

The Highest Chromosome Number and First Chromosome Fluorescent *in situ* Hybridization in the velvet worms of the family Peripatidae

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Received 19 December 2019 / Accepted 22 January 2020 / Published 3 March 2020
Communicated by John Wang

The diversity of Onychophora is poorly studied, despite there being nearly 200 described species divided in two families: Peripatidae and Peripatopsidae. Peripatid velvet worms are found mainly in the Neotropical region. The low morphological diversity in Peripatidae is an obstacle to determining its taxonomy, and chromosomal analyses can help clarify this. The aim of this work was to chromosomally analyze one species of *Epiperipatus* from Mato Grosso do Sul, Brazil. Conventional staining and telomeric fluorescent *in situ* hybridization (FISH) were performed with the gonads of three males of *Epiperipatus* sp. The specimens showed $2n \delta = 73$, the largest diploid number found in Onychophora to date, with the majority of chromosomes acro/telocentrics and the largest element submetacentric. The FISH marked the telomeric region of all elements and revealed one Interstitial Telomeric Site (ITS) on the proximal region of the long arm large submetacentric chromosome. The absence of male meiosis and female cell division in the analyzed specimens prevented us from determining whether the unpaired large submetacentric is a sex chromosome, which could lead to the description of a rare sex chromosome system (SCS) in Onychophora, or a case of fusion between autosomes. In either case, the presence of ITS is a clear indication of chromosomal fusion.

Key words: Cryptic species, *Epiperipatus*, Karyotype, Heteromorphic chromosome, Interstitial Telomeric Site.

BACKGROUND

Onychophora are ancient Panarthropod animals with soft bodies, lobopodial legs with claws and a peculiar hunting strategy by which they eject glue to capture prey (Monge-Najera 1995). The group has 201 species distributed within Peripatidae (81 species) and Peripatopsidae (120 species), 20 of which are considered *nomina dubia*; however, the biodiversity of these species is far from established (Oliveira et al. 2020).

Most peripatids are from Neotropical environments, and the Brazilian velvet worm fauna consists of only 16 species, four of which are considered vulnerable or

endangered (Oliveira et al. 2015; Instituto Chico Mendes de Conservação da Biodiversidade 2018). Furthermore, peripatids include several other Brazilian species that are undescribed (Sampaio-Costa et al. 2009; Oliveira et al. 2010). One of the main reasons they are undescribed is that their characters have low diversity, making it difficult to efficiently compare species (Sampaio-Costa et al. 2009; Oliveira et al. 2012a).

Besides the taxonomic gaps mainly in Peripatidae, several studies have found cryptic species of Onychophora, including in Brazilian species of *Epiperipatus* (Clark, 1913) (Reid et al. 1995; Reid 1996; Lacorte et al. 2011; Oliveira et al. 2011 2018).

Cryptic speciation and narrow geographical range are expected due to their low dispersal, cryptic habitat and reproductive biology (New 1995; Oliveira et al. 2011 2014).

Both families have few cytogenetic analyses, with Peripatidae being less studied, with only 6.17% of the species already karyotyped (Table 1) (Oliveira et al. 2012b). The lowest and highest diploid numbers found were $2n \delta = 8$ in *Eoperipatus* sp. and $2n \delta = \pm 60$ in *Epiperipatus biolleyi* (Bouvier, 1902), both belonging to Peripatidae (Mora et al. 1996; Jeffery et al. 2012; Oliveira et al. 2012b). Among species with a described Sex Chromosome System (SCS), the most common is the type δXY (Table 1), except for a population of *Euperipatoides rowelli* (Reid, 1996) that presented the type δX_1X_2Y (Rowell et al. 1995). Studies with differential chromosome techniques are virtually absent for velvet worms. The C-banding technique does not work for the group, and only one study employing chromosome fluorescent *in situ* hybridization (FISH) technique has been performed so far, showing $(TTAGGG)_n$ telomeric repeats only on terminal ends of pachytene bivalents of *Peripatopsis stelliporata* Sherbon & Walker, 2004 (Peripatopsidae), despite their importance for understanding the chromosome evolution in the clade (Rowell et al. 1995 2002; Vítková et al. 2005; Jeffery et al. 2012; Oliveira et al. 2012b 2018).

Cytogenetics provide a highly informative tool for distinguishing species of Onychophora, although it is not as well explored in Peripatidae (Oliveira et al. 2012b 2018). *Epiperipatus* is a genus with a monophyly that needs revision, and presents cryptic species (Oliveira et al. 2011 2012a). Thus, in this work, we described the karyotype of *Epiperipatus* sp. from Mato Grosso do

Sul, Brazil and discuss the cytotaxonomical value of the chromosomal data to the group.

MATERIALS AND METHODS

Five specimens (4 δ and 1 f) of *Epiperipatus* sp. (Fig. 1) were collected at the entrance zone of three caves and surrounding areas near the Gruta Manoel Cardoso ($56^\circ 43' 23.85'' W$; $20^\circ 34' 7.11'' S$, Bodoquena, Mato Grosso do Sul, Brazil). Only three males presented cell divisions, and were deposited in Coleção Zoológica da Universidade Federal do Mato Grosso do Sul with voucher (ZUFMS-00007).

We anesthetized the specimens in a chamber with ether, then dissected them by immersing individuals in a physiological solution based on Robson et al. (1966). The gonads were transferred to colchicine (Sigma Chemical CO.) solution in concentration of 0.16% (in the same physiological solution) for two hours. Then, we added an equal volume of hypotonic solution for 25 minutes and fixed the gonads in Carnoy I (3:1 methanol: acetic acid). We placed portions of gonadal tissue on a glass slide with a drop of acetic acid 60% and then, with the aid of a small metal rod, smashed the tissue to form a cell suspension before adding a few more drops of acetic acid solution to spread the material on the slide and then dry it on a metal plate at a temperature of 35 to 40°C. The slides were stained with 3% Giemsa solution (94 ml water, 3 ml phosphate buffer pH 6.8 and 3 ml Giemsa Merck-Darmstadt, Germany) for 10 minutes, except those used for FISH.

The FISH technique employed a peptidic nucleic acid (PNA) $(AATCCC)_3$ probe (PNA Bio, Inc) complementary to the $(TTAGGG)_n$ telomeric repeats of

Table 1. Review of Onychophora cytogenetics. Diploid number ($2n \delta$), Sex Chromosome System (SCS), chromosomal morphology, locality and references

Peripatidae					
Species	$2n \delta$	SCS	Chromosomal morphology	Locality	Reference
<i>Cerradopatus sucuriensis</i> ^a Oliveira et al., 2015 (cited as <i>Epiperipatus</i> sp.)	22	-	-	Brazil	Jeffery et al. 2012
<i>C. sucuriensis</i> Oliveira et al., 2015	22	-	6 acrocentric, 5 metacentric / submetacentric	Brazil	Oliveira et al. 2015
<i>Eoperipatus</i> sp.	8	-	-	Thailand	Jeffery et al. 2012
<i>Eoperipatus</i> sp.	8	-	-	Thailand	Oliveira et al. 2012b
<i>Epiperipatus biolleyi</i> (Bouvier, 1902)	$\pm 60^b$	-	-	Costa Rica	Mora et al. 1996
<i>Epiperipatus</i> sp.	73	-	1 submetacentric, 72 acro/telocentric	Brazil	Present study
<i>Principapillatus hitoyensis</i> Oliveira et al., 2013	54	XY	-	Costa Rica	Jeffery et al. 2012
<i>P. hitoyensis</i> Oliveira et al., 2013	54	XY	17 acrocentric, 9 metacentric/submetacentric and XY acrocentric	Costa Rica	Oliveira et al. 2012b

Table 1. (Continued)

Peripatopsidae					
Species	2n ♂	SCS	Chromosomal morphology	Locality	Reference
<i>Centorumis trigona</i> ^a Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	26	-	-	Australia	Rowell et al. 1995
<i>Cephalofovea cameroni</i> Reid et al., 1995	28	-	-	Australia	Reid et al. 1995
<i>Cephalofovea clandestina</i> Reid et al., 1995	28	-	-	Australia	Reid et al. 1995
<i>Cephalofovea pavimenta</i> Reid et al., 1995	34	-	-	Australia	Reid et al. 1995
<i>Cephalofovea tomahmontis</i> Ruhberg, 1988	34	XY	-	Australia	Reid et al. 1995
<i>C. tomahmontis</i> Ruhberg, 1988	34	XY	-	Australia	Rowell et al. 1995
<i>Diemenipatus mesibovi</i> Oliveira et al., 2018	18	XY	-	Tasmania	Oliveira et al. 2018
<i>Diemenipatus taiti</i> Oliveira et al., 2018	18	-	-	Tasmania	Oliveira et al. 2018
<i>Euperipatoides kanangrensis</i> ^a Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	32	XY	-	Australia	Rowell et al. 1995
<i>E. kanangrensis</i> Reid, 1996	32	XY	-	Australia	Jeffery et al. 2012
<i>Euperipatoides leuckartii</i> (Sänger, 1871)	32	XY	-	Australia	Rowell et al. 1995
<i>Euperipatoides rowelli</i> Reid, 1996	34	XY	-	Australia	Jeffery et al. 2012
<i>Euperipatoides rowelli</i> ^a Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	33	X ₁ X ₂ Y	-	Australia	Rowell et al. 1995
<i>E. rowellic</i> Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	34	-	-	Australia	Rowell et al. 1995
<i>E. rowellic</i> Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	34	XY	-	Australia	Rowell et al. 1995
<i>Euperipatoides</i> sp.	18	XY	-	Tasmania	Rowell et al. 1995
<i>Euperipatoides</i> sp.	18	-	-	Tasmania	Rowell et al. 1995
<i>Leucopatus anophthalmus</i> Oliveira et al., 2018	36	-	-	Tasmania	Oliveira et al. 2018
<i>Nodocapitus inornatus</i> ^a Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	30	-	-	Australia	Rowell et al. 1995
<i>Ooperipatellus insignis</i> (Dendy, 1890)	42	XY	Acrocentric, metacentric and telocentric	Australia	Rowell et al. 2002
<i>Ooperipatellus nickmayeri</i> Oliveira and Mayer, 2017	50	XY	-	Tasmania	Oliveira and Mayer 2017
<i>Ooperipatellus</i> sp. 1	42	-	Acrocentric, metacentric and telocentric	Australia	Rowell et al. 2002
<i>Ooperipatellus</i> sp. 2	42	-	Acrocentric, metacentric and telocentric	Tasmania	Rowell et al. 2002
<i>Ooperipatus hispidus</i> Reid, 1996	22	XY	-	Australia	Jeffery et al. 2012
<i>Peripatopsis balfouri</i> (Sedgwick, 1885) (cited as <i>Peripatus balfouri</i>)	28	-	-	South Africa	Montgomery 1900
<i>Phallocephale tallagandensis</i> Reid, 1996	18	XY	-	Australia	Jeffery et al. 2012
<i>Planipapillus biacinaces</i> Reid, 1996	40	-	Telocentric	Australia	Rowell et al. 2002
<i>Planipapillus bulgensis</i> Reid, 1996	24	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus cyclus</i> Reid, 2000	26	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus impacris</i> Reid, 2000	30	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus mundus</i> Reid, 1996	40	-	Telocentric	Australia	Rowell et al. 2002
<i>Planipapillus taylori</i> Reid, 1996	38	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 1	22	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 2	20	-	Metacentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 3	32	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 4	32	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 5	36	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 6	36–38	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 7	22	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Planipapillus</i> sp. 8	34	-	Metacentric, submetacentric and telocentric	Australia	Rowell et al. 2002
<i>Ruhbergia bifalcata</i> ^a Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	30	XY	-	Australia	Rowell et al. 1995
<i>Tasmanipatus barrette</i> Ruhberg et al., 1991	40	XY	-	Tasmania	Oliveira et al. 2018
<i>Tetrameraden meringosc</i> Reid, 1996 (cited as <i>Euperipatoides</i> sp.)	26	XY	-	Australia	Rowell et al. 1995

^aOliveira et al. (2015) described *Cerradopatus sucuriuensis* as a population of *Epiperipatus* sp. analysed by Jeffery et al. (2012). ^bThe author could not exactly define the diploid number, but used a photo of a mitotic cell with 2n = 60 and found meiotic cells varying from n = 28 to n = 32. ^cReid (1996) assigned to different genera the species chromosomally analyzed by Rowell et al. (1995) as populations of *Euperipatoides* sp.

vertebrates, labeled with Alexa fluor 488 (ThermoFisher Scientific), following the method of Genet et al. (2013), with a hybridization time of four hours at 37°C, without heat denaturing, and mounted using ProLong Diamond antifade with DAPI (ThermoFisher Scientific).

The cells were photographed with a Zeiss Axioimager D2 microscope holding a AxioCam 503 camera, using the ZEN Pro software. Chromosome morphology was determined with the free software IMAGEJ (Rasband 1997–2019) and the LEVAN plugin (Sakamoto and Zacaro 2009), according to Levan et al. (1964) and Green and Sessions (1991), using twenty-six metaphases of *Epiperipatus* sp.

RESULTS

Of the 47 mitotic metaphases, the three males of *Epiperipatus* sp. showed $2n \delta = 73$ (Fig. 2A–D) (Table 2, Fig. S1). Regarding chromosomal morphology, the majority of the elements was acro/telocentric and decreased gradually in size, except for the largest chromosome of the complement, which was a single submetacentric (4.26% of the karyotype \pm 0.49) and almost 50% longer than the second largest chromosome (2.91% of the karyotype \pm 0.30) (Table S1). The telomeric regions of all chromosomes were hybridized with the probe to the (TTAGGG)_n motif (Fig. 2B–D). The unpaired largest submetacentric chromosome has an interstitial telomeric site (ITS) in the proximal



Fig. 1. Specimen of *Epiperipatus* sp. from Mato Grosso do Sul, Brazil. Photo courtesy of Dr. Paulo Robson de Souza.

Table 2. Diploid numbers found in all analyzed metaphases of the three specimens of *Epiperipatus* sp.

Specimen	$2n \delta = 66$	$2n \delta = 67$	$2n \delta = 68$	$2n \delta = 69$	$2n \delta = 70$	$2n \delta = 71$	$2n \delta = 72$	$2n \delta = 73$
1	-	-	-	1	1	5	3	36
2	1	1	1	-	-	2	-	8
3	-	1	-	-	-	1	-	3

portion of the long arm (Fig. 2B, D–E; Fig. S2). No specimens presented cells during meiosis.

DISCUSSIONS

The $2n \delta = 73$ found in *Epiperipatus* sp. is the highest diploid number recorded for Onychophora up to now, with at least five more chromosomal pairs than

Epiperipatus biolleyi with $2n \delta = \pm 60$, which had the largest diploid number previously recorded (Mora et al. 1996). Despite the fact that peripatids are the most poorly studied onychophorans, it covers the karyotypes with the highest and lowest numbers of chromosomes ($2n \delta = 8$ and $2n \delta = 73$) for the onychophorans. Unfortunately, there is no Peripatidae phylogeny, but there is a cladogram for Peripatopsidae that uses four peripatid genera as external groups (Oliveira et al.

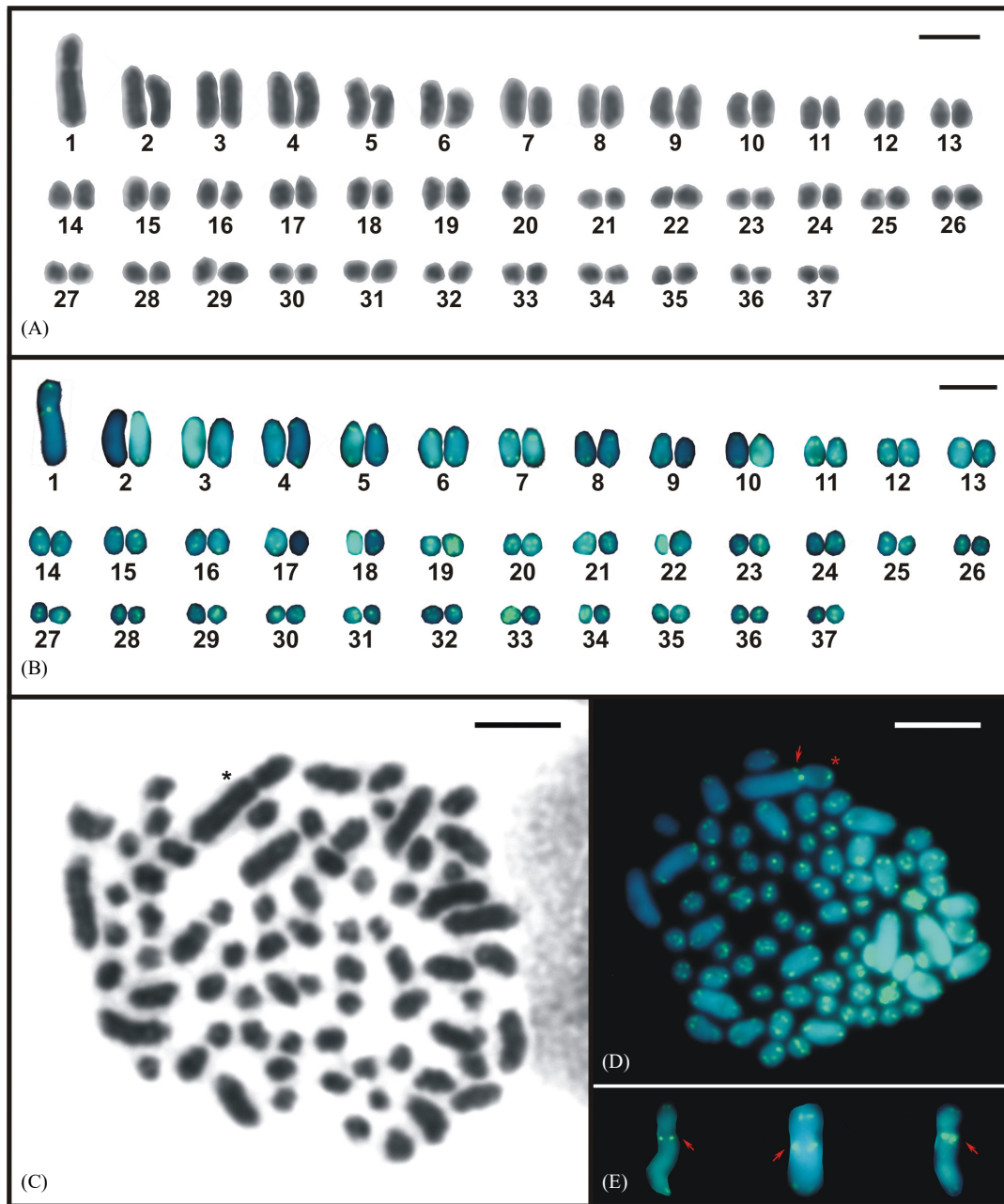


Fig. 2. Chromosomes of *Epiperipatus* sp. (A–B) Karyotype showing $2n \delta = 73$ in Giemsa staining (A) and telomeric fluorescent *in situ* hybridization (B). (C–D) Male mitosis used in the karyotype showed in A–B, respectively. Asterisk: large unpaired submetacentric. (E) Heteromorphic chromosome from three different metaphases. Arrow: Interstitial Telomeric Site (ITS). Scale bars = 5 μ m.

2018), revealing that *Epiperipatus* is closely related to *Principapillatus*, a genus that also has a high chromosome number ($2n \delta = 54$) (Jeffery et al. 2012; Oliveira et al. 2012b; present study). On the other hand, *Eoperipatus*, the genus with the lowest diploid number among onychophorans ($2n \delta = 8$), is basal within Peripatidae. Thus, chromosome number can be useful, along with molecular data, to reveal the evolutionary relationships in onychophoran. However, the scarcity of phylogenetic and cytogenetic data on Peripatidae reveals a weak point of this discussion.

Regarding the large submetacentric chromosome, one hypothesis is that it could be a sex chromosome. With $2n \delta = 73$, an odd chromosome number, there are at least three numerical possibilities for SCS (X_0 , X_1X_2Y or XY_1Y_2). The presumptive occurrence of an X_1X_2Y or XY_1Y_2 SCS was already discussed for one population of *Euperipatoides rowelli*, $2n \delta = 33$ (Peripatopsidae) (Rowell et al. 1995), but no details on chromosome size or morphology were presented for this population. The X_0 SCS may have originated from an XY SCS, already found in several onychophoran species (Table 1), through heterochromatinization and deletion of the Y chromosome (Kral et al. 2006). However, the presence of an ITS on the unpaired large submetacentric of *Epiperipatus* sp. supports an X_1X_2Y or XY_1Y_2 , originated from an XY by a fusion of an autosome and a sex chromosome SCS (see Araujo et al. 2012 for a review on SCS origins in spiders). If confirmed, the putative X_1X_2Y or XY_1Y_2 SCS would have originated at least twice within Onychophora, in Peripatopsidae and in Peripatidae. An analysis of male meiosis and female mitosis in *Epiperipatus* sp. would allow us to distinguish among an X_0 ($2n \delta = 73$, X_0 , sex univalent; $2n \text{♀} = 74$, XX), X_1X_2Y ($2n \delta = 73$, X_1X_2Y , sex trivalent; $2n \text{♀} = 74$, $X_1X_1X_2X_2$), or XY_1Y_2 ($2n \delta = 73$, XY_1Y_2 , sex trivalent; $2n \text{♀} = 72$, XX); however, no female cell division was found.

If the large unpaired element is not a sex chromosome, then this heteromorphism may have originated through the fusion between two autosomal chromosomes. Rowell et al. (2002) observed a high chromosome number diversity in species of *Planipapillus* Reid, 1996, which, according to Rockman and Rowell (2002), have undergone several centric fusion events, starting from a $2n \delta = 40$ ancestor karyotype with exclusively telocentric chromosomes. Therefore, the three individuals studied in this work would be heterozygous for a centric fusion, probably forming an autosomal trivalent on meiosis (the large submetacentric plus two smaller telocentrics). If this is the case, through the analysis of additional specimens, we may be able to find both homozygous individuals for the rearrangement and individuals that do not have the

rearrangement.

Regardless of whether it is a sex chromosome or autosome, the results from the telomeric FISH corroborate the rearrangement, where the proximal marking found indicates a probable centric fusion. This demonstrates the importance of the telomeric FISH technique in Onychophora, because besides being informative, other techniques such as C-banding are not usually effective in this group (Rowell et al. 1995 2002; Oliveira et al. 2018).

Cryptic speciation is common in Peripatopsidae, and molecular studies in this group have found several cryptic lineages that are not distinguishable through morphological analysis. The same occurs in Peripatidae; however, it is not possible to confirm this hypothesis due to the lack of studies with non-morphological methods (Lacorte et al. 2011; Oliveira et al. 2011). Chromosome data are already used in several groups to aid in the identification of cryptic species (Dobigny et al. 2005; Řezáč et al. 2018) and Oliveira et al. (2018) comment that chromosomes can illuminate several aspects of evolution in Onychophora. Although there are few studies on Peripatidae cytogenetics, a large karyotypic diversity within the group was noted (Table 1), and in future studies, the karyotype may be fundamental in the diagnosis of Peripatidae species, as in Peripatopsidae (Rowell et al. 2002; Oliveira et al. 2012b).

CONCLUSIONS

In conclusion, the present work shows that karyotypic data can be used to aid in taxonomic studies, principally in the polyphyletic *Epiperipatus*, which according to Oliveira et al. (2012a), possibly contains members of *Principapillatus* Oliveira et al., 2013, another genus with a high chromosome number (Jeffery et al. 2012). Additionally, telomeric FISH has been shown to be important in the detection of chromosomal rearrangements that may aid in our understanding of karyotype evolution in Onychophora.

Acknowledgments: The authors thank Dr. Paulo Robson de Souza of Universidade Federal de Mato Grosso do Sul (UFMS), Brazil for the photographs of the *Epiperipatus* sp. specimen from Mato Grosso do Sul, Brazil, and Matthijs Strietman for the language review of the manuscript. This work was supported by the BIOTA FUNDECT-CAPES under grant (SIAFEM 23519, termo de outorga 69/2014).

Authors' contributions: Specimens collection and identification: LMC. Specimens dissection and slide preparations: DA LMC. Chromosome analysis: DDD

DA. Manuscript writing: DDD DA LMC LHBS.

Competing interests: All authors declare that they have no conflict of interest.

Availability of data and materials: The material analyzed is available at the Coleção Zoológica da Universidade Federal do Mato Grosso do Sul.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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Supplementary Materials

Fig. S1. Pictures of all metaphases analyzed with score of diploid number. (download)

Fig. S2. Pictures of telomeric fluorescent in situ hybridization in ALEXA 488, DAPI and merged. (download)

Table S1. Relative average length of chromosomal pairs of *Epiperipatus* sp. (download)