2020. Journal of Arachnology 48:77–83

The first chromosomal analysis of bisexual populations of the Brazilian scorpion *Tityus serrulatus* (Scorpiones: Buthidae)

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Abstract. The scorpion *Tityus serrulatus* Lutz & Mello, 1922 (Buthidae) is endemic to Brazil and is considered the most toxic South American scorpion species. The ample distribution of this species and its occurrence in urban areas has been attributed primarily to the parthenogenetic mode of reproduction. For a long time, only all-female *T. serrulatus* populations were known. Male specimens are very rare and have been recently discovered in natural environments within the Brazilian semi-arid zone. In the present study, we investigated cytogenetically bisexual populations of *T. serrulatus*, and compared the data with those available for parthenogenetic specimens. The diploid number (2n=12) and the general configuration of the chromosomes in male and female specimens were similar to those recorded for parthenogenetic populations. Male postpachytene cells contained chromosomes in a parallel arrangement with no differentiation of the bivalents, indicating the absence of heteromorphic sex chromosomes. However, the number of 28S rDNA sites varied between male and female specimens. Females invariably presented only one rDNA cistron, while all the males had two. This finding seems to indicate the existence of a sex-specific heteromorphism in *T. serrulatus*, in which the females are heterogametic and the males are the homogametic sex.

Keywords: Cytogenetic, diploid number, meiosis, rDNA, sex chromosome system

Parthenogenesis is a relatively rare mode of animal asexual reproduction that has stimulated a great deal of interest in genetics, zoology, ecology, developmental biology and evolutionary studies (Simon et al. 2003; Kearney 2005; Morgan-Richard & Trewick 2005; Johnson 2006; D'Souza & Michiels 2010; Muñoz et al. 2010). Within the multicellular organisms, only one out of every 1,000 species reproduce parthenogenetically (Simon et al. 2003). Besides scorpions, among arachnids, parthenogenesis has been reported in Acari, Amblypygi, Araneae, Opiliones, Schizomida and Palpigradi (Lake 1986; Tsurusaki 1986; Lourenço & Cuellar 1994; Wrensch et al. 1994; Reddel & Cokendolpher 1995; Edwards et al. 2003).

The first recorded case of parthenogenesis in scorpions was that of a Brazilian species, *Tityus serrulatus* Lutz & Mello, 1922 (Matthiesen 1962) where the author discovered that unmated females born in captivity gave birth to young. This capacity was confirmed in subsequent studies, and *T. serrulatus* was considered to be an example of obligatory thelytokous parthenogenesis, in which virgin females produce all-female progeny without egg fertilization (Matthiesen 1962; San Martin & Gambardella 1966; Franke 2008; Lourenço 2008).

Parthenogenesis is now suspected to occur in at least 18 scorpion species, belonging to the families Buthidae, Hormuridae, Scorpionidae and Vaejovidae (for revision see Teruel 2004; Toscano-Gadea 2004; Yamazaki & Makioka 2004; Lourenço & Ythier 2007; Lourenço 2008; Ross 2010; Seiter 2012; Ayrey 2017; Rein 2017). In most of these cases, however, parthenogenetic reproduction was deduced from the lack of bisexual populations, rather than births from females known to be unmated (Francke 2008).

In a review of the evidence on parthenogenesis in the order Scorpiones, Francke (2008) concluded that multiple parturitions in captivity may often be the result of sperm storage and/or delayed ovule implantation, resulting in false evidence of parthenogenetic reproduction. Given this, robust evidence on parthenogenesis is available for only 10 species (Francke 2008; Ross 2010; Seiter 2012; Ayrey 2017). A number of other factors may also contribute to deviations of sex ratios in scorpion populations, such as differences between males and females in the age of sexual maturity, survival rates, and seasonal activity (Franke 2008).

Tityus serrulatus is endemic to Brazil, where it is found in 17 states in the central, southern and eastern regions of the country (Fig. 1). The wide distribution of this species and its rapid expansion throughout Brazil has been attributed to the parthenogenetic mode of reproduction (Bortoluzzi et al. 2007; Lourenço 2008, 2015; Souza et al. 2009). *Tityus serrulatus* is considered the most toxic of South American scorpions and is now associated primarily with urban areas (Soares et al. 2002; Lourenço, 2008, 2015). Since its taxonomic description in 1922, the first record of a supposed bisexual population of *T. serrulatus* was obtained by Lourenço & Cloudsley-Thompson (1999) from Irapé, in the Brazilian state of Minas Gerais. Nevertheless, the male described in that study was misidentified and did in fact correspond to *Tityus melici* Lourenço, 2003 (see Souza et al. 2009). In this same study, the authors, however, did record two male specimens of *T. serrulatus* from Januária, Minas Gerais (Souza et al. 2009).

The current knowledge on cytogenetics of *T. serrulatus* is based on the analysis of female specimens from two distant populations (approximately 550 km apart), in the states of São Paulo and Minas Gerais, in southeastern Brazil (Schneider &

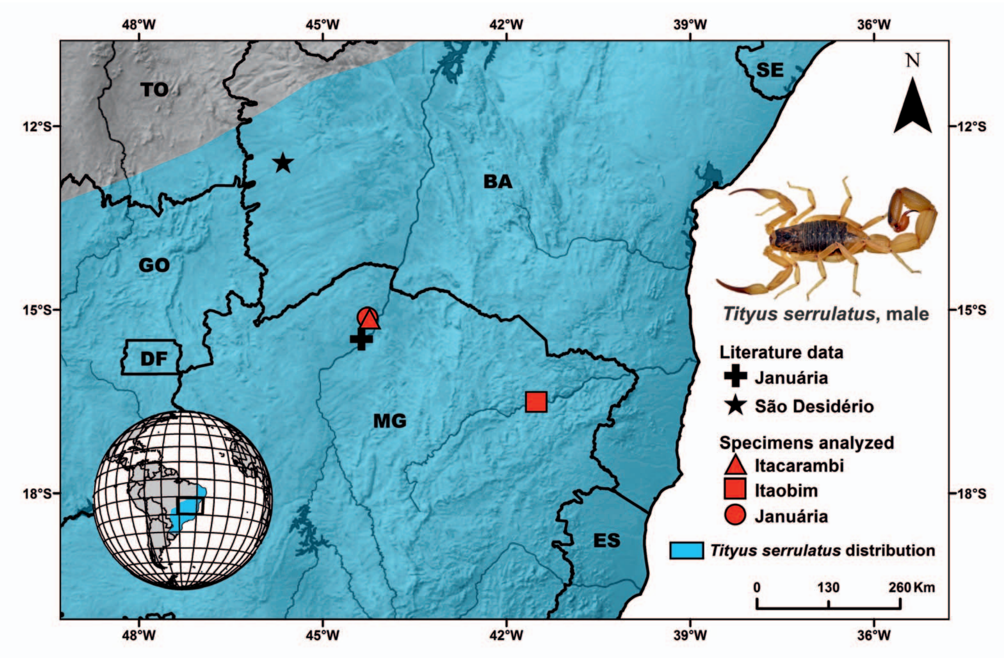

Figure 1.—Geographic distribution of the Brazilian scorpion *Tityus serrulatus* (blue shading) and the sites at which male specimens have been recorded. Published data: municipality of São Desidério, state of Bahia (black star–Santos et al. 2014) and Januária, state of Minas Gerais (black cross–Souza et al. 2009). Present study: municipalities of Itacarambi (red triangle), Itaobim (red square) and Januária (red circle; live specimen photograph), all in state of Minas Gerais. Brazilian states: BA = Bahia; DF = Distrito Federal; ES = Espírito Santo; GO = Goiás; MG = Minas Gerais; SE = Sergipe; TO = Tocantins.

Cella 2010). A conserved diploid number of 2n=12 was recorded, in addition to a single major ribosomal cistron, located in the terminal region of one chromosome. In the present study, we provide the first cytogenetic data on bisexual populations of *T. serrulatus*, which we compare with those from previous studies of parthenogenetic populations. In other animals, such as lizards and ticks, the investigation of bisexual and parthenogenetic populations had revealed differences in diploid number (parthenogenetic lineages are triploid) and contributed to the understanding of the origin of parthenogenesis (Laguna et al. 2010; Pellegrino et al. 2011; Cui et al. 2012).

In addition to the description of diploid number for males and females, we determined the behavior of the chromosomes during male meiosis, and the location of the nucleolar organizer regions (Ag-NORs), the 28S rDNA gene and telomeric sequence. Our findings seem to indicate the presence of sex-specific heteromorphism in this species.

METHODS

Our study included 15 specimens of *T. serrulatus* collected from three localities in Minas Gerais, Brazil (Fig. 1): one male from Itacarambi (15°9′17″S, 44°14′29″W), two males and eight females from Itaobim (16°30′22″S, 41°30′32″W), and four males from Januária (15°7′40″S, 44°16′26″W). The voucher specimens were deposited in the Coleções Taxonômicas of the Universidade Federal de Minas Gerais (UFMG – curator A.J. Santos), Belo Horizonte, Minas Gerais, Brazil.

The male and female chromosome preparations were obtained from the gonads, using the procedure described by Schneider et al. (2009). The slides were standard-stained with 3% Giemsa solution and silver-impregnated to detect the nucleolar organizer regions (Ag-NORs), according to Howell & Black (1980). For the rDNA-FISH (fluorescent *in situ* hybridization), the probes were obtained by PCR using the genomic DNA of *T. serrulatus* and the primers 28S-F 5′GACCCGTCTTGAAACACGGA and 28S-R 5′TCGGAAGGAACCAGCTACTA, described by Nunn et al. (1996). For the telomeric-FISH, the primers Tel-F 5′TAGGTTAGGTTAGGTTAGG and Tel-R 5′AACCTAACCTAACCTAACC were used as probes, without a DNA template. The probes were labelled with biotin-16-dUTP (1mM) by PCR. FISH was conducted according to Pinkel et al. (1986) with minor adjustments described by Almeida et al. (2010). The probes were detected with anti-biotin conjugated with Alexa Fluor-488 (200µg/mL). Chromosome spreads were counterstained with 4′-6-diamidino-2-phenylindole (DAPI)/Vectashield. The images were captured using a Zeiss Imager A2 microscope, coupled to a digital camera using Axio Vision software. To determine the relative length of each chromosome, 15 well-spread cells at mitotic metaphase or prophase I, submitted to FISH with rDNA probe or silver impregnation, were measured, using Image J (online at http://imagej.nih.gov/ij/)

RESULTS

All male specimens of *T. serrulatus* from the three localities presented a diploid number of 2n=12 and holocentric chromosomes (Fig. 2A). In both mitotic and meiotic cells, the chromosomes were easily distinguished, based on their size

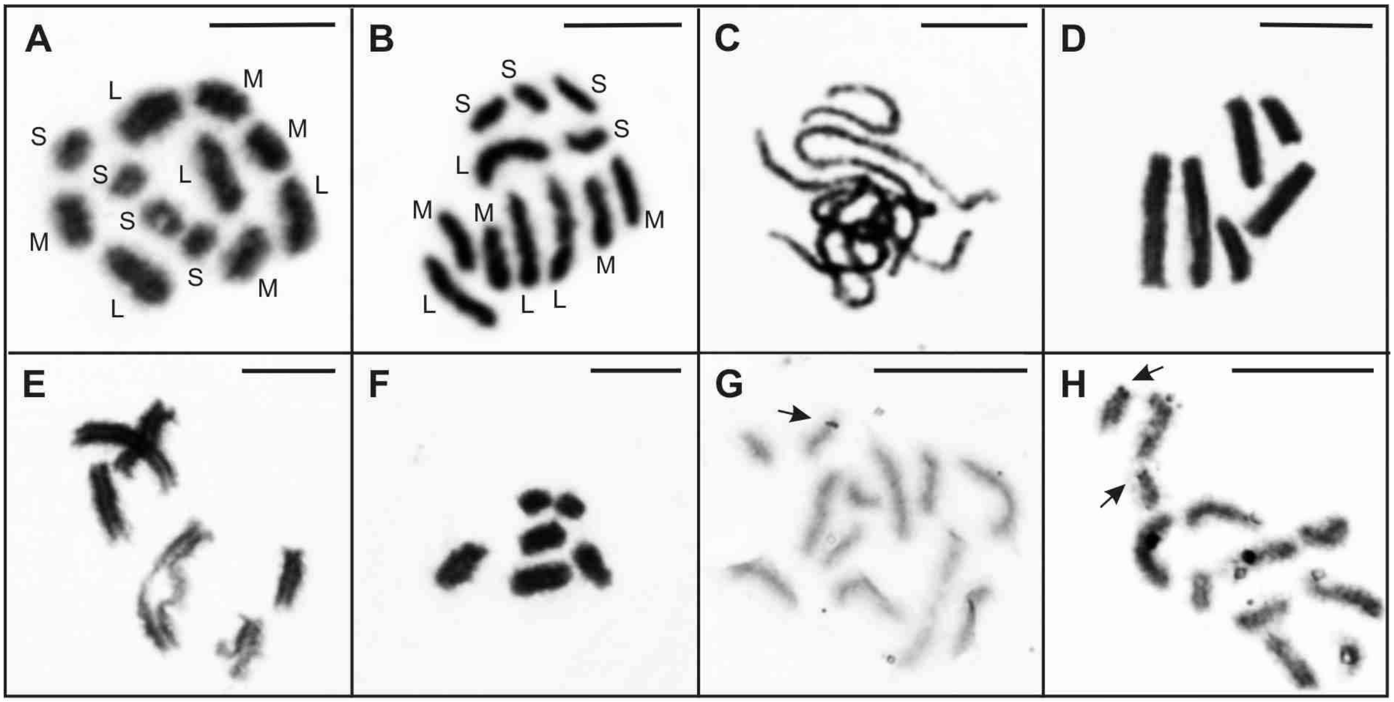

Figure 2.—Mitotic and meiotic cells of *Tityus serrulatus*. Male specimens: A, C–F, H; Female specimens: B, G. A, B. Mitotic metaphase, with 2n=12. C. Pachytene. D, E. Postpachytene, with six bivalents. F. Metaphase II, n=6. G, H. Silver–impregnated cells. The arrows point to nucleolar organizer regions on the terminal region of either one (G) or two (H) small-sized chromosomes. L = large; M = medium; S = small. Scale bars = 10μm.

(two large, two medium and two small pairs). The characteristics of the female chromosomes were similar to those of the males (Fig. 2B). The pachytene nuclei of the males exhibited the total synapsis of the chromosomes and the absence of a sexual vesicle (Fig. 2C). The postpachytene cells had six bivalents, with the chromosomes in a parallel arrangement. In this meiotic substage, all chromosomes were paired, although there was no evidence of chiasmata (Fig. 2D). In some cells, the interstitial region of the bivalents presented a gap or less-condensed chromatin (Fig. 2E). Metaphase II cells invariably contained the haploid number n=6 (Fig. 2F). The chromosomes of both male and female specimens were measured to determine the relative chromosome length and the precise localization of Ag-NORs/rDNA sites and heterochromatic region after the FISH (Fig. 3H). The size of the chromosomes and the location of these regions were similar to male and female specimens from all populations investigated.

In the silver-impregnated mitotic metaphase nuclei of the specimens from Itaobim, terminal NORs were found on only one small chromosome in the females, but on two small chromosomes in the males (Figs. 2 G,H). Two terminal NORs were observed in the males from Itacarambi and Januária, although only one silver-impregnated chromosome was observed in approximately 40% of the cells. The rDNA-FISH confirmed the Ag-NORs, indicating the presence of 28S genes in only one small mitotic chromosome in the females (Fig. 3D), but in two chromosomes in the males (Figs. 3A–C). In addition, in the pachytene and postpachytene spermatocytes, the rDNA probe hybridized with the terminal region of one small bivalent (Figs. 3 B,C). The FISH with the telomeric probe revealed discrete signals at the ends of all chromosomes in the males (Figs. 3 F,G). Male meiotic cells analysed with DAPI after FISH presented a bright heterochromatin region in the terminal region of one small-sized bivalent, which was associated with rDNA. In females, this heterochromatic region was easily visualized in only one chromosome (Fig. 3E).

DISCUSSION

The diploid number and the chromosome size recorded in the bisexual populations of *T. serrulatus* examined in this study were similar to those described previously for two parthenogenetic populations (Schneider & Cella 2010). This conservation of the karyotype is not typical for buthid scorpions, which frequently present variability in the chromosome complement both within and among populations (for a review, see Schneider et al. 2019).

In another parthenogenetic scorpion, *T. trivittatus* Kraepelin, 1898 (2n=6), Adilardi et al. (2014) recorded similar chromosomal characteristics in three Argentinean populations, and concluded that the conservation of the diploid number in this species may be related to the recent colonization of cities in the first half of the 20th century. Alternatively, the populations found in urban areas may have originated from a single lineage. The parthenogenetic and synantropic populations of *T. serrulatus* were probably derived from recently discovered bisexual populations inhabiting natural environments in the Brazilian semiarid zone. However, without any further phylogeographic studies and only based on the conserved macrokaryotype structure of the analysed populations, it would be premature to conclude that all parthenogenetic populations belong to a unique lineage.

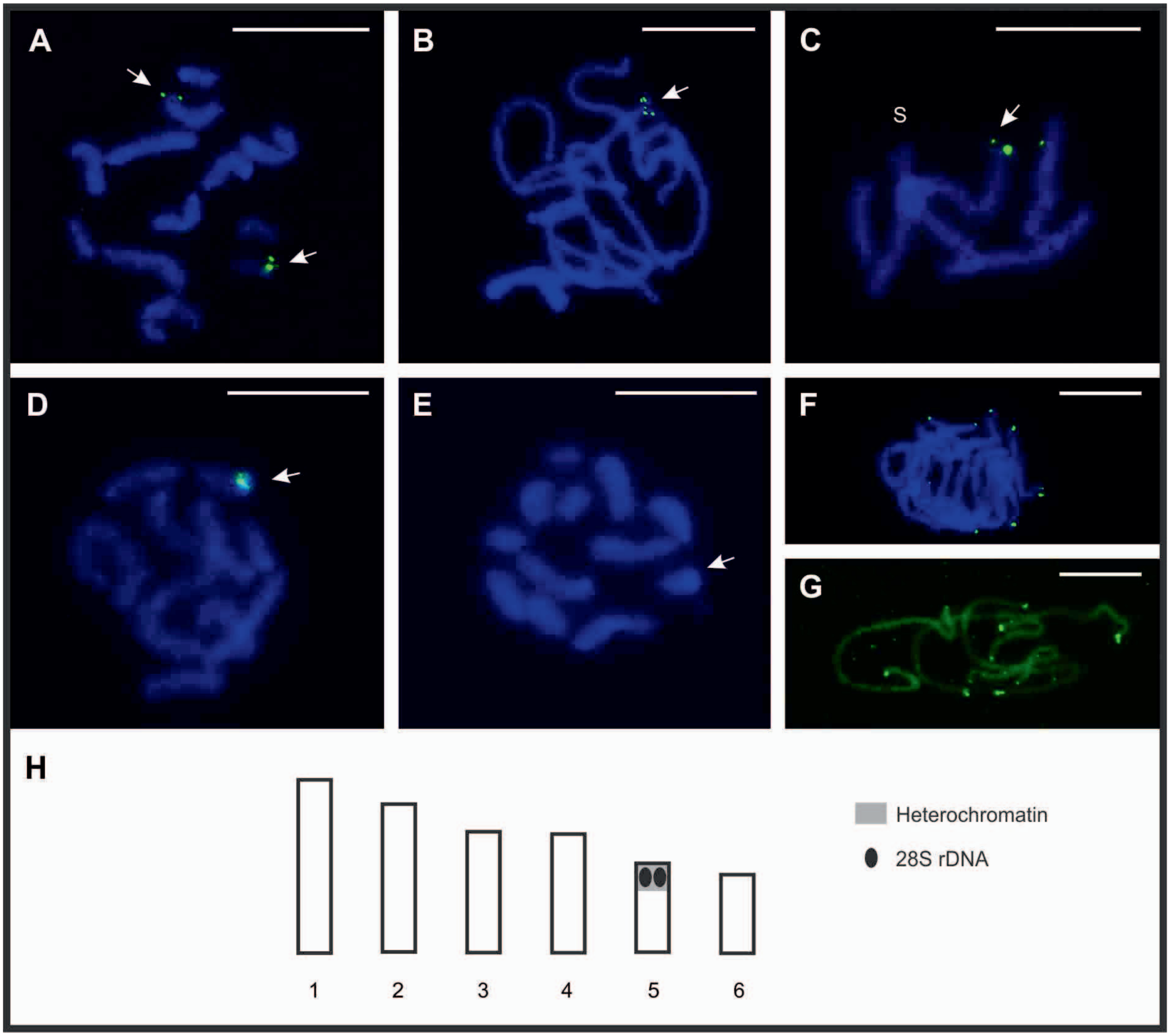

Figure 3.—Fluorescent *in situ* hybridization using 28S rDNA (A–D) and telomeric probes (F, G), DAPI after FISH (E) and male ideogram (H) of *Tityus serrulatus*. A. Male mitotic cell with ribosomal cistrons in the terminal regions of two small-sized chromosomes (arrows). B, C. Male pachytene and postpachytene, respectively, showing rDNA in one small-sized bivalent (arrow). D. Female mitotic metaphase cell, with only one rDNA site (arrow). E. Female mitotic cell with DAPI after FISH, showing terminal heterochromatin in one chromosome (arrow). F, G. Male pachytene, showing telomeric labelled signals at the ends of the bivalents. The cell in G was only analysed with filter for Alexa-Fluor-488. Ideogram showing the localization of heterochromatin and 28S rDNA sites. In female, only one chromosome of the pair 5 carries these regions. Scale bars = 10μm.

The chromosome behavior in the male meiotic cells of *T. serrulatus* was typical of that of all other scorpion species for which data are available, i.e., with chromosomes forming a parallel arrangement in meiosis I, indicating the absence of chiasmata; lack of bivalents with differential condensation and/or segregation. In analyses of the postpachytene cells of 17 *Tityus* species, the exclusive occurrence of bivalents, as observed in *T. serrulatus*, has been documented in two populations of *T. bahiensis* (Perty, 1833) and *T. obscurus* (Gervais, 1843), and in *T. fasciolatus* Pessôa, 1935, *T. magnimanus* Pocock, 1897, and *T. neglectus* Mello-Leitão, 1932 (Piza 1939, 1940; Kovarík et al. 2009; Mattos et al. 2013; Almeida et al. 2017). In these species, however, all the chromosomes were paired, with the exception of *T. fasciolatus* (2n=14), which presented unsynapsed interstitial or terminal regions in two of the bivalents. In *T. serrulatus*, the poorly-condensed interstitial regions observed in some bivalents may indicate the lack of complete synapsis, originating from minor heterozygous rearrangements. This was inferred for *T. fasciolatus* and confirmed through the analysis of the synaptonemal complex by transmission electron microscopy (Mattos et al. 2013; Schneider et al. 2015).

After detecting these differentially paired regions in some of the *T. serrulatus* bivalents, we accomplished FISH with telomeric probe, which may reveal the presence of interchromosomal telomere sequences (ITS) (Ocalewicz 2012). These ITSs have been observed in many vertebrate groups and may represent remnant telomeres left over from chromosomal rearrangements (Bertoni et al. 1996; Fagundes & Yonenaga-Yassuda 1998; Pellegrino et al. 1999; Go et al. 2000; Metcalfe et al. 2007). In *T. serrulatus*, we observed only terminal signals, similar to those found in other buthid and scorpionid species (Vítková et al. 2005; Adilardi et al. 2015, 2016; Almeida et al. 2017; Ojanguren-Affilastro et al. 2017; Mattos et al. 2018; Ubinski et al. 2018). Therefore, the ITSs may be lost during chromosomal rearrangements in *Tityus* species or they may not be detected by FISH due to the small number of repeats.

The presence of two terminal Ag-NORs and 28S rDNA genes, as observed in the males of *T. serrulatus*, is also the most common pattern in *Tityus* species (Mattos et al. 2013, 2014, 2018; Adilardi et al. 2014, 2016; Almeida et al. 2017; Ojanguren-Affilastro et al. 2017). This reinforces the conclusion that, in this genus, there is no clear relationship between the variation in the diploid number, which ranges from 2n=5 to 2n=32, and the number of rDNA sites. In relation to *T. serrulatus* males from Januária, the observation of only one Ag-NOR in some cells was certainly the result of the method used to obtain the data, which only identifies the regions with transcriptional activity. There was nevertheless a clear difference between female and male *T. serrulatus* specimens in the number of rDNA sites, with the females of both parthenogenetic (Schneider & Cella 2010) and bisexual populations presenting only one small-sized chromosome containing rDNA. This may indicate a sex-specific heteromorphism, pointing to the presence of cryptic sex chromosomes in this species. In this case, the females are heterogametic (ZW), with the 28S rDNA being located on the Z chromosome, while the males are homogametic (ZZ), with two rDNA sites. The macrostructure of these sex chromosomes has yet to be differentiated, and the only karyotype feature that distinguishes the sexes is the presence of one or two major rDNA sites. The occurrence of sex-specific location of the Ag-NORs and/or rDNA sites has also been reported in amphibian species with morphologically undifferentiated sex chromosomes of the XY/XX and ZZ/ZW types (Schmid et al. 1993; Schmid & Steinlein 2003). However, as emphasized by Schmid & Steinlein (2003), the presence of chromosomes with one (female) or two (male) rDNA cistrons probably has no influence on sex-determination, but is a reliable cytological marker for identifying the cryptic sex chromosomes. An alternative explanation for the location of the rDNA cistrons in only one chromosome of the females of *T. serrulatus* herein investigated is a deletion of these genes and its associated heterochromatin from one homologous chromosome. This hypothesis, however, is weakly supported, considering that all parthenogenetic females previously studied collected from two distinct populations also presented only one chromosome carrier of the rDNA (see Schneider & Cella 2010).

Until recently, morphologically differentiated sex chromosomes had not been found in scorpions. This appears to be the rule for Arachnida, given that homomorphic sex chromosomes have also been recorded in most acariform mites, Opiliones, Amplypygi and Palpigradi (Král et al. 2008; Paula-Neto et al. 2013; Tsurusaki et al. 2017). However, in an elegant study, Adilardi et al. (2016) demonstrated that males of *T. confluens* Borelli, 1899 are structurally heterozygotes for different chromosome rearrangements, while the females are invariably homozygotes. With this result, the authors provided the first evidence of cryptic sex chromosomes in *T. confluens*, with the males being the heterogametic sex (XY/XX system).

In a parthenogenetic population of *T. serrulatus*, the existence of only one chromosome with a rDNA cistron prompted Schneider & Cella (2010) to suggest that the eggs develop apomictically. Where the meiosis persists, oocytes lacking rDNA cistrons may be generated, reducing the fecundity of the individuals. The findings of the present study further reinforce this hypothesis. As the females are the heterogametic sex (ZW), the occurrence of meiosis and the recovery of the diploid number in the zygotes through the retention of a second polar body, as observed in the scorpion *Liocheles australasiae* (Fabricius, 1775) (Ischnuridae) (Yamazaki et al. 2001), could generate both WW (no rDNA genes) and male (ZW) offspring. But, neither type of offspring has yet been documented in *T. serrulatus*.

Many studies focusing on the evolution of sex chromosomes have demonstrated that progressive rearrangements, primarily through the accumulation of repetitive sequences and point mutations, established the morphological differentiation of the chromosomes (Reed & Philips 1997; Steinemann & Steinemann 1998; Ellegren 2011; Cioffi et al. 2012). In *T. serrulatus*, the difference between males and females, regarding the number of the 28S rDNA and its associated heterochromatin, may represent the early stage in the differentiation of sex chromosomes. In this context, the comparative investigation of the quantity and distribution of other repetitive sequences, such as the microsatellites and transposable elements, may reveal other sex-specific chromosome differences in scorpions.

Overall, our findings indicate that scorpions, in particular those of the genus *Tityus*, may provide excellent models for the investigation of chromosome evolution given the existence of species in which males are the homogametic or heterogametic sex. These species also contribute to the study of the evolution of the sex chromosomes in an unusual meiotic system, in which genetic recombination is absent in all chromosomes.

ACKNOWLEDGMENTS

The authors are grateful to two anonymous reviewers for critical reading and suggestion in the manuscript. Colleting permits were granted by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) through the Sistema de Autorização e Informação em Biodiversidade (SISBIO - #51395-1). This paper was also part of the "Programa de Pesquisas em Biodiversidade do Semiárido – PPBio Semiárido (CNPq - #558317/2009-0, 457471/2012-3).

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Manuscript received 17 April 2018, revised 30 August 2019.