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Unexpectedly high number of 18S rRNA gene clusters in *Miopsalis dillyi* (Opiliones: Cyphophthalmi: Stylocellidae) from Mindanao, Philippines

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Abstract. The suborder Cyphophthalmi (Arachnida: Opiliones) is the sister group to all remaining harvestmen. The group typically shows limited dispersal abilities, cryptic diversity and long-isolated populations. These facts make the group interesting for biogeographic, phylogenetic and cytogenetic studies. The suborder is divided into six families, all of them with a specific and long evolutionary history. However, many species are still undescribed, and their cytogenetic data are only fragmentary. This fact complicates the reconstruction of the main mechanisms of karyotype evolution in this harvestmen suborder, and utilization of the cytogenetic markers in the taxonomy of this morphologically uniform group of arachnids. Here, we present a cytogenetic study of one species of *Miopsalis* Thorell, 1890, of the family Stylocellidae from Mindanao (Philippines). Its karyotype consisted of mainly biarmed chromosomes (2n = 28). Interestingly, we found a multiplication of 18S rRNA gene clusters in up to seven pairs, which is one of the highest numbers in known harvestmen. These results support the likely presence of distinctive karyotype variability in an additional cyphophthalmid family, Stylocellidae (2n = 28–30).

Keywords: Chromosome, karyotype, FISH, meiosis, mite harvestmen

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Mite harvestmen of the suborder Cyphophthalmi (Arachnida: Opiliones) have limited dispersal abilities, long-isolated populations, a high number of cryptic species, and biogeographically constrained distribution patterns. These characteristics make cyphophthalmids an important model system for phylogenetic, biogeographic, and cytogenetic studies (e.g., Giribet et al. 2002; Boyer et al. 2007b; Svojanovská et al. 2016; Hiřman et al. 2018). More than 200 currently described species of mite harvestmen have been classified into six families (Kury 2018), with their distributions corresponding to the main landmass fragmentation of Earth's geological history (Boyer et al. 2007b; Giribet et al. 2012; Sharma & Giribet 2014; Fernández et al. 2017). For example, the family Stylocellidae (Infraorder Boreophthalmi) is endemic to Southeastern Asia, including the islands from Sumatra to New Guinea (Giribet et al. 2012). This family includes six described genera: *Fangensis* Rambla, 1994, *Giribetia* Clouse, 2012, *Leptopsalis* Thorell, 1882, *Meghalaya* Giribet, Sharma & Bastawade, 2007, *Miopsalis* Thorell, 1890, and *Stylocellus* Westwood, 1874 (Clouse 2012). Today, only two species, *Miopsalis tarumpitao* (Shear, 1993) and *M. dillyi* Schmidt, Clouse & Sharma, 2020, have been described from the Philippines (Shear 1993; Schmidt et al. 2020). Originally, Clouse et al. (2011) analysed two juveniles from Mindanao Island alongside other stylocellid genera, which resulted in their clear placement in the genus *Miopsalis*. However, the species has not been described yet (see Schmidt et al. 2020). Overall, the recent taxonomy of cyphophthalmids has been closely connected with DNA analyses in this morphologically very uniform group. For example, a recent study of the Australian genus *Karripurcellia* Giribet, 2003 (Pettalidae) identified possible cryptic species based on p-distances of only 1.3–2.5% among analyzed populations from south-western Australia (Schwentner & Giribet 2018). Gene flow among the populations is presumably very limited, or practically non-existent. The occurrence of numerous cryptic species of mite harvestmen that lack distinguishing morphological features, and frequently isolated populations with various degrees of genetic differentiation, complicate species delimitation in this group, and other lines of evidence are needed. For example, the application of cytogenetic markers is useful for the detection of cryptic diversity and species delimitation, in addition to genetic differentiation. This has already been shown in other groups of arachnids with similarly limited dispersal abilities such as pseudoscorpions (e.g., Zaragoza & Šťáhlavský 2008; Kotrbová et al. 2016), scorpions (e.g., Šťáhlavský et al. 2018b; Štundlová et al. 2019), and dysderid spiders (e.g., Řezáč et al. 2007).

The present study provides a cytogenetic characterisation for *Miopsalis dillyi*, one of only two species of *Miopsalis* from the Philippine islands (Shear 1993; Clouse 2012; Schmidt et al. 2020). This study of the species expands our knowledge about the karyotypes of mite harvestmen, which represents the sister group to all other harvestmen, and helps us to understand the possible ancestral state of the karyotypes of the entire order Opiliones. Cytogenetic data for the suborder Cyphophthalmi are still fragmentary (see Svojanovská et al. 2016; Hiřman et al. 2018). Karyotypes have been described for only a limited number of species in Sironidae (2n = 24–52, five species, see Juberthie 1956; Tsurusaki 2007; Svojanovská et al. 2016), Pettalidae (2n = 30–32, two species, see Šťáhlavský et al. 2012; Svojanovská et al. 2016), Neogoveidae (2n = 32, one species, see Hiřman et al. 2018) and Stylocellidae (2n = 30, one species, see Svojanovská et al. 2016). The only karyotype of a stylocellid, *Miopsalis* sp., from Brunei, contains a relatively low number of chromosomes of similar length, a predominance of biarmed chromosomes and one pair of the 18S rRNA gene clusters (Svojanovská et al. 2016). These characteristics seem to be typical for the majority of harvestmen, and probably represent the ancestral state for the entire order (Svojanovská et al. 2016). However, extension of the

cytogenetic analysis to another species is important to further test this hypothesis.

METHODS

Sampling.—Two males, one female, and two juveniles were collected from leaf litter on Mt. Malambo (GPS: 07.4813°N, 125.262°E) on Bemwa farm, Davao region, Philippines. For the cytogenetic analysis, we used live specimens. After dissection, the material was preserved in 96% ethanol and stored in -20°C for DNA and morphological analysis. In order to verify the correct identification of the specimens, we compared our sequences with published data derived from the species known from the region (Clouse & Giribet 2010; Clouse et al. 2011; Schmidt et al. 2019), which matched with *Miopsalis dillyi*. We also provide supplementary data about the morphological characterisation of one karyotyped male and one additional non-karyotyped male and female from the same locality (Supplementary File 1 and Supplementary Figs. S1–S4).

Chromosome preparation & karyotype analysis.—After 20 minutes of hypotonization in 0.075 M KCl, the tissues were fixed in methanol:glacial acetic acid (3:1) for 20 minutes. The "spreading" method (Traut 1976) with small modifications (see Svojanovská et al. 2016) was completed by the dissociation in 60% acetic acid on a microscope slide. The chromosomes were stained with 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 10 mins. Observations were made with an Olympus IX81 microscope and photographed with a ORCA-ER (Hamamatsu) camera. Measurements of chromosomes were made with ImageJ software (v.1.47 from Schneider et al. (2012) with the plugin Levan from Sakamoto & Zacaro (2009)) based on seven mitotic metaphases of the male holotype and one mitotic metaphase of one of the juveniles. Chromosome morphology classification follows Levan et al. (1964) and the relative chromosome length was calculated as a percentage of the diploid set.

Fluorescence in situ hybridization.—The distributions of major ribosomal 18S RNA genes were identified using FISH with a 18S rRNA gene probe and the methods already established for harvestmen (Šťáhlavský et al. 2018a). The probe was prepared from *Euscorpius sicanus* (Koch, 1837). A fragment of 18S rRNA gene was amplified by PCR using 18S-Gal forward: 5′-CGAGCGCTTTTATTAGACCA-3′ and 18S-Gal reverse: 5′-GGTTCACCTACGGAAACCTT-3′ primers (Fuková et al. 2005). The product was labelled with biotin-14-dUTP by nick translation using a Nick Translation Kit (Abbott Molecular). FISH was performed according to Fuková et al. (2005). The detection of the hybridized probe was performed with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs Inc.). The chromosomes were stained with DAPI (4′,6-diamidino-2-phenylindole) in Fluoroshield mountant (Sigma-Aldrich) and observed with an Olympus IX81 microscope. Photographs were taken with an ORCA-ER camera (Hamamatsu), pseudocolored (red for Cy3 and blue for DAPI), and superimposed with Cell^R software (Olympus).

DNA extraction, amplification & sequencing.—The DNA extraction was performed using gSYNC DNA Extraction Kit (Geneaid) following the protocol for tissue samples. A 700 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified by PCR using the following primers: forward LCO1490 (5′-GGTCCACAAATCATAAAGA TATTGG-3′), reverse HCO2198 (5′-TAAACTTCAGGAT GACCAAAAAATCA-3′) (Folmer et al. 1994). The PCR reaction mixture was prepared in a 25µl volume as follows: 7µl PCR H_2O, 12.5µl PPP Master Mix (Top-Bio s.r.o.), 1µl Forward primer, 1µl Reverse primer (each at 1 µM concentration), and 3.5µl DNA template. The PCR protocol included an initial step at 95°C for 3 min, followed by 40 cycles of three consecutive steps: denaturation at 95°C for 30 s, annealing at 45°C for 1 min and extension at 72°C for 1 min and 30 s. The PCR was terminated by a final extension step at 72°C for 10 min. Samples were sent for sequencing to Macrogen Inc. (Netherlands).

Phylogenetic analyses & DNA comparison.—The chromatograms of sequences were checked for quality, assembled and edited in Geneious R 9.1.8 (Kearse et al. 2012). Overall, 9 *Miopsalis* (3 from cytogenetically analysed *Miopsalis dillyi*, additional 6 from GenBank) specimens were included in phylogenetic reconstructions as the ingroup taxa and species *Meghalaya* sp. (GenBank) was used as the outgroup (Supplementary Table S1). The alignment was generated in MAFFT v.7 (Katoh & Standley 2013) using the 'Auto' settings with the 1PAM/K = 2 matrix parameter. The alignment was trimmed to the uniform length of 659 bp and translated into amino acids using the invertebrate mitochondrial genetic code to verify there were no stop codons that would indicate nuclear mitochondrial pseudogenes (numts) were present. The best-fit model of nucleotide substitution for COI was determined in PartitionFinder v.1.1 (Lanfear et al. 2012) under the Bayesian information criterion (BIC). The best model was GTR+G. The phylogenetic tree was reconstructed using Maximum Likelihood (ML). The ML analyses were conducted in raxmlGUI 1.3 (Silvestro & Michalak 2012), the nodal support was estimated from 1000 bootstrap replicates. The resulting ML tree was examined in FigTree v. 1.4.3 (Rambaut 2016). Pairwise uncorrected p-distances were calculated in MEGA7 (Kumar et al. 2018), in order to estimate the evolutionary divergence among the sequences (Table S2).

Morphological analysis.—The specimens were analysed and photographed using light microscopes Olympus SZX12 and Olympus AX70 Provis equipped with an Olympus camera (DP 72). Measurements were taken from photographs using software ImageJ v.1.47 (online at http://imagej.nih.gov/ij/) (Schneider et al. 2012). Some details were inspected using scanning electron microscopy (SEM). For SEM examination, specimens were drained and observed using a scanning electron microscope JEOL JSM-6380 LV.

RESULTS

Karyotype analysis.—We analysed chromosomes in the male holotype as well as in one juvenile of the same species. The male and juvenile karyotypes of *M. dillyi* consisted of 2n = 28 chromosomes (Figs. 1B,D). In the male holotype, the chromosomes gradually decreased in length from 4.64% to 2.78% of the diploid set (Fig. 1A). We detected 8 pairs of metacentric chromosomes (pairs Nos. 4, 7 and 9–14). The remaining six chromosome pairs were submetacentric. After FISH application we detected seven pairs of the 18S rRNA

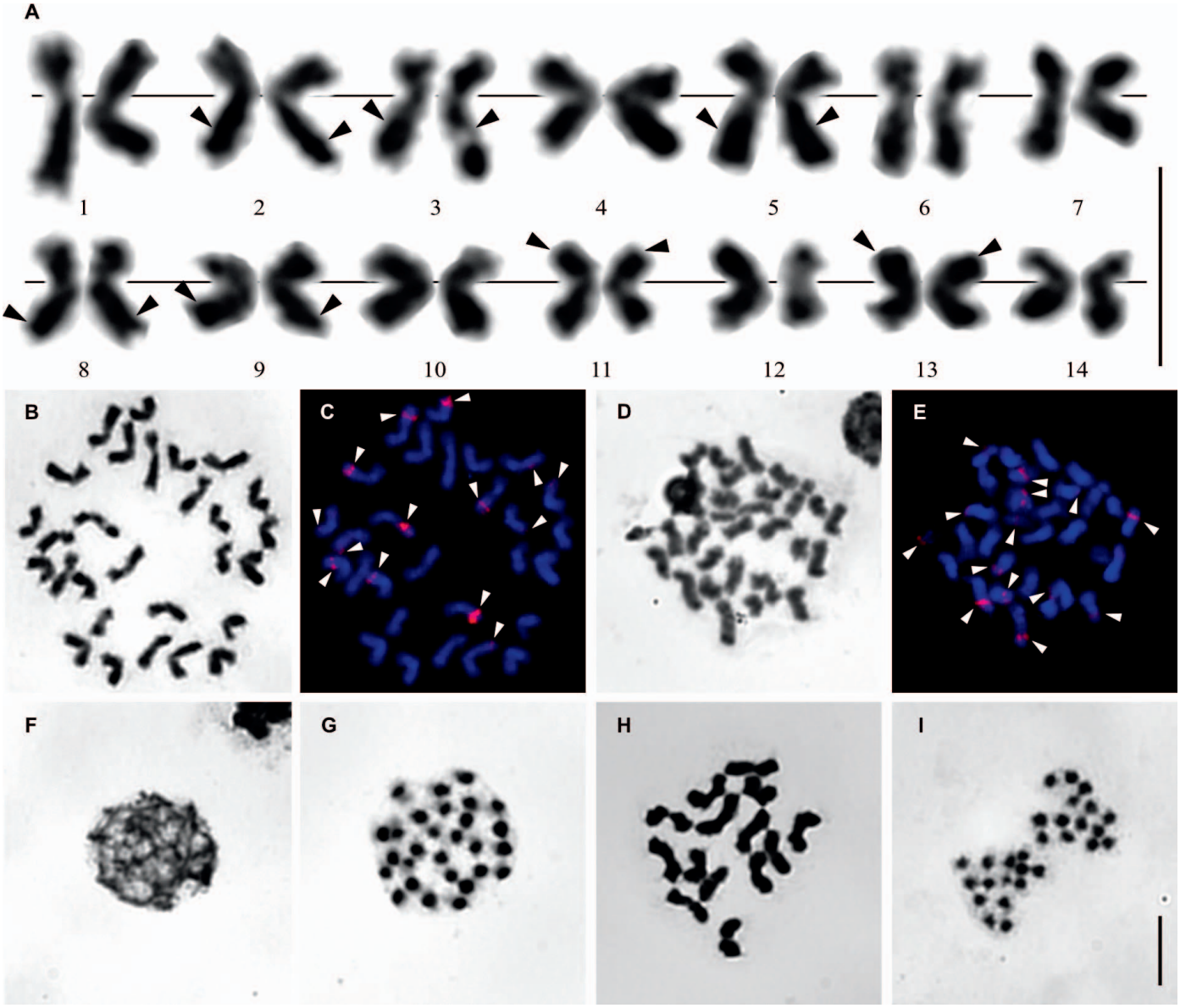

Figure 1.—The male karyotype and course of meiosis of *Miopsalis dillyi* (2n = 28) after Giemsa staining (A, B, D, F, G, H, I) and the corresponding cells after FISH with 18S rRNA genes, red signal (C, E): A. Karyotype based on spermatogonial metaphase, male; B, C. Mitotic metaphase, male; D, E. Mitotic metaphase, juvenile; F. Zygotene; G. Diplotene; H. Diakinesis; I. Metaphase II. Arrowheads indicate the position of 18S rRNA gene clusters. Bar = 10 μm.

gene clusters localized close to the centromeric region or terminal parts of chromatids on the long arm of homologous chromosomes of pair No. 2, 3, 5, 8 and 9 and short arm of homologous chromosomes of pair No. 11 and 13 (Figs. 1A,C). The same number of signals was detected also in one juvenile (Fig. 1E). During the early phases of the meiosis (e.g., zygotene) we observed large positive heteropycnotic parts of the chromosomes (Fig. 1F). Later during the diplotene (Fig. 1G), these heteropycnotic parts form substantial parts of the chromosomes and they are visible as conspicuous heteropycnotic blocks. The number of these blocks corresponds to the number of chromosomes (2n = 28). During diakinesis (Fig. 1H) and metaphase II (Fig. 1I), the spiralization is already uniform and the chromosomes are entirely isopycnotic. During the pairing of homologous chromosomes, we did not identify any conspicuous heteromorphic pairs that might correspond to the differentiated sex chromosomes (Fig. 1H).

DNA analysis.—Sequence data, namely the sequences for the COI gene from a total of 10 individuals (three of our own, and seven from GenBank) were used for molecular phylogenetic analysis. The Maximum Likelihood tree is structured and clustered into four main groups (Fig. 2A). The first group, containing species named Borneo 9 and Borneo 11, was monophyletic and sister to the remaining groups. This group had already been detected by Clouse et al. (2011). The second monophyletic group included Borneo 2 and Borneo 5 and was sister group to the remaining two groups. This group was also detected by Clouse et al. (2011). The relationship between the

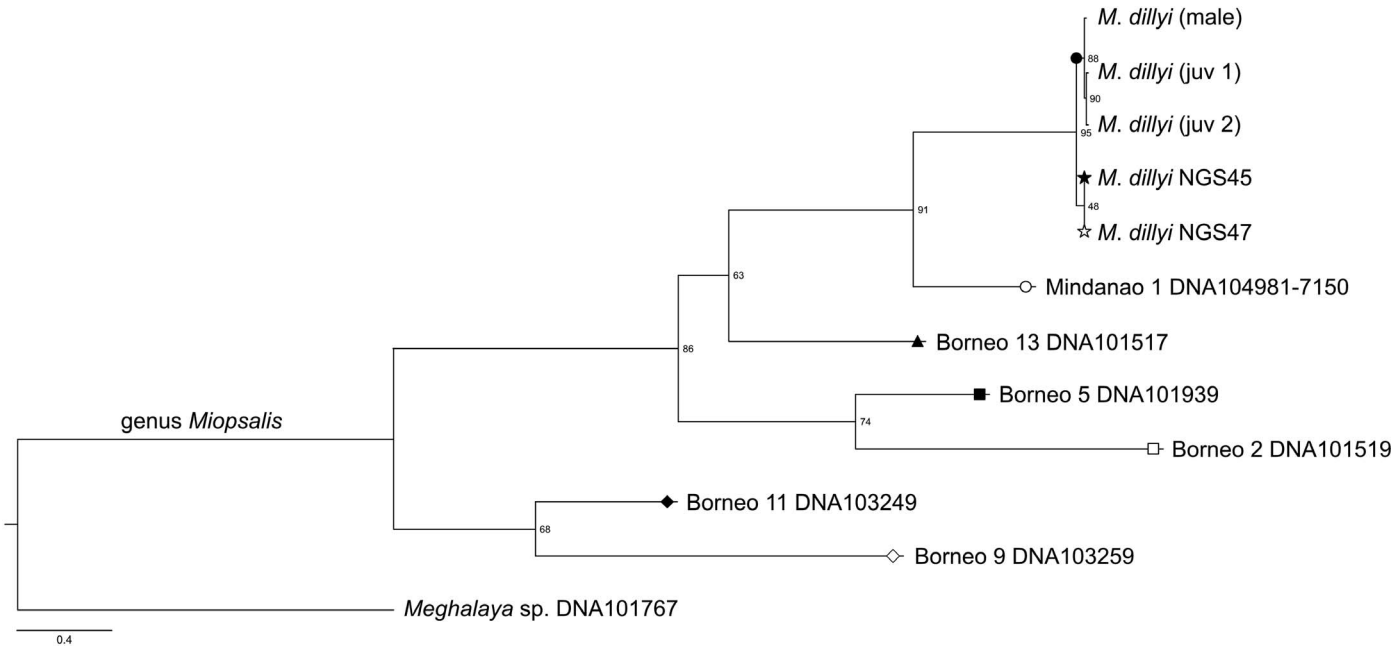

Figure 2A.—Maximum likelihood phylogenetic tree using COI for species of *Miopsalis*.

Figure 2B.—Distribution of analysed specimens on Borneo and Mindanao; symbols correspond to the clades on the tree in Figure 2A.

last two groups was not clear and had low support. The third group was monophyletic with two species from Mindanao. The species named Mindanao 1 from Clouse et al. (2011) is sister group to the species *M. dillyi*. Species Borneo 13 was probably sister group to the species from Philippines.

We also calculated pairwise uncorrected p-distances for *Miopsalis* species from the studied area (Supplementary Table S2). This comparison indicated similar evolutionary divergences between *M. dillyi* and the previously recognized species of this genus from the Philippines and Borneo (p-distances range from 4.4 up to 22.9%).

DISCUSSION

Harvestmen of the South Asian genus *Miopsalis* belong to the family Stylocellidae, which contains species of mite harvestmen found from the Eastern Himalayas to New Guinea and further, to Palawan and Mindanao, with recent biogeographic studies discovering a significant number of undescribed species from across the entire region (Clouse & Giribet 2010; Clouse et al. 2011; Clouse 2012). Regarding the Philippines only two species – *M. tarumpitao* from Palawan Island and *M. dillyi* from Mindanao – have been described from these islands (Shear 1993; Schmidt et al. 2020). Our analysis of *M. dillyi* extends our knowledge about the distribution and intraspecific variability of this species on Mindanao Island, and p-distance between the COI sequences known from Mindanao are comparable to those of different species from Borneo (p-distances >14%) (Table S2).

New insights into the diversity of stylocellids could be provided by the cytogenetic markers. The karyotype differences among cryptic species can be distinctive, especially in arachnid groups with limited dispersal abilities (e.g., Kotrbová et al. 2016; Šťáhlavský et al. 2018a), and can clearly help to delimit species boundaries (e.g., Řezáč et al. 2018; Štundlová et al. 2019). However, the application of cytogenetic methods, especially in the taxonomy of arachnids, has been complicated by the absence of documented cytogenetic characteristics in the majority of the known species, and so there has been little implementation of this feature into the description of new species (see discussion in Plíšková et al. 2016). The distinctive difference in the karyotypes has already been described in one genus of mite harvestmen: *Siro* Latreille, 1796 (2n = 30–52) (Svojanovská et al. 2016). Our cytogenetic analysis of *M. dillyi* (2n = 28) documents the interspecific variability in *Miopsalis* compared to the only other previously karyotyped species *Miopsalis* sp. (2n = 30) from Borneo (Svojanovská et al. 2016).

Interestingly, we found conspicuous differences in the number of 18S rRNA gene clusters between *Miopsalis* species. The species from Borneo possesses only one pair of these clusters, in a terminal position on the chromosomes (Svojanovská et al. 2016). This characteristic is proposed to be an ancestral state for the Class Arachnida (Forman et al. 2013). However, the number of 18S rRNA gene clusters increases to seven pairs in *M. dillyi*. Within harvestmen, similar multiplication of this gene cluster has been documented only in *Mitopus morio* (Fabricius, 1799) (Phalangiidae) (Jindrová et al. 2020). Two sites of rRNA loci per diploid chromosome set is the most frequent number in majority of animals (Sochorová et al. 2018). However, cytogenetic studies have also identified many exceptional cases of multiplication of rRNA genes within different phylogenetic lineages. It is considered as a result of an insertion of transposable elements, which potentially accelerates genomic reorganization after a speciation event or other specific stimuli, such as stress, etc. (e.g., Symonová et al. 2013). Increased numbers of rRNA gene clusters have also been documented in other mite harvestmen: *Parapurcellia amatola* de Bivort & Giribet, 2010 (Pettalidae, five pairs; Svojanovská et al. 2016). Among harvestmen, the multiplication of this gene cluster has also been documented recently in several phalangiid species: *Mitopus morio* (seven pairs), *Lacinius epiphiatus* (C.L. Koch, 1835) (five pairs; Jindrová et al. 2020), *Rilaena triangularis* (Herbst, 1799) (four pairs; Jindrová et al. 2020) and *Rhampsinitus leighi* (Pocock, 1903) (seven clusters; Šťáhlavský et al. 2018a). The fact that the multiplication of rRNA gene clusters occurred among unrelated taxa, suggests that the process is independent within harvestmen. In all the species with increased numbers of these clusters, they are situated mainly in terminal positions. Their proximity to the telomeric region facilitates the efficiency of ectopic recombination during rRNA gene cluster increase (Goldman & Lichten 1996), and the preferential replacement of rRNA genes into a new subtelomeric position (Nguyen et al. 2010). Three pairs of 18S rRNA gene clusters are also located at the interstitial position on long arms of chromosomes in *M. dillyi*. This location may be a consequence of chromosomal rearrangement (paracentric inversion) or an effect of transposable elements (see Cabrero & Camacho 2008). Our analysis demonstrates dynamic changes in the karyotypes in morphologically uniform mite harvestmen. It is possibly a consequence of low dispersal capability, as has been proposed for harvestmen from South Africa (Šťáhlavský et al. 2018a).

Recent studies have shown significant cryptic diversity in the suborder Cyphophthalmi (Boyer et al. 2007a; Giribet et al. 2012; Schwentner & Giribet 2018; Benavides et al. 2019). Despite the limited information about the karyotypes of mite harvestmen, cytogenetic markers seem to represent a promising approach that may supplement other commonly used species delimitation methods (e.g., morphology, DNA analyses). However, comparison of the karyotypes among a large number of closely related mite harvestmen species is still necessary to test this hypothesis.

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SUPPLEMENTARY FILES

Supplementary File 1.—Morphological characterization of *Miopsalis dillyi* from Mt. Malambo, Mindanao, Philippines. Online at https://doi.org/10.1636/JoA-S-20-028.s1

Supplementary Figure S1.—Analysed *Miopsalis dillyi*, habitus of the male: (A) dorsal view; (B) lateral view; (C) ventral view. Bar = 1 mm. Online at https://doi.org/10.1636/JoA-S-20-028.s2

Supplementary Figure S2.—Analysed *Miopsalis dillyi*, male: (A) sternal region; (B) anal region; (C) spiracle; (D) ozophore; (E) chelicera; (F) genital region. Bar = 100 μm. Online at https://doi.org/10.1636/JoA-S-20-028.s3

Supplementary Figure S3.—Analysed *Miopsalis dillyi*, male: (A – C) right leg I with details of tarsus and claw; (D – F) right leg II with details of tarsus and claw; (G – I) right leg III with details of tarsus and claw; (J – L) right mesal leg IV with details of adenostyle, tarsus and claw. Bar = 500 μm (A, D, G), 100 μm (B, E, H, K), 50 μm (C, F, I, J, L,). Online at https://doi.org/10.1636/JoA-S-20-028.s4

Supplementary Figure S4.—Analysed *Miopsalis dillyi*, dorsal (A) and ventral (B) view of male spermatopositor. Bar = 10 μm. Online at https://doi.org/10.1636/JoA-S-20-028.s5

Supplementary Table S1.—Summary of analysed species of genus *Miopsalis* including cytogenetically investigated *Miopsalis dillyi*. Online at https://doi.org/10.1636/JoA-S-20-028.s6

Supplementary Table S2.—Uncorrected p-distances between analysed species in percent. Online at https://doi.org/10.1636/JoA-S-20-028.s7

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