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Molecular cytogenetics of *Androctonus* scorpions: an oasis of calm in the turbulent karyotype evolution of the diverse family Buthidae

DAVID SADÍLEK[1]†, PETR NGUYEN[2,3]†, HALİL KOÇ[4], FRANTIŠEK KOVAŘÍK[1], ERSEN AYDIN YAĞMUR[5] and FRANTIŠEK ŠŤÁHLAVSKÝ[1]*

[1]*Department of Zoology, Faculty of Science, Charles University in Prague, Viničná 7, CZ-12844 Prague, Czech Republic*
[2]*Institute of Entomology, Biology Centre ASCR, Branišovská 31, 37005 České Budějovice, Czech Republic*
[3]*Faculty of Science, University of South Bohemia in České Budějovice, Branišovská 1760, 37005 České Budějovice, Czech Republic*
[4]*Biology Department, Science and Art Faculty, Sinop University, Sinop, Turkey*
[5]*Celal Bayar University, Alaşehir Vocational School, Alaşehir, Manisa, Turkey*

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Recent cytogenetic and genomic studies suggest that morphological and molecular evolution is decoupled in the basal arachnid order Scorpiones. Extraordinary karyotype variation has been observed particularly in the family Buthidae, which is unique among scorpions for its holokinetic chromosomes. We analyzed the karyotypes of four geographically distant species of the genus *Androctonus* Ehrenberg, 1828 (*Androctonus australis*, *Androctonus bourdoni*, *Androctonus crassicauda*, *Androctonus maelfaiti*) (Scorpiones: Buthidae) using both classic and molecular cytogenetic methods. The mitotic complement of all species consisted of $2n = 24$ elements. Fluorescence *in situ* hybridization with a fragment of the 18S ribosomal RNA gene, a cytogenetic marker well known for its mobility, identified a single interstitial rDNA locus on the largest chromosome pair in all species examined. Our findings thus support the evolutionary stasis of the *Androctonus* karyotype, which is discussed with respect to current hypotheses on chromosome evolution both within and beyond the family Buthidae. Differences in karyotype dynamics between *Androctonus* spp. and the other buthids can help us better understand the driving forces behind their chromosome evolution and speciation. *Biological Journal of the Linnean Society*, 2015, **115**, 69–76.

ADDITIONAL KEYWORDS: 18S rDNA – FISH – holocentric chromosomes – holokinetic drive – multivalent association.

INTRODUCTION

The genome architecture of many related organisms has often diverged from ancestral karyotypes as a result of chromosomal rearrangements (e.g. inversions, translocations, fusions, and fissions). The extent and rate of evolutionary karyotype changes differ in different groups of organisms and most likely depend on physical characteristics of the karyotype and specific chromosome structures. For example, the minimum-interaction hypothesis suggests that a high number of small elements minimizes deleterious chromosome mutations originating in erroneous resolutions of chromosome interlockings in meiosis. Chromosome numbers should thus increase as a result of chromosome fission over the course of karyotype evolution (Imai *et al*., 1986). In organisms with holokinetic chromosomes, the absence of a localized

*Corresponding author. E-mail: stahlf@natur.cuni.cz
†These authors contributed equally to this work.

centromere is assumed to facilitate karyotype evolution via fusions and fissions by reducing the risk of the formation of dicentric and/or acentric chromosomes (Wrensch, Kethley & Norton, 1994; Melters *et al*., 2012; Bureš & Zedek, 2014). Furthermore, holokinetic drive (i.e. a mechanism analogous to centromere drive) has been invoked to explain considerable variation in chromosome size and number, as well as the negative correlation between chromosome number and genome size observed in some taxa with holokinetic chromosomes (Bureš & Zedek, 2014). However, Melters *et al*. (2012) noted that holokinetic clades in general do not show an increase in karyotype diversity. For example, comparative chromosome mapping revealed highly conserved genome organization in moths and butterflies (Lepidoptera) with $N = 31$ being both the modal and ancestral chromosome print (Baxter *et al*., 2011; Van't Hof *et al*., 2013). Although chromosome numbers range from $N = 5$ to $N = 223$ in Lepidoptera (Marec, Sahara & Traut, 2010), chromosomal instability is exceptional and is restricted only to several genera (Kandul *et al*., 2004; Lukhtanov *et al*., 2011; Talavera *et al*., 2013).

Scorpions of the family Buthidae are assumed to belong to the few holokinetic clades with extremely labile karyotypes (Melters *et al*., 2012). Buthids are basal and the most diverse family of the arachnid order Scorpiones (Prendini & Wheeler, 2005), which comprises 1016 species in 90 genera (Rein, 2014). Members of the family Buthidae have many interesting cytogenetic peculiarities. That explains why they belong to the most frequently cytogenetically studied scorpions, with 50 species from the 17 genera analyzed, which represents more than half of the scorpion species karyotyped so far (Schneider, Mattos & Cella, 2014). As in all the other scorpions, male meiosis is achiasmatic in buthids (Mattos *et al*., 2013). However, they are unique among scorpions as due to the holokinetic nature of their chromosomes. Buthids have lower number of chromosomes compared to other scorpions. Nevertheless, as noted above, their chromosome numbers are highly variable, ranging from $2n = 5$ to $2n = 56$ (Schneider *et al*., 2009a). Remarkable intraspecific variability in karyotypes has been reported in almost one-third of buthids examined so far (Schneider *et al*., 2009a). Yet, genome organization is exceptionally stable in some taxa, such as the genus *Androctonus* Ehrenberg, 1828 in which all five examined species possess $2n = 24$ chromosomes (Chovet, Deloince & Goyffon, 1971; Goyffon *et al*., 1971; Moustafa *et al*., 2005). This genus comprises 23 described species distributed in arid and semi-arid regions from north-western Africa and western Asia to India (Rein, 2014). However, an analysis of DNA indicated the existence of cryptic *Androctonus* species in north-western Africa (Coelho *et al*., 2014).

Tandem arrays of genes for major ribosomal RNAs (rDNA) was found to be a useful marker for exposing concealed karyotype variation in both monocentric and holokinetic taxa with an identical chromosome number (Cabral de Mello, Moura & Martins, 2011; Grzywacz *et al*., 2011; Panzera *et al*., 2012). The numbers and distribution of rDNA clusters in scorpions were examined mostly by classic silver staining (Schneider *et al*., 2009b; Schneider & Cella, 2010; Mattos *et al*., 2013) because molecular cytogenetic methods have been only recently applied in this group (Schneider & Cella, 2010; Adilardi *et al*., 2014; Mattos *et al*., 2014). In the search for hidden karyotype variation, we analyzed the karyotypes of four geographically distant *Androctonus* species by means of standard cytogenetic methods and fluorescence *in situ* hybridization (FISH) with an 18S rDNA probe. Our findings emphasize the high stability of the *Androctonus* genome, which is discussed in terms of current hypotheses on karyotype evolution and speciation.

MATERIAL AND METHODS

We analyzed four species of the genus *Androctonus*; namely, *Androctonus australis* (Linnaeus, 1758) (one male) (Egypt), *Androctonus bourdoni* Vachon, 1948, stat. n. (one male) (Morocco, 32.30°N 09.18°W), *Androctonus crassicauda* (Olivier, 1807) (two males) (Turkey, 36.95°N 38.02°E), and *Androctonus maelfaiti* Lourenço, 2005 (one male) (Pakistan, app. 24°40′N 70°15′E). Species were determined by F. Kovařík and are deposited in his private collection (*A. maelfaiti*), the collection of Department of Zoology, Charles University in Prague, Czech Republic (*A. australis* and *A. bourdoni*), and in the collection of Alaşehir Vocational School, Celal Bayar University, Turkey (*A. crassicauda*).

Preparations

Chromosomal preparations were made using the spreading technique by Traut (1976) described in detail in arachnids by Šťáhlavský & Král (2004). Briefly, testes were dissected and hypotonized in 0.075 M KCl solution for 20 min and then fixed in glacial acetic acid: methanol (1 : 3) for 20 min. Fixed material was macerated in a drop of 60% acetic acid and spread on a microscope slide on histological plate at 45 °C. The chromosome slides were stained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 20 min. The images of Giemsa stained chromosomes were observed in an Olympus Provis AX 70 light microscope and documented with Olympus DP 72

(Olympus Europa Holding) and QuickPHOTO CAMERA, version 2.3 (Promicra).

FISH detection of 18S rDNA

The 18S rDNA probe for FISH was prepared from the arachnid *Dysdera erythrina* (Walckenaer, 1802) (Dysderidae), as described by Forman *et al*. (2013). A fragment of the 18S rRNA gene was amplified by polymerase chain reaction (PCR) from genomic DNA obtained by standard phenol–chloroform–isoamyl alcohol extraction using forward and reverse primers 5′-CGAGCGCTTTTATTAGACCA-3′ and 5′-GGTTCA CCTACGGAAACCTT-3′, respectively. The PCR product of approximately 1000 bp was extracted from an agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega). The 18S rDNA fragment was re-amplified by PCR and then labelled with biotin-14-dUTP by nick translation using a Nick Translation Kit (Abbott Molecular).

FISH with biotinylated 18S rDNA probe was performed as described by Fuková, Nguyen & Marec (2005). Briefly, chromosome preparations were pretreated with 100 μg mL^{-1} RNase A in 2 × SSC buffer for 1 h at 37 °C and then washed twice for 5 min in 2 × SSC. Chromosomes were denatured at 68 °C for 3 min 30 s in 70% formamide in 2 × SSC. The probe cocktail for one slide contained 20 ng of probe and 25 μg of salmon sperm DNA (Sigma-Aldrich) in 10 μl of 50% formamide, 10% dextran sulphate, and 2 × SSC. Hybridization was carried out overnight and biotin was detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs Inc.), followed by one round of amplification with biotinylated anti-streptavidin and Cy3-conjugated streptavidin (Vector Labs Inc.).

The preparations were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich) and mounted in antifade based on DABCO (1,4-diazabicyclo[2,2,2]octane; Sigma-Aldrich). Preparations were observed in an Olympus IX81 microscope equipped with an ORCA-AG monochromatic charge-coupled device camera (Hamamatsu). The images were pseudocoloured (red for Cy3 and blue for DAPI) and superimposed with Cell^R software (Olympus Soft Imaging Solutions GmbH).

Karyotype analysis

The relative position of the 18S rDNA signal and relative diploid set length (DSL) were measured and calculated for each specimen using IMAGEJ, version 1.45r (Schneider, Rasband & Eliceiri, 2012) with the plug-in Levan (Sakamoto & Zacaro, 2009) based on 10 postpachytene spermatocyte nuclei or mitotic metaphase nuclei of spermatogonia. The DSL was calculated for each chromosome as a percentage of the diploid set. In the case of *A. crassicauda*, the results of FISH were not satisfying as a result of artefacts of DAPI staining. However, comparison of the 18S rDNA signal with the same cell after Giemsa staining with prominent constriction in the same location allowed the precise detection of the position of the rDNA cluster.

RESULTS

Karyotype analysis

In accordance with previously published data, the mitotic complement of all analyzed *Androctonus* species consisted of $2n = 24$ holocentric chromosomes (Fig. 1A, B, C, D, E, F, G, H). The size of the chromosomes is similar in all observed species (see Supporting information, Table S1). The first pair of chromosomes is always considerably larger than the rest of the complement. The difference between the first and the second chromosome pair forms approximately 2% of DSL in all species analyzed (Fig. 2). The differences between the rests of chromosomes are lower than 1% of DSL (see Supporting information, Table S1).

In addition to mitotic chromosomes, we also analyzed meiotic nuclei in all *Androctonus* species except *A. bourdoni*, aiming to detect possible rearrangements and unusual pairing of chromosomes. Male meiosis was considered as achiasmatic because no crossing-over was observed in postpachytene and metaphase I bivalents. We also did not detect any heteromorphic bivalent, which suggests that no morphologically differentiated sex chromosomes occur in the *Androctonus* spp. examined. In *A. australis* (26 cells), *A. maelfaiti* (59 cells), and one male of *A. crassicauda* (22 cells), we observed only bivalents in the postpachytene nuclei (Fig. 1I). However, in addition to cells with only bivalents (137 cells) (Fig. 1J), multivalent associations were found at low frequency in the second male of *A. crassicauda* as one cell with a tetravalent (Fig. 1K), two cells with clear octovalents, and one cell with unspecified multivalents (Fig. 1L) were detected.

Position of 18S rDNA

One cluster of 18S rDNA was revealed by FISH in all studied species (Figs 1B, D, F, H and 2). The 18S rDNA probe signal was localized interstitially at approximately one-third of the largest chromosome pair. The ribosomal cistron is localized almost identically in all species. The *A. australis* 18S rDNA signal was the closest to the middle of the first chromosome (28.7%, SD = 8.77) ($N = 10$), whereas the

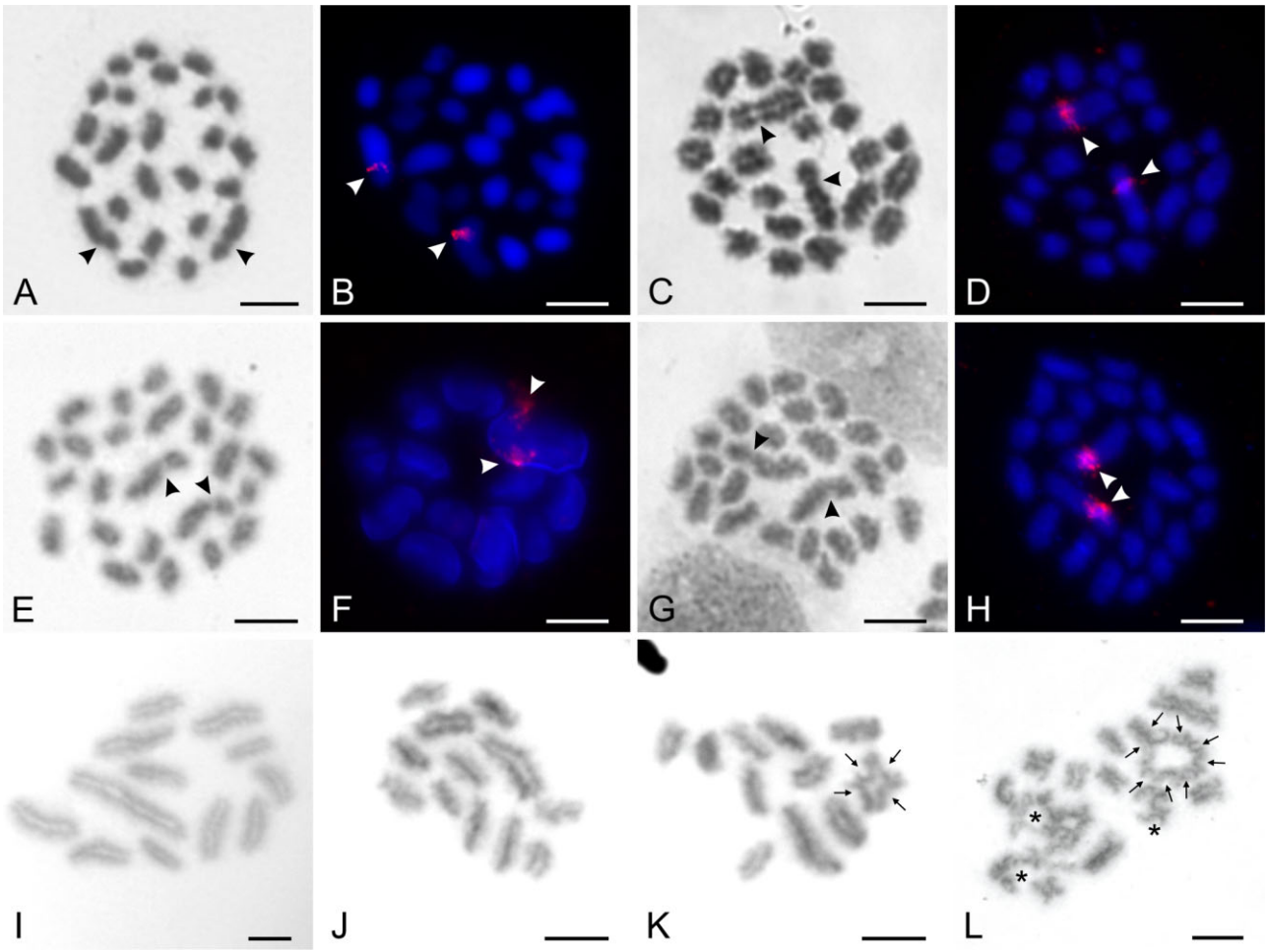

Figure 1. The location of the 18S rDNA cluster (red signal) in mitotic metaphases (A, B, C, D, E, G, H) and in meiotic postpachytene (F) and the pairing of chromosomes during meiosis in *Androctonus* species (I, J, K, L). The chromosomes are counterstained with DAPI (blue) or Giemsa. A, B, *Androctonus australis*. C, D, *Androctonus bourdoni*. E, F, *Androctonus crassicauda*. G, H, *Androctonus maelfaiti*. White arrowheads indicate the 18S rDNA cluster; black arrowheads indicate the constriction that corresponds to nucleolar organizing regions. I, *Androctonus maelfaiti*, cell with bivalents only. J, K, L, *Androctonus crassicauda*. J, cell with bivalents only. K, cell with one tetravalent. L, fragment of the cells with octovalent and unclear multivalents (asterisks). Arrows indicate chromosomes in multivalents. Scale bar = 5 μm.

most terminal 18S rDNA signal was observed in *A. bourdoni* (25.65%, SD = 4.51) ($N = 10$). The rDNA loci of *A. crassicauda* and *A. maelfaiti* was detected in 27.16%, SD = 6.76 ($N = 10$) and 27.55%, SD = 5.03 ($N = 10$) of the first chromosomal pair, respectively (Fig. 2). Furthermore, we noted that the 18S rDNA locus corresponds to a prominent constriction observed under Giemsa staining (Fig. 1C, D).

DISCUSSION

Despite their evolutionary antiquity, scorpions have not changed much morphologically in last 400 MYR, which has earned them the label 'living fossil' (Prendini, 2005; Cao *et al.*, 2013). Recent cytogenetic and genomic studies suggest that morphological and molecular evolution is decoupled in scorpions (Schneider *et al.*, 2009a; Cao *et al.*, 2013). Extraordinary variation in genome organization has been observed in the family Buthidae, which is unique among scorpions because it possesses holokinetic chromosomes assumed to facilitate chromosome rearrangements (Schneider *et al.*, 2009b; Mattos *et al.*, 2013). In the present study, we analyzed the karyotypes of four geographically distant species of the *Androctonus* genus, which appears to be the exception proving the rule.

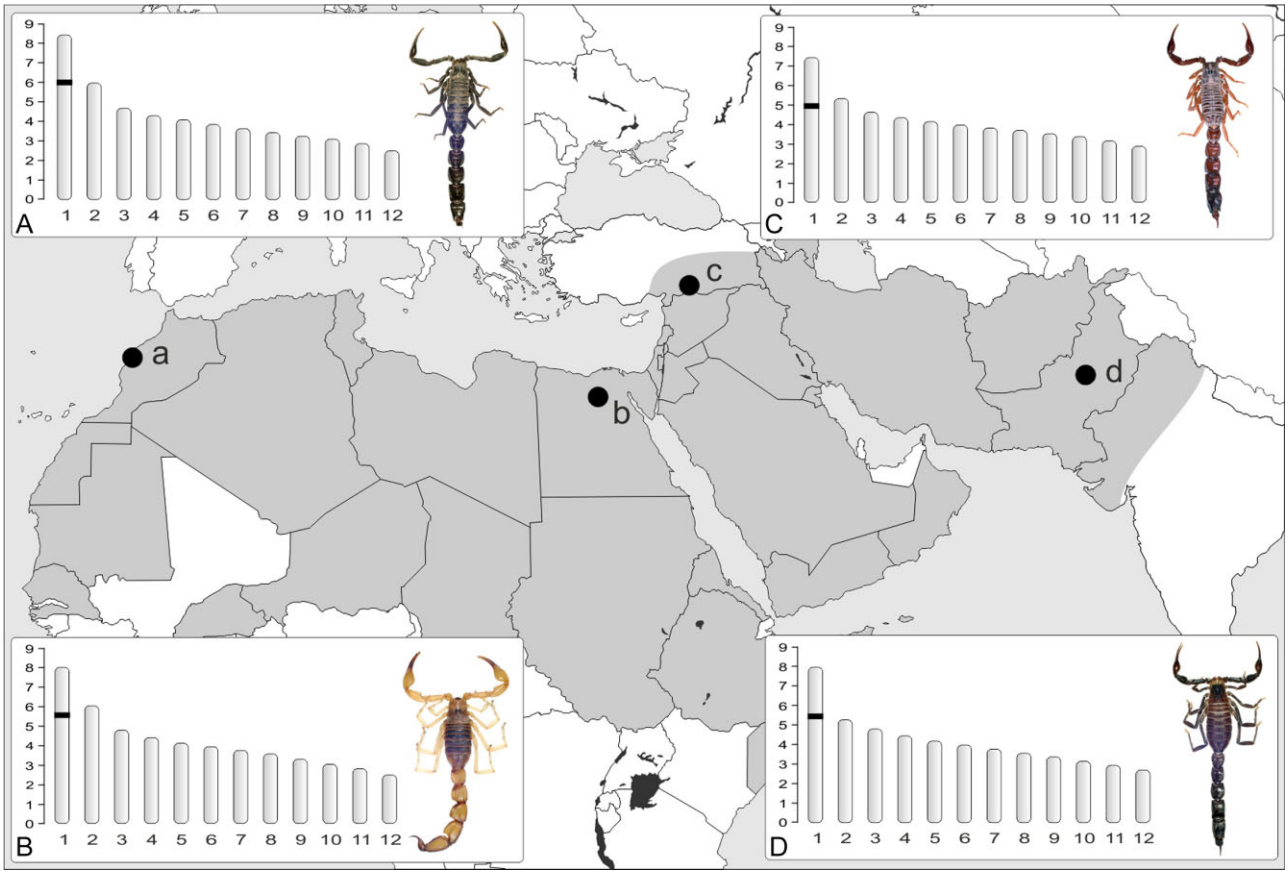

Figure 2. Idiograms for haploid set and the locality of analyzed *Androctonus* species. The length of chromosomes is calculated as a percentage of diploid set. The black line indicates the position of 18S rDNA cluster. In the map, the grey colour indicates the approximate distribution of the genus. A, *Androctonus bourdoni*. B, *Androctonus australis*. C, *Androctonus crassicauda*. D, *Androctonus maelfaiti* (lowercase letters indicate locations).

The *Androctonus* scorpions under study share general cytogenetic features, such as the presence of holokinetic chromosomes and male achiasmatic meiosis, observed in other members of the family Buthidae (Mattos *et al*., 2013). In accordance with previous studies (Chovet *et al*., 1971; Goyffon *et al*., 1971; Moustafa *et al*., 2005), the karyotypes of all four species investigated in the present study consisted of $2n = 24$ chromosomes. In all the *Androctonus* species examined, FISH with the 18S rDNA probe detected a single interstitial rDNA locus localized at approximately one-third of the largest chromosome pair. Furthermore, the relative position of ribosomal genes (Fig. 2) and the size of the rDNA-bearing chromosome pair (pair number 1; see Supporting information, Table S1) suggest its homology among all *Androctonus* species examined.

So far, clusters of ribosomal genes were mapped by means of FISH only in representatives of two buthid genera, *Tityus* and *Rhopalurus* Thorell, 1876. The whole rDNA repeat unit of *Drosophila melanogaster* revealed only one rDNA locus in diploid complement of *Tityus serrulatus* Lutz & Mello, 1922 (Schneider & Cella, 2010) and two terminal clusters of rDNA in seven other buthids (Mattos *et al*., 2014). The same rDNA distribution was observed also in *Tityus trivittatus* Kraepelin, 1898 using a partial sequence of its 28S rRNA gene as a probe (Adilardi *et al*., 2014). However, tandem arrays of major ribosomal RNA genes have been traditionally detected using the classic silver impregnation technique (Howell, 1977), which selectively stains argyrophylic proteins associated mainly (but not exclusively) with active nucleolar organizing regions (NORs) (Imai *et al*., 1992). Mitotic complements of 12 buthid species of the *Ananteris* Thorell, 1891, *Rhopalurus*, and *Tityus* genera studied so far generally contained two homologous chromosomes bearing terminally located NORs (Schneider *et al*., 2009b; Mattos *et al*., 2013; Adilardi *et al*., 2014). However, it should be noted that the homology of NOR-bearing chromosomes across buthids is questionable as a result of considerable

differences in their karyotypes (Mattos *et al*., 2014). Indeed, three NORs observed on three distinct loci (two terminal and one interstitial) in the aberrant *R. rochai* Borelli, 1910 male suggest that rDNA clusters can be mobile in buthids (Mattos *et al*., 2013). Beyond the family Buthidae, NOR distribution was studied in scorpions only in two representatives of the *Bothriurus* Peters, 1861 genus (Bothriuridae) with three NORs (Schneider *et al*., 2009a). The findings reported for 18S rDNA FISH thus do not provide any evidence of karyotype variation in the genus *Androctonus* and confirm the remarkable stability of its genome architecture conserved despite the high levels of genetic divergence reported recently from northwestern Africa (Coelho *et al*., 2014). The observed evolutionary stasis is exceptional in buthids and holds true across the whole distribution area (Fig. 2) of the *Androctonus* genus, spanning from Morocco (Chovet *et al*., 1971; Goyffon *et al*., 1971; present study) via Egypt (Moustafa *et al*., 2005; present study) and Turkey (present study) to Pakistan (present study).

Static chromosome evolution has been reported in several other taxa of plants (Stuessy & Crawford, 1998; Mandáková, Heenan & Lysak, 2010) and animals (Aprea *et al*., 2004; Ellegren, 2010; Neto *et al*., 2011; Van't Hof *et al*., 2013). In birds, genome stability was explained by the low density of interspersed repeats being assumed to mediate chromosomal rearrangements (Ellegren, 2010). In *Tityus* scorpions, epigenetic silencing of repeats appears to preserve genome integrity because chromosomes with conspicuous blocks of heterochromatin have a low rate of chromosome rearrangements (Mattos *et al*., 2013, 2014). Moreover, evolutionary stasis observed in *Androctonus* spp. is in stark contrast with the predicted tendency of holokinetic chromosomes to fuse and fission, as well as the aforementioned holokinetic drive hypothesis. The latter postulates that differences in the size and number of holokinetic chromosomes are propelled by the proliferation or removal of transposable elements driven by asymmetry of the meiotic spindle. It has been emphasized that holokinetic drive would not operate in telokinetic taxa, where microtubules attach to chromosome ends in meiosis (Bureš & Zedek, 2014). Also noteworthy, there were speculations on telokinetic activity in buthid *Tityus bahiensis* (Perty, 1833) (Piza, 1939, 1943). However, these ideas were rejected and holokinetic chromosomes were confirmed in this species (Brieger & Graner, 1943; Benavente, 1982). Thus, stable karyotypes of *Androctonus* species may suggest a low amount of repeats in their genome.

Chromosome rearrangements have been reported in the genus *Androctonus*. Moustafa *et al*. (2005) observed multivalents with three, four or seven chromosomes involved in meiotic nuclei of *A. australis*, *Androctonus bicolor* Ehrenberg, 1828, *Androctonus amoreuxi* Audouin, 1826, and *A. crassicauda*. In present study, we observed multivalents only in metaphase I of one *A. crassicauda* male. This was most likely due to their low frequency as 141 nuclei had to be scored to reveal four cells containing multivalents. Unfortunately, the frequency of multivalents observed in a previous study by Moustafa *et al*. (2005) is not known. Multivalent associations were documented also in ten more buthid genera (e.g. Schneider *et al*., 2009b; Mattos *et al*., 2013, Šťáhlavský, Koç & Yağmur, 2014). It was suggested that multivalent chromosome associations in *Tityus* represent fusion and fission rearrangements in the heterozygous state (Schneider *et al*., 2009b; Mattos *et al*., 2013). A stable karyotype and the low frequency of these rearrangements in *Androctonus* scorpions support this hypothesis. Furthermore, it is reasonable to assume that a few multivalents observed in *Androctonus* spp. formed *de novo* and, in contrast to other buthids, do not spread in their populations.

In general, the spread of new chromosome rearrangements in a species can be explained either by selection or genetic drift in small populations (Veltsos, Keller & Nichols, 2008; Pannell & Pujol, 2009). Schneider *et al*. (2009b) hypothesized that the occurrence and maintenance of multivalents in *T. bahiensis* can indicate an evolutionarily selected mechanism, which permits balanced segregation of all chromosomes during meiosis. However, karyotype stability along with a low frequency of multivalent associations observed in *Androctonus* spp. would suggest that chromosome rearrangements are associated with a fitness cost rather than benefit in this genus (Schneider *et al*., 2009b; Mattos *et al*., 2013). Alternatively, chromosome rearrangements can be fixed as a result of a bottleneck or founder effect in small populations or upon expansion of a species range, respectively (Lande, 1985; Veltsos *et al*., 2008; Pannell & Pujol, 2009). Static chromosome evolution would then suggest that the *Androctonus* genus diversified because of vicariance rather than dispersal. This is in agreement with Coelho *et al*. (2014), who hypothesized that fragmentation of xeric habitats caused by climatic oscillations may be responsible for diversification of the genus *Androctonus*. Hence, the genome stability observed in the *Androctonus* scorpions might reflect its evolutionary history.

In conclusion, in the present study, we performed cytogenetic analysis of four geographically distant *Androctonus* species. FISH with the 18S rDNA probe did not detect any karyotype variation because it revealed a single interstitial rDNA locus in all species examined. The findings of the present study thus support an extraordinary stability of the *Androctonus* karyotypes, which provides an unique opportunity for

contrasting current hypotheses on the chromosome evolution both within and beyond the family Buthidae. Moreover, we suggest the *Androctonus* spp. as a stepping stone to comparative genomic studies in scorpions due to their genome stability and chromosome number ($2n = 24$) shared with the sequenced scorpion model *Mesobuthus martensii* (Schneider *et al*., 2009a, Cao *et al*., 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Relative diploid set length (%) of chromosomes (± SD) in analyzed *Androctonus* species including the number of measured cells from mitotic metaphase (mit) and meiotic postpachytene (ppach).