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Application of cytogenetic markers in the taxonomy of flat rock scorpions (Scorpiones: Hormuridae), with the description of *Hadogenes weygoldti* sp. n.☆

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ABSTRACT

In the present study, we performed the first comparative cytogenetic study in *Hadogenes* species using both standard and molecular cytogenetic approaches. Information about the diploid set, number and distribution of 18S rDNA and telomeric sequences was obtained from three South African species, *Hadogenes trichiurus* (Gervais, 1843), *H. zuluanus* Lawrence, 1937 and *H. weygoldti* sp. n.. All species analysed differ considerably in the number of chromosomes (*H. trichiurus* 2n = 48, *H. zuluanus* 2n = 80, *H. weygoldti* sp. n. 2n = 113). In contrast, the number of 18S rDNA clusters and distribution of telomeric sequences represent rather stable cytogenetic characters in *Hadogenes*. Within all karyotypes, we identified one pair of 18S rDNA clusters. The telomeric signals were exclusively on the terminal chromosomal regions. Interestingly, the chromosomal location of 18S rDNA clusters varied from terminal to interstitial in species karyotypes, indicating the presence of hidden structural chromosomal changes. Additionally, the present comparative study is complemented by the description of a new species, *H. weygoldti* sp. n., based on specific karyotype features and morphological characters. Finally, our cytogenetic results are compared with known chromosomal data of other *Hadogenes* species, and the use of cytogenetic approaches in the taxonomy of scorpions is discussed.

1. Introduction

The scorpions represent ancient arachnids known by their typical appearance, which has not changed much compared to 400 million year old fossils (Dunlop, 2010). This uniform external morphology has often complicated the higher classification and reconstruction of their phylogenetic relationships, which are still the subject of discussion (see Prendini and Wheeler, 2005; Sharma et al., 2015; Soleglad and Fet, 2003a). It is therefore not surprising that the morphological uniformity of certain structures presents difficulties in species delimitation in this order.

The analysis of DNA is a powerful tool for detecting phylogenetically separated lineages, and its use facilitates correct interpretation of morphological variability. This approach helps to detect cryptic genetic diversity, as was shown in widespread taxa, such as the genera *Euscorpius* (Parmakelis et al., 2013) or *Buthus* (Sousa et al., 2012). Knowledge of the genetic structure of analysed species may also help to understand intraspecific variability that may be erroneously interpreted as interspecific differences (see e.g. Kovařík et al., 2017). It should be noted that the delimitation of species based only upon DNA analysis also is rather disputable and may overestimate species diversity, especially in sedentary organisms (e.g. Opatova and Arnedo, 2014; Parmakelis et al., 2013).

Another useful method that can provide insight into cryptic diversity is karyotype analysis. In some morphologically uniform groups karyotypes may vary considerably and have helped to detect cryptic species in different arachnid orders (e.g. Ojanguren-Affilastro et al., 2017; Řezáč et al., 2007; Zaragoza and Šťáhlavský, 2008). The scorpions display great variability in diploid numbers

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that range from 5 up to 175 (see Schneider et al., 2017). Such distinctive karyotype differences suggest the potential use of cytogenetic markers in solving intricate taxonomic problems. Moreover, the broad variability of cytogenetic markers is magnified by the holocentric or monocentric chromosome organization in this order. These two types of chromosomes basically differ in the distribution pattern of the centromeric areas. While monocentric chromosomes have kinetic activity restricted to a single chromosomal area (i.e. centromere), holocentric chromosomes lack such a localized centromeric region and display kinetic activity diffused along the length of the chromosome (e.g. Melters et al., 2012). The holocentrics are typical for Buthidae, the best cytogenetically explored scorpion family (e.g. Schneider et al., 2009a). This type of chromosome has evolved independently only a few times in arachnids: in some mites (Wrensch et al., 1994) and two families of spiders (Král et al., 2006). Due to their holocentric nature, buthid chromosomes may tolerate frequent fragmentation or fusion, and this is probably a factor underlying high intraspecific variability observed mainly in some *Tityus* species, e.g. *T. bahiensis* (2n = 5–19) (Schneider et al., 2009a) or *T. obscurus* (2n = 11–16) (Almeida et al., 2017). This high intraspecific variability and similar appearance of holocentric chromosomes may complicate direct application of cytogenetic data to taxonomy of this group. On the other hand, the cytogenetic characterization, such as number and position of nucleolar organizing regions (NORs), seems to be stable in buthid scorpions (e.g. Mattos et al., 2014; Sadílek et al., 2015), and differences in this marker can assist in distinguishing species with similar holocentric chromosomes (Ojanguren-Affilastro et al., 2017).

In contrast to the Buthidae, the chromosomes in the other studied families are monocentric (Schneider et al., 2009b), and these scorpions also exhibit distinctive karyotype differences. In the better explored genus *Urodacus* (Urodacidae) from South Australia, multiple chromosomal differences were found not only between seven species examined, but also within their populations. The highest intraspecific variability was found in *U. novaehollandiae* (2n = 66–175) and *U. manicatus* (2n = 29–64), and the karyotypes differing in chromosome numbers were treated as 'cytotypes' (Shanahan, 1989). Shanahan (1989) assumed that populations with different 'cytotypes' may represent several cryptic species. Unfortunately, the cytogenetic data were not used in any subsequent study on morphology or variability of DNA sequences to test this hypothesis.

Within the scorpions, only the genus *Hadogenes*, endemic to the southern part of Africa with 19 recognized species (Rein, 2017), was subject to such a complex analysis that demonstrated that the karyotypes of twelve species differ considerably (2n = 36–174) and this data has taxonomic potential (Newlands, 1980; Newlands and Cantrell, 1985). The utilization of cytogenetic markers in the taxonomy of this genus is limited by the fact that neither Newlands (1980) nor Newlands and Cantrell (1985) specified the locality of the karyotyped samples, and only presented the diploid numbers of chromosomes without providing details about their morphology.

For this reason, we analysed three remaining unkaryotyped species of *Hadogenes* from the southern limit of distribution of the genus of flat rock scorpions to expand our knowledge about the karyotype differences in this genus. Moreover, we also used fluorescence *in situ* hybridization (FISH) to identify the number and position of the 18S rRNA genes and the telomeric sequence, which is the first implementation of this method in the family Hormuridae. Both markers are frequently used for the analysis of karyotype differences and identification of specific chromosomal changes during the karyotype evolution of different taxa (e.g. Grzywacz et al., 2011; Nguyen et al., 2010; Panzera et al., 2012; Rovatsos et al., 2015). However, within scorpions they were only used frequently in buthids with holocentric chromosomes (Adilardi et al., 2014, 2015, 2016; Almeida et al., 2017; Mattos et al., 2014; Ojanguren-Affilastro et al., 2017; Sadílek et al., 2015; Schneider and Cella, 2010).

2. Material and methods

2.1. Sampling

We analysed three species of flat rock scorpion belonging to the family Hormuridae from different localities in South Africa:

Hadogenes trichiurus (Gervais, 1843): Free State: Bankfontein (30.073683°S, 24.883627°E), 1 female, 2 juveniles.

Hadogenes weygoldti sp. n.: Northern Cape: Sutherland (32.289692°S, 20.599503°E), 1 male holotype.

Hadogenes zuluanus Lawrence (1937): KwaZulu-Natal: Ophathe Game Reserve (28.374147°S, 31.389749°E), 1 male, 1 female, 1 juvenile.

2.2. Chromosome preparation and karyotype analysis

Chromosome slides were made from male and female gonads or from mesenteron tissue of juveniles as described by Plíšková et al. (2016). The testes were dissected and exposed to initial hypotonic treatment in 0.075 M KCl for 20 min, then fixed in methanol:acetic acid solution (3:1) for 30 min. Thereafter, tissue fragments were macerated in 60% acetic acid and the resultant cell suspension was spread on slides using a preheated histological plate (45 °C). The chromosome preparations were stained with 5% Giemsa solution in Sörensen buffer (pH 6.8) for 30 min. Additionally, we used the C-banding technique according to the standard protocol of Sumner (1972). In this case, the chromosome slides were stained with DAPI (FluoroshieldTM; Sigma-Aldrich) and the images were inverted in Adobe Photoshop CS4 11.0 for higher contrast. Chromosome morphology was documented by an Olympus AX70 Provis microscope using an Olympus DP72 camera and QuickPHOTO CAMERA v2.3 software (Promicra). For *Hadogenes weygoldti* sp. n. and *H. trichiurus*, five mitotic metaphases were selected for karyotype analysis. In *H. zuluanus* we measured five meiotic postpachytenes and we computed the mean values for homologue chromosomes within pairs. Chromosome measurements were performed for the diploid set length (DSL) using ImageJ v1.45r (Schneider et al., 2012) with the plugin Levan (Sakamoto and Zacaro, 2009) (Table A.1). Morphology of chromosomes was classified based on the nomenclature presented by Green and Sessions (1991).

2.3. Probes

Total genomic DNA was extracted from *Euscorpius sicanus* using the Tissue Genomic DNA kit (Geneaid). Obtained gDNA was used as a template for amplification of the 18S rDNA fragment. Unlabelled 18S rDNA probe for FISH was generated by the polymerase chain reaction (PCR) with the use of the 18S-Gal forward (5′-CGAGCGCTTTTATTAGACCA-3′) and 18S-Gal reverse primers (5′-GGTTCACCTACGGAAACCTT-3′), following the conditions described by Fuková et al. (2005). Unlabelled telomeric probe $(TTAGG)_n$ was performed through non-template PCR using the $(TTAGG)_4$ and $(CCATT)_4$ primers, according to protocol described by Sahara et al. (1999). Both probes for FISH were labelled with biotin-14-dUTP by nick translation using a Nick Translation Kit (Abbott Molecular).

2.4. *Fluorescence* in situ *hybridization (FISH)*

For both probes prepared, FISH was carried out according to Fuková et al. (2005), with minor modifications. Briefly, the chromosome slides were initially treated with RNase A (200 μg/ml in 2 x saline-sodium citrate (SSC) for 60 min (37 °C), then denatured

in 70% formamide in 2 x SSC for 3 min 30 s (68 °C). Afterwards, a probe mixture containing 20 ng of probe and 25 ng of salmon sperm DNA in 10 μL of 50% formamide, 10% dextran sulfate in 2 x SSC (per slide) was denatured, applied to slides, and the hybridization process took place overnight (37 °C). The following day, all non-specifically bound probe was removed in a series of stringent washes. The probes were detected with Cy3-conjugated streptavidin and resulting signals were subsequently amplified by biotinylated anti-streptavidin and Cy3-conjugated streptavidin. Chromosomes were counterstained with DAPI (FluoroshieldTM; Sigma-Aldrich) and observed by 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 CellR̂ software (Olympus Soft Imaging Solutions GmbH).

2.5. Taxonomy

Nomenclature and measurements follow Stahnke (1971), Kovařík (2009), and Kovařík and Ojanguren-Affilastro (2013), except for trichobothriotaxy (Vachon, 1974) and sternum (Soleglad and Fet, 2003b). Hemispermatophore terminology follows the revised, unified scheme of Monod et al. (2017), except instead of 'stem' we retained the equivalent term 'trunk' for: (i) preservation of nomenclatural stability, as it is widely used in taxonomic and zoological literature, and (ii) consistency with usage of the term 'truncal flexure'. The capsule region was optically cleared by proteolytic digestion (1 mg/ ml bovine trypsin, 4 mM $CaCl_2$, 200 mM Tris-NaOH, pH 7.8, 50 °C, 45 min) (Lowe, 2001). Images of serial focal planes were acquired in 15 μm steps and stereoscopic imagery was rendered by Zerenestacker 1.02.

3. Results

3.1. Karyotype analysis

The mitotic metaphases of *Hadogenes weygoldti* sp. n. contain the highest diploid number (2n = 113) compared to the other species analysed in this study (Fig. 1A). Chromosomes in the karyotype gradually decrease in size from 1.96 to 0.36% DSL (Table A.1). The morphology of individual chromosomes could not be determined due to the absence of clearly recognizable centromeres in mitotic chromosomes even after the application of C-banding technique. The pair of 18 rDNA clusters is located in the interstitial region of chromosomes that differ slightly in length, 0.9 and 0.6% DSL (Fig. 1B). The repetitive telomeric sequences $(TTAGG)_n$ revealed two signals at the terminal regions of all the chromosomes without any interstitial location (Fig. 1C).

Hadogenes zuluanus possesses 80 chromosomes in the mitotic metaphase (Fig. A.1G) and 40 bivalents in postpachytene (Fig. 1D). Analysis of meiotic phases (late postpachytene, metaphase II), with chromosomes possessing a well-recognized centromere, enabled us to determine their morphology (Fig. A.1B and C). The karyotype consists of 7 pairs of metacentric (pairs No. 2, 3, 5, 8, 9, 12, 20), two pairs of submetacentric (pairs No. 7, 11), one pair of subtelocentric (pair No.1) and 30 pairs of acrocentric chromosomes (Table A.1). The C-banding confirmed the position of constitutive heterochromatin in centromeric region in the majority of large chromosomes in late postpachytene. Moreover, we identified one additional large block of constitutive heterochromatin on short arm of one pair of chromosomes (Fig. A.1F and H). However, we did not identify conspicious blocks of constitutive heterochromatin in centromeric region especially in small chromosomes during postpachytene (compare Fig. A.1E and F) and in the majority of chromosomes during mitotic meptaphase (compare Fig. A.1G and H). The first pair of chromosomes is considerably longer (2.70% DSL) than the remaining chromosomes, which gradually decrease in length from 1.91 to 0.69% DSL (Table A.1). During meiosis we did not detect any heteromorphic pairs of chromosomes (Fig. A.1A and B) or any atypical chromosome behaviour (Fig. A.1A–D). One pair of 18S rDNA clusters is present at the terminal region of metacentric chromosome pair No. 5 (Fig. 1E). FISH with the repetitive telomeric sequences $(TTAGG)_n$ revealed two signals at the terminal regions in all of the chromosomes without any interstitial locations (Fig. 1F).

The mitotic metaphases of *Hadogenes trichiurus* exhibits the lowest number of chromosomes in this study, 2n = 48 (Fig. 1G). Chromosomes in the karyotype gradually decrease in size from 3.24 to 1.31% DSL (Table A.1). As in the case of *H. weygoldti* sp. n., the analysed C-banded mitotic metaphases did not allow us to establish the morphology of the chromosomes in the karyotype. One pair of the 18S rDNA signals is located at the terminal region of the long chromosome pair (Fig. 1H). The repetitive telomeric sequences $(TTAGG)_n$ are located at the terminal regions in all of the chromosomes without any interstitial signal (Fig. 1I).

3.2. Taxonomy

Hadogenes weygoldti sp. **n**. (Figs. 2–5, A.2–A.5)

Type locality: RSA, Sutherland, 32.289692°S 20.599503°E.

Type material: RSA, Sutherland, 32.289692°S 20.599503°E, 1♂ (holotype) ♀ (paratype), leg. local collector. Types are deposited in the public collection of Faculty of Science, Charles University in Prague.

Etymology: A patronym in honor of Prof. Peter Weygoldt, Germany, for his friendship and lifelong dedication to arachnids.

Diagnosis: Total length 44–48 mm. Color yellowish brown. External trichobothria on pedipalp patella number 35–37; ventral trichobothria on patella number 11–13; femur with three trichobothria, of which only one is dorsal. Ventral edge of cheliceral movable finger without large basal denticle. Sternum longer than wide, type 2 (Soleglad and Fet, 2003b) with posterior emargination and convex lateral lobes. Metasomal segment V with single ventral carina. Male has markedly longer metasoma than female. All metasomal segments longer than wide in both sexes. Telson elongate. Pectinal teeth number 6–7 in female and 11 in male. Legs with one pedal spur; retrolateral spur absent; lateroapical margins of tarsi straight; setation formula of tarsomere II is 3/3.

Description: The adults are 44.3 mm (male) and 48 mm (female) long. The habitus is shown in Fig. 2A–D.

3.2.1. Sexual dimorphism

The mesosoma is matte in the male and glossy in the female; the male has markedly longer metasomal segments (Figs. A.3A–F) and strong pedipalp finger lobe/socket, and the proximal gap is missing in the female (Fig. 3B and K).

3.2.2. Trichobothria (Fig. 3A–G)

Neobothriotaxic major, type C. Pedipalp patella with 35–37 external and 11–13 ventral, one internal, and two dorsal, totally with 49–53 trichobothria; chela with *dt*, *dst*, *dsb*, *db*, *Dt*, *Db*, *et*, *est*, *esb*, *eb*, *Et* 1–5, *Est*, *Eb* 1–3, *Esb*, *it*, *ib*, 18–20 additional external and 19–23 ventral, totally with 59–65 trichobothria; femur with *i*, *d*, *e*, totally with three trichobothria. Total number of trichobothria is 111–121.

3.2.3. Coloration (Fig. 2A–D)

The base color is uniformly yellowish brown. The pedipalp fingers and internal carinae of femur and patella of pedipalp and legs are black.

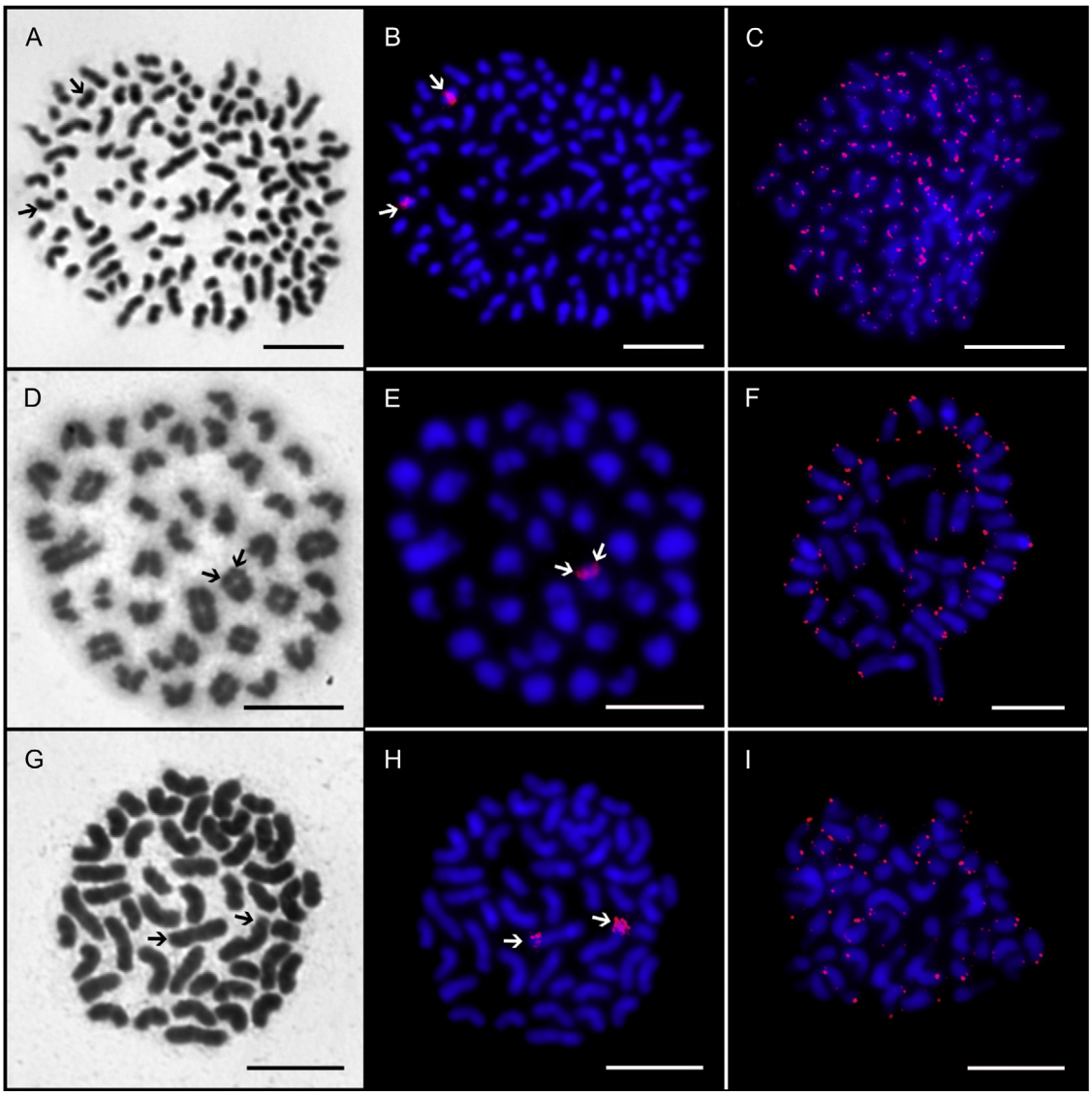

Fig. 1. Chromosomes of *H. weygoldti* sp. n. (2n = 113) (A–C), *H. zuluanus* (2n = 80) (D–F), *H. trichiurus* (2n = 48) (G–I) after Giemsa staining (A, D, G) and after FISH with 18S rDNA (B, E, H) and telomeric sequences $(TTAGG)_n$ (C, F, I). (A–C) Mitotic metaphase (A and B correspond to the same cell). (D and E) The same cell of late postpachytene. (F) Early postachytene. (G–I) Mitotic metaphase (G and H correspond to the same cell). Arrows indicate the position of 18S rDNA. Bar = 10 μm.

3.2.4. Pedipalps (Fig. 3A–L)

The pedipalps are sparsely hirsute and finely granulated. The femur and patella bear four granulate carinae; the ventroexternal and ventrointernal carinae are incomplete; the dorsoexternal and dorsointernal carinae are completely developed. The chela bears two smooth externoventral and externodorsal carinae. The dentate margin of the movable finger is armed with two parallel rows of denticles extending the entire length of the finger.

3.2.5. Metasoma and telson (Fig. A.3A–F)

The metasoma and telson are sparsely hirsute and finely granulated. The metasomal segments I–IV bear a total of 6 often incomplete carinae. The four ventral carinae are reduced on metasomal segments I and more developed on metasomal segments II–IV. The dorsolateral carinae are reduced on metasomal segments I–II and more developed on metasomal segments II–IV; on the third and mainly fourth metasomal segments they terminate in a distinct tooth mainly in the male. The first segment is wider than deep posteriorly. The fifth segment bears five carinae (two dorsal and three ventral), ventral carinae are composed of coarse pronounced granules. The telson is elongated, with the aculeus shorter than the vesicle.

3.2.6. Carapace (Fig. A.2A–B)

The carapace is densely finely granulated, longer than wide, lacks carinae but has a deep sagittal furrow with forked, V-shaped furrow on each side of the posterior part. The anteromedial margin of the carapace is strongly concave. There is a pair of median eyes and three lateral eyes. The carapace and mesosoma are densely, finely granulated.

3.2.7. Mesosoma (Fig. A.2A–C, H)

The mesosoma is densely, finely granulated. Tergites I–II can lack carinae, whereas tergites III–VI bear a carina. Tergite VII bears three incomplete carinael traces. Sternites are smooth with no carinae but bear two symmetric furrows. The sternite V lacks posterior lateral depressions. The pectinal tooth count is 6–7 in female and 11 in male.

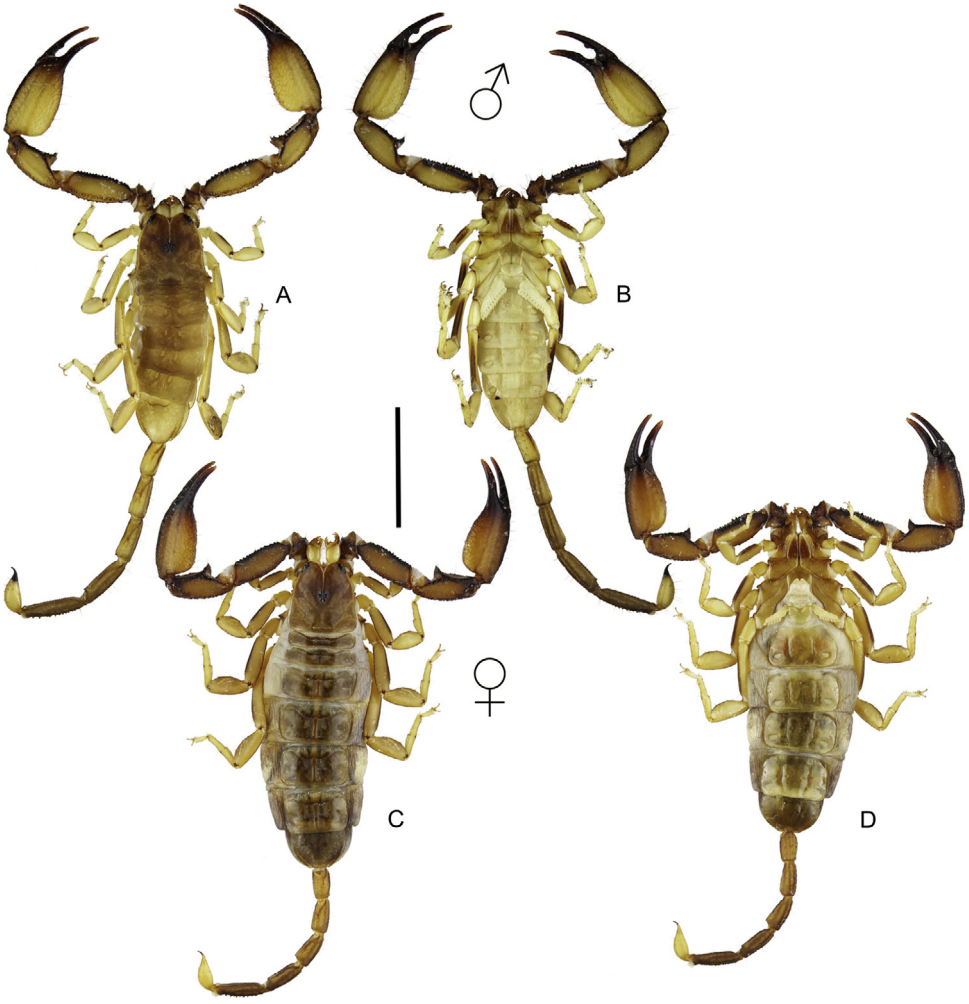

Fig. 2. Habitus of *Hadogenes weygoldti* sp. n. (A) Holotype male, dorsal view. (B) Holotype male, ventral view. (C) Paratype female, dorsal view. (D) Paratype female, ventral view. Bar = 10 mm.

3.2.8. Legs (Fig. A.2D–G)

The legs with one pedal spur, retrolateral spur absent. The lateroapical margins of tarsi straight. The tarsomeres are hirsute with setae and macrosetae. Spiniform formula of tarsomeres II is 3/3.

3.2.9. Hemispermatophore (Fig. 4A–F)

Lamelliform, long and narrow, distal lamina longer than trunk, with conspicuous double hook along basal anterior margin. Section of lamina distal to hook bent anteriorly by angle of about 30° relative to basal section. Apex of lamina rounded, antero-distal margin smooth without a pronounced crest. Hemisolenos tapered, apical margin rounded; clasper prominent, strongly curved with blunt tip. This overall hemispermatophore morphology and structure of the capsule region are similar to what has been reported for other *Hadogenes* spp. (Lamoral, 1979; Monod and Lourenço, 2005; Monod et al., 2017; Newlands and Prendini, 1997; Prendini, 2001, 2005, 2006). However, a distinctive feature is the proximal section of the distal lamina below the hook being exceptionally short, comprising only 14.4% of the total distal lamina length (Fig. 4A–B). In 11 other species of *Hadogenes*, the range was 15.8% – 26.3% (based on published illustrations or photos). In a subset of 11 species, the relative lengths of proximal sections could be either short or long in larger hemispermatophores (taking stalk length as a size metric), but was only short in smaller hemispermatophores (c.f. plot of Fig. 5B with empty upper diagonal area). Length of stalk (i.e. portion of hemispermatophore distal to truncal flexure) was well correlated with carapace length (Fig. 5A; R = 0.901, F = 34.61, P = 0.00037; N = 10, linear regression based on published data), and was thus a good proxy for size of males. The relatively abbreviated proximal distal lamina of *H. weygoldti* sp. n. appears to be related to the fact that it is the smallest member of the genus.

3.2.10. Measurements in mm

Total length of male holotype 44.3; carapace length 6.05, posterior width 5.8; metasoma and telson length 25.23; first metasomal segment length 3.25, width 1.55; second metasomal segment length 3.8, width 1.4; third metasomal segment length 4.0, width 1.2; fourth metasomal segment length 4.75, width 1.2; fifth metasomal segment length 5.23, width 1.17; telson length 4.2; telson width 1.3; telson depth 1.16; pedipalp femur length 6.45, width 2.45; pedipalp patella length 6.1, width 2.65; chela length 11.15; manus width 4.0; manus depth 1.98; movable finger length 5.4.

Total length of female paratype 48; carapace length 6.2, posterior width 6.12; metasoma and telson length 21; first metasomal segment length 2.65, width 1.8; second metasomal segment length 3.03, width 1.2; third metasomal segment length 3.2, width 1.25; fourth metasomal segment length 3.8, width 1.2; fifth metasomal segment length 4.4, width 1.07; telson length 3.9; telson width 1.22; telson depth 1.13; pedipalp femur length 6.1, width 2.25; pedipalp patella length 5.85, width 2.5; chela length 10.9; manus width 3.7; manus depth 1.87; movable finger length 5.4.

Fig. 3. Morphology of *Hadogenes weygoldti* sp. n. (A–C) Male holotype, right pedipalp chela dorsal (A), external (B) and ventrointernal (C). (D–F) Right patella dorsal (D), external (E) and ventral (F). (G–I) Right femur dorsal (G), external (H) and ventral (I). Trichobothrial pattern is indicated (terminology according to Vachon (1974). (J–L) Female paratype, right chela dorsal (J) and external (K), and granulation of movable finger (L). (M and N) Female paratype, chelicera in dorsal (M) and ventral (N) views.

3.2.11. Affinities

H. weygoldti sp. n. is the smallest species of the genus *Hadogenes*. Most of the *Hadogenes* species have total length from 90 to 210 mm. Only *H. tityrus* (total length 50–80 mm) and *H. lawrencei* (total length 47–49.5 mm) have a similar length to *H. weygoldti* sp. n. Both of these species differ from *H. weygoldti* sp. n. morphologically by the shape of the pedipalp segments, which are extremely elongated and narrow, and by sexual dimorphism in the length of the metasoma, which is present in *H. weygoldti* sp. n. and absent in *H. lawrencei* and *H. tityrus* which have the metasoma the same length in both sexes (Fig. A.5A–D). An important difference is in the number of chromosomes: *H. lawrencei* has 2n = 132 and *H. tityrus* 2n = 168 (Newlands, 1980; Newlands and Cantrell, 1985), whereas in *H. weygoldti* sp. n. there is 2n = 113.

3.2.12. Ecological notes (Fig. A.4A–C)

Terrain at the type locality consists of barely vegetated rocky hills with abundant rock slabs and crevices. The surrounding areas are flat and uniformly covered with shrubs. This habitat is characterized by hot summers with sporadic rainfall and cool winters without rain. The type specimens were located in dense populations at the tops of the hills under large and very flat rocks. Their habits differed from other *Hadogenes* species in that they also sheltered under rocks situated on bare soil, not just between rocks and in rock crevices. In this dense population, we some-

Fig. 4. Hemispermatophore of *Hadogenes weygoldti* sp. n. (A and B) Male holotype, left hemispermatophore, contralateral (interior, concave) (A), and lateral (exterior, convex) (B) views. (C–F) Male holotype, capsule region of right hemispermatophore (enzymatically cleared), contralateral (interior, concave) (C), anterior (sperm duct margin) (D), lateral (exterior, convex) (E), and posterior (F) cross-stereoscopic views. Bar = 1 mm (A and B), 500 µm (C–F).

times observed several juvenile individuals of different instars living together under the same rock, which indicates a very low level of intraspecific aggression. During the summer, females were observed to be heavily gravid or to have recently given birth. The size of litters carried by females ranged between 12 and 15. Individuals at all stages of development were observed. Males were very rare compared to juveniles and females. These were freshly molted to adulthood or waiting directly next to a gravid female, which indicates a mate-guarding behaviour. Other scorpions species observed in this area were *Opistophthalmus austerus* and *Parabuthus capensis* on the flats and *Uroplectes carinatus* at the foothill.

4. Discussion

The scorpions have a wide range of chromosome numbers (2n = 5–175), and the diploid numbers of chromosomes very often differ considerably between species (see Schneider et al., 2017). This suggests that even the basic characteristics of karyotypes seem to be useful for application in scorpion taxonomy, at least in some groups of this morphologically uniform order. However, the direct application of specific cytogenetic markers in delimitation of new scorpion species is still not routinely implemented. Moreover, we have still only limited information about intraspecific variability in this order. The scorpions represent the third best explored arachnid order from the cytogenetic point of view, with 122 karyotyped species, but for taxonomic purposes we do not have enough karyotyped species suitable for comparison. At present, there are only ten genera that have more than three species karyotyped (see Schneider et al., 2017).

Another significant limitation on the application of the older cytogenetic information for taxonomy use is that many of the older studies did not specify sampling localities very precisely,

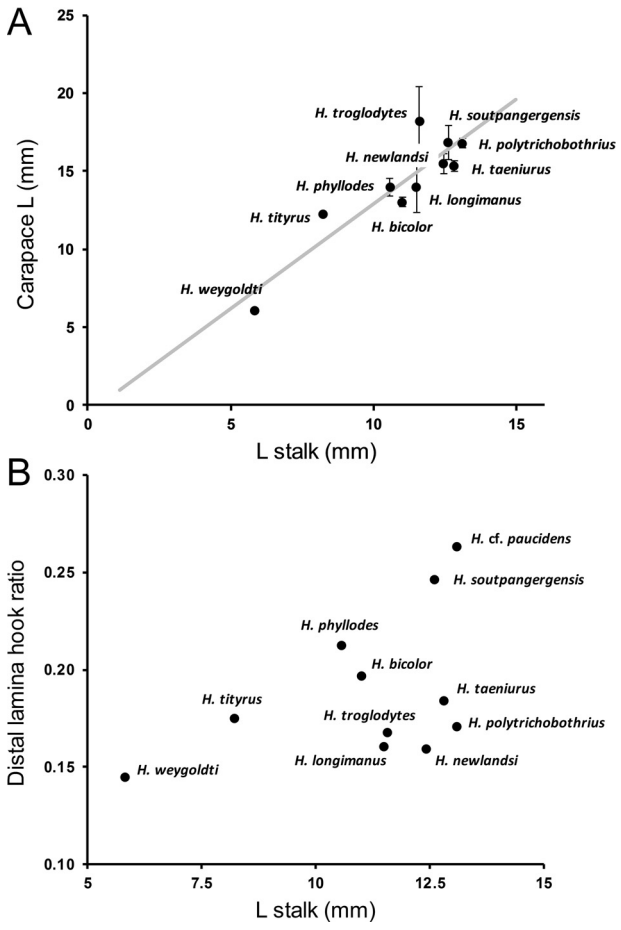

Fig. 5. Comparative biometrics of hemispermatophore distal lamina of *Hadogenes* spp. (A) Scatter plot of carapace length vs. stalk length for hemispermatophores of 10 species of *Hadogenes*. Gray line is a least squares regression. (B) Scatter plot of distal lamina hook ratio (=length of distal lamina from transverse ridge to base of hook/total length of distal lamina) vs. stalk length for hemispermatiophores of 11 species of *Hadogenes*. We computed total length of distal lamina = length of distal lamina from transverse ridge to base of hook + length of distal lamina from base of hook to apex (along an oblique axis, if angled). Measurements from other *Hadogenes* spp. were estimated from published figures (Lamoral, 1979; Monod and Lourenço, 2005; Monod et al., 2017; Prendini, 2001, 2005, 2006). We omitted *H. zumpti* from these plots because Newlands and Prendini (1997) neglected to label the scale bar in their hemispermatophore figure. However, if its hemispermatophore size is correlated with body size as in other species, we predict that it would be consistent with the distribution of points in Fig. 5B. Indeed, it is larger than *H. weygoldti* sp. n., and it has a larger distal lamina hook ratio.

and did not preserve specimens for subsequent taxonomic revision (e.g. Shanahan, 1989; Venkatanarasimhaiah and Rajasekarasetty, 1964a,b). This problem is evident especially in genus *Heterometrus*. In this genus we have information about karyotypes in nine species: *H. bengalensis* (2n = 64) (Sharma et al., 1962), *H. fulviceps* (2n = 86 and 88) (Sharma et al., 1962; Venkatanarasimhaiah and Rajasekarasetty, 1964a), *H. gravimanus* (Venkatanarasimhaiah and Rajasekarasetty, 1964b), *H. longimanus* (2n = 64) (Srivastava and Agrawal, 1961), *H. minotaurus* (2n = 54) (Plíšková et al., 2016), *H. scaber* (2n = 96) (Venkatanarasimhaiah and Rajasekarasetty, 1964a), *H. spinifer* (2n = 56) (Vítková et al., 2005), *H. swammerdami* (2n = 60) (Venkatanarasimhaiah, 1965), *H.* sp. (2n = 62) (Rajasekarasetty et al., 1979). However, the descriptions of karyotypes were published mainly during the 1960s and the definition of some *Heterometrus* taxa has changed significantly since then (e.g. Couzijn, 1981; Kovařík, 2004). Therefore, in this genus matching the karyotypes to the correct species is not possible in some cases, and the utilization of older cytogenetic data in taxonomy is consequently disputable (see Plíšková et al., 2016).

The situation is different in the genus *Hadogenes*. We have available information about the karyotypes of 14 species (Newlands, 1980; Newlands and Cantrell, 1985; present study), which makes it the best explored genus of scorpions with monocentric chromosomes (see Schneider et al., 2017). Newlands (1980) and Newlands and Cantrell (1985) demonstrated considerable differences of the diploid numbers between species (2n = 36–174), and these interspecific traits were also supported by electrophoretic data from venom proteins. They recognized the importance of species-specific chromosome characteristics in the taxonomy of this genus, and incorporated their cytogenetic data into their determination key as one of the valid species characteristics. These results were later revised with the study of additional material, and some species were formally described or redescribed (Newlands and Prendini, 1997; Prendini, 2005, 2006).

Our results support the importance of cytogenetic data in the taxonomy of this genus. We identified another three species-specific karyotypes that differ considerably, and cytogenetic markers helped us to recognize the new species *H. weygoldti* sp. n. The distribution pattern of *Hadogenes* generally displays allopatric or parapatric distribution (Newlands, 1980) (Fig. 6), and the differentiation of the karyotypes may ensure an effective postzygotic barrier (King, 1993). Based on the data obtained here, we are not able to precisely determine the mechanisms of karyotype differentiation in *Hadogenes* species. Limited information about the morphology of individual chromosomes in the karyotypes and the unknown phylogenetic structure of *Hadogenes* species do not allow us to track multiple chromosomal changes. Unfortunately, the C-banding method did not allow us to specify the positions of all centromeric regions in analysed species. Absence of visible centromeres complicated the specification of chromosomal morphology, especially in karyotypes described only after mitotic metaphases. Considering this fact, the analysis of meiotic stages with visible centromere (postpachytene or metaphase II) seems to be more precise for the description of karyotypes. The blocks of constitutive heterochromatin in centromeric regions are probably very small in scorpions. It is one reason why they were not visibile on all chromosomes in other scorpions which also have monocentric chromosomes such as *Urodacus* (Urodacidae) (Shanahan, 1989), *Bothriurus* (Bothriuridae) (Schneider et al., 2009b), and *Heterometrus* (Scorpionidae) (Plíšková et al., 2016). Nevertheless, in view of the fact that the majority of the biarmed chromosomes in *H. zuluanus* represent the long chromosomes, the fusions/fissions of chromosomes seem to be the main mechanism of differentiation of karyotypes. These types of chromosomal rearrangements may be heterozygous and produced multivalents in scorpions with monocentric chromosomes (Plíšková et al., 2016; Schneider et al., 2009b; Shanahan, 1989). The heterozygous fusions/fissions of chromosomes may also explain the odd number of chromosomes in *Hadogenes weygoldti* sp. n. (2n = 113) seen in our study as it was also proposed in *Euscorpiops orioni* (2n = 103) (Euscorpiidae) (Kovařík et al., 2015) or *Urodacus* species (Shanahan, 1989). In contrast to the high variability of diploid numbers, the number of NORs seems to be stable in flat rock scorpions. We identified only one pair of 18S rDNA clusters in all three analysed species. This number is typical for majority of analysed scorpions (e.g. Adilardi et al., 2014, 2015; Mattos et al., 2014; Sadílek et al., 2015) and the one pair of NORs in a terminal position on chromosomes seems to be ancestral for all arachnid orders (Forman et al., 2013). Interestingly, *H. weygoldti* sp. n. has 18S rDNA clusters placed in an interstitial position, which may be a consequence of chromosome rearrangements (peri- or paracentric inversions, tandem fussions), transposable element insertions or ectopic recombination in this species (see Cabrero and Camacho, 2008). We identified telomeric

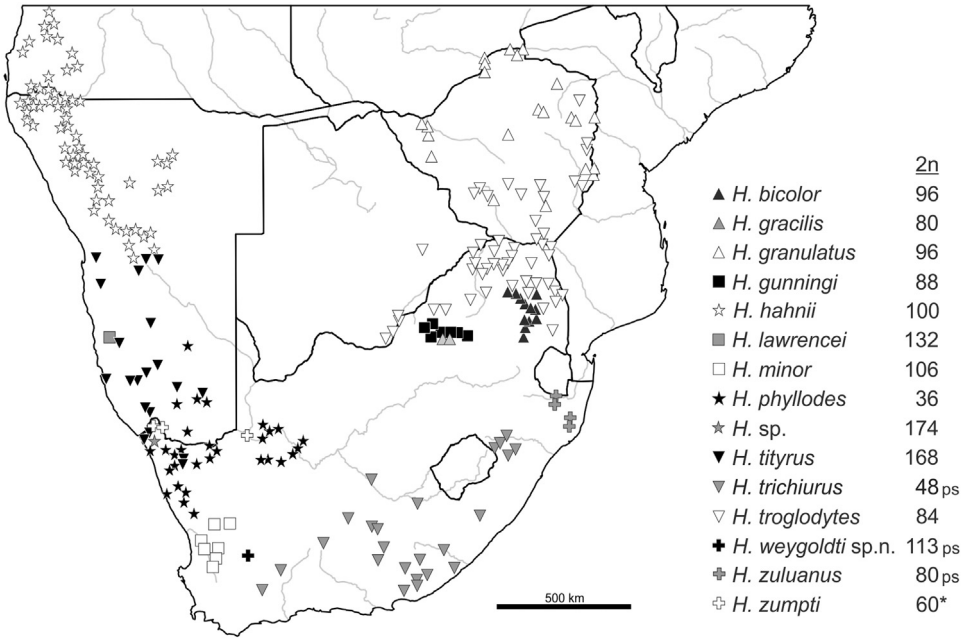

Fig. 6. Geographical distributions of *Hadogenes* species with the known diploid number of chromosomes, based on material examined in present study (ps), Newlands (1980) and Prendini (2005, 2006). The asterisk describes approximate number of chromosomes.

signals exclusively on terminal chromosomal regions in all three analysed species. We did not detect any intestitial telomeric clusters that may reflect the remnants of chromosomal fusion events as was documented in some other organisms (e.g. Pellegrino et al., 2009). The interstitial telomeric sequence is probably eliminated rapidly after chromosomal fusions or is lost during breakage preceding the fusions in scorpions. This is supported by the fact that interstitial signals were not detected even in *Tityus confluens*, a species with intensive chromosomal fusions (Adilardi et al., 2016).

Our results indicate the importance of cytogenetic analysis in scorpions and demonstrate that cytogenetic characterization may represent a valuable tool in the taxonomy of this morphologically uniform order.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcz.2018.01.007.

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