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Molecular systematics of the neotropical scorpion genus *Tityus* (Buthidae): The historical biogeography and venom antigenic diversity of toxic Venezuelan species☆

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ABSTRACT

We provide a mitochondrial DNA-based phylogenetic hypothesis for 21 *Tityus* species collected in Venezuela, Trinidad, Brazil and Panama, including 12 taxa known to be toxic to humans. Our phylogenetic reconstruction is based on 850 nucleotides of the combined cytochrome oxidase subunit I and 16S rRNA genes for most species, and centered on Venezuelan scorpions owing to the detailed taxonomic and biogeographic information available for *Tityus* in this region. The principal phylogenetic result was the strong support for mtDNA clades representing geographical groupings associated with the Perijá mountain range, the Mérida Andes, or the central and eastern coastal ranges in Venezuela, suggesting that vicariance has been a potent force in the diversification of local scorpions. Venezuelan *Tityus* species have been organized by González-Sponga into three artificial morphological groups, "androcottoides", "discrepans", and "nematochirus", based on the array of ventral carinae in metasomal segments II–IV. We also incorporated a fourth morphological group ("*Tityus clathratus*"), recently documented in Venezuela. Our results do not support the clustering of the species in the "androcottoides" and "discrepans" morphological groups, which include the majority of taxa of medical importance, but provided support for the "nematochirus" species group. *T. clathratus* was found to cluster with the Brazilian *T. serrulatus* and *T. bahiensis*. Divergence times of most clades are consistent with major events in the geological history of northern Venezuela and suggest that many Venezuelan *Tityus* species formed in the late Miocene and the Pliocene. In turn, we used the *Tityus* mtDNA phylogeny to determine the potential utility of phylogenetic systematics to predict *Tityus* venom antigenic reactivity by testing the recognition of *T. nororientalis*, *T. discrepans*, *T. zulianus*, *T. perijanensis*, and *T. clathratus* venoms by anti-*T. discrepans* horse antibodies. Cross-reactivity was significantly higher for the closely

Abbreviations: COI, subunit I of cytochrome oxidase; ELISA, enzyme-linked immunosorbent assay; mtDNA, mitochondrial deoxyribonucleic acid; PCR, polymerase chain reaction; 16S rRNA, large subunit (16S) ribosomal ribonucleic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

☆ The authors wish to dedicate this article to the memory of the late Professor Manuel A. González-Sponga (1929–2009), a Venezuelan arachnologist whom described most of the known Venezuelan scorpion fauna.

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related eastern (*T. nororientalis*) and central coastal (*T. discrepans*) species in comparison to the distantly related Andean (*T. zulianus*) and Perijá (*T. perijanensis*) species. Reactivity of *T. clathratus* low mol. mass toxic components towards anti-*T. serrulatus* and anti-*T. discrepans* antivenoms was low, suggesting that venom components produced by the subgenus *Archaeotityus* (which encompass "clathratus" species) diverge antigenically from other *Tityus* scorpions.

1. Introduction

Tityus is the largest scorpion genus, with almost 170 known species (Fet and Lowe, 2000; González-Sponga, 1996a, 2001a; Lourenço, 2002a, 2006). The genus is exclusively Neotropical, with species that range from Costa Rica and the Lesser Antilles plus Puerto Rico to northern Argentina. *Tityus* reaches its greatest diversity in northwestern South America, with roughly half the species described from Colombia, Ecuador and Venezuela (Borges et al., 2006a; De Sousa et al., 2006; González-Sponga, 1981, 1984, 1985, 1987, 1994a,b, 1996a,b, 1997a,b, 2001a,b, 2002, 2004, 2005; González-Sponga et al., 2001; Lourenço, 1997a,b, 1998, 2000, 2002b, 2007; Lourenço and Otero-Patiño, 1998; Quiroga et al., 2004; Rojas-Runjaic and de Armas, 2007; Rojas-Runjaic and De Sousa, 2007). The genus occurs principally in mesic environments (Lourenço, 2001), and is often found in habitats shared with humans. Co-distribution with humans coupled to the toxicity of the species in this genus accounts for the fact that most severe and lethal cases of scorpion envenoming in South America and the Caribbean region are due to stings by *Tityus* (Lourenco and Cloudsley-Thompson, 1996; Becerril et al., 1997; Borges and De Sousa, 2006; Borges et al., 2006b; Daisley et al., 1999; De Sousa et al., 2000; Gómez and Otero, 2007; Otero et al., 2004).

Considerable attention has been focused on *Tityus* species descriptions and taxonomy in the time since C.L. Koch erected the genus in 1836. Pocock (1894) provided the first synopsis of Caribbean *Tityus* species; Kraepelin (1911) reviewed the species belonging to the "*Tityus bolivianus*" group; Mello-Campos (1924) reviewed the Brazilian species and Mello-Leitão (1934) revised the species from Argentina. However, it was not until Mello-Leitão's monograph (1945) that a comprehensive study of the genus and its distribution was published, distinguishing 15 morphological groupings ('Formenkreise') of *Tityus* species. More recent investigations by González-Sponga (1978, 1981, 1984, 1985, 1987, 1994a,b, 1996a,b, 1997a,b, 2001a,b, 2002, 2004a,b, 2005), González-Sponga et al. (2001), Lourenço (1984a, 1992, 1996a, 1997a,b, 1998, 2000, 2002a,b, 2005, 2006, 2007), Lourenço and Bruehmueller-Ramos (2004), Lourenço and Otero-Patiño (1998), Lourenço and Pézier (2002), Lourenço and Ponce de Leão Giupponi (2004), Lourenço et al. (2005), Francke and Stockwell (1989), Prendini (2001), Quiroga et al. (2004), De Sousa et al. (2006), Borges et al. (2006a), Rojas-Runjaic and de Armas (2007), and De Souza et al. (2009) have not only increased the species count in the genus but have also underscored differences in opinion regarding the systematic relationships of *Tityus* species groups. The study of such relationships is fully warranted in order to provide new insights into biogeographical patterns and a framework for analyses of venom diversity in this medically important lineage.

Contemporary *Tityus* systematists have used different aspects of the morphological diversity of the genus in their analysis and description of species groups (Lourenço, 2000, 2002a; Lourenço, 2006; González-Sponga, 2001a, 2002), and have also studied different regional assemblages of scorpions. With the intent to further organize the genus using already available genus/group names, Lourenço (2006) has recently proposed a subdivision of *Tityus* into five subgenera, namely *Archaeotityus* Lourenço, *Atreus* Gervais, *Brazilotityus* Lourenço, *Caribetityus* Lourenço, and *Tityus* C. L. Koch, mainly in order to maintain the stability of the used names. Some authors have suggested that a thorough revision of the genus still remains urgently needed given the ample morphological variations in *Tityus* (Rojas-Runjaic and de Armas, 2007). Such variations are highlighted by the fact that phenotypic differences among the species comprising named species groups exceeds the morphological diversity described for many genera of Old World scorpions (Fet and Lowe, 2000).

We have focused our attention on Venezuelan *Tityus* in order to take advantage of the research of González-Sponga (1981, 1984, 1985, 1987, 1994a,b, 1996a,b, 2001a,b, 2002, 2004a,b, 2005) who has organized the Venezuelan species currently recognized into three morphological groups, depending on the number and array of ventral carinae on metasomal segments II–IV: the "androcottoides" and "nematochirus" groups (already proposed by Mello-Leitão in 1945) and the group "discrepans", erected to accommodate those species characterized by the presence of a single ventromedian carina in the above segments. This latter group has also been documented for the island of Trinidad by Prendini (2001). According to González-Sponga (1996a), the "androcottoides" group is recognized on the basis of paired carinae in the proximal portion of metasomal segments II–IV, and single or convergent carinae in the distal portion of these segments, whereas species belonging to the "nematochirus" group are characterized by paired ventrosubmedian carinae on the same segments.

We have also included in our study a fourth morphological group, termed "*Tityus clathratus*" by Mello-Leitão (1945) and Lourenço (1984a), as Rojas-Runjaic and de Armas (2007) have recently documented its systematics, distribution, and species composition in Venezuela. Species belonging to this group are clearly distinguished from the other *Tityus* on the basis of their smaller (18–40 mm-long) size, pigmentation throughout their bodies, and presence of a rhomboidal subaculear tubercle. Taxa from this group were not assigned by González-Sponga (1996a) to any of the above Venezuelan groupings given "its dubious systematic position", although

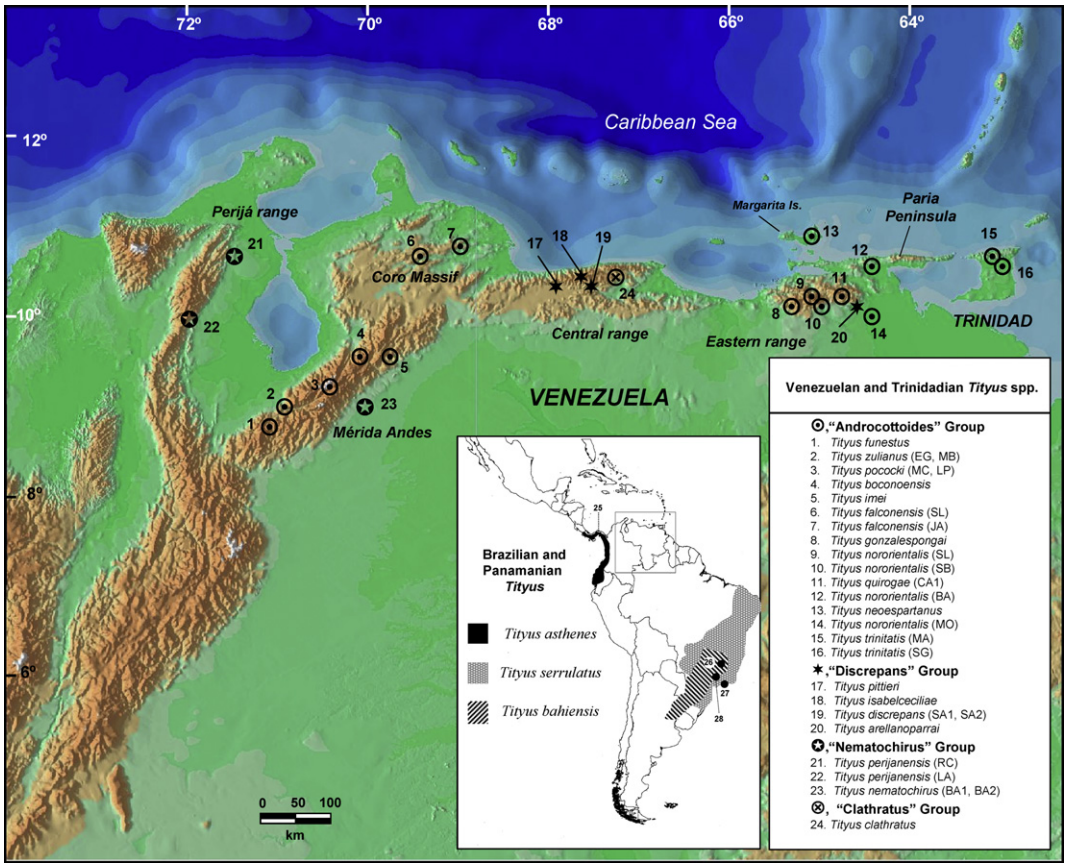

Fig. 1. Map showing the collection localities of *Tityus* species in Venezuela and Trinidad. Inset map shows the collection localities and geographic distribution of the *Tityus* species from Brazil and Panama included in this study. Distribution data for the two Brazilian species, *Tityus bahiensis* and *Tityus serrulatus*, are taken from Lourenço (2002a) and De Souza et al. (2009), respectively.

"clathratus" species possess the double metasomal carinae characteristic of the "nematochirus" group.

As González-Sponga's systematic hypothesis (González-Sponga, 1996a, 2001a, 2002) suggests that the described metasomal characters are synapomorphic for the named morphological groups, one of the objectives of our *Tityus* study is to test such proposal cladistically. Our phylogenetic analysis utilizes 17 Venezuelan, two Brazilian, one Panamanian, and one Trinidadian species of *Tityus* scorpions (Fig. 1), and is based on mitochondrial DNA (mtDNA) sequences represented by 540 bp region of the cytochrome oxidase subunit I gene (COI) and a 318–332 bp region of the large ribosomal subunit RNA (16S rRNA) gene. The Venezuelan species represent each of the four named species groups defined above (Fig. 2). Furthermore, the selected species represent four different geographical regions in Venezuela: the Perijá mountain range in the western part of the country, the Mérida Andes, the central (Cordillera de la Costa) coastal range, and the eastern (Serranía Oriental del Interior/Paria Peninsula) coastal range. In this sense, this study also intends to assess the role played by geography and earth history in the diversification of Venezuelan *Tityus* scorpions.

Our sampling design includes species that are toxic to humans, as well as species whose lethality or reactivity towards the only available Venezuelan scorpion antivenom (prepared against *Tityus discrepans*) has not been determined. Specific antivenoms remain the only effective therapeutic measure for treating scorpion envenomation (Espino-Solís et al., 2009), which clinical manifestations include functional exacerbation of the sympathetic and parasympathetic nervous systems (Amitai, 1998). Previous studies have recognized that *Tityus* species are highly diverse from the stand point of the antigenicity of their venom components (Borges et al., 1999, 2008). The venom neurotoxic components are low mol. mass (*ca.* 3–8 kDa) proteins with high affinity towards their pharmacological receptors in excitable tissues and immune cells (i.e. voltage-gated ion channels), which need to be neutralized rapidly in humans envenomed by *Tityus* spp. to prevent death mainly from respiratory distress (Amaral and Rezende, 2000; Gazarian et al., 2005; Espino-Solís et al., 2009). In this respect, Borges et al. (2008) in a study on the antigenicity of *Tityus* venoms from Brazil and Venezuela concluded that the extent of antigenic cross-reactivity among species depends on the studied taxa rather than the geographical distance between their habitats. As there is a need to establish the antigenic cross-reactivity for those *Tityus* species that have yet to be characterized epidemiologically and to provide a rationale for the use of available

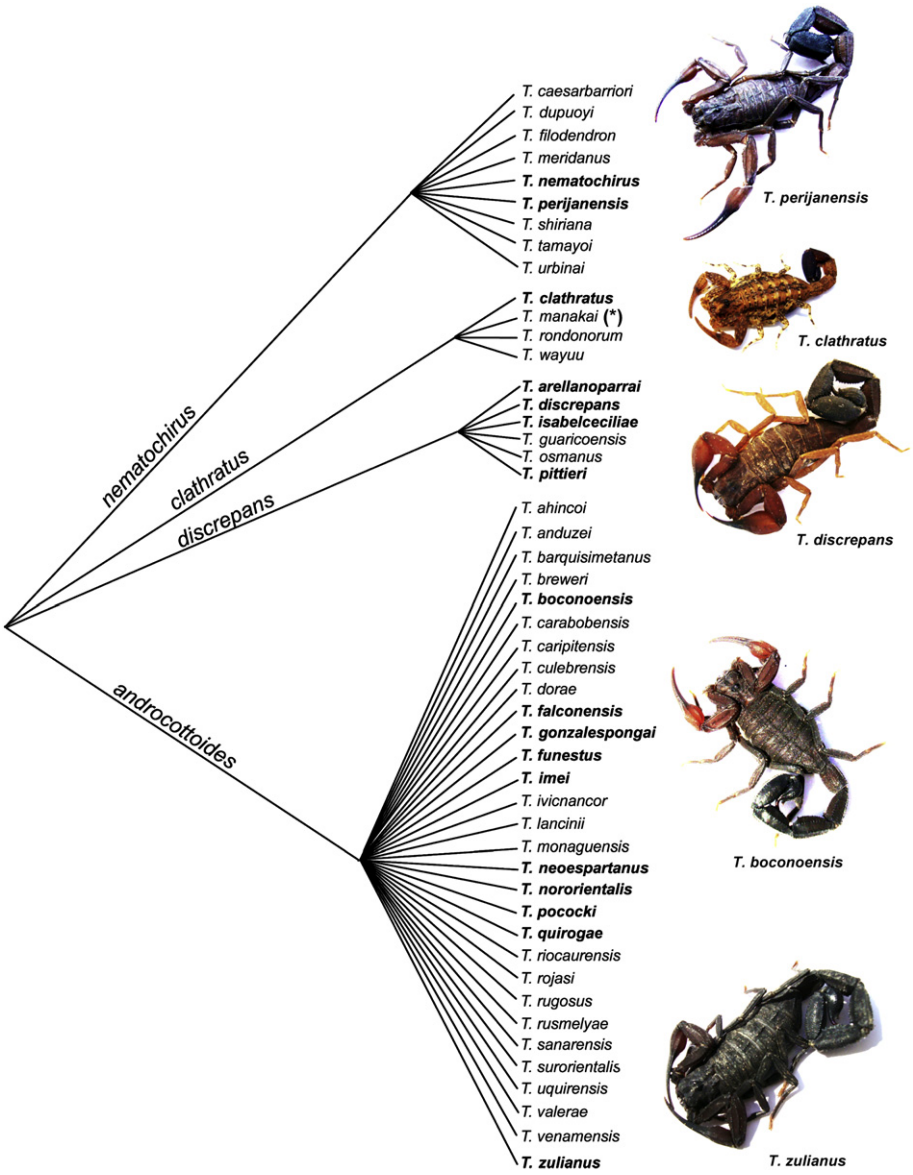

Fig. 2. Hypothesis of phylogenetic relationships among Venezuelan *Tityus* species based on the morphological groups of González-Sponga (1996a, 2001a). A fourth group of species ("*Tityus clathratus*") has been included based on the evidence provided by Rojas-Runjaic and de Armas (2007). Species names in bold have been sequenced for the molecular systematic analysis of *Tityus*. Representative species of each group are shown on the right (photographs not to scale). (*) *Tityus manakai* González-Sponga, a species not previously assigned to a morphological group (González-Sponga, 2004a), belongs to the "clathratus" group (F. Rojas-Runjaic, pers. com.).

antivenoms (Borges and De Sousa, 2006; De Sousa et al., 2000), a third objective of this study is to determine the potential utility of mtDNA-based phylogenetic systematics to predict antigenic reactivity for *Tityus* venoms.

2. Materials and methods

2.1. Study species

A list of the scorpion species sampled for this study is provided in Table 1 and Fig. 1 shows the geographic distributions of the 21 *Tityus* species analyzed in this work, of which 17 were collected in Venezuela (see Section 2.2). We also included *Tityus asthenes* Pocock from Panama, which ranges into the Pacific coast of Colombia and also Ecuador (Lourenço, 1997a,b, 2002b), *Tityus trinitatis* Pocock from the island of Trinidad, and two Brazilian species, *Tityus serrulatus* Lutz and Mello, and *Tityus bahiensis* Perty.

Excluding specimens provided as gifts (see Acknowledgements), adult scorpions were collected at night from the localities listed in Table 1 using ultraviolet light (Stachel et al., 1999). The specimens were transported live from the field and the Venezuelan species were identified according

Table 1
Species names, sample sizes (*N*), and collection locations of the *Tityus* species and outgroups analyzed in this study. Numbers and abbreviations in brackets after the species names indicate map locations (Fig. 1) and collection sites (Fig. 3), respectively.

Species	*N*	Morphological group[a]	Collection site	Geographical coordinates
T. arellanoparrai (20)	1	1	Cerro Boquerón (near Yurucual), Monagas, VE	10°10′40″N, 63°25′30″W
T. asthenes (25)	1	3	Barro Colorado Island, Panama Canal, PA	9°9′28″N, 79°50′49″W
T. bahiensis (26)	1	N.D.	Ouro Preto, Minas Gerais, BR	20°23′05″S, 43°30′14″W
T. boconoensis (4)	1	2	Pampán, Trujillo, VE	9°24′42″N, 70°31′00″W
T. clathratus (24)	1	4	El Hatillo, Miranda, VE	10°25′59″N, 66°49′10″W
T. discrepans (19, SA1,2)	2	1	San Antonio de los Altos, Miranda, VE	10°23′01″N, 66°56′58″W
T. falconensis (7, JA)	1	2	Jacura, Falcón, VE	11°04′04″N, 68°50′58″W
T. falconensis (6, SL)	1	2	San Luis, Falcón, VE	11°06′59″N, 69°42′12″W
T. funestus (1)	1	2	Bailadores, Mérida, VE	8°15′13″N, 71°49′40″W
T. gonzalespongai (8)	2	2	La Orquídea, Anzoátegui, VE	10°00′44″N, 64°22′39″W
T. imei (5)	1	2	Hacienda La Guayana (near Biscucuy), Portuguesa, VE	9°21′30″N, 69°55′30″W
T. isabelceciliae (18)	1	1	El Junko, Federal District, VE	10°28′49″N, 67°03′39″W
T. nematochirus (23, BA1,2)	2	3	Barinitas, Barinas, VE	8°46′22″N, 70°25′49″W
T. neoespartanus (13)	2	2	Cerro Copey, Margarita Island, VE	11°03′14″N, 63°53′46″W
T. nororientalis (12, BA)	1	2	Barcelona (near Morro Puerto Santo), Sucre, VE	10°41′05″N, 63°08′12″W
T. nororientalis (14, MO)	1	2	Mosú (near Quiriquire), Monagas, VE	9°58′47″N, 63°13′38″W
T. nororientalis (10, SB)	1	2	Sector Belén (near Cocollar), Monagas, VE	10°10′16″N, 63°46′13″W
T. nororientalis (9, SL)	1	2	San Lorenzo (near Cumanacoa), VE	10°13′00″N, 63°55′06″W
T. perijanensis (21, RC)	1	3	Fundo La Orchila, Mara Municipality, Zulia, VE	10°48′44"N, 72°21′13"W
T. perijanensis (22, LA)	1	3	Ipika, Machiques de Perijá Municipality, Zulia, VE	9°52́53N, 72°5102"W
T. pittieri (17)	1	1	Rancho Grande Biological Station, Aragua, VE	10°22′00″N, 67°41′02″W
T. pococki (3, MC)	1	2	Mérida City, Mérida, VE	8°36′03″N, 71°09′52″W
T. pococki (3, LP)	1	2	La Pedregosa (near Mérida City), Mérida, VE	8°37′04″N, 71°07′11″W
T. quirogae (11, CA1)	2	2	Caripe, Monagas, VE	10°09′55″N, 63°30′14″W
T. serrulatus (28, RP)	1	N.D.	Ribeirão Preto, São Paulo, BR	21°09′09″S, 47°49′05″W
T. serrulatus (27, SP)	1	N.D.	São Paulo, São Paulo, BR	22°50′01″S, 45°14′02″W
T. trinitatis (15, MA)	1	2	Matelot, TT	10°49′02″N, 61°07′06″W
T. trinitatis (16, SG)	1	2	Near Sangre Grande, TT	10°35′43″N, 61°07′28″W
T. zulianus (2, EG)	1	2	El Guayabal, Mérida, VE	8°20′59″N, 71°38′58″W
T. zulianus (2, MB)	1	2	Mesa Bolívar, Mérida, VE	8°20′59″N, 71°38′58″W
Rhopalurus laticauda (CH)	1	Outgroup	Charallave, Miranda, VE	10°14′18″N, 66°51′36″W

[a] Morphological groups for Venezuelan *Tityus* are: 1. "discrepans", 2. "androcottoides", 3. "nematochirus", and 4. "clathratus." N.D., Morphological group not assigned to the Brazilian species by González-Sponga (1996a). The Panamanian *T. asthenes* and the Trinidadian *T. trinitatis* correspond to the groups "nematochirus" and "androcottoides," respectively, according to the arrangement of metasomal ventral carinae. BR, Brazil; PA, Panama; TT, Trinidad and Tobago; VE, Venezuela.

to González-Sponga (1996a,b), Quiroga et al. (2004), De Sousa et al. (2006) and Borges et al. (2006a), and the Panamanian and Trinidadian species were identified following Lourenço (2000) and Lourenço (1984b), respectively. Two or more individuals were sequenced for 12 of the 25 study species (including outgroups).

2.2. *Venezuelan* Tityus *systematic hypothesis*

In Fig. 2 we present the systematic hypothesis based on morphology derived from González-Sponga's 1996 compendium of the Venezuelan scorpion fauna, in which he recognized three monophyletic artificial *Tityus* groupings, namely "androcottoides", "discrepans", and "nematochirus". We have included a fourth morphological group in Fig. 2 to accommodate the species *Tityus clathratus* C.L. Koch.

To test such systematic hypothesis, we included in our analyses 11 species belonging to the "androcottoides" group, four "discrepans" species, three species belonging to the "nematochirus" group, and one "clathratus" species. In addition to the Venezuelan species shown in Fig. 2 (in bold face), the Panamanian *T. asthenes* displays the paired, metasomal ventral carinae characteristic of the Venezuelan "nematochirus" group and the Trinidadian *T. trinitatis* conforms to the "androcottoides" metasomal pattern described by González-Sponga (1996a, 2001a). It should be noted that the Venezuelan scorpions *Tityus zulianus* González-Sponga, *Tityus nororientalis* G.-S., *Tityus neoespartanus* G.-S., *Tityus quirogae* De Sousa et al., and *Tityus falconensis* G.-S. in the "androcottoides" group, *Tityus discrepans* (Karsch) and *Tityus isabelceciliae* González-Sponga et al. in the "discrepans" group, *Tityus perijanensis* González-Sponga in the "nematochirus" group as well as the Panamanian, Trinidadian, and Brazilian *Tityus* species are all reported toxic to humans, with varying levels of lethality (Bucaretchi et al., 1995; Borges, 1996; Daisley et al., 1999; De Sousa et al., 2000; González-Sponga et al., 2001; Borges et al., 2002, 2004a,b, 2006b; Guinand et al., 2004; Borges and De Sousa, 2006; Borges and Rojas-Runjaic, 2007; De Sousa et al., 2007; Gómez and Otero, 2007).

2.3. *DNA extraction, amplification, and sequencing*

DNA was extracted by digesting 0.1–0.5 g of fresh or preserved (in 95% ethanol) muscle tissue from pedipalps or caudal segments in 500 μl of lysis buffer (10 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA, pH 8.0, 0.5% SDS) containing 0.2 mg/ml proteinase K (Promega) for 6–14 h at 37 °C, according to Delabre et al. (1995). After phenol/chloroform/isoamyl alcohol extraction of digested tissue, DNA was precipitated with isopropanol and resuspended in nuclease-free water to a final concentration of 50–100 ng/μL.

Amplification of mtDNA genes was carried out in 50-μl reactions containing 1 μl DNA (~50–100 ng), 1X PCR Buffer II (Perkin–Elmer, Forest City, CA), 2 mM $MgCl_2$, 2 μM each of dATP, dCTP, dGTP, and dTTP, 2 pM of each primer, and 1.25 Units of AmpliTaq DNA polymerase (Perkin–Elmer, Forest City, CA). We used published primers for the COI and 16S rDNA PCR amplifications and sequencing. The forward primer for COI amplification was C1-J-2183 (5′-CAACAT TTATTTTGATTTTTTGG-3′), designed by Simon et al. (1994) for a conserved region at the C-terminus of insect COI. The reverse primer was COIKG-R2 (5′-GATATTAATCCTAAAAAA TGTTGAGG-3′), designed by Tanaka et al. (2001) for amplification of *Apis* COI. COI amplification conditions were as follows: (1) initial denaturation at 94 °C for 5 min; (2) four cycles of denaturing at 94 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min; (3) 29 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min; and (4) a final extension phase at 72 °C for 5 min.

For 16S rRNA amplifications, the forward primer (16SF, 5′-CGATTTGAACTCAGATCA-3′, Gantenbein et al., 1999) was a scorpion-specific version of the universal primer LR-J-12887 of Simon et al. (1994). The reverse primer (16SR, 5′-GTGCAAAGGTAGCATAATCA-3′) was a 16S rRNA scorpion-specific sequence reported by Gantenbein et al. (1999). These primers amplify a 400 bp region at the 3′ end of the 16S rRNA gene, encompassing the conserved peptidyl transferase center (Hedin and Maddison, 2001; Smith and Bond, 2003). Amplification conditions were as follows: (1) initial denaturation at 94 °C for 5 min; (2) 30 cycles of denaturing at 94 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 30 s; and (3) a final extension phase at 72 °C for 7 min.

Typically we observed a single amplification product for both COI (540 bp) and 16S rRNA (approximately 330 bp), which was excised from 1% low-melting agarose gels and extracted using GELase™ (Epicentre Technologies, Madison, WI) for 12–15 h at 45 °C. Purified products were cycle-sequenced using the Taq-DyeDeoxy Terminator Cycle Sequencing kit, which uses D-Rhodamine-based dideoxy-chain termination reactions (Applied Biosystems, Foster City, CA). COI PCR fragments were sequenced using primers C1-J-2183 and C1-J-2441 (described by Simon et al., 1994). Sequencing of 16S rRNA PCR fragments was performed using the PCR primers. The protocol for the sequencing reactions was: 94 °C for 15 s, decrease temperature 1 °C per second to 50 °C, 50 °C for 1 min, increase temperature 1 °C per second to 60 °C, 60 °C for 4 min for 25 cycles. The reactions were then centrifuged through Sephadex™ (Princeton Separations) columns, and dried down on a Savant Speed-Vac for 30 min. Samples were resuspended in loading dye and sequenced to obtain bidirectional sequences using an ABI 377 automatic DNA sequencer.

Following gel electrophoresis, chromatograms were imported into Sequencher 4.1 (Genes Codes) and aligned by eye. Nucleotide sequences were checked for reading-frame errors, and improbable amino acid replacement substitutions. COI protein-coding sequences were translated using MacClade (Version 3.07, Maddison and Maddison, 1992) and compared to the amino acid sequence for the homologous region of the COI gene published for spiders of the subfamily Dendryphantinae (Hedin and Maddison, 2001) and for insects (Lunt et al., 1996). 16S rRNA sequences were aligned taking into account the secondary structure prediction for 16S rRNA (Gutell, 1996), including the conserved regions forming stems and the hypervariable regions containing loops and inner stems in other arachnids (Smith and Bond, 2003). Location of conserved and variable regions in the 16S *Tityus* alignment was optimized further by comparing it to the alignment (ALIGN_000479-481) established by Gantenbein and Largiadèr (2003) for *Buthus occitanus* 16S rRNA sequences, which we downloaded from the EMBL nucleotide sequence database (http://www.ebi.ac.uk). Due to difficulties in the alignment of the 16S data set and to avoid introducing noise in the data, we excluded 20 bp (8 bp between nucleotides 113–120 and 12 bp between nucleotides 187–198 in our alignment) and placed gaps in the hypervariable region to maximize the alignment.

2.4. Phylogenetic analysis

A Nexus file was created with each gene region designated as a separate partition, exported from Sequencher 4.1. Maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) analyses were performed separately for both COI and 16S using PAUP* Version 4.0b10 (Swofford, 2002). Our ME analyses were based on genetic distances calculated under the LogDet model (Steel, 1994) in order to account for any nucleotide composition bias among the scorpion lineages analyses. In MP analyses, we used stepwise addition with 1000 randomized input orders and the Goloboff fit criterion with $k = 2$ (Goloboff, 1993), which weights against homoplasies. Results using $k = 2$ are presented, given that analyses using different weighting schemes did not show variations among tree topologies. For the 16S data, gaps placed within hypervariable regions were treated as fifth characters. We used TBR based heuristic searches in all analyses. We used 1000 bootstrap replications to assess confidence for the phylogenetic hypotheses presented here. Average divergence within and between mtDNA clades was calculated using Sequencer version 6.1.0 (http://nmg.si.edu/Sequencer.html).

An evaluation of data partition incongruence using the partition homogeneity test (Farris et al., 1995) implemented in PAUP* revealed no conflicting nodes ($p = 0.93$). Thus, we also analyzed the combined COI-16S data using the above approaches, and also a Bayesian approach using the Markov chain Monte Carlo method (Steel, 1994) implemented in *MrBayes* version 3.0b5 (Huelsenbeck and Ronquist, 2001). This analysis employed the general time reversible model (nst = 6). Four Monte Carlo Markov chains (MCMC, Yang and Rannala, 1997) were run 6,000,000 generations and sampled every 100 generations. Inspection of the resulting ML scores suggested that likelihood stationarity was reached by 3000 generations; we discarded the trees sampled from the first 5000 generations. A majority rule consensus of the remaining sampled trees was generated with PAUP* to provide a phylogenetic hypothesis with associated marginal posterior probability values for internal branches. The posterior probability indicates the frequency

of a given clade among the trees sampled in the Bayesian analyses (Huelsenbeck and Ronquist, 2001).

We used the SH test (Shimodaira and Hasegawa, 1999) implemented in PAUP* to test a null hypothesis of no difference between a tree based on the morphological hypothesis of González-Sponga (1996a, 2001a, 2002) that underlies the named species groups in the genus (including "clathratus") and the best mtDNA phylogenetic tree.

2.5. Rate constancy and estimation of divergence time

Maximum likelihood scores (Huelsenbeck et al., 1996) were estimated for trees with and without a molecular clock enforced to test for rate constancy. Scores were statistically compared using the likelihood ratio test (LRT). We used the Akaike Information Criterion (AIC) in the program Modeltest v.3.06 (Posada and Crandall, 1998) to select the model of nucleotide substitutions for the combined COI and 16S dataset. ML analyses were performed using the general time reversible (GTR) + Γ (gamma distribution of rates among sites) + I (proportion of invariant sites) model of sequence evolution with the following parameters settings: R-Matrix = (R(a) [A–C] = 0.6118; R(b) [A–G] = 14.8404; R(c) [A–T] = 3.0657; R(d) [C–G] = 0.7054; R(e) [C–T] = 5.4744; R(f) [G–T] = 1.0000); base frequencies = (A = 0.2941; C = 0.1106; G = 0.1699; T = 0.4254); proportion of invariant sites = 0.4708; and shape parameter of gamma distribution = 1.0199.

For estimation of divergence times among clades of *Tityus*, we used two different approaches. First, we applied a calibration of 2.9% sequence divergence per million years (Ma) estimated for populations of *Buthus occitanus* Amoreux separated by the re-opening of the Strait of Gibraltar 5.2 Ma ago (Gantenbein and Largiadèr, 2003). These authors also used combined COI and 16S mtDNA data.

We also estimated divergence times and evolutionary rates using paleogeographic information. We employed a Markov chain Monte Carlo (MCMC) approach to estimate posterior probability distributions of absolute divergence times following the method of Thorne et al. (1998) and Kishino et al. (2001), and implemented for multigenic data (Thorne and Kishino, 2002) in Thorne's software package "multidistribute" version 05/Aug/03 (ftp://statgen.ncsu.edu/pub/thorne/). Divergence times were estimated using all sequences of both genes, assuming the Bayesian consensus topology based on the combined data. We temporally constrained two nodes on the tree: (1) the divergence time of *T. asthenes* and *T. perijanensis* was tied to the interval 11.8–2.7 Ma based on the formation of the Eastern Cordillera of the Andes to which the two species are restricted (Díaz de Gamero, 1996; Gregory-Wodzicki, 2000); and (2) the divergence of *Tityus* "central" and "eastern" clades was constrained by the formation of the Cariaco basin at approximately 2 Ma ago (Schubert, 1982).

These analyses were based on the following parameters: *sampfreq* = 100; *numsamps* = 10,000; *burnin* = 100,000. The following priors were used: *brownmean* = 0.5; *brownsd* = 0.5; *rttm* = 3.5 (i.e. 35 Ma); *rttmsd* = 1.0; *bigtime* = 1000. Since the value chosen for *rtrate* has a significant effect on the time estimate for nodes near the base, the prior value was assumed to represent the average branch length from ingroup root to ingroup tips based on the output from *estbranches* (*rtrate* = 0.22, *rtratesd* = 0.22). We also performed analyses assuming a rate two times faster (*rtrate* = 0.44, *rtratessd* = 0.44), to test the effect of a higher prior rate on divergence time estimate.

2.6. Venom extraction and immunoreactivity tests

We used enzyme-linked immunosorbent assays (ELISA) to compare the reactivity of venoms extracted from *T. clathratus*, *T. discrepans*, *T. nororientalis*, *T. perijanensis*, and *T. zulianus* against anti-*T. discrepans* horse antivenom (Biotecfar, Facultad de Farmacia, Universidad Central de Venezuela), available for human use. Scorpions collected at the same localities as for the genetic study (Table 1) were milked manually following the procedure of Zlotkin and Shulov (1969) by forcing individuals to sting repeatedly onto Parafilm® sheets. After lyophilization, venom was dissolved in double-distilled water and protein concentration was determined by the method of Lowry et al. (1951). Three hundred nanograms of venom representing each scorpion species (in 100 μl) (pooled from approximately 50 individuals) was adsorbed to the surface of the wells of Hemobag microtitration plates at 4 °C for 12–14 h, and blocked with 3% bovine serum albumin containing Tween-20 for 3 h at room temperature. Plates were washed with saline containing 0.05% Tween-20 and 100 μl of different antivenom dilutions (1:100 to 1:10,000) were added to wells and the plates incubated for 45 min at room temperature. The plates were washed again, and 100 μl of horseradish peroxidase (HRP)-conjugated anti-horse immunoglobulin (Sigma) diluted 1:1000 was added to each well and incubated as described. Wells were washed a third time and 100 μl of ortho-phenylenediamine (Sigma) (1 mg/ml) plus 4 μl of hydrogen peroxide were added and the plates placed at room temperature for 15–20 min before spectrophotometric determination of color change at 495 nm. The significance of statistical differences between absorbance readings was evaluated using a Student's *t*-test.

Immunoblotting was carried out basically as described by Borges et al. (1999) to evaluate the reactivity of *T. clathratus* venom components against anti-*T. discrepans* and anti-*T. serrulatus* (Fundaçao Ezequiel Dias, Belo Horizonte, Brasil) horse antivenoms, the latter also used for human treatment. *T. serrulatus* (from Sigma) and *T. discrepans* venoms were used as controls. Briefly, equal amounts of protein venom samples (30 μg) were electrophoresed on 15% polyacrylamide gels containing sodium dodecyl sulfate and subsequently transferred onto nitrocellulose membranes, which were then incubated with appropriate antivenom dilutions. Successful protein transfer was verified using pre-stained protein standards (BioRad) and also Ponceau S (Sigma) staining of blotted membranes. Secondary antibody was the HRP-conjugated anti-horse immunoglobulin used in ELISA tests. Detection of immobilized antigens was achieved by using the enhanced chemiluminescence method (Amersham) where luminol is oxidised by HRP in the presence of phenol.

3. Results

The mitochondrial COI ($n = 25$) and 16S rRNA ($n = 32$) gene sequences were determined for 21 *Tityus* species. In order to select an outgroup for our study, we performed a Bayesian analysis of the 16S rRNA sequence data in which we included, in addition to the abovementioned *Tityus* species, the following confamilial (Buthidae) taxa (accession numbers in brackets): one species of *Rhopalurus* (*R. laticauda* Thorell, from Charallave, Miranda State, Venezuela; AY586785), two species of *Alayotityus* (*A. delacruzi* Armas (DQ990826) and *A. nanus* Armas (DQ990828)), seven species of *Centruroides* (*C. balsaensis* Ponce and Francke (AF439758), *C. bani* Armas and Marcano Fondeur (AJ288644), *C. exilicauda* (Wood) (AJ288636), *C. infamatus infamatus* C.L. Koch (AF439753), *C. limpidus limpidus* (Karsch) (AF439764), *C. sculpturatus* Ewing (AJ288640), and *C. vitattus* (Say) (AJ288643)), and *Microtityus fundorai* Armas (DQ990830). We selected *R. laticauda* as the outgroup for our analyses based on the results of the Bayesian analysis of alternative topologies, which indicated that *R. laticauda* is sister to the *Tityus* clade *sensu stricto*, since the posterior probability of the monophyly of the latter is 100%. Therefore, *R. laticauda* is as good as any alternative taxon examined as the outgroup for *Tityus*.

All sequences have been deposited in GenBank and have the following accession numbers: AY586753–AY586785 for 16S rRNA sequences, and AY586786–AY586812 for COI sequences. Trees rooted using *R. laticauda* as an outgroup identified mtDNA lineages representing *T. serrulatus, T. clathratus* and *T. bahiensis* (in the 16S tree) as sister taxa to a monophyletic clade comprising all remaining *Tityus* species. These three species also demonstrated high levels of mtDNA sequence divergence from the remaining Venezuelan, Panamanian, and Trinidadian *Tityus*. The two Brazilian species, *T. serrulatus* and *T. bahiensis*, and the Venezuelan *T. clathratus* were used as outgroups in our focused phylogenetic analyses of all remaining *Tityus* species.

3.1. Characterization of the Tityus mitochondrial COI and 16S rRNA genes

The length of the 16S sequences ranged from 314 to 322 bp with a nucleotide composition of 13.6% guanine, 32.3% adenine, 40.2% thymine, and 13.8% cytosine. A chi-square (χ^2) homogeneity test of base frequencies indicated a lack of significant heterogeneity ($p = 0.99$). We used the *Buthus occitanus* 16S rRNA sequence alignment (Gantenbein and Largiadèr, 2003) and the secondary structure posited for other arachnids (Hedin and Maddison, 2001; Smith and Bond, 2003) to guide our alignments. The *Tityus* alignment revealed three hypervariable regions comprising the loops and inner-loops (*sensu* Smith and Bond, 2003) located between the structurally conserved stem and connecting areas that form the peptidyl transferase center identified in other arachnids (Hedin and Maddison, 2001). All nucleotide sequences were completely conserved across species in the stem regions. Alignment was particularly difficult in the loop regions due to high levels of sequence variation; therefore, we decided to exclude 20 bp from our alignment of 16S sequences (as indicated in Section 2). The average number of transitions in the loop regions was 20.11, whereas the average number of transversions was 26.92.

COI sequences were 543 bp in length. Sequence alignment was unambiguous and encoded the C-terminal amino acid region comprising transmembrane segments M7 through M10, extramembrane loops E4 and E5, and intramitochondrial membrane loops I4 and I5, following the nomenclature of Lunt et al. (1996) for the insect COI gene. The correspondence between the structural domains and the patterns of nucleotide variability was similar to that reported for insects (Lunt et al., 1996) and spiders in the subfamily Dendryphantinae (Hedin and Maddison, 2001). In this sense, the most variable areas in the *Tityus* COI alignment corresponded to loops I4 and I5. None of the sequences exhibited stop codons when translated into amino acid sequences; 69.9% of the variable sites and 72.5% of the informative sites are third position substitutions. Nucleotide composition for this region of the COI gene was 22% guanine, 25.1% adenine, 41.8% thymine, and 11.1% cytosine. A chi-square (χ^2) homogeneity test of base frequencies at all positions was not significant ($p = 0.70$); however, focused analyses of base frequencies by codon position demonstrated a significant deviation of expected base frequencies at third positions ($p < 0.01$). We found a high proportion of thymine at third positions (53%) compared to the other bases (adenine = 30%, cytosine = 2%, guanine = 13%).

Because transition saturation is common in mitochondrial DNA sequence data sets, and because our study includes some distantly related taxa, we were concerned about the high proportion of thymine. We plotted the ratio of transitions to transversions against ML genetic distance for each *Tityus* DNA sequence, because as transitions become saturated over time, the Ti:Tv values should steadily decline with genetic distance until a slope of zero is reached. The transition:transversion ratio in *Tityus* COI and 16S leveled off at a ML distance of approximately 0.15, indicative of saturation at this level of divergence (data not shown).

3.2. Molecular systematics of Venezuelan Tityus

Independent analyses of the partitioned mitochondrial genes and combined data produced congruent phylogenies with NJ, MP, or ML methods. A strict parsimony analysis of the 16S data with all positions (stem and loops) weighted equally resulted in ten MP trees, 452 steps in length (Consistency Index = 0.5265, Homoplasy Index = 0.4735, Retention Index = 0.7464). A majority rule consensus of ten trees is presented in Fig. 3. Parsimony analysis of the COI haplotypes yielded minimum-length trees highly congruent with the 16S tree. Tree statistics are: tree length = 663; CI = 0.496; RI = 0.667.

Fig. 4 presents a hypothesis of relationships for *Tityus* scorpions based on a Bayesian analysis of the combined mitochondrial data. Whether based on single gene trees or the combined data, our phylogenetic analyses identified four mtDNA clades (Figs. 3 and 4). The first clade comprises *T. serrulatus* from the state of São Paulo, Brazil, and *T. clathratus* collected from El Hatillo, Central Venezuela, but representing a nationwide distribution (González-Sponga, 1996a; Rojas-Runjaic and de Armas, 2007), with

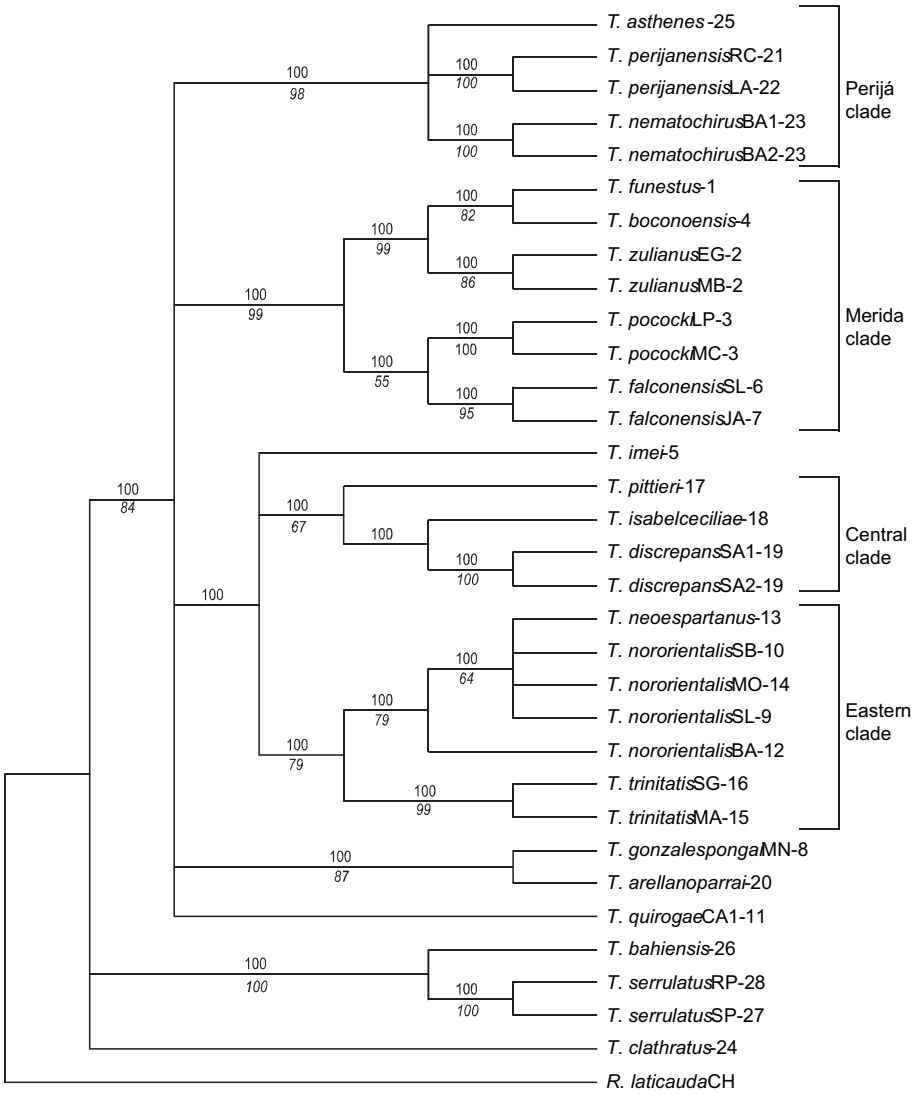

Fig. 3. Hypothesis of phylogenetic relationship among *Tityus* species based on 318–325 nucleotides of the mitochondrial 16S rRNA gene. The *Tityus* cladogram is based on MP analysis (95% Majority Rule consensus of 10 trees). Numbers above branches represent frequencies; numbers below branches represent bootstrap confidence estimates greater than 70%. Tree statistics as follows: tree length, 452; Consistency Index, 0.5265; Homoplasy Index, 0.4735; Retention Index, 0.7464. The tree is rooted to *Rhopalurus laticauda*. Numbers and initials after species names indicate locations in map shown in Fig. 2 and collection sites indicated in Table 1.

a divergence from the other identified clades ranging between 52.92 ± 4.49 and 56.73 ± 3.03%.

A second, well-supported clade (100% bootstrap in the MP tree, 100% posterior probability value in the Bayesian tree), referred to here as the "Perijá" mtDNA clade, included *T. perijanensis* from the Perijá mountain range, *Tityus nematochirus* (Mello-Leitão) from the Llanos (southern) slope of the Mérida Andes in southwestern Venezuela (also known from both slopes of the Eastern Colombian Cordillera [Lourenço, 1997a,b]), and *T. asthenes*, with a range extending from Ecuador to the Pacific coast of Colombia and Panamá (Lourenço, 2000, 2002b; Flórez, 2001).

The third clade, named the "Mérida Andes" clade, has also strong node support and included *Tityus* individuals ($n = 8$) distributed from the highlands of Mérida and Trujillo States (*Tityus funestus* (Hirst), *T. zulianus*, *Tityus pococki* (Hirst), *Tityus boconoensis* González-Sponga) and *T. falconensis* from the Coro Massif, in the border between Lara and Falcón States.

The fourth, "central-eastern" clade, comprised *Tityus* individuals inhabiting the central ($n = 4$) and eastern ($n = 7$) coastal ranges in Venezuela, and includes *T. trinitatis* from the Northern Range in Trinidad. Additional geographic structure is evident among *Tityus* species belonging to this clade, which can be separated into "central" and "eastern" mtDNA sub-clades. The "central" sub-clade includes most "discrepans"-group species described for Venezuela, ranging from Rancho Grande National Park (Aragua State) in the east to the western-most limit of Cordillera de la Costa (Miranda State and Capital District). Support for the "central" mtDNA clade is low in the 16S MP tree (50%), but has a 100% posterior probability in the COI – 16S Bayesian tree.

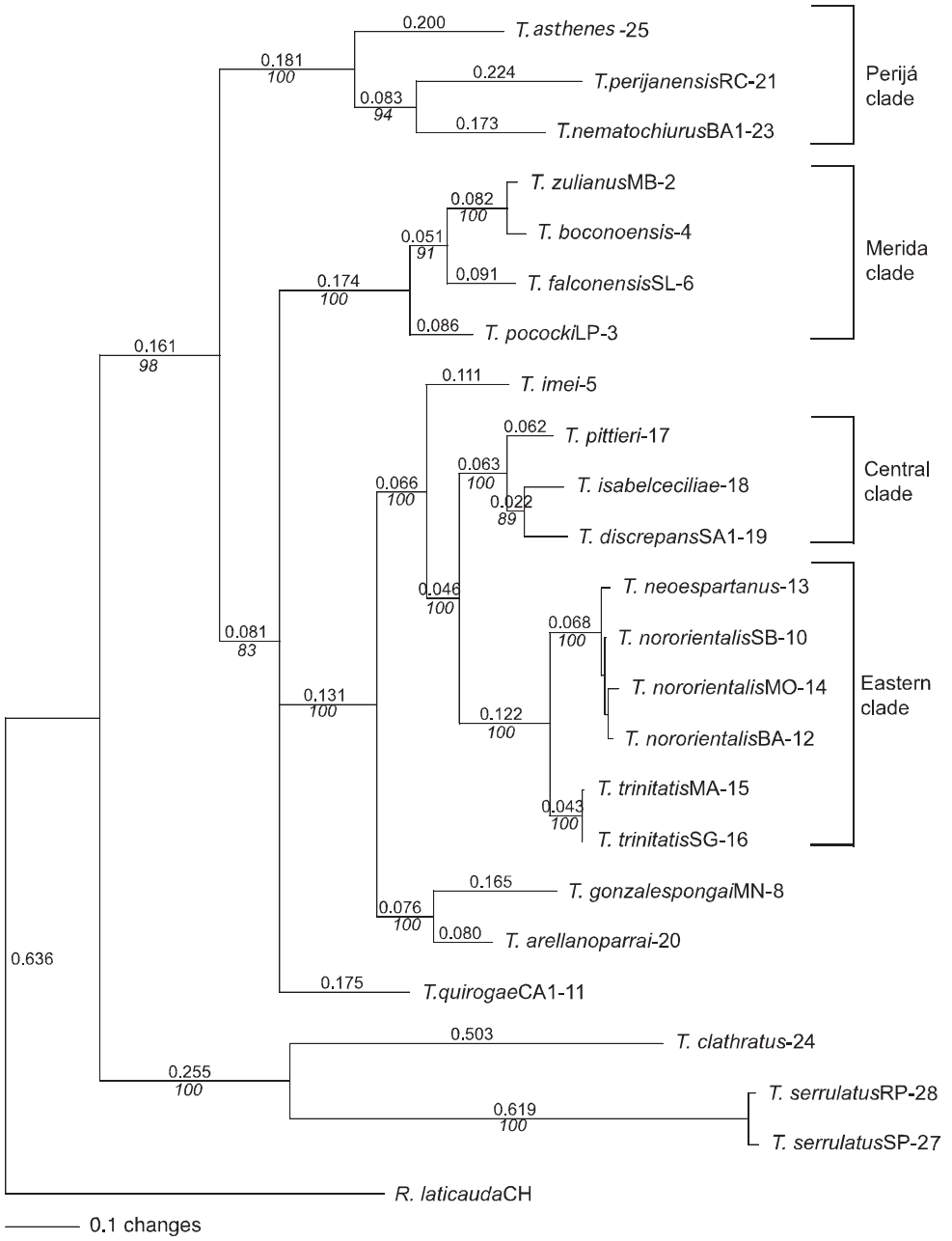

Fig. 4. Hypothesis of phylogenetic relationship among *Tityus* species based on 852 bp nucleotides representing the combined mitochondrial 16S rRNA and COI genes. The tree shown resulted from Bayesian analysis using the GTR model of nucleotide substitution. Gene tree distances according to the model are provided above the branches and posterior probability support values greater than or equal to 95% are presented below the branches. Numbers and initials after species names indicate locations in map shown in Fig. 2 and collection sites indicated in Table 1.

The "eastern" sub-clade has strong bootstrap support in all trees and includes "androcottoides"-group individuals from northeastern Venezuela such as *Tityus neoespartanus* G.-S from Margarita Island and *Tityus nororientalis* G.-S collected from the Paria Peninsula and the northern and southern valleys of Serranía Oriental del Interior. Given its wide distribution, the abundant records of severe envenomation cases, and variations in venom composition across its range (De Sousa et al., 2000; Borges and De Sousa, 2006; Borges et al., 2008), we sampled *T. nororientalis* from various localities in the northeast. *T. trinitatis* samples, endemic to Trinidad and Tobago, form a well-supported cluster within the "eastern" sub-clade. The mean within-group divergence of the "eastern" sub-clade was the lowest of all resolved *Tityus* mtDNA haplotypes, $3.32 \pm 2.56\%$. The mtDNA divergence among *T. neoespartanus* and *T. nororientalis* from mainland sites is extremely low, with a single nucleotide substitution in 16S and four substitutions in COI.

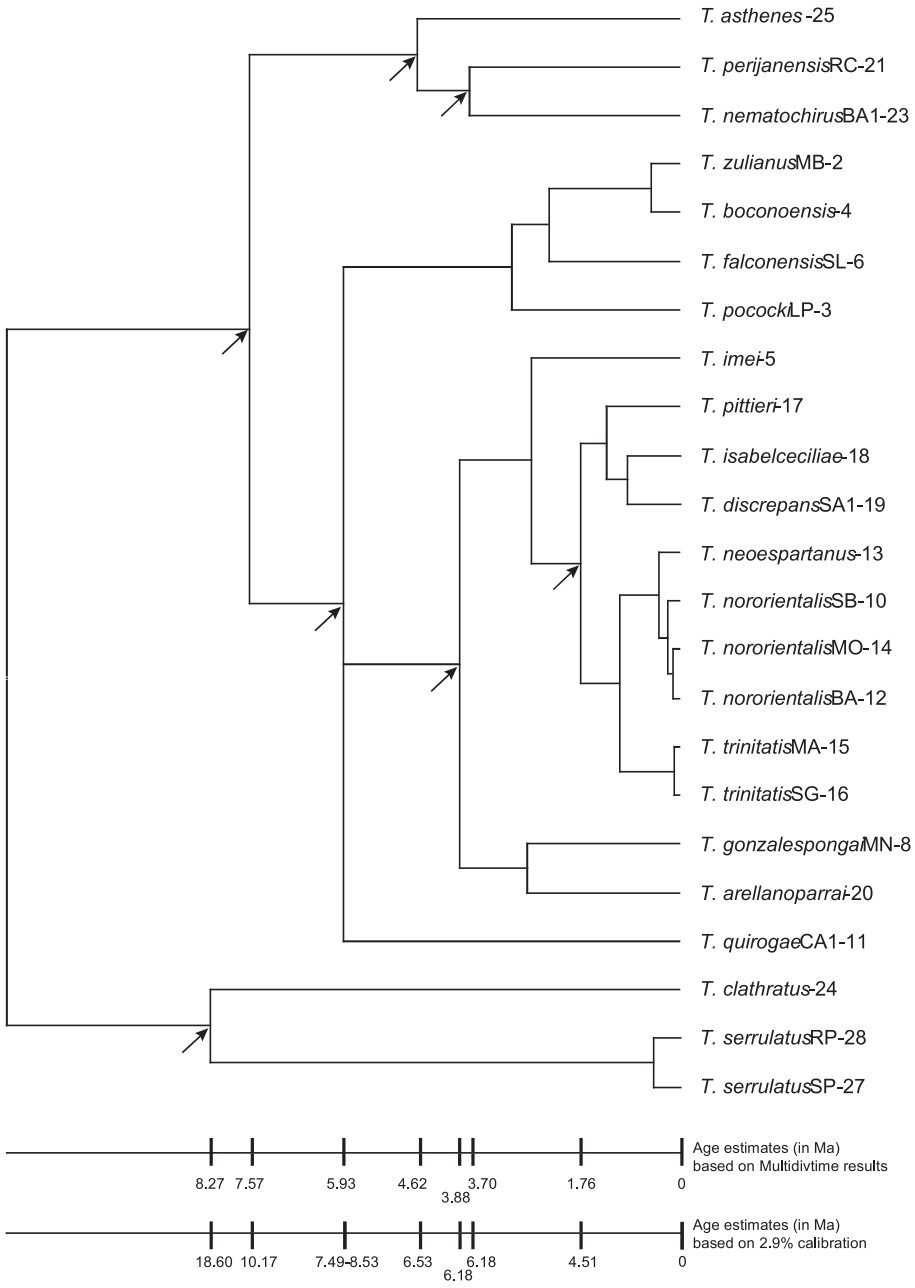

Fig. 5. Divergence time estimates for *Tityus* species. The topology is the same as that shown in Fig. 4.

Tityus gonzalespongai Quiroga et al. (an eastern "androcottoides") and *Tityus arellanoparrai* G.-S. (an eastern "discrepans"), both distributed along the eastern coastal range but restricted to the southern slopes of the Bergantín and Caripe Massifs, respectively, constitute another subclade within the central-eastern clade. Finally, *Tityus imei* Borges et al., from Sierra Portuguesa, was placed into the central-eastern clade in all analyses (50% support for MP, 100% posterior probability for Bayesian analyses). The phylogenetic position of *T. quirogae* De Sousa et al. (range Anzoátegui and Monagas States, southern slope of the eastern range) was unresolved given the lack of support of its association to any of the identified clades in Venezuelan *Tityus*.

Of particular note is the mtDNA divergence between Andean and eastern "androcottoides" ($25.26 \pm 1.39\%$) when compared to the $13.09 \pm 0.89\%$ divergence between eastern "androcottoides" and the "central" group containing "discrepans" species. To test the morphological hypothesis of González-Sponga (1996a), which predicts the monophyly of the named species groups (Fig. 2), the ML tree was compared to tree which constrained species to their named groups. The SH test rejected the null hypothesis of no difference between the morphologically

constrained tree and the unconstrained mtDNA ML tree ($p < 0.05$).

3.3. Rate constancy and divergence time inferences

The LRT failed to reject the null hypothesis of rate constancy ($-\ln = 5620.89$ for the not enforced tree and 5636.28 for the enforced tree, $\chi^2 = 30.78$, df $= 21$, $p > 0.05$). Under the assumption of a molecular clock, we applied a calibration of 2.9% sequence divergence per Ma (estimated for *Buthus occitanus*, Gantenbein and Largiadèr, 2003) to estimate times of divergence (Fig. 5).

We also used the MCMC approach to obtain divergence calculated using two prior rates (rttm = 0.22; rttm = 0.44). We found no major differences between the time estimates using these two priors.

Fig. 5 presents a comparison of estimated divergence times based on the 2.9% calibration and the MCMC approach. Divergence times estimated using the 2.9% calibration are earlier than those obtained using the MCMC approach.

Our estimates of divergence times provided the following results based on the 2.9% calibration and the MCMC approach. The *T. clathratus/T. serrulatus* clade shared a common ancestor with the rest of the Venezuelan *Tityus* during early to middle Miocene (18.66 Ma using the 2.9% calibration; 11.85 Ma using the MCMC approach, 95% confidence interval, 6.67–19.50). Species of *Tityus* in the Perijá clade shared a common ancestor with species in the Mérida/Central/Eastern clade in the late Miocene (10.17 Ma using 2.9% calibration; 7.57 Ma using the MCMC approach).

One of the constrained nodes was the split between *T. asthenes* and *T. perijanensis/T. nematochiurus* clade at 11.8–2.7 Ma. Our results suggest that these species diverged during the late Miocene–early Pliocene, possibly during the last period of the formation of the eastern Andes (6.53 Ma using the 2.9% calibration; 4.62 Ma using the MCMC approach).

The Central and Eastern clades shared a most recent common ancestor (MRCA) during the Pliocene (4.51 Ma according to the 2.9% calibration; 1.76 Ma using the MCMC approach), corresponding to the formation of the Cariaco basin (Schubert, 1982).

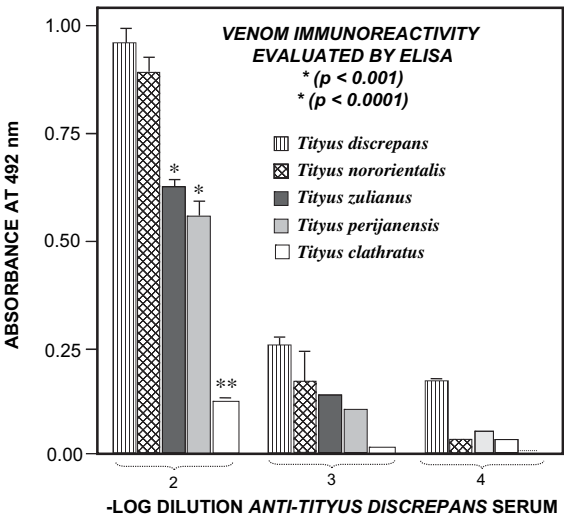

Fig. 6. Antigenic reactivity of Venezuelan *Tityus* scorpion venoms against antibodies anti-*Tityus discrepans* venom as evaluated by ELISA. Dilutions of serum from 1/100 to 1/10,000 were tested for reactivity against representative venoms from Venezuelan mtDNA haplotypes. Statistical significance (*, $p < 0.001$ or **, $p < 0.0001$) between absorbance measurements is indicated.

3.4. Antigenic reactivity of representative Venezuelan Tityus venoms

Fig. 6 shows the results of an ELISA-type assay comparing the reactivity of Venezuelan *Tityus* venoms against anti-*T. discrepans* horse antibodies. Binding of venom components (including low mol. mass peptides) to microtitration plates was confirmed upon detection of negligible amounts of protein during the washing procedure. This result agrees with previous observations documenting the effective immobilization of scorpion venom peptides onto ELISA plates and nitrocellulose membranes and their recognition by specific antibodies (Alvarenga et al., 2005; Mendes et al., 2008; Oukkache et al., 2008).

The following venoms were tested: *T. discrepans* ("central" sub-clade, control venom), *T. nororientalis* ("eastern" sub-clade), *T. zulianus* ("Mérida Andes" clade), *T. perijanensis* ("Perijá" clade), and *T. clathratus*. Absorbance at 495 nm corresponding to the product formed *via* horseradish peroxidase-driven reaction was used to estimate the binding of anti-*T. discrepans* antibodies per immobilized venom antigen. At 1/100 antibody dilution, reactivity of venoms from *T. zulianus* and *T. perijanensis* ($p < 0.001$) and also *T. clathratus* ($p < 0.0001$) was significantly different to the reactivity of *Tityus nororientalis* venom and the control, *T. discrepans*, venom.

As *T. clathratus* was found to cluster with the Brazilian *T. serrulatus* and *T. bahiensis* in our mtDNA analyses, we tested the immunoreactivity of *T. clathratus* low mol. mass components against the Brazilian (anti-*T. serrulatus*) and Venezuelan (anti-*T. discrepans*) commercial antivenoms using immunoblotting after SDS-PAGE. Fig. 7 shows the results of immunoblots to evaluate the reactivity of *T. clathratus* 6–8 kDa components, which co-electrophorese with *T. discrepans* and *T. serrulatus* neurotoxic fractions (Borges et al., 1999). Recognition of *T. clathratus* toxins by both antibodies was significantly less intense than for the control venoms, indicating low reactivity.

4. Discussion

4.1. Molecular phylogeny and divergence time for Tityus species

The two different sets of molecular data gathered for this project (16S rRNA and COI) are known to differ in rates and patterns of nucleotide substitution (Hedin and Maddison, 2001). Nonetheless COI has a rate of *Tityus* mtDNA evolution roughly equal to 16S when averaged over all pairwise comparisons. Relationships among *Tityus* species inferred from mtDNA 16S data lack resolution due to the fact that the highly variable sites appear to be saturated with substitutions, while too few conserved sites exhibit

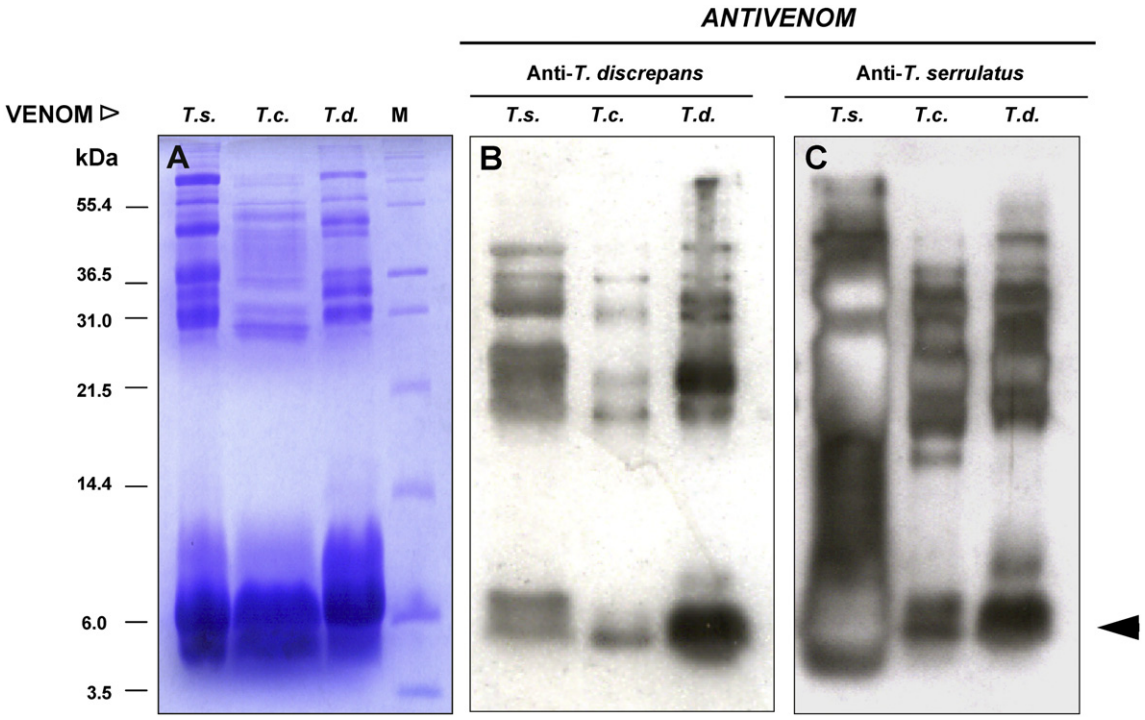

Fig. 7. Antigenic reactivity of *T. clathratus* venom components as evaluated by inmunoblotting. (**Panel A**) SDS-PAGE of *T. clathratus* (T.c.), *T. discrepans* (T.d.) and *T. serrulatus* (T.s.) venoms using equal amounts of protein (30 μg). (**Panel B**) Venom reactivity towards anti-*T. discrepans* antibodies. (**Panel C**) Venom reactivity towards anti-*T. serrulatus* antibodies. Arrow indicates migration of the venom neurotoxic, low mol. mass components as shown by Borges et al. (1999).

variation (Simon et al., 1994). Moreover, most variable sites in the *Tityus* 16S gene are located upstream of the peptidyl transferase center, where alignment was uncertain. We improved the phylogenetic information content of the 16S gene region by optimizing our alignment according to the secondary structure of the scorpion 16S gene, and by excluding regions of sequence that we could not align with confidence. The fact that 16S and COI MP analyses resulted in similar phylogenetic consistency indexes, and that the same and reasonably well-supported mtDNA clades were obtained in the analyses of the separate gene partitions is reassuring, even if anticipated.

The results presented in this article provide the first molecular phylogenetic hypothesis for species relationships across any regional group of the scorpion genus *Tityus*. Only one previous study by Teruel et al. (2006) demonstrated the monophyly of the clade formed by the Cuban genus *Alayotityus* and the *T. serrulatus* and *T. bahiensis* 16S rRNA sequences reported in this work. Our experimental design centered a purposefully biogeographic focus on Venezuelan *Tityus* scorpions to take advantage of the thorough taxonomic characterization of the Venezuelan scorpion fauna by González-Sponga (1984, 1996a, 2001a,b). Our sampling of species diversity in *Tityus* and outgroup genera in the family Buthidae suggested that the genus *Tityus* is monophyletic.

The degree of phylogenetic separation of *T. clathratus*, widespread in northern South America and recorded from Curaçao, Trinidad, Guyana, Suriname, French Guiana, Brazil, and the Colombian Amazonia in addition to Venezuela, was somewhat anticipated by earlier research. Indeed, González-Sponga (1996a) noted the difficulty of placing this species, together with *Tityus melanostictus* Pocock, into any of the proposed Venezuelan *Tityus* groups given their lack of morphological affinity. Mello-Leitão (1945), and later Lourenço (1984a, 1992, 2000, 2002a,b), assigned *T. clathratus* to a group of small body-sized, highly pigmented *Tityus* scorpions, which include both *cis*- and *trans*-Andean species, considered by both authors to occupy a plesiomorphic position among its congeneric groups. Furthermore, Lourenço (1999) posited that members of the "*Tityus clathratus*" group were the ancestors of the so-called "proto-*Tityus*", e.g. the Antillean genera *Alayotityus* Armas and *Tityopsis* Armas from Cuba and also *Caribetityus* Lourenço from Hispaniola, and more recently, proposed the erection of the subgenus *Archaeotityus* to accommodate the species of this group (Lourenco, 2006b). Our data substantiates this proposal although more *Archaeotityus* species need to be analyzed molecularly to support the monophyly of the subgenus. Additional support for the degree of phylogenetic distance of *Archaeotityus* from other *Tityus* in South America comes from venom immunoreactivity data as discussed in Section 4.3.

The phylogenetic distinctiveness of *T. quirogae* was not anticipated by earlier studies, although the fact that males have the long and slender pedipalps typical of the "nematochirus" group (De Sousa et al., 2006), rather than the "androcottoides" type, might have signaled its isolated placement in the mtDNA phylogeny. The restricted distribution of this species in the mountainous areas of Anzoátegui, Monagas, and Sucre States (De Sousa et al., 2006) is

particularly remarkable in view of its apparent phylogenetic separation from other species.

The degree of biogeographic structure in the remaining Venezuelan *Tityus* species studied here, and the deep molecular divergence amongst the biogeographically restricted groups, was not anticipated by earlier investigations of the genus. Mitochondrial DNA relationships among scorpion lineages clearly identify clades associated with geologically and geographically distinct features, suggesting that vicariance has been a potent force in the diversification of Venezuelan *Tityus*. Mitochondrial DNA haplotypes grouped into regional clades representing *Tityus* from the Perijá mountain range and the Mérida Andes in western Venezuela, and the central and eastern coastal ranges, including Trinidad. The level of mtDNA divergence between these regional mtDNA clades indicates geographic range expansion and diversification dating to the Miocene, or earlier.

The genetic divergence between *Tityus* from the "Mérida Andes" and the "central-eastern" clade suggests that the ancestors of these two groups were separated by the uplift of the eastern Andean Cordillera. Mid-Miocene activity in the Andean axis uplifted much of the northern Cordillera to elevations above 1000 m approximately 14–11 Ma (Potts and Behrensmeyer, 1992), followed by a second more dramatic uplift to elevations above 4000 m during the Pliocene and Pleistocene (Potts and Behrensmeyer, 1992; Gregory-Wodzicki, 2000). The initial mid-Miocene uplift of the Andes was associated with wide-scale changes in the Neotropical flora (Gentry, 1982; Van der Hammen, 1989), and major physiographic changes in the Amazon Basin (Hoorn, 1994). An additional barrier limiting dispersal and separating the "Mérida Andes" from the "central-eastern" *Tityus* clades was the Proto-Orinoco River, which existed in the earliest Miocene in the Falcón Basin, situated to the east of the Maracaibo Basin (Díaz de Gamero, 1996). The course of the Orinoco was deflected to its present position in the eastern Venezuelan Maturín Basin, in the latter part of the Miocene, as a consequence of the final uplift of the Mérida Andes (Díaz de Gamero, 1996; Audemard, 2003). The change in Orinoco drainage pattern may have permitted invasion of the newly formed Coro Massif by the Mérida Andes ancestor of *T. falconensis*, presently endemic in the mountainous border between the States of Falcón and Lara, western Venezuela (Fig. 1).

Our data suggest that the "central" and "eastern" coastal *Tityus* sub-clades split during the Pliocene. This separation time is somewhat older than the 2 Ma formation of the Cariaco Basin (Schubert, 1982), the most plausible biogeographic event causing the disjunction of "central" and "eastern" *Tityus* clades. Schubert (1982) based his dating on the sedimentary thickness of the Cariaco Basin and assumed a constant sedimentation rate; however, his earlier work (Schubert, 1981) recognized that right-slip along El Pilar and Morón faults separating the central and eastern ranges began in the late Tertiary. So perhaps the separation of the "central" and "eastern" *Tityus* scorpion clades began somewhat earlier than the formation of the Cariaco Basin, or perhaps the *Buthus occitanus* mtDNA calibration has led to an overestimate of the age of these clades, a possibility strongly supported by the MCMC-based estimation of the separation between the "central" and "eastern" scorpions (Fig. 5).

The phylogenetic association of *T. trinitatis* scorpions from Trinidad with the northeastern Venezuela *Tityus* species is strongly consistent with the fact that Trinidad's northern mountain range and the Venezuelan Paria Peninsula are part of the same geological province (Erlich and Barrett, 1990), and have been connected a minimum of five times in the past 140,000 years (Murphy, 1997). It is clear from our phylogenetic results that the separation of *T. trinitatis* postdated the separation of the "central" and "eastern" *Tityus* clades.

It is worth noting the low level of mtDNA divergence among our sample of *T. neoespartanus* and *T. nororientalis* individuals. The original description by González-Sponga (1996b) reports that *T. neoespartanus* and *T. nororientalis* can be distinguished by their body size, dimensions of the pedipalp chelae, and the number of denticle rows on the movable finger of the pedipalp. These species are found on Isla Margarita and the mainland respectively, and the lack of informative mtDNA divergence between the species indicates recent separation into allopatric isolates. It would be of interest to determine if the species have attained reproductive isolation.

Tityus imei Borges et al. from the southern foothills of Sierra Portuguesa, western Venezuela, morphologically resembles *Tityus sanarensis* G.-S., an "androcottoides" species from the northern piedmont of the same range (González-Sponga, 1997), but has a sequence divergence of 23.57% from its nearest Andean neighbor, *T. boconoensis*. Our analyses, and also the results of Borges et al. (2006a), clearly show that *T. imei* shares more overall sequence similarity with central-eastern *Tityus* species (13.0% divergence) and thus represents the western-most limit of the central-eastern coastal mtDNA clade. *T. imei* also exhibits the marked sexual dimorphism (elongated male caudal segments) typical of the eastern, not western, Venezuelan species in the "androcottoides" group (Borges et al., 2006a). The region inhabited by *T. imei* belongs to the same geological province as the central coastal mountains (Campos et al., 1979; González de Juana et al., 1980), and supports the idea that this region of northern Venezuela had formed by the middle to late Eocene (Erlich and Barrett, 1990).

4.2. Molecular phylogeny and morphological hypothesis for Venezuelan Tityus

Our mtDNA phylogeny of Venezuelan *Tityus* provides an informative contrast to the previous hypotheses of systematic relationship for the group based on morphological characters. The morphology-based studies have postulated the existence of four *Tityus* species groups in Venezuela, namely "androcottoides", "discrepans", "nematochirus", and "clathratus". The mtDNA phylogeny for the separate and combined data set, however, rejects the grouping of *Tityus* taxa studied here into the "androcottoides" and "discrepans" groups. This result mainly owes to the morphological grouping of a geographically diverse array of scorpion species into the "androcottoides" group, but not even the well-sampled "discrepans" group was fully supported in the mtDNA phylogenetic analyses. While most

"discrepans" species fell within the "central" clade, *T. arellanoparrai* clustered separately with *T. gonzalespongai*, an "androcottoides" species also from the eastern range. On the other hand, our study provides support for the "nematochirus" group as *T. asthenes*, *T. nematochirus*, and *T. perijanensis* clustered together in all analyses, suggesting the monophyly of this clade. Future studies should sample and sequence more species belonging to this group to confirm such monophyly, considering its broad distribution in the Mérida Andes, the Serranía Oriental del Interior, the Guiana Massif and Venezuelan Amazonia (González-Sponga, 1996a, 2001a,b, 2002). Therefore, our phylogenetic results indicate that the arrangement of ventral carinae in metasomal segments II–IV does not provide sufficient morphological resolution to place the Venezuelan *Tityus* belonging to the "androcottoides" and "discrepans" groups into monophyletic clades.

It remains to be determined whether the use of additional characters such as body size, cuticle pigmentation, shape of subaculear spine, and dilation of middle lamellae of female pectines, originally used by Lourenço (1997a,b, 1998, 2002a,b) to erect the "asthenes", "bahiensis", "adisi," and "clathratus" groups within the genus and more recently utilized to subdivide it into subgenera (Lourenço, 2006), would produce a morphological hypothesis for the Venezuelan *Tityus* that was not in such strong conflict with our molecular systematic hypothesis for the group. In fact, Lourenço (2006) have included the Venezuelan "androcottoides" and "discrepans" taxa analyzed in this work under the subgenus *Atreus*, a grouping confirmed by our results. However, the "nematochirus" species studied herein, *T. asthenes*, *T. nematochirus*, and *T. perijanensis*, are also placed in this subgenus. The fact that these species clustered with a strong node support in all analyses (the "Perijá" mtDNA clade) suggests that *Atreus* might need to be revised. All in all, additional evidence from other data sets (nuclear/mtDNA markers, toxin structure/activity) should be sought, in conjunction with morphological evidence, to help clarify systematic relationships among *Tityus* scorpions.

4.3. Venom antigenicity

One of the aims of this study was to explore the potential utility of mtDNA-based phylogenetic systematics to predict scorpion venom antigenic reactivity for those *Tityus* species that have yet to be characterized epidemiologically. The number of described *Tityus* species increase steadily and so do the number of envenomation cases in many endemic areas across Tropical America, as humans increasingly disturb their habitats (Lourenço, 1996b). Toxic *Tityus* species can also be introduced into areas where they are not prevalent (De Sousa et al., 2008). Health authorities dealing with the issue of manufacturing effective antivenoms have encountered for other toxic taxa, such as snakes, the problem of limited neutralization efficacy depending upon geographic locale. For scorpions, at least in the case of Venezuela, venom antigenic cross-reactivity is dependent on the species (Borges et al., 2006b, 2008), and therefore there is need to catalog the fauna in terms of their venom recognition by available commercial antivenoms.

The "Mérida Andes" clade and the "central" and "eastern" sub-clades found in this work coincide with the Andean, north central and northeastern areas of endemism for scorpion envenomation in Venezuela defined by De Sousa et al. (2000) and Borges and De Sousa (2006). Since differences have been noted in terms of severity and clinical outcome between *Tityus* envenomation cases from these areas (Borges et al., 2006b; Borges and De Sousa, 2006), we reasoned that *Tityus* phylogeography might help define regional toxin repertoires possibly responsible for differential toxicity and antigenicity.

We compared the reactivity towards anti-*T. discrepans* horse antibodies (the commercial antivenom available in Venezuela) of venoms from representative species belonging to the "central" (*T. discrepans*), "eastern" (*T. nororientalis*), "Mérida Andes" (*T. zulianus*), "Perijá" (*T. perijanensis*) mtDNA clades, and the cosmopolitan *T. clathratus*, using ELISA tests (Fig. 6). These five species also represent the four different named morphological groups, and the morphologically-based hypothesis of *Tityus* species relationship predicts similar levels of venom antigenic reactivity between *T. discrepans* and the members of the other three morphological groups. However, *T. discrepans* and *T. nororientalis* shared a higher degree of venom antigenic reactivity when compared to *T. zulianus*, *T. perijanensis* or *T. clathratus* (Fig. 6). Thus, our results predict that venom immunological cross-reactivity will be greater between *Tityus* species that are closely related in the mtDNA phylogeny.

Our data suggest that the venom of *T. zulianus* and *T. perijanensis* contain lower amounts of proteins antigenically similar to those produced by *T. discrepans*, and/or contain components with different antigenic reactivity. These results confirm the observation that the anti-*T. discrepans* antivenom only partially neutralizes the *in vivo* toxic effect of *T. zulianus* and *T. perijanensis* venoms (Borges et al., 2005, 2006b) and substantiates similar immunoreactivity data obtained with rabbit antivenom antibodies (Borges et al., 2008). Taken together, the differential venom antigenicity found here, and the known dissimilarities in venom action and composition between *T. discrepans* and *T. zulianus* (Borges et al., 2004, 2006b) are indicative of structural and/or functional diversity among toxins produced by different mtDNA Venezuelan *Tityus* clades. In fact, we have shown, using mass spectral and toxin cDNA sequencing analyses that the venoms of *T. zulianus* and *T. discrepans* differ at the transcriptome and proteome levels (Borges et al., 2006b). A previous report showing that the anti-*T. discrepans* antivenom is unable to recognize the toxin-containing fraction of the Brazilian *Tityus serrulatus* venom (Borges et al., 1999) further supports the existence of different regional sets of toxins with unique antigenic properties within South America.

The fact that the low mol. mass fraction (*ca*. 6–8 kDa) of *T. clathratus* venom is poorly recognized by either the anti-*T. discrepans* or anti-*T. serrulatus* commercial antibodies (Fig. 7), adds support to the evolutive distance of this morphological group from other South American species and suggests that the antigenic epitopes of its toxins diverge significantly with respect to other *Tityus* groups/subgenera. On the other hand, ongoing analyses of *T. clathratus* toxin genes have revealed a close relationship between these toxins and those expressed by *T. serrulatus* and *T. bahiensis* (A. B., in preparation), further supporting

the phylogenetic relationship between *T. clathratus* and these Brazilian species found in our mtDNA analyses.

In spite of a broadly similar three-dimensional structure (Martin-Eauclaire and Couraud, 1995), long-chain scorpion toxins responsible for up to 90% of the venom lethality display sequence variations in their antigenic epitopes, producing lack of cross-reactivity even if the toxins belong to the same structural group (Granier et al., 1989; De Lima et al., 1993). It could be hypothesized that genetic isolation, as a result of vicariant or ecological barriers, may have led to the production of toxin repertoires containing species- and/or group-specific functional/antigenic epitopes. In this sense, differential expression of toxin-encoded gene-family members in the gastropod genus *Conus* has been reported to play a role during shifts to new ecological niches (Duda and Remigio, 2008).

An improved systematic classification of *Tityus* species based on multiple criteria [venom antigenicity and toxicity (Becerril et al., 1997; Borges et al., 2008), toxin transcriptomic/proteomic analyses (Batista et al., 2004, 2006, 2007; Borges et al., 2006b; D'Suze et al., 2009; Nascimento et al., 2006), and molecular phylogeography (this work)] would be of help in the characterization of toxins from different areas and for improving serotherapy. Additional genetic and biochemical work carried out on the toxins produced by representative species should shed light on the possibility that, in parallel to the strong biogeographic structuring of *Tityus* species, toxinological partitioning exists, which may have important epidemiological as well as clinical consequences.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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