

Male and female meiosis in the mountain scorpion *Zabius fuscus* (Scorpiones, Buthidae): heterochromatin, rDNA and TTAGG telomeric repeats

Renzo Sebastián Adilardi¹ · Andrés Alejandro Ojanguren-Affilastro² · Camilo Iván Mattoni³ · Liliana María Mola¹

Received: 20 August 2014 / Accepted: 14 April 2015 / Published online: 22 April 2015
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Abstract All cytogenetically studied scorpions present male achiasmatic meiosis and lack heteromorphic sex chromosomes. In contrast, information about female meiosis in scorpions is scarce due to the difficulty of finding meiotic cells. The genus *Zabius* includes three described species and no chromosome studies have been performed on it until now. We analyzed the constitutive heterochromatin distribution, NORs and telomeric sequences in mitosis and meiosis of males and females of different populations of *Zabius fuscus*. All specimens presented $2n = 18$ holokinetic chromosomes that gradually decreased in size. Male meiosis presented nine bivalents and a polymorphism for one reciprocal translocation in one population. Telomeric signals were detected at every terminal region, confirming also the presence of a $(TTAGG)_n$ motif in Buthidae. Constitutive heterochromatin was found in three chromosome pairs at a terminal region; moreover, NORs were embedded in the heterochromatic region of the largest pair. Chromosome size and landmarks allowed us to

propose the chromosomes involved in the rearrangement. In four females, cells at different prophase I stages were analyzed. We describe a diffuse stage and the presence of ring-shaped bivalents. We discuss the possible origin of these bivalents in the framework of chiasmatic or achiasmatic female meiosis. These results contribute to increase the scarce evidence of female meiosis in scorpions and raise new questions about its mechanism.

Keywords Holokinetic chromosomes · Achiasmatic meiosis · Telomere · FISH · Female diffuse stage

Introduction

Zabius Thorell 1893, belonging to family Buthidae C. L. Koch 1837, occurs in southern South America, in xeric and hilly areas of southern Brazil, Paraguay and central Argentina (Ábalos 1953; Teruel 2002; Ojanguren-Affilastro 2005; Acosta et al. 2008). This is the southernmost genus of the family Buthidae, reaching latitude 41°S in the north of the Argentinean Patagonia. Currently, *Zabius* only includes three described species: *Zabius birabeni* Mello-Leitão 1938, *Zabius gaucho* Acosta, Candido, Buckup and Brescovit 2008, and *Zabius fuscus* (Thorell 1876); however, at least two further undescribed species of the genus are known from Argentina and Paraguay (Mattoni and Ojanguren-Affilastro, unpublished data).

Zabius fuscus has been widely studied, since it is quite a common species in the highly populated hilly areas of central Argentina. Because of this, its systematics, ecology and reproductive strategies are very well known (Peretti 1991, 1993; Acosta and Rosso de Ferradás 1996; Peretti and Carrera 2005). Nevertheless, until now no cytogenetic studies on the genus have been performed.

✉ Renzo Sebastián Adilardi
rsadilardi@yahoo.com.ar

¹ Laboratorio de Citogenética y Evolución, Departamento de Ecología, Genética y Evolución, IEGEBA (CONICET-UBA), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, C1428EGA CABA, Argentina

² División de Aracnología, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, CONICET, Ángel Gallardo 470, C1405DJR CABA, Argentina

³ Laboratorio de Biología Reproductiva y Evolución (CONICET-UNC), Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, X5000JJC Córdoba, Argentina

Buthidae scorpions present holokinetic chromosomes and, as the rest of the cytogenetically studied scorpions, achiasmatic male meiosis and a lack of heteromorphic sex chromosomes. Chromosome numbers are highly variable within this family, from $2n = 5$ in some individuals of *Tityus bahiensis* (Perty 1833) to $2n = 48$ in *Uroplectes carinatus* (Pocock 1890), and a remarkable incidence of structural heterozygosis for translocations is present, evidenced as multivalent associations during meiosis I (see Supplementary Table 1 in Schneider et al. 2009a; Mattos et al. 2013; Kovářik et al. 2013; Adilardi et al. 2014).

The achiasmatic meiosis is usually restricted to the heterogametic sex in bisexual species (White 1973). In arachnids it is found in males of acariform mites (Keyl 1957), pseudoscorpions of the family Chthoniidae Daday 1888 (Štáhlavský and Král 2004) and it was proposed for spiders of the families Dysderidae C. L. Koch 1837 and Segestriidae Simon 1893, although the presence of chiasmata was also stated (Benavente and Wettstein 1980; Rodríguez Gil et al. 2002; Král et al. 2006).

The presence of achiasmatic male meiosis in scorpions has been vastly confirmed in all cytogenetic studies of different families (overviewed in Schneider et al. 2009a). In contrast, information about female meiosis is scarce due to the difficulty of finding meiotic cells from ovariuteri preparations, as mentioned by Piza (1939), Shanahan (1989a) and Schneider et al. (2009b). In only five species of scorpions some female meiotic characteristics were mentioned: *Heterometrus madraspatensis* Pocock 1900 (sub *Palamnaeus fulvipes madraspatensis*) (Scorpionidae Latreille 1802), *Urodacus manicatus* (Thorell 1876) (Urodacidae Pocock 1893), *Liocheles australasiae* (Fabricius 1775) (Hormuridae Laurie 1896), *Lychas mamoreus* (C. L. Koch 1844) and *Lychas variatus* (Thorell 1876) (Buthidae) (Nath 1925; Shanahan 1989a, b; Yamazaki et al. 2001).

In this work we analyzed male and female mitosis and meiosis of *Zabius fuscus* by Giemsa staining, C-banding, Ag-NOR staining and FISH with 28S rDNA and telomeric (TTAGG)_n probes. Furthermore, we present new features of female meiosis in scorpions and discuss about its chiasmatic or achiasmatic nature.

Materials and methods

Specimens were manually collected by the authors under rocks during the day and with the aid of ultraviolet flashlights at night, in hilly areas of Córdoba province, central Argentina. The studied sample consisted of five males and nine females collected near the city of La Falda (31°4.818'S, 64°28.379'W), two males and one female from Dique El Cajón (30°51.306'S, 64°33.922'W), two

males, two females and 11 embryos (of one of these females) from Flor Serrana (31°21.461'S, 64°34.892'W) and one male from Chancaní (31°25.109'S, 65°27.448'W).

All specimens were carried alive to the laboratory. The testes, ovariuteri and embryos were dissected in saline solution and cytogenetic preparations were performed according to Adilardi et al. (2014). Conventional staining was carried out with 5 % Giemsa solution in distilled water for 12–15 min. The C-banding was performed according to the protocol described by Sumner (1972) and then stained with DAPI (4',6-diamidino-2-phenylindole). The study of the nucleolar organizer regions (NORs) was carried out by silver-staining technique according to Howell and Black (1980).

Fluorescence in situ hybridization (FISH) technique was performed with 28S rDNA and (TTAGG)_n telomeric probes. Unlabeled 28S ribosomal probes were obtained by PCR, using as a template genomic DNA from *Tityus trivittatus* Kraepelin 1898 as described by Adilardi et al. (2014). Digoxigenin-11-dUTP was used in a PCR for labelling. Telomeric probe was generated by PCR in absence of template (Ijdo et al. 1991). The protocol was performed according to López Fernández et al. (2004) with minor modifications in the design of primers and PCR mix. In brief, PCR was set up in 50 µl of reaction mixture containing 1.5 mM MgCl₂, 0.02 mM of each dNTP, 0.5 µM of each of the two primers (5'-TTAGGTTAGGTTAGGTTAGGTTAGG-3' and 5'-CCTAACCTAACCTAACCTAACCTAA-3') and 1.5 U *Taq* DNA polymerase. Labelling was carried out by PCR with biotin-16-dUTP. PCR was done in a GenePro (Bioer) thermal cycler.

FISH was performed as described by González et al. (2004) with slight modifications. The hybridization mixture per slide (50 µl) contained 200 ng of each probe, 50 % deionized formamide, 10 % dextran sulfate and 2XSSC. After overnight hybridization the probes were detected with anti-digoxigenin-fluorescein Fab fragments (Roche Applied Science, Mannheim, Germany) and streptavidin-Cy3 conjugate (Sigma, St Louis, MO, USA). The preparations were counterstained with DAPI and mounted in Vectashield (Vector, Burlingame, CA, USA).

The slides were examined under a Leica DMLB microscope equipped with a Leica DFC350 FX monochrome digital camera. Pictures were pseudo-coloured and processed with Adobe Photoshop CS5. To study the meiotic karyotype, chromosome measurements of well-spread C-banded male prometaphase I cells were performed using ImageJ software (<http://imagej.nih.gov/ij/>). For this purpose two males from La Falda, one from Dique El Cajón and one from Flor Serrana were employed, and at least five cells per specimen were measured. The relative length of each bivalent was calculated as a percentage of total haploid complement length (%TCL). Also, the bivalents and

the quadrivalent of seven cells from the male with the reciprocal translocation were measured.

Results

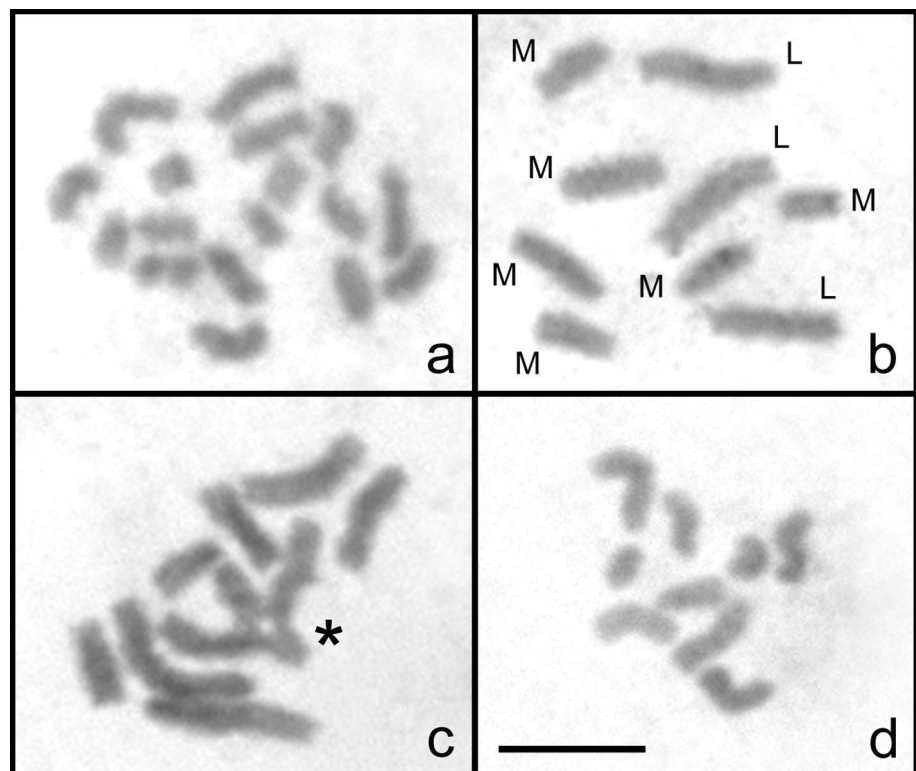
Mitotic cells of *Zabius fuscus* presented $2n = 18$ holokinetetic chromosomes in all the studied males, females and embryos (Fig. 1a). At the first meiotic division seven males (three from La Falda, and the males from Dique El Cajón and Flor Serrana) showed nine homomorphic bivalents that gradually decrease in size. The nine bivalents were separated for practical purposes in two groups: the three larger bivalents (number 1–3, ranging from 16 to 13 % of the TCL) and six middle-sized bivalents (number 4–9, ranging from 12 to 7 % of the TCL) (Fig. 1b). One male from La Falda showed seven bivalents and one quadrivalent. The quadrivalent was large and there were two other large bivalents of different size and five middle-sized bivalents decreasing in size (Fig. 1c). The males with nine bivalents as well as the male with the quadrivalent presented nine chromosomes at the second meiotic division in all the cells (Fig. 1d). In one male from La Falda and the male from Chancaní only mitotic cells were found.

FISH with $(TTAGG)_n$ telomeric repeats on mitotic cells revealed two signals on each terminal region of all chromosomes (Fig. 2a). The localization of terminal

chromosomal regions by the telomere probes facilitated a detailed study of male meiosis. At leptotene/early zygotene all the telomeric signals were grouped in one region of the nucleus in a *bouquet* configuration (Fig. 2b). Later at zygotene some telomeric regions were disposed in a broader region (Fig. 2c). At mid-pachytene the telomeric signals were already dispersed along the nucleus (Fig. 2d). At postpachytene and prometaphase I the four telomeric signals of each terminal region (one per sister chromatid) could be identified in many bivalents as well as in the quadrivalent (Fig. 2e, f). The telomeric signals at one terminal region of the largest bivalent were frequently weaker than the other signals (Fig. 2f). At late prophase II the chromatids of each chromosome were indistinguishable except for the double telomere signal at the terminal regions (Fig. 2g). At metaphase II the chromosomes presented the highest degree of condensation, each chromatid was visible with its telomeric signals, and some chromosomes seemed to be bent (Fig. 2h). At early anaphase II all the chromatids were bent and showed a parallel migration (Fig. 2i). No interstitial telomeric signal was observed at any stage.

Silver staining on mitotic cells revealed the presence of NORs at one terminal region of the largest pair of chromosomes (Fig. 3a). Hybridization with 28S rDNA probe showed terminal ribosomal clusters in the largest bivalent, both in the males with bivalents of all populations as in the

Fig. 1 Giemsa staining of *Zabius fuscus* ($2n = 18$). **a** Female mitotic metaphase; **b** Male meiotic prometaphase I with nine bivalents of large (L) and medium (M) sizes; **c** Male meiotic prometaphase I with seven bivalents and one quadrivalent. **d** Male meiotic late prophase II. *Asterisk* indicates the quadrivalent. *Scale bar* 10 μ m



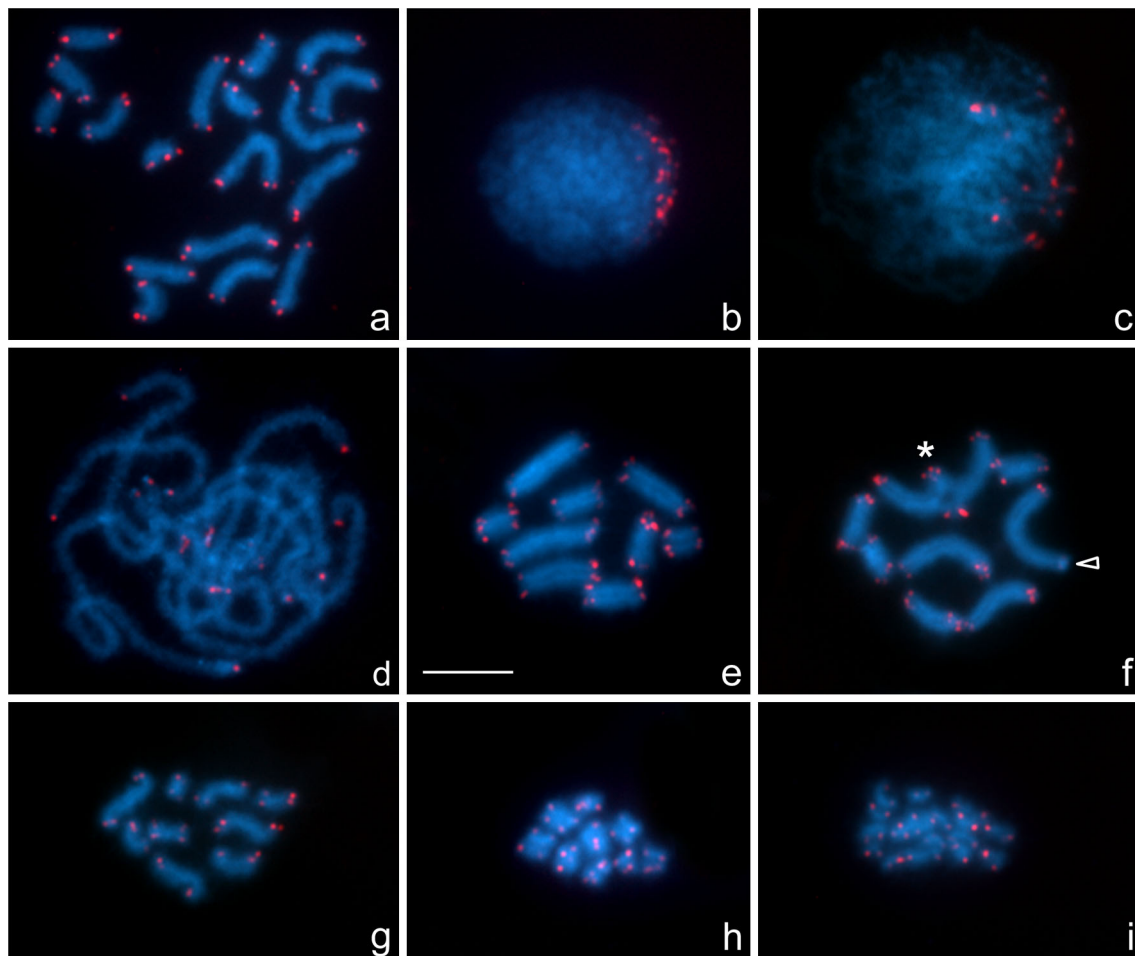


Fig. 2 Fluorescence in situ hybridization with $(TTAGG)_n$ telomeric probes in embryonic mitosis (**a**) and male meiosis (**b–i**) of *Zabius fuscus*. **a** Late mitotic prophase; **b** Leptotene/early zygotene showing a bouquet configuration; **c** Zygotene; **d** Pachytene; **e** Prometaphase I with nine bivalents; **f** Late postpachytene with seven bivalents and

one quadrivalent; **g** Late prophase II; **h** Metaphase II; **i** Early anaphase II. Chromosomes are counterstained with DAPI. Asterisk indicates the quadrivalent. Empty arrowhead indicates weak telomeric signals. Scale bar 10 μ m

male with the quadrivalent (Fig. 3b, c). These terminal regions were the ones that presented weaker telomeric signals. In the male with the translocation the rDNA signal was fainter in one homologue, probably indicating different number of ribosomal cluster repeats (Fig. 3c). C-banding with DAPI staining showed terminal bands at three chromosome pairs. The largest pair presented conspicuous C-bands at one terminal region and sequential C-banding and silver staining revealed that the NORs were in these same terminal regions (Fig. 3d, g). Pairs number seven and nine showed small C-bands at one terminal region (Fig. 3d, g). Both in the males with bivalents of all populations and in the male with quadrivalent the C-banding pattern was present in three bivalents: the largest (number 1), one medium-sized (number 7) and the smallest (number 9) (Fig. 3e, f).

The analysis of ovariuteri preparations of one adult and three sub-adult females revealed a total of 61 cells at

different stages of meiotic prophase I. Moreover, in two of these females mitotic cells with 18 chromosomes were also observed (Fig. 4a). Most of the meiotic cells were at diffuse stage and presented variable degree of chromatin decondensation and increased cellular size (Fig. 4b–d). DAPI staining and telomeric probes allowed us to determine the number and general morphology of the bivalents. At early diffuse stage it was not possible to differentiate all the bivalents. However, in the bivalents that could be analyzed, the telomeric signals were at the terminal regions, which often presented a fork-like structure. Some bivalents showed a medial separation between homologous chromosomes, while others remained paired (Fig. 4b). At a later diffuse stage nine bivalents could be identified; they showed more defined boundaries due to chromatin condensation. At this stage the large and medium-sized bivalents showed meiotic configurations resembling

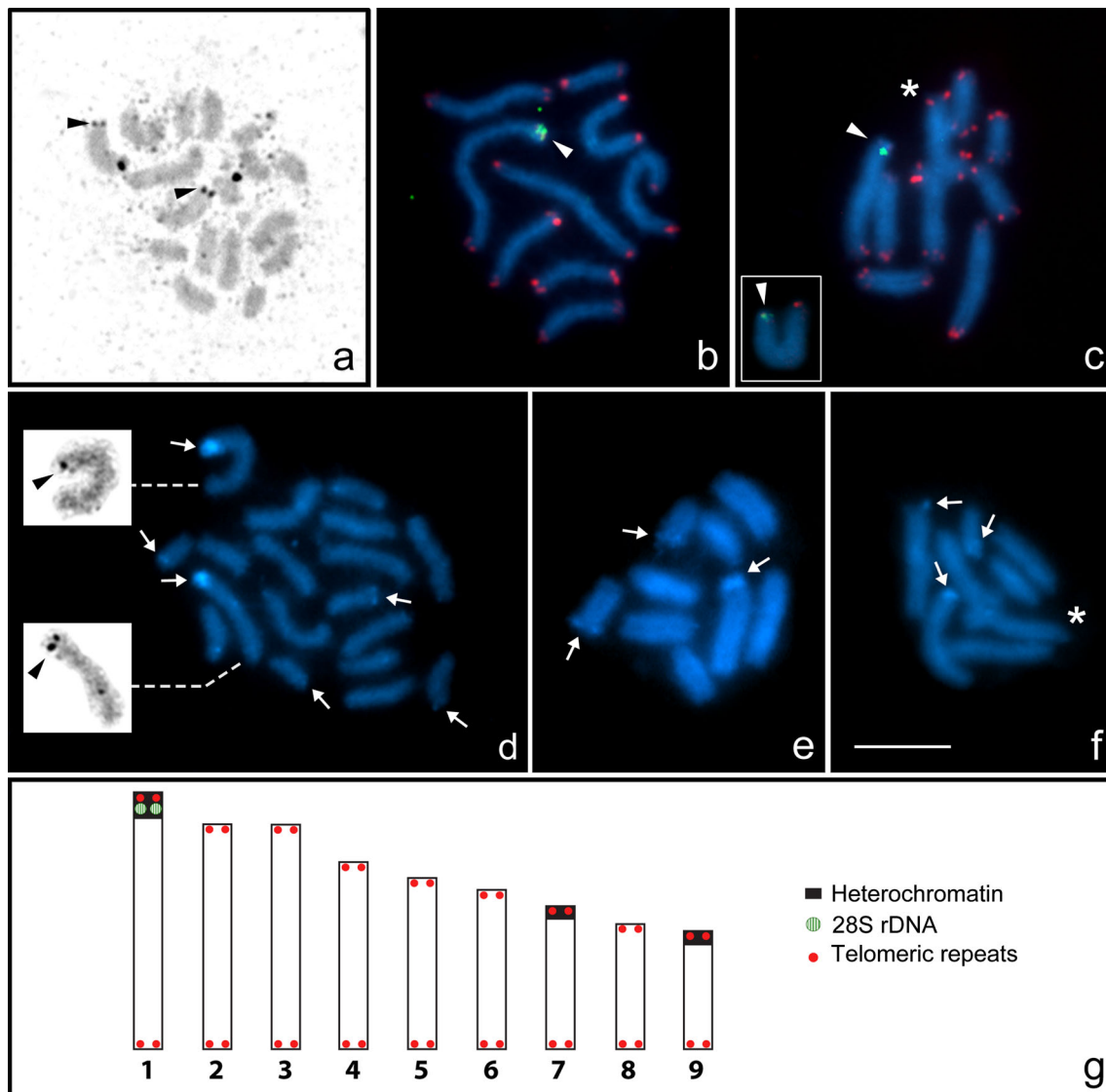


Fig. 3 NORs, FISH, C-banding stained with DAPI and meiotic idiogram of *Zabius fuscus*. **a** Silver staining of female mitotic prometaphase; **b** Male late postpachytene with nine bivalents after FISH with 28S rDNA and telomeric (TTAGG)_n probes; **c** Male prometaphase I with seven bivalents and one quadrivalent after FISH with 28S rDNA and telomeric (TTAGG)_n probes. *Inset* bivalent number 1 from the same male showing difference in 28S rDNA signal size between homologous chromosomes; **d** C-banding of embryonic

mitotic prometaphase. *Insets* same chromosomes after silver staining; **e** C-banding of male prometaphase I with nine bivalents; **f** C-banding of male prometaphase I with seven bivalents and one quadrivalent; **g** Idiogram showing distribution of constitutive heterochromatin and FISH results. *Black arrowheads* indicate Ag-NORs. *White arrowheads* indicate 28S rDNA hybridization signals. *White arrows* indicate heterochromatic bands. *Asterisks* indicate the quadrivalent. *Scale bar* 10 μm

ring-shaped bivalents. In these bivalents the homologous chromosomes were associated in the subterminal regions and their terminal regions were separated in a fork-like structure, while the smaller bivalents did not show any clear medial separation (Fig. 4c, d). Only five cells presented more condensed bivalents at a diakinesis-like stage. Most of the bivalents showed telomeric signals at both terminal regions and some large bivalents presented the ring-shaped morphology (Fig. 4e). No hybridization signals for the 28S rDNA probes could be obtained from these preparations.

Discussion

In ten genera of Buthidae for which meiosis has been studied (*Ananteris* Thorell 1891, *Androctonus* Ehrenberg 1828, *Gint* Kovařík, Lowe, Plíšková, Štáhlavský 2013, *Isometroides* Keyserling 1885, *Isometrus* Ehrenberg 1828, *Lychas* C. L. Koch 1845, *Mesobuthus* Vachon 1950, *Odontobuthus* Vachon 1950, *Rhopalurus* Thorell 1876 and *Tityus* C. L. Koch 1836) some or all species showed individuals with one or more reciprocal translocations in heterozygosis. This was evidenced in meiosis by the

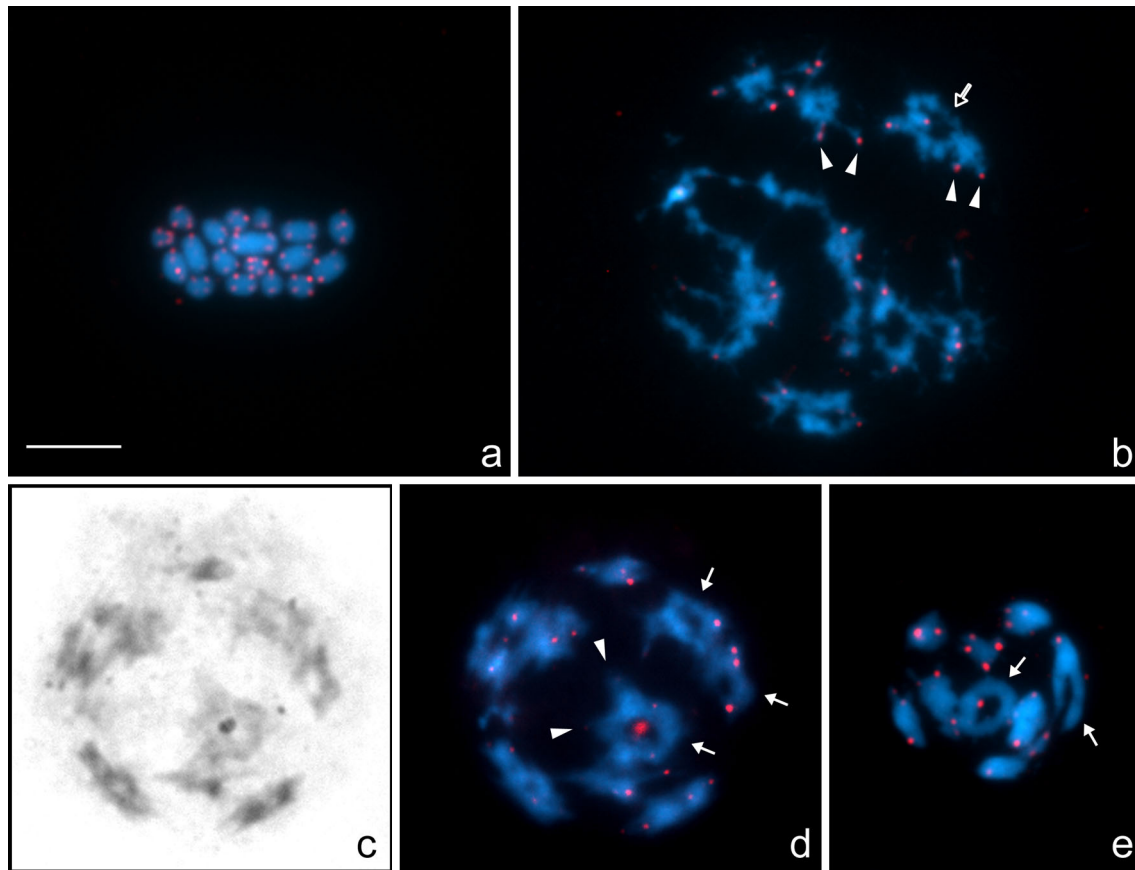


Fig. 4 Female mitosis and meiosis of *Zabius fuscus* after FISH with telomeric probes (**a**, **b**, **d**, **e**) and Giemsa staining (**c**). **a** Mitotic metaphase. **b** Diffuse stage. **c** Late diffuse stage. **d** Late diffuse stage (same cell shown in **c**). **e** Diakinesis-like stage. *Arrowheads* show

fork-like terminal chromosomal regions. *Empty arrow* shows bivalent with medial separation. *Arrows* show ring-shaped bivalents. *Scale bar* 10 μ m

occurrence of multivalents composed of different number of chromosomes: from four in many species, up to 28 in *Rhopalurus agamemnon* (C. L. Koch 1839) (Sato 1940; Sharma et al. 1959; Shanahan 1989a; Moustafa et al. 2005; Schneider et al. 2009b; Kovařík et al. 2013; Mattos et al. 2013). In other two genera only bivalents were found until now: in *Buthus occitanus* (Amoreux 1789) and in two species of *Centruroides* Marx 1890) (Wilson 1931; Guénin 1961; Yoshida and Toshioka 1964). *Zabius fuscus*, herein studied, is the first species of the genus cytogenetically analyzed and showed a polymorphism for one reciprocal translocation in specimens from La Falda. This result provides new data about the occurrence of heterozygosity for translocations in another genus of the family. Shanahan (1989a) stated that the establishment of interchanges is favored by the presence of achiasmatic male meiosis and alternate segregation of multivalents, which ensures balanced gametes, and proposed that interchange heterozygosity is of adaptive significance in Buthidae.

Until now, telomeric sequence in scorpions has only been studied in *Heterometrus spinifer* (Ehrenberg 1828) (Scorpionidae), where (TTAGG)_n motif was detected (Vítková et al. 2005). In this work we confirm the presence of (TTAGG)_n telomeric repeats in one species of Buthidae. Taking into account the phylogenetic distance between these two families (Soleglad and Fet 2003), TTAGG could be a conserved motif in the order Scorpiones. Our results support the hypothesis that this is an ancestral motif in arthropods (Vítková et al. 2005). Moreover, FISH with telomere probes allowed a more detailed analysis of early male prophase I in *Z. fuscus*. The clear identification of the clustering of telomeric regions on a restricted area of the nuclear envelope in a *bouquet* configuration was possible, as well as its disassembly at pachytene stage.

Buthidae presented differences in constitutive heterochromatin amount and distribution (Shanahan 1989a; Mattos et al. 2013). In *Zabius fuscus* constitutive heterochromatin was at the terminal regions and restricted to only a few pairs of chromosomes. A similar pattern is also

present in *Rhopalurus agamemnon*, *Tityus bahiensis*, *T. maranhensis* Lourenço, de Jesus Junior and Limeira-de-Oliveira 2006, *T. paraguayensis* Kraepelin 1895 and *T. stigmurus* (Thorell 1876) (Mattos et al. 2013). In *Zabius fuscus* the terminal NORs were located at the conspicuous blocks of constitutive heterochromatin of the largest pair. The association between ribosomal clusters and heterochromatin is also present in other species of Buthidae and seems to be a shared feature within this family (Schneider et al. 2009b; Schneider and Cella 2010; Mattos et al. 2013; Adilardi et al. 2014).

A comparative analysis of the normal karyotype of *Zabius fuscus* versus the one with the translocation was conducted to propose a possible origin for the quadrivalent. Taking into account the identical features of heterochromatin and NORs distribution, we can conclude that pairs 1, 7 and 9 were not involved in the translocation. Moreover, based on the size of the quadrivalent and the number of large and middle-sized bivalents in this male, we propose that the chromosomes involved in the rearrangement belong to one member of pairs 2 or 3 (large) and one of the larger pairs of middle-sized chromosomes of the normal karyotype. Additional chromosome landmarks would be necessary to unequivocally identify the pairs involved in the rearrangement.

Female meiosis in scorpions has been scarcely studied, with data available only from five species. Nath (1925) shows two drawings of oocytes chromosomes from *Heterometrus madraspatensis* (Scorpionidae), but there is no discussion about the meiotic process in the text. Shanahan (1989a, b) describes a few “putative metaphase I” on females of *Lychas mamoreus*, *Lychas variatus* (Buthidae) and *Urodacus manicatus* (Urodacidae) with chromosomal bodies that were similar to the achiasmate bivalents found on males of these species. Finally, in a detailed histological work of the parthenogenetic scorpion *Liocheles australasiae* (Hormuridae), Yamazaki et al. (2001) describe the course of meiosis in serial sections, but in the karyological observations there is no mention about the chiasmatic or achiasmatic nature of the meiotic division.

In the present study we found meiotic cells in four females of *Zabius fuscus*. The chromatin decondensation and the shape of the bivalents in most cells led us to propose that they were at diffuse stage. Diffuse stage has not been described yet in scorpions; nevertheless, it has been described in males of almost all groups of arachnids: spiders (Suzuki 1954; Benavente and Wettstein 1980; Rodríguez Gil et al. 2002; Král et al. 2006, 2013), ticks (Kahn 1964), mites (Haineman and Hughes 1970), harvestmen (Sharma and Dutta 1959), pseudoscorpions (Štáhlavský et al. 2006) and palpigrades (Král et al. 2008).

The ring-shaped morphology observed in the larger bivalents at late diffuse stage and at a diakinesis-like stage

(Fig. 4c–e) could indicate the presence of bivalents with two terminal or subterminal chiasmata. If this were the case, we would be in the presence of chiasmatic females and achiasmatic males, as seen in most bisexual species, in which achiasmatic meiosis occurs only in one sex (White 1973).

Shanahan (1989a) proposed that female and male scorpions possess a similar system, with achiasmatic meiosis and interchange heterozygosity, as the presence of achiasmatic meiosis could facilitate the regular disjunction of multivalents in both sexes. Considering this possibility, another explanation is that the terminal or subterminal associations between homologous chromosomes in *Zabius fuscus* are not really of chiasmatic nature. Alternative mechanisms are present in different organisms with achiasmatic meiosis to maintain homologous chromosomes associated until metaphase I, ensuring the regular disjunction of the bivalents (e.g. Debus 1978; Oakley 1982; Marec 1996; Kořínková and Král 2011; McKee et al. 2012).

Current results on female meiosis do not allow us to confirm the existence of either chiasmatic or achiasmatic meiosis in this cytogenetically uncommon group. Further studies are required to know the complete course of meiosis in female scorpions.

Acknowledgments This work was supported by grants from the National Council of Scientific and Technological Research (CONICET) (PIP 0342), University of Buenos Aires (UBA) (Ex 0859) and National Agency for Scientific and Technological Promotion (ANPCyT) (PICT 2010-1665) to Drs L. Poggio and L. Mola, ANPCyT (PICT 2010-0906) to Drs A. V. Peretti and C. I. Mattoni and ANPCyT (PICT 2010-1764) to Dr A. A. Ojanguren-Affilastro. The authors wish to thank to Gonzalo Rubio, Andrés Porta, Ernesto Costa-Schmidt and Hernán Iuri for their help during the field work.

Conflict of interest The authors declare that they have no conflict of interest.

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