tityus bahiensis* Tbah00729 mRNA sequence (Fragment) |
| | | | | | | 612.3328 | 2 | K.C(+57.02)VEHLLSLPR.A | |
| | | | | | | 401.2473 | 2 | K.AQVIGITTPFKK.V | |
| | | | | | | 451.2544 | 2 | K.KC(+57.02)VEHLLSLPR.A | |
| | | | | | | 748.8595 | 2 | N.DGIDMSGNNKVN.F | |
| | | | | | | 504.9067 | 2 | N.DGIDIM(+15.99)GSSGNKVNK.F | |
| | | | | | | 597.5231 | 4 | K.DC(+57.02)PENGYMSGNNKVNKF.F | |
| | | | | | | 601.5221 | 4 | K.DC(+57.02)PENGYMSGNNKVNKF.F | |
| | | | | | | 596.6284 | 3 | N.DGIDIM(+15.99)GSSGNKVNKF.F | |
| | | | | | | 586.2556 | 3 | N.DGIDIM(+15.99)GSSGNK.V | |
| | | | | | | 783.8533 | 2 | K.VGQAQDDDDNYER.V | |
| | | | | | | 448.1848 | 2 | R.ASC(+57.02)VLADC(+57.02) | |
| | | | | | | 502.2550 | 2 | E.ETGLSGPGAK.D | |
| | | | | | | 632.7864 | 2 | N.KVAQQDDSDDNYERVDTVAHEAHL | |
| | | | | | | 841.3672 | 2 | K.VGQAQDDDDNYERVDTVAH | |
| | | | | | | 466.6990 | 2 | K.YYC(+57.02)NNAK.G | |
| A0A0C9RFK9 | 183.09 | 20 | 11 | 11 | 44944 | 470.9696 | 4 | K.SHTFC(+57.02)TPSTC(+57.02)KIEAGG.K.V | Putative metalloproteinase, TSA: *Tityus bahiensis* Tbah00905 mRNA sequence |
| | | | | | | 500.2694 | 4 | K.VTESDKKTLTDDTHNQLL.N | |
| | | | | | | 422.5527 | 3 | K.LASGKENQYQL.L | |
| | | | | | | 663.2826 | 2 | K.SHTFC(+57.02)TPSTC(+57.02)K.I | |
| | | | | | | 404.2213 | 3 | K.TILDTHNQLR.N | |
| | | | | | | 522.2868 | 3 | K.VGC(+57.02)VGAVYGVENV.R.V | |
| | | | | | | 503.2654 | 3 | R.NKLASGKENQYQL.L | |
| | | | | | | 454.5795 | 3 | K.IEAGGKVTESDKK.T | |
| | | | | | | 535.2839 | 2 | K.SVTIDPGQIR.R | |
| | | | | | | 498.7635 | 2 | I.LDTHNQL.R.N | |
| | | | | | | 404.5492 | 2 | K.TILDTHN(+57.02)QLR.N | |
| | | | | | | 545.7296 | 2 | K.FEHDGSDQRA | |
| A0A0C9QKW3 | 169.79 | 17 | 12 | 8 | 42480 | 486.5908 | 4 | D.PREDGTVDINTAGIANSAGVC(+57.02)KPC(+57.02)KPC(+57.02) | Putative metalloproteinase, TSA: *Tityus bahiensis* Tbah00248 mRNA sequence (Fragment) |
| | | | | | | 549.6150 | 3 | L.IA | |
| | | | | | | 776.6194 | 3 | N.TAGIANSAGVC(+57.02)KPC(+57.02)L.IA | |
| | | | | | | 780.6169 | 3 | R.GMIDPREDGTVDINTAGIANSAGVC(+57.02)KPC(+57.02)L.IA | |
| | | | | | | 617.3062 | 2 | R.GM(+15.99)GDPREDGTVDINTAGIANSAGVC | |
| | | | | | | 663.6675 | 3 | R.GM(+15.99)GDPREDGTVDINTAGIANSAGVC | |
| | | | | | | 492.2530 | 2 | A.NSAGVC(+57.02)KPC(+57.02)L.IA | |
| | | | | | | 498.2787 | 2 | A.NSAGVC(+57.02)KPC(+57.02)L.IA | |
| | | | | | | 560.2844 | 2 | V.DINTAGIANSAGVC(+57.02)KPC(+57.02)L.IA | |
| | | | | | | 412.2187 | 2 | K.YYC(+57.02)NNAK.G | |

(Continued)
| Accession | %10lgP | % Cov | # Pept | # Unique | Avg. mass | m/z  | z | Matching peptide sequences* | Description |
|-----------|--------|-------|--------|----------|-----------|------|---|----------------------------|--------------|
| A0A218QX25 | 163.62 | 17 | 12 | 1 | 45537 | 833.8954 | 833.6491 | R.TITIAHEAGHM(+15.99)LGVPHDGHESTEVGVPN(+.98)GPAG.K.S | Putative metalloproteinase (Fragment) Tityus serrulatus |
| A0A218QX15 | 162.04 | 17 | 11 | 1 | 44723 | 830.9042 | 831.1525 | R.TITIAHEAGHM(+15.99)LGVPHDGHESTEVGVPN(+15.99)GPAG.K.S | Putative metalloproteinase Tityus serrulatus |
| A0A218QX19 | 147.71 | 14 | 9 | 9 | 37523 | 831.8368 | 540.7810 | K.EGYIM(+15.99)GNDYGENER.K | Metalloserulase 18 Tityus serrulatus |
| A0A1S5QN52 | 147.71 | 12 | 9 | 9 | 43885 | 831.8368 | 540.7810 | K.EGYIM(+15.99)GNDYGENER.K | Metalloserulase 18 Tityus serrulatus |
| A0A0C9RP91 | 107.95 | 10 | 3 | 3 | 29546 | 492.9221 | 549.9507 | R.LGTVDRQSGPQYR.F | Putative metalloproteinase, TSA: Tityus bahiensis Tbah01003 mRNA sequence |
| A0A076L876 | 105.83 | 9 | 5 | 1 | 42691 | 491.2649 | 427.2144 | K.FSTC(+57.02)SVENIK.Y | Hyaluronidase Tityus bahiensis |
| A0A1S5QN67 | 95.13 | 10 | 4 | 1 | 41560 | 487.7220 | 440.7540 | M.YFLGKPR.A | Metalloserulase 20 (Fragment) Tityus serrulatus |
| A0A0C9RAM3 | 90.92 | 10 | 5 | 5 | 46533 | 650.2638 | 482.2693 | K.DEPSQFSC(+57.02)SSR.I | Hyaluronidase Tityus bahiensis |
| A0A218QXX3 | 71.45 | 5 | 2 | 2 | 37728 | 592.7829 | 452.7415 | K.FSTC(+57.02)SVENIK.Y | Putative metalloproteinase Tityus serrulatus |

(Continued)
Catamarca, where *T. trivittatus* is prevalent and associated with stings, mainly in children, with incidence rates ranging from 5.62 to 32.51 cases per 100,000 inhabitants [47] (Fig 8). Although the overall risk of mortality per envenomation is relatively low, the wide distribution and the synanthropic behavior of *T. trivittatus* make it a significant public health risk [4]. Specimens representing the local Paraguayan population of *T. trivittatus* were mainly found in crevices and pipelines inside human dwellings. A population of *T. confluens*, a species of medical importance in Argentina [46], also inhabits Great Asunción, but its local sanitary importance remains to be determined.

As severe scorpion envenomations have been reported from Asunción and neighbouring areas in Paraguay [10], it was important to evaluate the neutralizing capacity of therapeutic scorpion AVs from Brazil and Argentina against local populations of *T. trivittatus*. These anti-venoms are proven therapeutic tools in both countries against human envenomation by *T. serrulatus* and *T. trivittatus* [9,4]. We did not assay the AV produced in Mexico against species in the genus *Centruroides* as its lower immunoreactivity towards *Tityus* spp. venom components has been demonstrated [1,12]. The antivenom produced against *T. discrepans* in Venezuela was not used either considering its low recognition towards venoms from southern South American *Tityus* spp. and that it does not abolish the action of *T. serrulatus* NaTxs and KTxs on ion channels [48,1]. Our preliminary assessment using a single dose of antivenom suggested that the Brazilian (anti-*T. serrulatus*) AV offered the best protection in the mouse neutralization assay. Eventhough the amount of AV protein used per mouse was similar in these assays in the case of the Brazilian (281 μg) and Argentinean (331 μg) AVs, protection provided by the INPB AV from Argentina was 50% (Table 1). To investigate whether there were variations in AV reactivity that could account for such in vivo differences, we carried out immublotting assays. Western blots indicated that a greater number of protein components from both *T. trivittatus* populations cross-reacted with components from *T. serrulatus* AV, compared to the reactivity observed after probing membranes with the INPB AV, particularly in the region where low molecular mass neurotoxins migrate (< 10 kDa). This could contribute, at least in part, to the higher in vivo neutralization provided by the Brazilian AV. Importantly, INPB antibodies recognized components from the Paraguayan population at a lesser extent compared to Argentinean samples, including neurotoxic peptides (< 10 kDa) associated with lethality, which was unanticipated considering that these populations have been historically regarded as conspecific [25,11]. To evaluate quantitatively the antigenic differences between venoms from both populations, titration and competitive ELISA assays were carried out using the INPB AV, which is the only antivenom used in Paraguay to treat envenomed victims [10]. Titration ELISA tests showed that *T. trivittatus* venoms from Paraguay and Argentina were similarly recognized by the anti-*T. trivittatus* (INPB) AV, probably as a result of the contribution of high molecular mass components (Fig 3A). Tested using the same concentrations in

| Accession  | % Cov | # Pept | # Unique | Avg. mass | m/z  | z  | Matching peptide sequencesa | Description                                      |
|-----------|-------|--------|----------|-----------|------|----|-----------------------------|-----------------------------------------------|
| A0A218QXF3 | 5     | 2      | 2        | 42121     | 592.7829 | 452.7415 | K.FSTC(+57.02)SVENIK.Y K.SDPPFIFTK.S | Putative metalloproteinase (Fragment) *Tityus serrulatus* |
| A0A1S5QN57 | 4     | 2      | 2        | 44932     | 592.7829 | 452.7415 | K.FSTC(+57.02)SVENIK.Y K.SDPPFIFTK.S | Metalloserrulase 16 *Tityus serrulatus* |

a Peptide spectral matching search performed against the Uniprot Arachnida database, using Peaks X software
b m/z and z values correspond to listed peptide sequences.

https://doi.org/10.1371/journal.pntd.0008899.t003
Table 4. Protein matches obtained by nESI-MS/MS of tryptic peptides from the SDS-PAGE 30 kDa band of *Tityus trivittatus* (Argentina) venom\(^a,b\).

| Accession   | -10lgP | % Cov | #Pept | #Unique | Avg. mass | m/z   | g  | Matching peptide sequences                              | Description                                                                 |
|-------------|--------|-------|-------|---------|-----------|-------|-----|---------------------------------------------------------|-----------------------------------------------------------------------------|
| A0A0C9QKU3  | 250.12 | 32    | 51    | 20      | 44081     | 838.4091 |     | R.TITIAHEAGHLGVPHDQESTEAEPNGPAK.S                      | Putative metalloprotease, TSA: *Tityus bahiensis* Tbah00944 mRNA sequence   |
|             |        |       |       |         |           | 1122.872 3 |     | R.TITIAHEAGHM(+15.99)                                  |                                                                             |
|             |        |       |       |         |           | 1117.8669 3 |     | LGVPHDQESTEAEPNGPAK.S                                  |                                                                             |
|             |        |       |       |         |           | 725.8853 2 |     | K.ESDVQNGKYVHSDIYK.A                                   |                                                                             |
|             |        |       |       |         |           | 1117.8693 3 |     | R.TITIAHEAGHLGVPHDQ(+.98)                             |                                                                             |
|             |        |       |       |         |           | 569.2993 2 |     | ESTEAEVPNGPAK.S                                       |                                                                             |
|             |        |       |       |         |           | 713.5822 4 |     | K.YVHSIDIYK.A                                         |                                                                             |
|             |        |       |       |         |           | 577.7931 4 |     | R.TITIAHEAGHLGVPHDQESTEAEPVN(+.98)                    |                                                                             |
|             |        |       |       |         |           | 843.3818 2 |     | GPGAK.S                                                |                                                                             |
|             |        |       |       |         |           | 1123.2007 3 |     | K.YVHSIDIYK.A                                         |                                                                             |
|             |        |       |       |         |           | 628.8071 2 |     | A.HEAGHLGVPHDQESTEAEPNGPAK.S                          |                                                                             |
|             |        |       |       |         |           | 785.8694 2 |     | K.ESDVQNGKYVHSDIYKAN.K                                 |                                                                             |
|             |        |       |       |         |           | 473.7244 2 |     | H.DQESTEAEPNGPAK.S                                     |                                                                             |
|             |        |       |       |         |           | 470.2470 4 |     | R.TITIAHEAGHM(+15.99)LGVPHDQ(+.98)                    |                                                                             |
|             |        |       |       |         |           | 585.2924 2 |     | ESTEAEVPNGPAK.S                                       |                                                                             |
|             |        |       |       |         |           | 487.7671 2 |     | E.STEAEVPNGPAK.S                                      |                                                                             |
|             |        |       |       |         |           | 576.9368 3 |     | D.GQESTEAEPNGPAK.S                                    |                                                                             |
|             |        |       |       |         |           | 786.3618 2 |     | K.ANKYYC(+57.02)K.N                                   |                                                                             |
|             |        |       |       |         |           | 499.7454 4 |     | E.AEVPNGPAK.S                                         |                                                                             |
|             |        |       |       |         |           | 730.3516 3 |     | S.TEAEVPNGPAK.S                                       |                                                                             |
|             |        |       |       |         |           | 693.3278 2 |     | Y.VHSDIYK.A                                           |                                                                             |
|             |        |       |       |         |           | 786.3539 2 |     | S.DIYKANKYYC(+57.02)K.N                               |                                                                             |
|             |        |       |       |         |           | 842.6499 4 |     | D.GQ(+.98)ESTEAEVPNGPAK.S                             |                                                                             |
|             |        |       |       |         |           | 717.5781 4 |     | K.ESDVQNY(+15.99)ESTEAEVPNGPAK.S                      |                                                                             |
|             |        |       |       |         |           | 692.6572 3 |     | M.LGVPHDQESTEAEPNGPAK.S                               |                                                                             |
|             |        |       |       |         |           | 681.8154 2 |     | Q.STEAEVPNGPAK.S                                      |                                                                             |
|             |        |       |       |         |           | 629.2960 4 |     | D.GQESTEAEPVN(+.98)GPAGK.S                            |                                                                             |
|             |        |       |       |         |           | 470.7375 2 |     | R.TITIAHEAGHM(+15.99)LGVPHDQ(+15.99)ESTEAEVPNGPAK.S    |                                                                             |
|             |        |       |       |         |           | 482.7752 2 |     | (+.98)GPAGK.S                                         |                                                                             |
|             |        |       |       |         |           | 838.9047 4 |     | A.HEAGHM(+15.99)                                      |                                                                             |
|             |        |       |       |         |           | 633.2950 4 |     | LGVPHDQESTEAEPNGPAK.S                                  |                                                                             |
|             |        |       |       |         |           | 717.8291 4 |     | GPGAK.S                                                |                                                                             |
|             |        |       |       |         |           | 1123.5376 3 |     | K.ESDVQNGKYVHSDIYKAN.K K.GHHMLGVPHDQESTEAEPNGPAK.S     |                                                                             |
|             |        |       |       |         |           | 471.4928 2 |     | E.AEVPNGPAK.S                                         |                                                                             |
|             |        |       |       |         |           | 569.2993 2 |     | S.DIYKANKYYC                                           |                                                                             |
|             |        |       |       |         |           | 470.2470 4 |     | R.TITIAHEAGHM(+15.99)LGVPHDQ(+.98)ESTEAEVPNGPAK.S      |                                                                             |
|             |        |       |       |         |           | 482.7752 2 |     | (+.98)GPAGK.S                                         |                                                                             |
| A0A218QX15  | 204.73 | 26    | 29    | 2       | 44723     | 834.9000 |     | R.TITIAHEAGHLGVPHDQESTEVGPAK.S                        | Putative metalloproteinase OS = *Tityus serrulatus* OX = 6887              |
|             |        |       |       |         |           | 550.2784 2 |     | L.GLVPHDQESTEVGPAK.S                                  |                                                                             |
|             |        |       |       |         |           | 829.6457 4 |     | R.TITIAHEAGHLGVPHDQESTEAEPNGPAK.S                      |                                                                             |
|             |        |       |       |         |           | 829.8973 4 |     | N.AVGIALGASAC(+57.02)NK+(+57.02)EK.V                   |                                                                             |
| A0A218QX25  | 205.56 | 21    | 28    | 1       | 45537     | 829.6457 |     | R.TITIAHEAGHLGVPHDQESTEAEPNGPAK.S                      | Putative metalloproteinase (Fragment) OS = *Tityus serrulatus* OX = 6887   |
|             |        |       |       |         |           | 829.8973 4 |     | R.TITIAHEAGHLGVPHDQESTEAEPNGPAK.S                      |                                                                             |
|             |        |       |       |         |           | 1111.5361 3 |     | R.TITIAHEAGHLGVPHDQESTEAEPNGPAK.S                      |                                                                             |

(Continued)
| Accession | -10gP | % Cov | #Pept | #Unique | Avg. mass | m/z | z | Matching peptide sequences | Description |
|-----------|-------|-------|-------|---------|-----------|-----|---|---------------------------|-------------|
| A0A0C9RFM9 | 186.62 | 31 | 21 | 1 | 21496 | 427.7211 | 2 | A.DVPNGP GAK.S | Putative metalloproteinase, TSA: Tityus bahiensis Tbah02152 mRNA sequence (Fragment) OS = Tityus bahiensis Ox = 50343 PE = 2 SV = 1 |
| A0A0C9S3A4 | 178.48 | 23 | 16 | 16 | 43397 | 734.3187 | 2 | K.VGAQDDSD YNER.V | Putative metalloproteinase, TSA: Tityus bahiensis Tbah00729 mRNA sequence (Fragment) OS = Tityus bahiensis Ox = 50343 PE = 2 SV = 1 |
| A0A0C9R FK9 | 176.57 | 27 | 15 | 15 | 44944 | 470.9690 | 4 | K.SHTFC(+57.02)TPSTC(+57.02)KIEAGK.V | Putative metalloproteinase, TSA: Tityus bahiensis Tbah00905 mRNA sequence OS = Tityus bahiensis Ox = 50343 PE = 2 SV = 1 |
| A0A218QWW8 | 156.90 | 24 | 18 | 1 | 34355 | 607.9504 | 3 | K.EN(+98)EPSFI KESDVQNGK.Y | Putative metalloproteinase (Fragment) OS = Tityus serrulatus Ox = 6687 PE = 4 SV = 1 |
| A0A0C9QKW7 | 103.50 | 20 | 9 | 1 | 25764 | 656.9733 | 3 | I.HN(+98)AN(+98)NYC(+57.02)KNATGLAQ.K.A | Putative metalloproteinase, TSA: Tityus bahiensis Tbah00001 mRNA sequence (Fragment) OS = Tityus bahiensis Ox = 50343 PE = 2 SV = 1 |
competitive ELISA assays, venom from Paraguay did not reproduce the inhibition curve obtained with venom from Argentina in the ability to prevent binding of INPB antibodies to immobilized control venom, with 77.2 ± 4.2% antibodies remaining free in solution (Fig 3B).

### Table 4. (Continued)

| Accession   | -10lgP | % Cov | #Pept | #Unique | Avg. mass | m/z   | z − | Matching peptide sequences                                                                 | Description                                                                 |
|-------------|--------|-------|-------|---------|-----------|-------|-----|--------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| A0A0C9QKW3  | 98.80  | 15    | 5     | 5       | 42480     | 515.249|     | E.GSPGAANC(+57.02)PAK.A                                                                    | Putative metalloproteinase, TSA: Tityus bahiensis Tbah00248 mRNA sequence   |
|             |        |       |       |         |           |       |     | K.AGYIMGRN.N                                                                               | Fragment OS = Tityus bahiensis OX = 50343 PE = 2 SV = 1                    |
|             |        |       |       |         |           | 515.753|     | K.YKFSPC(+57.02)TK.K                                                                      |                                                                             |
|             |        |       |       |         |           | 449.214|     | K.AGYIM(+57.02)GNR.N                                                                       |                                                                             |
|             |        |       |       |         |           | 434.2216|    | K.FSPC(+57.02)TKK.C                                                                         |                                                                             |
|             |        |       |       |         |           | 780.621|     | R.GM(+15.99)GDPREDGTVDINTAGIANSAGVC(+57.02)KPC(+57.02)JK.A                                 |                                                                             |
| A0A0C9RM5   | 97.68  | 13    | 7     | 6       | 46533     | 474.2709|     | 2 K.MPVPFEPK.I                                                                               | Hyaluronidase OS = Tityus bahiensis OX = 50343 PE = 2 SV = 1               |
|             |        |       |       |         |           | 650.2643|     | K.DEPSQFSC(+57.02)SSR.I                                                                    |                                                                             |
|             |        |       |       |         |           | 482.2698|     | K.M(+15.99)PYFKPTK.I                                                                        |                                                                             |
|             |        |       |       |         |           | 439.2124|     | R.IQMENSRL.I                                                                                |                                                                             |
|             |        |       |       |         |           | 513.5931|     | 3 S.KHGEWPSDRVEK.V                                                                           |                                                                             |
|             |        |       |       |         |           | 470.8954|     | K.HQEWDPSDRVEK.V                                                                            |                                                                             |
|             |        |       |       |         |           | 509.7636|     | 2 K.YAKEEW.EK                                                                               |                                                                             |
| A0A218QX9    | 95.40  | 11    | 5     | 4       | 37523     | 442.5718|     | 3 K.ANIM(+15.99)YFLGKPR.A                                                                   | Putative metalloproteinase, (Fragment) OS = Tityus serrulatus OX = 6887 PE = 1 |
|             |        |       |       |         |           | 831.8405|     | K.EGYIM(+15.99)GNDYGENER.K                                                                  |                                                                             |
|             |        |       |       |         |           | 696.9644|     | 3 K.C(+57.02)PGKEGYIMGNDYGENER.K                                                             |                                                                             |
|             |        |       |       |         |           | 554.9958|     | 3 K.C(+57.02)PGKEGYIMGNDYGENERK.F                                                              |                                                                             |
| A0A1S5QN52  | 95.40  | 9     | 5     | 4       | 43885     | 442.5718|     | 3 K.ANIM(+15.99)YFLGKPR.A                                                                   | Metalloeluralase 18 OS = Tityus serrulatus OX = 6887 PE = 2 SV = 1         |
|             |        |       |       |         |           | 831.8405|     | 2 K.EGYIM(+15.99)GNDYGENER.K                                                                 |                                                                             |
|             |        |       |       |         |           | 696.9644|     | 3 K.C(+57.02)PGKEGYIMGNDYGENER.K                                                             |                                                                             |
|             |        |       |       |         |           | 554.9958|     | 3 K.C(+57.02)PGKEGYIMGNDYGENERK.F                                                              |                                                                             |
| A0A0C9RP91  | 89.83  | 8     | 3     | 3       | 29546     | 492.9219|     | 3 R.LGTVDQRSQPGYR.F                                                                         | Putative metalloproteinase, TSA: Tityus bahiensis Tbah01461 mRNA sequence   |
|             |        |       |       |         |           | 418.2051|     | 2 R.QLSQPQYR.F                                                                              | Fragment 1) OS = Tityus bahiensis OX = 50343 PE = 2 SV = 1               |
|             |        |       |       |         |           | 451.2379|     | G.DSGGPLVTR.N                                                                               |                                                                             |
| A0A1S5QN67  | 87.09  | 14    | 4     | 1       | 41560     | 719.3635|     | 3 R.TMTQKPKPSVGANAGAGYKG.V                                                                   | Metalloserulase 20 (Fragment) OS = Tityus serrulatus OX = 6887 PE = 2 SV = 1 |
| A0A0C9RPA3  | 86.26  | 10    | 4     | 1       | 43099     | 487.1898|     | 2 K.VC(+57.02)DEC(+57.02)YK.V                                                              | Putative metalloproteinase, TSA: Tityus bahiensis Tbah01003 mRNA sequence   |
|             |        |       |       |         |           |           |     | Fragment 1) OS = Tityus bahiensis OX = 50343 PE = 2 SV = 1                                   |                                                                             |
| A0A1E1WVW9  | 63.40  | 5     | 5     | 2       | 44401     | 592.7829|     | 2 K.FSTC(+57.02)SVENIK.Y                                                                    | Putative metalloproteinase OS = Tityus obscura OX = 122124 PE = 4 SV = 1   |
|             |        |       |       |         |           | 452.7415|     | K.SDPFFIK.T.S                                                                               |                                                                             |
| A0A1Y3BFR2  | 60.55  | 4     | 2     | 2       | 36493     | 433.2403|     | 3 E.NFRPVQPLNQR.Q                                                                           | Carbonic anhydrase 2-like protein OS = Euroglyphus maynei                  |
|             |        |       |       |         |           | 440.7520|     | 2 R.PVQPLNQR.Q                                                                               | OX = 6958 GN = BLA29_002815 PE = 3 SV = 1                                |

*a Peptide spectral matching search performed against the Uniprot Arachnida database, using Peaks X software.

b m/z and z values correspond to listed ion peptide sequences.

https://doi.org/10.1371/journal.pntd.0008899.t004
Taken together with the immunoblotting results and in vivo data, such partial competition reinforces the suggestion that venom components with significant antigenic differences exist across the geographic distribution of T. trivittatus, some of which could account for the venom toxicity of the Paraguayan population. In addition, SDS PAGE showed differences in both type and relative abundance of venom proteins between Argentinean and Paraguayan T. trivittatus (Figs 2 and 4). Previous work has shown that the INPB AV effectively neutralizes venom from Argentinean T. trivittatus whereas a fourfold amount of Brazilian anti-T. serrulatus AV is needed for neutralization of the same venom challenge dose [49]. Taken together with our results, these previous findings suggest that southern South American Tityus spp. produce toxins antigenically more diverse than envisaged in previous studies [7].

Venom proteins are shared between these populations in the high molecular mass range (20–60 kDa), as determined by SDS PAGE (Fig 4), cross-recognition in blots (Fig 2), and ELISA titrations (Fig 3A). Some of these proteins have been identified in scorpion venoms as metallopeptidases and hyaluronidases, which contribute to the severity of the envenomation process. Some T. serrulatus metallopeptidases are capable of hydrolyzing neuropeptides in vitro,
releasing mediators that could interact with ion channels and promote indirect neurotoxicity [50]. In particular, a group of scorpion metallopeptidases named Antareases have been postulated to play a role in the development of scorpion venom-induced pancreatitis as they cleave SNARE (N-ethylmaleimide-Sensitive factor Attachment protein Receptors) isoforms associated with zymogen granule membranes in exocrine pancreas, disrupting the normal vesicular traffic [51,52]. Scorpion venom hyaluronidase activity significantly enhances bioavailability of low molecular mass neurotoxins as has been shown in *T. serrulatus* [53]. Notably, transcriptomic studies have indicated that proteases are the most abundant transcripts in Brazilian scorpions from the genus *Tityus*, representing 48%, 38%, and 33% of the venom glands transcripts of *T. obscurus* [54], *T. bahiensis* [35], and *T. serrulatus* [54], respectively. Thus, we explored proteolytic (gelatinolytic) and hyaluronidase activities in *T. trivittatus* venoms. Fig 4 (right panel) shows a major band with hyaluronic acid-degrading activity migrating between 40–50
kDa, which is in the range reported for other scorpion venom hyaluronidases [53,22]. Distinct hyaluronidases have been reported from *T. serrulatus* and *T. bahiensis* but little is known about the potential existence of catalytic differences among isoforms [55,35]. Fig 4 (middle panel) shows that the majority of proteins with gelatin-degrading activity were population-specific, with a major component in the Argentinean population migrating around 37 kDa, and a

Table 5. *LD*\textsubscript{50} comparison of venoms from *Tityus* species in assays using 20–22 g mice and intraperitoneal injection route, including 95% CI (in brackets).

| Species/Geographic origin                  | *LD*\textsubscript{50} (mg/kg), i.p. | Reference     |
|------------------------------------------|-------------------------------------|---------------|
| *Tityus asthenes* (Colombia, Antioquia)  | 6.08 (5.19–6.98)                    | [42]          |
| *Tityus pachyurus* (Colombia, Tolima)    | 4.80 (4.40–5.20)                    | [43]          |
| *Tityus fuhrmanni* (Colombia, Antioquia) | 3.90 (3.00–4.90)                    | [44]          |
| *Tityus trivittatus* (Argentina, Córdoba) | 1.45 (1.15–1.80)                    | [12]          |
| *Tityus serrulatus* (Brasil, Minas Gerais) | 1.30 (0.99–1.65)                    | [45]          |
| *Tityus trivittatus* (Paraguay, Asunción) | 1.19 (0.89–1.71)                    | This work     |
| *Tityus trivittatus* (Argentina, Entre Ríos) | 1.03 (1.00–1.05)                    | [12]          |
| *Tityus trivittatus* (Argentina, Catamarca/La Rioja) | 0.90 (0.60–1.15) | [12] |
| *Tityus confluens* (Argentina, Jujuy/Catamarca) | 0.70 (0.45–1.05) | [46] |
| *Tityus trivittatus* (Argentina, Entre Ríos/Santa Fé) | 0.70 (0.50–1.00) | [12] |

Fig 8. Geographic distribution of *T. trivittatus* in southeast South America (based on [11], in gray). Localities (red squares) are shown in Argentina where *T. trivittatus* venom lethal medium doses have been determined in mice using the intraperitoneal route of injection (mg/kg, in boldface) [12], including that reported in this study from Asunción, Paraguay (95% confidence limits in brackets). (*, dose reported is from a venom mixture from Catamarca and La Rioja).
protein around 110 kDa in the population from Paraguay. Differences in the proteolytic enzymes of both *T. trivittatus* populations are apparent from these results.

To further explore the differences in protease content between *T. trivittatus* populations we subjected the 30 kDa component, which is present at different abundances in these two samples and is within the mass range of other scorpion proteases, to trypsin digestion and proteomic analysis through nESIMS/MS. Sequences of most tryptic peptides derived from both populations matched Brazilian *T. bahiensis* and *T. serrulatus* metalloproteinases. However, component from the *T. trivittatus* Paraguayan population contained peptides similar to additional *T. serrulatus* metalloproteinases (metalloserrulases 1, 16, 18, 20) compared with the Argentinean population (metalloserrulases 18, 20). Distinct metalloserrulases have a preference for cleaving neupeptides with high specificity, implying that they are neuropeptidases with different biological targets and roles in the envenoming process [37]. Considering the differences in venom gelatin zymograms and electrophoretic mobility, it is feasible that proteolytic proteins with differential properties exist in these *T. trivittatus* populations that could influence their toxicity. However, a full proteomic/transcriptomic study is needed in both cases for a proper comparison of their proteolytic components.

To gain further knowledge into the protein composition of both *T. trivittatus* populations, MALDI TOF MS was used to determine their venom fingerprint in the NaTx and KTx ranges (Fig 5). In the NaTx range, the populations shared components of masses 6606.1 Da and 6940.2 Da. The latter closely resembles the calculated mass for toxin Tt1g (6938.12), a β-toxin isolated and characterized from the Argentinean *T. trivittatus*, which acts on the sodium current activation component in excitable tissues [7]. The fact that three components in NaTx mass range were unique to the Paraguayan population and six were exclusive to Argentinean *T. trivittatus*, together with the observation that no shared components were detected in the mass range of antimicrobial peptides or KTx provides additional evidence for their toxinological divergence. Identification of population-specific *T. trivittatus* NaTxs by proteomic analysis is currently ongoing. Considering that NaTxs are the most lethal components of buthid scorpion venoms [8], such identification is crucial for the design of therapeutic antivenoms that aid in the neutralization of specific toxic components of Paraguayan *T. trivittatus*. Previous studies have shown that antibodies prepared against recombinant NaTxs effectively neutralize venom lethality and that NaTxs could be used as immunogens in antivenom manufacture [56].

In regard to their toxinological differences, we asked whether there could be evolutionary differences between these *T. trivittatus* populations as well. Amplification of a fragment encoding COI, which is used for DNA barcoding, allowed sequence analysis, both at the amino acid and nucleotide levels. A Bayesian analysis revealed that the Paraguayan and Argentinean *T. trivittatus* populations are 8.14% divergent at the nucleotide level (Fig 7) and may represent distinct species as this is within the range of COI divergence for other scorpion species in the family Buthidae [57,32]. Additionally, *T. trivittatus* from Paraguay exhibits more amino acid replacements in this COI segment with respect to the Argentinean population in comparison to *T. confluentus* (Fig 6). Divergence time estimates for these populations correspond to the middle to late Miocene, between 5 and 15 Ma, based on our time-calibrated phylogeny. This timeframe overlaps closely with the estimated age of the inland sea that existed in southern South America, named the Paranaense sea, between 15 and 13 Ma [58]. The sea occupied most areas of northern Argentina and Uruguay [59], and could have isolated the genetically divergent Paraguayan population of *T. trivittatus*. The same mechanism has been postulated for frogs in the genus *Lepidobatrachus*, armadillos in the genus *Calyptophractus*, and geckos in the genus *Homonota* [60–62]. Given the divergence date estimates, genetically differentiated *Tityus*
populations could have originated by vicariance as Miocene marine incursions along the Paraná river basin fragmented their ancestral range.

*T. trivittatus* was described in 1898 based on specimens collected in San Salvador (presently in Guairá department, eastern Paraguay), 120 km southeast from Asunción, within the current distribution range for this species in Paraguay [63,25]. As such, our sampled Paraguayan population represents *T. trivittatus sensu stricto* which warrants further research to uncover the true taxonomic identity of the supposedly conspecific population inhabiting northern-central and eastern Argentina, historically identified as *T. trivittatus* [11]. Morphological differences between these populations would confirm our findings.

Taken together, our results suggest that further venom and taxonomic diversity exists in southern South American *Tityus* than previously thought. Further research is being carried out in our laboratories to determine the true extent of the toxinological relationships between *T. trivittatus* populations inhabiting urban areas in Paraguay and its synanthropic Argentinean and Brazilian congeners, both in venom composition and function. Importantly, such studies would aid in the design of more effective therapeutic tools against scorpionism in the region.

**Supporting information**

S1 Data. Venom reactivity of *Tityus trivittatus* populations from Paraguay and Argentina towards INPB (anti-*T. trivittatus*, Argentina) horse antibodies measured at 495 nm using ELISA titrations.

(XLSX)

S2 Data. Binding of free antivenom (INPB, Argentina) (%) to immobilized *Tityus trivittatus* (Argentina) venom in the presence of competing *T. trivittatus* venoms from Paraguay and Argentina.

(XLSX)

**Acknowledgments**

We acknowledge the help of Dr. Dayane Naves de Souza during operation of the MALDI TOF MS spectrometer and Mr. David J. Guerrero for scorpion identification. AB, ARdA, and CC are thankful to the PRONII system for scientific categorization (Consejo Nacional de Ciencia y Tecnología, Paraguay).

**Author Contributions**

**Conceptualization:** Adolfo Borges, Antonieta Rojas de Arias, Bruno Lomonte, Carlos Chávez-Olórtegui.

**Data curation:** Adolfo Borges, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Matthew R. Graham, Evanguedes Kalapothakis.

**Formal analysis:** Adolfo Borges, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Matthew R. Graham, Evanguedes Kalapothakis.

**Funding acquisition:** Adolfo Borges.

**Investigation:** Adolfo Borges, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Carlos Chávez-Olórtegui, Matthew R. Graham, Evanguedes Kalapothakis, Cathia Coronel, Adolfo R. de Roodt.
Methodology: Adolfo Borges, Antonieta Rojas de Arias, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Carlos Chávez-Olórtegui, Matthew R. Graham, Evanguedes Kalapothakis, Cathia Coronel, Adolfo R. de Roodt.

Project administration: Adolfo Borges.

Resources: Adolfo Borges, Antonieta Rojas de Arias, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Carlos Chávez-Olórtegui, Matthew R. Graham, Evanguedes Kalapothakis, Cathia Coronel, Adolfo R. de Roodt.

Software: Adolfo Borges, Bruno Lomonte, Matthew R. Graham.

Supervision: Adolfo Borges.

Validation: Adolfo Borges, Antonieta Rojas de Arias, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Carlos Chávez-Olórtegui, Matthew R. Graham, Evanguedes Kalapothakis.

Visualization: Adolfo Borges, Bruno Lomonte, Cecilia Díaz, Matthew R. Graham.

Writing – original draft: Adolfo Borges.

Writing – review & editing: Adolfo Borges, Antonieta Rojas de Arias, Sabrina de Almeida Lima, Bruno Lomonte, Cecilia Díaz, Carlos Chávez-Olórtegui, Matthew R. Graham, Evanguedes Kalapothakis, Cathia Coronel, Adolfo R. de Roodt.

References

1. Borges A, Lomonte B, Angulo Y, de Patiño HA, Pascale JM, Otero R et al. Venom Diversity in the Neotropical Scorpion Genus Tityus: Implications for antivenom design emerging from molecular and immunochemical analyses across endemic areas of Scorpionism. Acta Tropica. 2020; 204:105346. https://doi.org/10.1016/j.actatropica.2020.105346 PMID: 31982434

2. Chippaux JP, Goyffon M. Epidemiology of scorpionism: A global appraisal. Acta Tropica. 2008; 107:71–9.

3. Torrez PPQ, Dourado FS, Bertani R, Cupo P, França FOS. Scorpionism in Brazil: exponential growth of accidents and deaths from scorpion stings. Revista de la Sociedad Brasileira de Medicina Tropical. 2019; 52:e20180350.

4. de Roodt AR, García SI, Salomón OD, Segre L, Dolab JA, Funes RF et al. Epidemiological and clinical aspects of scorpionism by Tityus trivittatus in Argentina. Toxicon. 2003; 41(8):971–7. https://doi.org/10.1016/S0041-0101(03)00066-7

5. de Roodt AR, Lanari LC, García SI, Costa de Oliveira V, Damín CF, de Titto EH. Accidents and deaths by venomous animals in Argentina during the period 2000–2011. Revista Ecuatoriana de Ciencia, Tecnología e Innovación en Salud Pública. 2017; 1(1):1–24.

6. Martínez PA, Andrade MA, Bidau CJ. Potential effects of climate change on the risk of accidents with poisonous species of the genus Tityus (Scorpiones, Buthidae) in Argentina. Spatial and Spatio-temporal Epidemiology. 2016; 25:67–72.

7. Coronas F, Diego-García E, Restano-Cassulini R, de Roodt AR, Possani LD. Biochemical and physiological characterization of a new Na+-channel specific peptide from the venom of the Argentinean scorpion Tityus trivittatus. Peptides. 2015; 68:11–6.

8. Rodríguez de la Vega RC, Possani LD. Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure-function relationships and evolution. Toxicon. 2005; 46(8):831–44. http://dx.doi.org/10.1016/j.toxicon.2005.09.006.

9. Bucaretchi F, Fernandes LCR, Fernandes CB, Branco MM, Prado CC, Vieira RJet al. Clinical consequences of Tityus bahiensis and Tityus serrulatus scorpion stings in the region of Campinas, southeastern Brazil. Toxicon. 2014.

10. Borges A, Rojas de Arias A. El Accidente por Escorpiones Tóxicos en el Paraguay: Mitos y Realidad en el contexto de la Emergencia por Escorpionismo en el Sudeste de la América del Sur. Revista de la Sociedad Científica del Paraguay. 2019; 24(Special Issue):27–35. https://doi.org/10.32480/rsclp.2019-24-1.27–35
1. Ojanguren Affilastro AA. Estudio monográfico de los escorpiones de la República Argentina. Revista Ibérica de Aracnología. 2005; 11:75–241.

2. de Roodt AR, Coronas FIV, Lago N, González ME, Laskowicz RD, Beltramino JC et al. General biochemical and immunological characterization of the venom from the scorpion Tityus trivittatus of Argentina. Toxicon. 2010; 55(2–3):307–19. http://dx.doi.org/10.1016/j.toxicon.2009.08.014.

3. Oukkache N, Chgoury F, Lalaoui M, Alagon A, Ghalim N. Comparison between two methods of scorpion venom milking in Morocco. Journal of Venomous Animals and Toxins including Tropical Diseases. 2013; 19(1):5.

4. Lowry OH, Rosebrough NJ, Farr AL, Randall AJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951; 193(1):265–75.

5. World Health Organization. Progress in characterization of venoms and standardization of antivenoms. Geneva, 1981, No. 58:28.

6. Fan HW, Vigilato MAN, Pompei JCA, Gutierrez JM, Red de Laboratorios Públicos Productores de Antivenenos de América Latina. Situación de los laboratorios públicos productores de antivenenos en América Latina. Rev Panam Salud Publica. 2019; 43:1–9.

7. de Roodt AR. Comments on Environmental and Sanitary Aspects of the Scorpionism by Tityus trivittatus in Buenos Aires City, Argentina. Toxins. 2014; 6:1434–52.

8. World Health Organization. WHO Guidelines for the Production, Control and Regulation of Snake Antivenoms. Geneva, 2010.

9. Itzhakii RF, Gill DM. A Micro-Biuret Method for Estimating Proteins. Analytical Biochemistry. 1964; 9:401–10.

10. Borges A, García CC, Lugo E, Alfonzo M, Jowers MJ, Op den Camp HJM. Diversity of long-chain toxins in Tityus zulianus and Tityus discrepans venoms (Scorpiones, Buthidae): Molecular, immunological, and mass spectral analyses. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2006; 142(3–4):240–52.

11. King TP, Joslyn A, Kochoumian MS. Antigenic cross-reactivity of venom proteins from hornets, wasps, and yellow jackets. Journal of Allergy and Clinical Immunology. 1985; 75:621–8.

12. Cevallos MA, Navarro-Duque C, Varela-Julia M, Alagon AC. Molecular mass determination and assay of venom hyaluronidases by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Toxicon. 1992; 30:925–30. PMID: 1523685

13. Ojanguren-Affilastro AA, Adilardi RS, Cajade R, Ramírez MJ, Ceccarelli FS, Mola LM. Multiple approaches to understanding the taxonomic status of an enigmatic new scorpion species of the genus Tityus (Buthidae) from the biogeographic island of Paraje Tres Cerros (Argentina). PLOS ONE. 2017; 12(7):e0181337. https://doi.org/10.1371/journal.pone.0181337 PMID: 28746406

14. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology. 1994; 3(5):294–9.

15. Arango CP, Wheeler WC. Phylogeny of the sea spiders (Arthropoda, Pycnogonida) based on direct optimization of six loci and morphology. Cladistics 2007; 23:255–93. https://doi.org/10.1111/j.1096-0031.2007.00143.x

16. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol. 2012; 29(8):1969–73. https://doi.org/10.1093/molbev/mss075 PMID: 22367748

17. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research. 2004; 32(5):1792–7. https://doi.org/10.1093/nar/gkh340 PMID: 15034147

18. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 2016 33(7):1870–4.

19. Gantenbein B, Bet V, Gantenbein-Ritter IA, Balloux F. Evidence for recombination in scorpion mitochondrial DNA (Scorpiones: Buthidae). Proceedings of the Royal Society of London B: Biological Sciences. 2005; 272(1564):697–704.
33. Graham MR, Myers EA, Kaiser RC, Vet V. Cryptic species and co-diversification in sand scorpions from the Karakum and Kyzylkum deserts of Central Asia. Zoologica Scripta. 2019; 48(6):801–12.

34. Quintero-Hernández V, Jiménez-Vargas JM, Gurrola GB, Valdivia HH, Possani LD. Scorpion venom components that affect ion-channels function. Toxicon. 2013; 76:328–42. https://doi.org/10.1016/j.toxicon.2013.07.012 PMID: 23891887

35. de Oliveira UC, Candido DM, Coronado Dorce VA, Junqueira-de-Azevedo IdLM. The transcriptome recipe for the venom cocktail of Tityus bahiensis scorpion. Toxicon. 2015; 95:52–61. https://doi.org/10.1016/j.toxicon.2014.12.013.

36. Carvalho D, Kuniyoshi A, Kodama R, Oliveira A, Serrano S, Tambourgi De et al. Neuropeptide Y family-degrading metallopeptidases in the Tityus serrulatus venom. Toxins 2019; 11:194.

37. Fletcher PJ, Fletcher M, Weninger K, Anderson T, Martin B. Vesicle-associated membrane protein (VAMP) cleavage by a new metalloprotease from the Brazilian scorpion Tityus serrulatus. Journal of Biological Chemistry. 2010; 285:7405–16.

38. Ministerio de Salud de la Republica Argentina. Guı´a de Prevención, Diagnóstico, Tratamiento y Vigilancia Epidemiológica del Envenenamiento por Escorpiones. Buenos Aires: Programa Nacional de Prevención y Control de las Intoxicaciones—Precotox; 2011.

39. Borges A, Tsushima RG, Backx PH. Antibodies against Tityus discrepans venom do not abolish the effect of Tityus serrulatus venom on the rat sodium and potassium channels. Toxicon. 1999; 37:867–81.

40. Ortega-E, Rendón-Anaya M, Rego SC, Schwartz EF, Possani LD. Antarease-like Zn-metalloproteases are ubiquitous in the venom of different scorpion genera. Biochimica et Biophysica Acta (BBA)—General Subjects. 2014; 1840:1738–46.
53. Pessini AC, Takao TT, Cavaleiro EC, Vichnewski W, Sampaio SV, Giglio JRet al. A hyaluronidase from *Tityus serrulatus* scorpion venom: isolation, characterization and inhibition by flavonoids. Toxicon. 2001; 39(10):1495–504. http://dx.doi.org/10.1016/S0041-0101(01)00122-2.

54. de Oliveira UC, Nishiyama M.Y. Jr., Dos Santos M.B.V., Santos-da-Silva A.P., Chalkidis H.M., Souza-Imberg A. et al. Proteomic endorsed transcriptomic profiles of venom glands from *Tityus obscurus* and *T. serrulatus* scorpions. PLoS One. 2018 13(3):e0193739. https://doi.org/10.1371/journal.pone.0193739 PMID: 29561852

55. Guerra-Durarte C, Rebelo Horta CC, Ribeiro-Oliveira-Mendes BB, de Freitas Magalhães B, Costal-Oliveira F, Stransky Set al. Determination of hyaluronidase activity in *Tityus* spp. Scorpion venoms and its inhibition by Brazilian antivenoms. Toxicon. 2019; 167:134–43.

56. Mendes TM, Dias F, Horta CCR, Pena LF, Arantes EC, Kalapothakis E. Effective *Tityus serrulatus* antivenom produced using the Ts1 component. Toxicon. 2008; 52:787–93.

57. Sousa P, Froufe E, Alves PC, Harris DJ. Genetic diversity within scorpions of the genus *Buthus* from the Iberian Peninsula: mitochondrial DNA sequence data indicate additional distinct cryptic lineages. Journal of Arachnology. 2010; 38:206–11.

58. Hernández RM, Jordan TE, Dalenz Farjat A, Echavarria L, Idleman BD, Reynolds JH. Age, distribution, tectonics, and eustatic controls of the Paranense and Caribbean marine transgressions in southern Bolivia and Argentina. Journal of South American Earth Sciences. 2005; 19(4):495–512. https://doi.org/10.1016/j.jsames.2005.06.007.

59. Brea M, Zucol A. The Paraná-Paraguay Basin: Geology and Paleoenvironments Overview of the Geology and Geography. In: Albert J.S., Reis RE, editors. Historical biogeography of Neotropical freshwater fishes. Berkeley: University of California Press; 2011. p. 69–87.

60. Delsuc F, Superina M, Tilak M-K, Douzery EJP, Hassanin A. Molecular phylogenetics unveils the ancient evolutionary origins of the enigmatic fairy armadillos. Molecular Phylogenetics and Evolution. 2012; 62(2):673–80. http://dx.doi.org/10.1016/j.ympev.2011.11.008.

61. Morando M, Medina CD, Avila LJ, Perez CHF, Buxton A, Sites JW Jr. Molecular phylogeny of the New World gecko genus *Homonota* (Squamata: Phyllodactylidae). Zoologica Scripta. 2014; 43(3):249–60. https://doi.org/10.1111/zsc.12052

62. Brusquetti F, Netto F, Baldo D, Haddad CFB. What happened in the South American Gran Chaco? Diversification of the endemic frog genus *Lepidobatrachus* Budgett, 1899 (Anura: Ceratophryidae), Molecular Phylogenetics and Evolution. 2018; 123:123–36. doi:https://doi.org/10.1016/j.ympev.2018.02.010.

63. Fet V, Lowe G. Buthidae. In: Fet V, Sissom W.D., Lowe G, Braunwalder M.E., editor. Catalog of the Scorpions of the World (1758–1998). New York: New York Entomological Society; 2000. p. 54–286.