ORIGINAL ARTICLE

Distribution of 18S rDNA clusters in Central European harvestmen of the suborder Eupnoi (Arachnida: Opiliones)

Hana JINDROVÁ[1], Matyáš HIŘMAN[1,*], David SADÍLEK[1], Pavel BEZDĚČKA[2] and František ŠŤÁHLAVSKÝ[1]

[1] Charles University, Faculty of Science, Department of Zoology, Viničná 7, CZ-12844 Praha, Czech Republic; e-mails: svojanovska.hanka@seznam.cz, hirmanm@natur.cuni.cz, david.sadilek@natur.cuni.cz, frantisek.stahlavsky@natur.cuni.cz

[2] Museum of the Highlands Jihlava, Masarykovo náměstí 55, CZ-58601 Jihlava, Czech Republic; e-mail: bezdecka@muzeum.ji.cz

Abstract. rDNA clusters are an important cytogenetic marker for studying karyotype evolution and chromosomal changes. The variability of this cytogenetic characteristic is, however, still almost unknown in the karyotypes of the entire class Arachnida (Arthropoda: Chelicerata). This situation is particularly evident in harvestmen (Arachnida: Opiliones), with 97 species studied cytogenetically, for which there is information on the number and position of rDNA clusters for only 13. Moreover, previous studies indicate that the number of rDNA loci is highly variable in the species analysed, ranging from one to five pairs of rDNA clusters. Based on this fragmentary information, which is for rare species mainly from the limits of the distribution of their families, it is still not possible to reconstruct the ancestral state for this important cytogenetic feature in this order. Building upon recent research in Central Europe, we analysed the number and position of 18S rDNA in 13 species belonging to the suborder Eupnoi. This revealed that their karyotypes were variable in terms of the diploid number (2n = 16–36) and number of 18S rDNA clusters (from one to seven pairs). For the first time, an 18S rDNA cluster was detected on B chromosomes in harvestmen. Our study sheds new light on the karyotype evolution and 18S rDNA distribution in harvestmen and provides an improved understanding of the ancestral state of karyotypes in the order Opiliones.

INTRODUCTION

The Eupnoi is one of the four suborders of the order Opiliones (Arthropoda: Arachnida), which together with the suborders Dyspnoi, Cyphophthalmi and Laniatores, constitute the third-largest order of the class Arachnida (e.g. Giribet & Kury, 2007; Pinto-da-Rocha et al., 2007). Despite their high diversity (6500 described species: Kury, 2017), only 97 species of harvestman have been cytogenetically analysed (Tsurusaki et al., 2020). Despite this paucity of data, some harvestmen are very variable in their basic cytogenetic characteristics, such as chromosome number (2n = 10–109), degree of differentiation between the sex chromosomes, the presence of B chromosomes and possible polyploidy (see Tsurusaki & Cokendolpher, 1990; Tsurusaki, 2007).

The most general ancestral state indicate morphologically undifferentiated sex chromosomes in harvestmen (e.g. Tsurusaki, 2007; Svojanovská et al., 2016). The XY sex chromosome system is reported only in the harvestman *Sabacon makinoi* Suzuki, 1949 (Dyspnoi: Sabaconidae) (Tsurusaki, 1989) and 13 species of the Sclerosomatidae

(Eupnoi) (see Tsurusaki et al., 2020). The exception, in the whole Class Arachnida, is one population of *Mitopus morio* (Fabricius, 1799) (Eupnoi: Phalangiidae), which potentially has a WZ system (Tsurusaki & Cokendolpher, 1990). However, the differences in chromosome length in some bivalents and their interpretation as sex chromosomes, may reflect the heterozygous nature of the nucleolus organiser regions (NORs) (Šťáhlavský et al., 2018).The NORs include the major ribosomal RNA (45S rRNA: i.e. 18S, 5.8S, and 28S rRNA) gene clusters, which are variable in harvestmen. Moreover, there is little data on this cytogenetic marker in this group of arachnids. This is the reason why it is currently not possible to specify precisely an ancestral state for this order. Silver staining has identified only one NOR, in *Psathyropus tenuipes* (L. Koch, 1878) (Eupnoi: Sclerosomatidae) (Gorlov & Tsurusaki, 2000) and *Dyscocyrtus pectinifemur* (Mello-Leitão, 1937) (Laniatores: Gonyleptidae) (Schneider et al., 2008) and one pair of NORs, in *Goniosoma spelaeum* (Mello-Leitão, 1932) (Laniatores: Gonyleptidae) (Oliveira et al., 2006). However, fluorescence in-situ hybridisation (FISH), using

* Corresponding author; e-mail: hirmanm@natur.cuni.cz

an 18S rDNA probe, frequently reveals one or two pairs of 18S rDNA clusters in different families of the suborder Cyphophthalmi and *Parapurcellia amatola* de Bivort & Giribet, 2010 (Cyphophthalmi: Pettalidae), with the number of such clusters sometimes being up to five pairs (Svojanovská et al., 2016). An increase in the number of 18S rDNA clusters from two to seven and the variability of the heterozygosity in the number and size of such clusters, have also been identified in five species of phalangiid from South Africa (Šťáhlavský et al., 2018). All previously analysed species have considerably limited dispersal abilities, which has resulted in distinct genetic differentiation (e.g. Giribet et al., 2016) and most likely in rapid changes in the distribution of NORs. FISH, using the 5S rDNA probe, revealed that in the harvestman *Psathyropus tenuipes* L. Koch, 1878 (Eupnoi: Sclerosomatidae) there is only one pair of this minor cluster of the rRNA gene (Watanabe et al., 2009). However, the position of this 5S rRNA gene cluster evolved independently of that of the major rRNA gene clusters (see Sochorová et al., 2018) and, therefore, this characteristic cannot help us understand the evolutionary dynamics of NORs in harvestmen. This is the reasons why we are focusing on the distribution of 18S rDNA in phalangiids in Central Europe in the present study. Species from this area are generally widely distributed and gene flow in these taxa is usually not limited, unlike in the previously analysed groups. The objective of this analysis was to better identify the ancestral state of the NORs and the main evolutionary changes in this cytogenetic marker in this suborder.

MATERIALS AND METHODS

Material

We analysed 13 species belonging to the families Phalangiidae and Sclerosomatidae from different localities in Central Europe (Table S1). The harvestmen were collected individually and transported alive to the laboratory for subsequent cytogenetic analyses. Analysed specimens are deposited in the public collection of the Faculty of Science, Charles University in Prague.

Chromosome preparations and karyotype evaluation

We used the "plate-spreading" technique (Traut, 1976) for chromosome preparations according to Šťáhlavský & Král (2004). During this procedure, dissected gonads were incubated in a hypotonic solution of 0.075 M KCl for 20 min then fixed in methanol : glacial acetic acid (3 : 1) for at least 20 min. Finally, the tissue was dissolved in a drop of 60% acetic acid on a microscope slide and spread on the surface of the microscope slide and left to evaporate. The chromosome preparations were stained with 5% Giemsa solution in Sörensen phosphate buffer for 15 min. The chromosomes were photographed using an ORCA-AG monochromatic camera (Hamamatsu Photonics Europe, Herrsching, DE) attached to an Olympus IX81 microscope. The karyotypes were described based on at least five sister metaphase II (the morphology of the chromosomes is unclear in the mitotic metaphases) nuclei using the software ImageJ 1.45r (Schneider et al., 2012) with the plugin Levan (Sakamoto & Zacaro, 2009).

FISH detection of 18S rDNA

The probe for 18S rDNA was prepared according to Šťáhlavský et al. (2018) and labelled with biotin-14-dUTP using the Nick Translation Kit (Abbott Molecular, Chicago, Illinois, USA). The overall hybridisation procedure followed the protocol described by Fuková et al. (2005) including the initial treatment with RNase A (200 μg/ml in 2 × SSC) for one hour (37°C). Denaturation of chromosomes was done at 68°C for 3 min and 30 s in 70% formamide in 2 × SSC. Then a probe mixture (20 ng of the probe, 25 μg of salmon sperm DNA, 10 μl of 50% formamide, 20% dextran sulphate in 2 × SSC) was put on each slide and allowed to hybridize overnight (37°C). Then the probe was detected using Cy3-conjugated streptavidin, followed by the application of biotinylated antistreptavidin and another dose of Cy3-conjugated streptavidin. Chromosomes were counterstained with DAPI (FluoroshieldTM; Sigma-Aldrich, St. Louis, Missouri, USA) and observed under an Olympus IX81 microscope equipped with an ORCA-AG monochromatic charge-coupled device camera (Hamamatsu). The images were pseudo coloured and superimposed using CellˆR software (Olympus Soft Imaging Solutions GmbH).

RESULTS

Family Phalangiidae

We analysed seven species belonging to following genera: *Egaenus*, *Lacinius*, *Mitopus*, *Oligolophus*, *Opilio*,

Table 1. Summary of the cytogenetic characteristics of the Eupnoi analysed. a – acrocentric chromosomes, B – B chromosomes, c – centromeric region, m – metacentric chromosomes, sm – submetacentric chromosomes, s – subterminal region, t – terminal region, ? – unclear chromosome pairs with 18S rDNA cluster.

| Family / Species	2n	Karyotype formula	Length of the haploid set (%)	Number of 18S rDNA clusters	Position of 18S rDNA pair + morph / pos
Phalangiidae					
Oligolophus tridens	16	12m, 4sm	9.66–4.17	1 pair	7. pair sm / t
Egaenus convexus	24	10m, 14sm	8.96–4.04	1 pair	1. pair m / c
Opilio canestrinii	24	18m, 6sm	12.70–5.16	1 pair	1. pair m / s
Phalangium opilio	24	18m, 6sm	10.90–4.94	1 pair	3. pair m / c
Lacinius epiphiatus	30	24m, 6sm	10.86–4.92	5 pairs	8.sm, 9.sm, 12.m, 13.m, 14.sm pair / t
Mitopus morio	32	22m, 10sm	8.88–3.8	7 pairs	? / t
Rilaena triangularis	36	30m, 6sm	9.00–3.8	4 pairs	? / s–t
Sclerosomatidae					
Leiobunum blackwalli	20	16m, 4sm	13.10–6.90	1 pair	1. pair m / t
Leiobunum rotundum	20+0–3B	14m, 6sm, 0–3Ba	11.56–8.56	1 pair + 0–3B	2. pair m / t
Leiobunum limbatum	22	14m, 8sm	11.11–6.91	1 pair	3. pair m / t
Leiobunum rupestre	22	22m	13.68–6.64	1 pair	3. pair m / t
Leiobunum gracile	22	14m, 8sm	12.75–7.02	1 pair	4. pair m / t
Nelima semproni	24	18m, 6sm	10.77–6.51	2 pairs	3. and 5. pair m / t
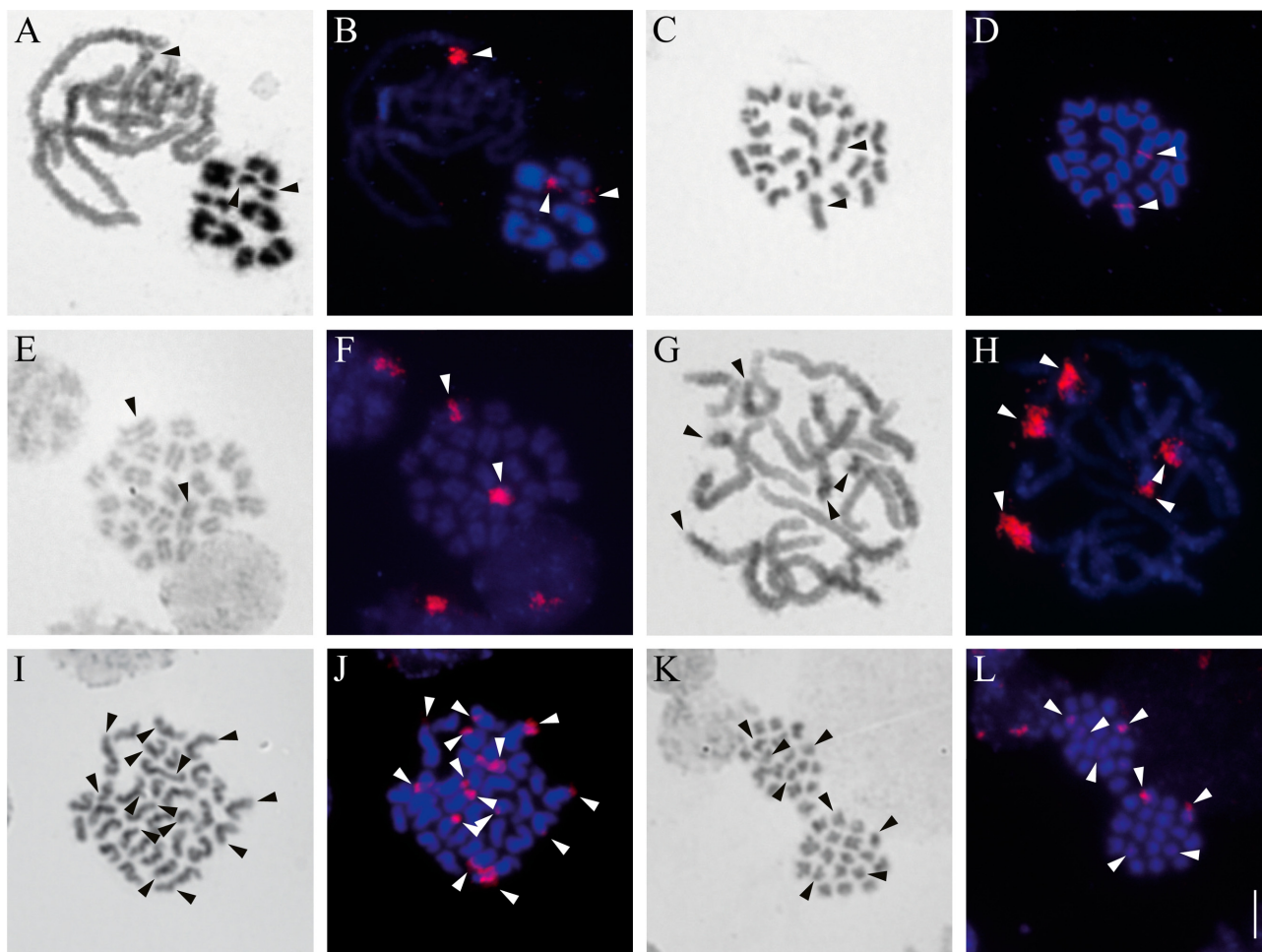
Fig. 1. The chromosomes of the family Phalangiidae. The chromosomes are counterstained with Giemsa (A, C, E, G, I, K) and the same nuclei after FISH with DAPI (blue) (B, D, F, H, J, L). *Oligolophus tridens* (2n = 16): A, B – pachytene and diakinesis; *Egaenus convexus* (2n = 24): C, D – mitotic metaphase; *Opilio canestrinii* (2n = 24): E, F – mitotic metaphase; *Lacinius ephipiatus* (2n = 30): G, H – late pachytene; *Mitopus morio* (2n = 32): I, J – mitotic metaphase; *Rilaena triangularis* (2n = 36): K, L – two sister cells of metaphase II. Arrowheads indicate the position of 18S rDNA clusters (red signals). Bar = 5 μm.

Phalangium and *Rilaena*. Their karyotype diploid numbers range from 2n = 16 to 2n = 36 and consist of mainly bi-armed chromosomes that gradually decrease in length and the number and positions of 18S rDNA differ considerably (Table 1).

The lowest number of chromosomes (2n = 16) occurred in the species *Oligolophus tridens* (C.L. Koch, 1836) (Fig. 1A). In this species, we detected one pair of 18S rDNA clusters at the ends of the seventh pair of chromosomes (Fig. 1B). The karyotypes of *Egaenus convexus* (C.L. Koch, 1835) (Fig. 1C), *Opilio canestrinii* (Thorell, 1876) (Fig. 1E) and *Phalangium opilio* (Linnaeus, 1761) (Fig. S1A) consisted of 2n = 24 with one pair of 18S rDNA clusters in the males of these species. These clusters were close to the centromere of the first (*E. convexus*, Fig. 1D) and third (*P. opilio*, Fig. S1B) pair of chromosomes and at a subterminal position on the first pair of chromosomes (*O. canestrinii*, Fig. 1F). Several 18S rDNA gene clusters occurred in the last three species analysed. Karyotype of *Lacinius epiphiatus* (C.L. Koch, 1835) (2n = 30) included five pairs of these clusters at terminal positions on the eighth, ninth, twelfth, thirteenth and fourteenth pair of chromo-

somes (Fig. 1G–H). In *Mitopus morio* (Fabricius, 1799) (2n = 32) there were seven pairs of clusters of 18S rDNA also always at a terminal position (Fig. 1I–J). Karyotype of the species *Rilaena triangularis* (Herbst, 1799) (2n = 36) included four pairs of 18S rDNA clusters at subterminal or terminal positions (Fig. 1K–L). For the last two mentioned species it was not possible to specify the chromosome pairs bearing 18S rDNA clusters in their karyotype.

Family Sclerosomatidae

We analysed six species belonging to the genus *Leiobunum* and one to the genus *Nelima*. The karyotypes (2n = 20–24) and the number and positions of 18S rDNA differ slightly between genera. In all species biarmed chromosomes predominate and gradually decrease in size (Table 1).

In one male of *L. blackwalli* (Meade, 1861), we identified 2n = 20 (Fig. 2A) same as in the species *L. rotundum* (Latreille, 1798). However, in one male of *L. rotundum* from České Budějovice we identified a variable 2n caused by the presence of 0–3 B chromosomes (Fig. 2C). Three other species, *L. limbatum* L. Koch, 1861 (Fig. 2E), *L. rupestre* (Herbst, 1799) (Fig. 2G) and *L. gracile* Thorell, 1876 (Fig.
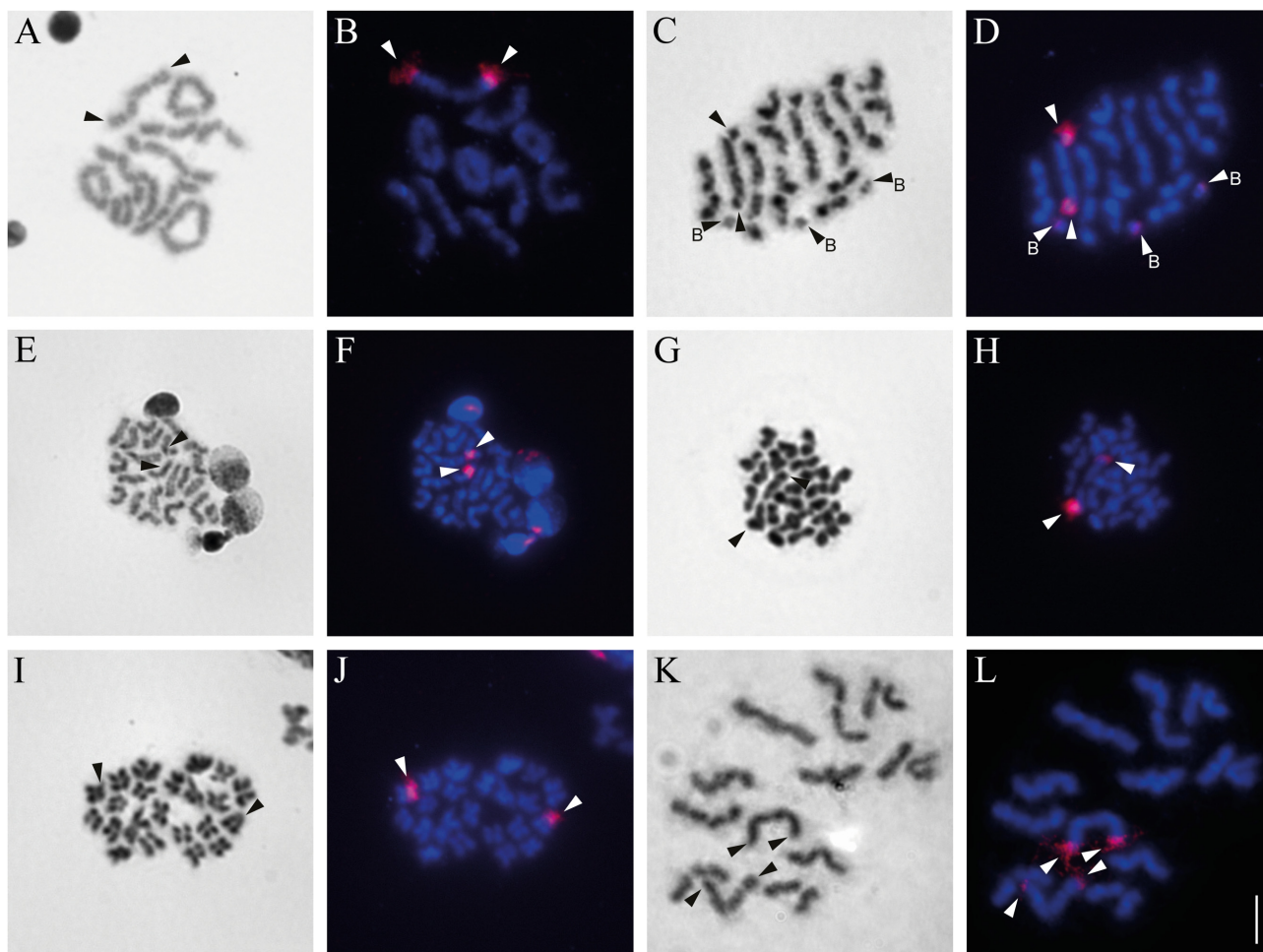
Fig. 2. The chromosomes of the family Sclerosomatidae. The chromosomes are counterstained with Giemsa (A, C, E, G, I, K) and the same nuclei after FISH with DAPI (blue) (B, D, F, H, J, L). *Leiobunum blakwalli* (2n = 20): A, B – diplotene; *Leiobunum rotundum* (2n = 20 + 3B): C, D – late metaphase I; *Leiobunum limbatum* (2n = 22): E, F – mitotic metaphase; *Leiobunum rupestre* (2n = 22): G, H – diakinesis; *Leiobunum gracile* (2n = 22): I, J – two sister cells of metaphase II; *Nelima semproni* (2n = 24): K, L – diplotene. Arrowheads indicate the position of 18S rDNA clusters (red signals) and the position of B chromosomes by arrowheads with "B". Bar = 5 µm.

2I) had 2n = 22. The number and position of 18S rDNA is very consistent in all of the five species of *Leiobunum* analysed. We identified only one pair of 18S rDNA signals always at a terminal position and mostly on one of the first three chromosome pairs, see Table 1 (Fig. 2B, D, F, H, J). Interestingly, we identified an 18S rDNA cluster on all three B chromosomes in the species *Leiobunum rotundum* (Fig. 2D). Compared to that in the species *Nelima semproni* (Szalay, 1951) (2n = 24) (Fig. 2K) two pairs of 18S rDNA clusters were detected by FISH on terminal parts of the third and fifth chromosome pair (Fig. 2L).

DISCUSSION

NORs are one of the most commonly observed markers in animal karyotypes. Previously, this region was identified mainly by silver staining, which can underestimate the number of NORs (Miller et al., 1976). FISH is currently a better and more frequently used tool for mapping the major ribosomal RNA (i.e. 45S: 18S, 5.8S and 28S) gene clusters (e.g. Nguyen et al., 2010; Mattos et al., 2014; Štundlová et al., 2019). Recent reviews of the literature and an online database indicate that, despite the wide range in numbers

of 45S rDNA loci (from one to 54 loci/2C) in animals, about 60% of the karyotypes have only a single 45S locus (Sochorová et al., 2018) and 45S rDNA is most frequently distally located (Sochorová et al., 2018). These characteristics correspond with the predicted ancestral state of 45S rDNA in arachnids in general (Forman et al., 2013) and harvestmen in particular (Svojanovská et al., 2016). Only a few species of harvestmen, from only two suborders, have been analysed, the Cyphophthalmi (Svojanovská et al., 2016; Hiřman et al., 2018) and the Eupnoi (Šťáhlavský et al., 2018). These studies reveal numbers of rDNA clusters that differ from the predicted ancestral state; for example, *Parapurcellia amatola* (Cyphophthalmi) has 10 signals of 18S rDNA (Svojanovská et al., 2016) and *Rhampsinitus leighi* (Pocock, 1903) (Eupnoi) has seven (Šťáhlavský et al., 2018). However, *Miopsalis* sp. (Styllocelidae), which has a basal position in the harvestmen phylogeny, with a diploid number 2n = 30, has one pair of 18S rDNA clusters in a terminal position and no acrocentric pairs of chromosomes in the karyotype (Svojanovská et al., 2016).

Our study has increased the knowledge based on the number and position of rDNA clusters in the Suborder

Eupnoi (Šťáhlavský et al., 2018). We detected one pair of 18S rDNA clusters in most of the species analysed, whilst showing that there is some difference in localisation on chromosome; for example, pericentromeric in *Egaenus convexus*, subterminal in *Opilio canestrinii* and terminal in *Rilaena triangularis* (all family Phalangiidae). Also, we detected 18S rDNA multiplication in three species of the family Phalangiidae: *Lacinius epiphiatus* (2n = 30, five pairs), *Mitopus morio* (2n = 32, seven pairs) and *Rilaena triangularis* (2n = 36, four pairs), and in one species of the family Sclerosomatidae: *Nelima semproni* (2n = 24, two pairs). These results indicate that, despite the recent finding of high variability in 18S rDNA clusters in species from South Africa (Šťáhlavský et al., 2018), 18S rDNA may be conserved in some species of the Suborder Eupnoi. High numbers of 18S rDNA clusters are frequently recorded in specimens inhabiting regions with extreme conditions and may thus represent an adaptation to such conditions, as has previously been postulated for other taxa, such as fish (Symonová et al., 2013; Sember et al., 2015). However, we need more data to confirm this in harvestmen. B chromosomes are already reported in some species of the Suborder Eupnoi (Tsurusaki, 2007; Watanabe et al., 2009), but here, for the first time, 18S rDNA was detected on B chromosomes in harvestmen, specifically in the species *Leiobunum rotundum*. B chromosomes carrying rDNA (active or inactive) have already been detected in vertebrates, such as rats (Stitou et al., 2004) and fish (Baroni et al., 2009; Poletto et al., 2010), and also in invertebrates, such as the grasshopper *Dichroplus pratensis* Bruner, 1900 (Bidau et al., 2004). Further investigation is necessary to obtain a better understanding of the function of rDNA on B chromosomes in harvestmen.

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Supplementary material follows (Table S1, Fig. S1).

Table S1. Sex, location and date collected for the species included in this study.

Species	Locality	Date	GPS	Males
Phalangiidae				
Egaenus convexus (C.L. Koch, 1835)	Havraníky	5. 5. 2014	48.8174N, 15.9824E	1
Lacinius ephippiatus (C.L. Koch, 1835)	Horní Vltavice	17. 7. 2014	48.9778N, 13.8076E	1
	Horní Vltavice	17. 7. 2014	48.9533N, 13.7720E	1
	Zátoň	18. 7. 2014	48.9470N, 13.8269E	1
	Jamné nad Orlicí	22. 7. 2014	50.0534N, 16.6667E	1
Mitopus morio (Fabricius, 1799)	Hřensko	24. 6. 2012	50.8770N, 14.2829E	1
	Orličky	6. 10. 2012	50.0481N, 16.6890E	1
	Nová Pec: Plešné jezero	17. 7. 2014	48.7773N, 13.8679E	1
	Nová Pec: Rakouská louka	17. 7. 2014	48.7708N, 13.8528E	1
	Říčky v Orlických Horách	7. 10. 2012	50.2177N, 16.4633E	1
	Říčky v Orlických Horách	7. 10. 2012	50.2306N, 16.4854E	1
	Trhová Kamenice	7. 8. 2014	49.8113N, 15.8082E	3
Oligolophus tridens (C.L. Koch, 1836)	Orlík	20. 10. 2013	49.5118N, 14.1500E	1
	Přeseka	19. 10. 2013	49.0465N, 14.7420E	2
	Těptín	19. 10. 2013	49.8870N, 14.5627E	2
	Třeboň	19. 10. 2013	48.9855N, 14.8456E	1
Opilio canestrinii (Thorell, 1876)	České Budějovice	14. 10. 2014	48.9638N, 14.4792E	1
	Jílové u Prahy	20. 9. 2014	49.8992N, 14.4901E	1
	Písty	30. 9. 2014	50.1587N, 15.0107E	1
	Praha	21. 10. 2013	50.0704N, 14.4215E	2
	Praha	21. 10. 2013	50.0649N, 14.4300E	1
	Žabonosy	3. 8. 2014	50.0336N, 15.0288E	2
Phalangium opilio (Linnaeus, 1761)	České Budějovice	14. 10. 2014	48.9799N, 14.4640E	1
	Pardubice	30. 9. 2013	50.0418N, 15.7776E	2
	Praha	21. 10. 2013	50.0821N, 14.4701E	1
Rilaena triangularis (Herbst, 1799)	Káraný	6. 5. 2014	50.1802N, 14.7595E	2
	Milovice	27. 5. 2014	48.8476N, 16.6813E	2
	Pavlov	28. 5. 2014	48.8754N, 16.6607E	2
Sclerosomatidae				
Leiobunum blackwalli (Meade, 1861)	Veselí nad Lužnicí	19. 10. 2013	49.1505N, 14.6930E	1
Leiobunum limbatum L. Koch, 1861	Pardubice	30. 9. 2013	50.0418N, 15.7776E	1
	Praha	6. 10. 2012	50.0719N, 14.4233E	1
	Praha	6. 10. 2012	50.0654N, 14.4220E	1
	Říčky v Orlických Horách	7. 10. 2012	50.2177N, 15.4633E	2
Leiobunum rotundum (Latreille, 1798)	České Budějovice	14. 10. 2014	48.9512N, 14.4859E	2
	Praha	21. 10. 2013	50.0704N, 14.4215E	1
	Veselí nad Lužnicí	19. 10. 2013	49.1505N, 14.6930E	1
Leiobunum rupestre (Herbst, 1799)	Říčky v Orlických Horách	6. 10. 2012	50.2177N, 15.4633E	1
Leiobunum gracile Thorell, 1876	Křída	20. 10. 2013	49.3512N, 14.5114E	1
	Říčky v Orlických Horách	6. 10. 2012	50.2177N, 15.4633E	1
Nelima semproni (Szalay, 1951)	Veselí nad Lužnicí	19. 10. 2013	49.1505N, 14.6930E	3
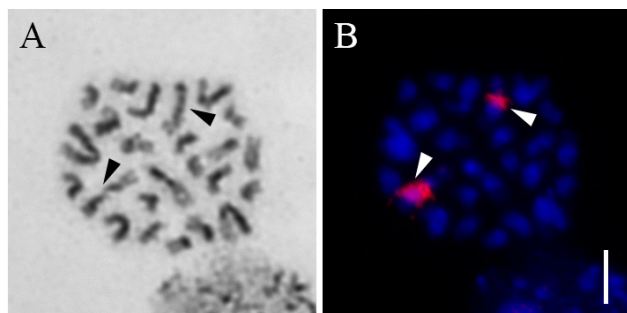
Fig. S1. The chromosomes (mitotic metaphase) of *Phalangium opilio* (2n = 24): A – Giemsa staining; B – the same nuclei after FISH with DAPI (blue). Arrowheads indicate the position of 18S rDNA clusters (red signals). Bar = 5 µm.