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FISH PARASITOLOGY - ORIGINAL PAPER

Chromosomal study of *Khawia abbottinae* (Cestoda: Caryophyllidea): karyotype and localization of telomeric and ribosomal sequences after fluorescence in situ hybridization (FISH)

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Abstract

An original cytogenetic study combining classical karyotype analysis and modern fluorescence in situ hybridization using telomeric $(TTAGGG)_n$ and ribosomal sequences (18S rDNA) was performed in *Khawia abbottinae* (Cestoda, Caryophyllidea), a parasite of Chinese false gudgeon (*Abbottina rivularis*) from China. Analyses based on conventional Giemsa staining, DAPI, YOYO-1 dye, and silver (Ag) staining were also carried out. The karyotype is composed of eight pairs of metacentric and telocentric chromosomes ($2n = 16$, $n=5m + 3t$). Constitutive heterochromatin was mainly positioned at pericentromeric regions, and telomeric sequences $(TTAGGG)_n$ were restricted to the end of all chromosomes. In mitotic preparations stained with Giemsa, both homologues of chromosome pair 4 showed a distinct secondary constriction. FISH with rDNA probe confirmed that this secondary constriction contains a nucleolar organizer region (NOR). The process of spermatocyte meiosis and the dynamics of nucleolus degradation in dividing cell were scrutinized. The present study and its results enhance the limited knowledge on basic karyotype characteristics and 18S rDNA clusters location in caryophyllidean tapeworms.

Keywords Cestoda · Karyotype · FISH · 18S rDNA mapping · Telomeres

Introduction

Tapeworms of the order Caryophyllidea represent a unique group among the "true" cestodes (Eucestoda) in that they are monopleuroid, i.e. their body is monozoic and contains only one set of reproductive organs (Mackiewicz 1994). Recent studies revealed several unusual molecular and genetic phenomena (Kráľová-Hromadová et al. 2012; Brabec et al. 2012; Scholz et al. 2014; Hanzelová et al. 2015; Špakulová et al. 2019), such as the lineage-specific nuclear paralogs of mtDNA, intraindividual divergence of ribosomal internal transcribed spacers 2 (ITS2) along with multiple rDNA loci, cryptic species diversity, phenotypic variability but genetic uniformity, and the triploid character of some species. Knowledge on the chromosome sets of caryophyllidean tapeworms is rather scanty; thus far, 23 tapeworm species have been studied cytogenetically, and the karyotypes complements of only 14 species have been recognized in all details (for a review, see Špakulová et al. 2011; Orosová and Oros, 2012).

The genus *Khawia* Hsü, 1935, the most specious genus of the order Caryophyllidea, contains eight valid species parasitizing cyprinid fishes with worldwide distribution, except for the neotropical region (Scholz et al. 2011; Scholz and Oros 2017). *Khawia abbottinae* Xi, Oros, Wang, Scholz and Xie, 2013 was originally described as a specific parasite of the Chinese false gudgeon, *Abbottina rivularis* (Cyprinidae,

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Gobioninae), from the Yangtze River basin in China (Xi et al. 2013). In total, the authors reported five species of the genus *Khawia* (*K. abbottinae*, *K. japonensis*, *K. rossittensis*, *K. saurogobii* and *K. sinensis*) in China.

Within the family Lytocestidae, chromosomes have been studied only sparsely and the knowledge of karyotype organization has been limited to 6 species of 3 genera to date (for review see Špakulová et al. 2011; Orosová and Oros 2012). The majority of previous studies were based exclusively on a description of the diploid number, with low emphasis on chromosome morphology. For the genus *Khawia*, karyological analysis of 4 species, namely *K. japonensis* (under synonym of *K. iowensis*, Grey 1979), *K. sinensis*, *K saurogobii* and *K. rossittensis* revealed a constant diploid number, $2n = 16$, and similar chromosome morphology (Grey 1979; Petkevičiūtė 1998; Mutafova and Nedeva 1999; Orosová et al. 2010a; Orosová and Oros 2012). Repetitive DNA analysis has shown only a very low variation in the arrangement of heterochromatin along the chromosomes among the studied species. Thus, it is difficult to identify chromosome rearrangements and to study chromosome evolution within this genus based on classical karyotype analysis. Ribosomal DNAs are valuable cytogenetic markers in plant and animal genome research, as they are presented in numerous repeated units, generally organized in clusters, and thus are easily visualized in the chromosomes. Fluorescent in situ hybridization (FISH) has been used for decades for physical mapping of repeated sequences, such as ribosomal DNAs on mitotic and meiotic chromosomes in various organisms (Nguyen et al. 2010; Sheng and Wang 2010; Cabral-de-Mello et al. 2011; Bombarová et al. 2015; Solovyeva et al. 2016; Teixeira et al. 2017; Supiwong et al. 2019). There are rarely any molecular cytogenetic studies that use FISH to map the ribosomal genes of caryophyllidean tapeworms, and such data are available for only four species. In the genus *Khawia*, a single locus bearing a nuclear organizer region (NOR) was detected at the homologues of pair 7 in *K. saurogobii* and of pair 6 in *K. sinensis* (Orosová et al. 2010a; Orosová and Oros 2012). In both species, FISH with the 18S rDNA probe identified major rDNA clusters located in the pericentromeric region on the small metacentric chromosome.

Cytogenetic studies can contribute an array of information independent from morphological and molecular characteristics, which are routinely used for phylogenetic analysis. The differences and similarities may not be apparent at the molecular or morphological level. However, they could be revealed through karyological data (Sessions 1996). The intention of the current work was to extend the limited knowledge of chromosomes in caryophyllidean tapeworms. An in-depth chromosomal analysis (number, size, morphology) in a newly described species of the family Lytocestidae, *K. abbottinae*, was carried out for the first time. Number and location of NORs were analysed through Ag staining and FISH with 18S ribosomal genes. Additionally, FISH with the telomeric probe $(TTAGGG)_n$ was performed to the examined karyotype for the possible presence of interstitial telomeric sequences (ITS). Moreover, the present study also focused on a description of heterochromatin distribution (DAPI-banding) and the occurrence of nucleoli during meiosis by staining with fluorescent dye YOYO-1.

Materials and methods

Specimens

Fifteen alive specimens of *Khawia abbottinae* were obtained from the intestine of the Chinese false gudgeon (*Abbottina rivularis*) from water bodies of the middle and lower reaches of the Yangtze River basin (Yangtze River in Wuhan, Hubei province, and Taihu Lake in Wuxi, Jiangsu province) in April 2011 and April 2018. Tapeworms were dissected from the intestine of freshly killed fish, rinsed several times in saline solution, and processed immediately for chromosome slide preparations, and subsamples of specimens were fixed in 96% ethanol for DNA extraction.

Chromosome preparations

For cytogenetic analysis, whole living tapeworms were incubated in 0.025% colchicine solution (in saline) for 1 h at room temperature (RT). Thereafter, they were placed into a hypotonic solution of 0.75 M KCl for 2 h at RT and torn gently at the testicular body area using the insulin syringes. The torn worms were placed into freshly prepared cold fixative solution (methanol to acetic acid, 3:1) with two changes, 15 min each. The fixed material was stored at − 20 °C.

Spread chromosome preparations were made using the method according to Orosová and Špakulová (2018). Shortly, small pieces containing medullary parenchyma with testes were transferred into a drop of 60% acetic acid on a clean slide and torn into fine pieces with the help of tungsten needles. The slide was then placed on a heating plate at 45 °C, and the drop of cell suspension was slowly drawn along the slide until the liquid evaporated. The preparations were passed through an ethanol series (70, 80, and 100%, 1 min each) and stored at − 20 °C until further use. Before each kind of staining, preparation was removed from the freezer, dehydrated in an ethanol series, and air-dried.

Karyological analysis

The preparations were stained with 5% solution of Giemsa (Merck, NJ, USA) in phosphate buffer (pH 6.8) for 25 min and rinsed with distilled water. Detailed analysis (length, morphology and centromeric index of chromosomes) was

performed in 10 best mitotic spreads (with clearly distinguishable chromosomes) out of 112 evaluated dividing cells from 9 worms. The chromosomes were classified following the nomenclature of Levan et al. (1964).

Silver nitrate ($AgNO_3$) staining

$AgNO_3$ staining was performed according to Howell and Black (1980): six drops of 50% $AgNO_3$ solution and three drops of 2% gelatine in 1% formic acid were mixed on the surface of chromosome slides and coverslip. The slide was then incubated on a heating plate at 45 °C until the purple Giemsa-colour of the chromosome preparation changed to light brown. Slides were rinsed with distilled water and air-dried.

YOYO-1 staining

To visualize the nucleoli, chromosome preparations were stained with the DNA and RNA binding dye YOYO-1, (1,1′-[1,3propanediyl-bis[(dimethylimino)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-quinolinum tetraiodide) (Molecular Probes, Eugene, OR, USA), at a concentration of 4 nmol/L in PBS buffer containing 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Finally, the preparations were mounted in antifade based on DABCO (Sigma Aldrich, St. Louis, MO, USA).

DAPI staining

The chromosome preparation was cleaned in phosphate-buffered saline (1× PBS) containing 1% Triton X-100 for 5 min at RT, stained with 0.5 μg/mL DAPI (4′,6-diamino-2-phenylidole; Sigma-Aldrich) in PBS containing 1% Triton X-100 for 15 min, washed in 1% Kodak-PhotoFlo (Kodak Alaris Inc., Rochester, NY, USA) for 3 min at RT, and then in 1% Kodak-PhotoFlo in miliQ water for 1 min at RT. Finally, the slides were mounted in antifade based on DABCO (Sigma-Aldrich; for details, see Traut et al. 1999).

Preparation of telomeric and 18S rDNA probes

Telomeric probe $(TTAGGG)_n$ was generated by means of non-template PCR as described previously (Sahara et al. 1999) and labelled with Cy3-dUTP using the improved nick translation procedure of Kato et al. (2006) with slight modifications. The modified 20 μL reaction contained 1 μg unlabelled DNA; 50 μM dATP, dCTP, and dGTP; 10 μM dTTP; 20 μM labelled nucleotides; 1× nick translation buffer (50 mM Tris-HCl, pH 7.5; 5 mM MgCl2; 0.005% BSA); 10 mM β-mercaptoethanol; 0.005 U DNase I and 20 U DNA polymerase I (ThermoFisher, Waltham, MA, USA). The reaction time was 1 h at 15 °C.

The unlabeled, about 2100-bp-long 18S rDNA probe was prepared by PCR from the *K. abbottinae* genomic DNA, using two primers, 18S-WormA forward (5′-GCGAATGGCTCATTAAATCAG-3′) and 18S-WormB reverse (5′-CTTGTTACGACTTTTACTTCC-3′). The procedure described by Littlewood and Olson (2001) was used. The genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The probe was labelled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) either by PCR with dNTP mix containing 0.35 mM biotin-16-dUTP or using the improved nick translation procedure (for details, see above). The time of incubation was 50 min at 15 °C in a thermocycler.

FISH with biotin-labelled 18S rDNA probe

For FISH the procedure described by Sahara et al. (1999) with slight modifications was used. Briefly, chromosome preparations were treated with proteinase K (20 mg/mL) in 1 × PBS for 5 min at 37 °C, washed twice in 1 × PBS for 5 min each, and then digested with 100 μg/mL RNase A in 2 × SSC for 1 h at 37 °C and washed twice in 2 × SSC for 5 min each. The slides were incubated in 5 × Denhard's solution for 30 min at 37 °C. After denaturation at 68 °C the chromosome were hybridized with the probe cocktail containing: 10 μl; 50% deionized formamide, 10% dextran sulfate in 2 × SSC, ~ 50 ng of biotinylated 18S rDNA probe and 25 μg of sonicated salmon sperm DNA for one slide (Sigma-Aldrich, St. Louis, MO, USA). The probe was denatured at 90 °C for 5 min. Hybridization at 37 °C for 20 h was followed by stringent washes, which included 50% formamide in 2 × SSC (3 × 5 min, 46 °C) (Fluka, Buchs, Switzerland), 2 × SSC (5 × 2 min, 46 °C), 0.1 × SSC (3 × 5 min, 62 °C), and 4 × SSC containing 0.1% Tween 20 (3 × 3 min, 37 °C). Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA), amplified with biotinylated anti-steptavidin (Vector Labs. Inc., Burlingame, CA, USA), and Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 μg/mL DAPI (4′,6-diamino-2-phenylidole; Sigma-Aldrich) and mounted in DABCO- based antifade (Sigma-Aldrich).

FISH with tyramide signal amplification (TSA-FISH)

The telomeric probe was prepared by nick translation as described above and purified using Sephadex (Illustra Sephadex G-50 fine DNA grade, GE Healthcare, Chicago, IL, USA). For TSA-FISH, we used the procedure described in Zrzavá et al. (2018). In particular, chromosome slides were treated with 10 mM HCl for 10 min at 37 °C and incubated in 1%

hydrogen peroxide for 30 min at RT to quench endogenous peroxidase activity. Then, the preparations were digested with 100 μg/mL RNase A for 1 h at 37 °C and blocked with 5× Denhard's solution for 30 min at 37 °C. Chromosomes were denatured in a probe mix containing 10–30 ng of the labelled telomeric probe, 50% deionized formamide, and 10% dextran sulfate in 2 × SSC for 5 min at 70 °C and hybridized overnight. Hybridization signals were enhanced by antifluorescein-HRP (horseradish peroxidase) conjugate (PerkinElmer, Waltham, MA, USA) diluted 1:1000 and incubated with tyramide solution (TSA Plus Fluorescein system, PerkinElmer) for 5–7 min. The preparations were counterstained and mounted in DABCO-based antifade containing 0.5 μg/mL of DAPI.

Microscopy and image processing

The stained slides were inspected under conventional light and fluorescence microscopes Leica DM 4000 B equipped with colour digital camera DFC 450 C and Olympus B51 equipped with DP70 CCD camera. Black-and-white fluorescent photographs were captured, pseudocoloured (light blue for DAPI, green for YOYO-1 and red for Cy3), and processed with Adobe Photoshop, version 7.0.

Results

Basic karyotype characteristics

Evaluating of 112 dividing cells from nine worms showed exclusively a modal diploid number $2n = 16$ and a fundamental number FN = 28 (Fig. 1). In meiotic pachytene and diplotene/diakinesis nuclei, clumps of eight bivalents ($n = 8$) was commonly observed (Figs. 2c–f and 3d). The *K. abbottinae* karyotype consists of eight chromosome pairs, five metacentric (pair number 1, 2, 3, 4, and 5), and three telocentric (pair number 6, 7, and 8) that are shown in Fig. 1c, d. The karyotype formula can be summarized as $2n = 16$; $n = 5m + 3t$. The chromosomes were comparatively large; in 10 mitotic spreads used for karyometrical analysis, the largest chromosome 1 measured 10.2 μm, being thus markedly longer than the second metacentric pair in all the evaluated chromosome complements (for karyometrical measurements, see Table 1). Gradual length decrease of the remaining seven pairs was observed. The mean total complement length (TLC) was 56.8 μm. At the short arm of both homologues of metacentric chromosome pair 4, a noticeable secondary constriction was observed in Giemsa-stained slides (see the inset in Fig. 1), pointing out the presence of a NOR.

The processes of meiotic division of spermatocytes were documented by Giemsa staining (Fig. 2) and using fluorescent YOYO-1 staining, which also allowed a nucleolus to be highlighted (Fig. 3a–f). One large compact nucleolus was usually observed in interphase nuclei using YOYO-1 staining (Fig. 3a). During the first meiotic prophase, a single nucleolus was regularly detected from early leptotene to pachytene nuclei, where the nucleolus was visible as a lightly stained mass on the lateral site of the bivalent (Figs. 2d and 3b–e). Nucleolar remnants were sometimes observed during early diplotene (Figs. 2e and 5c, d) but not in late diplotene (Figs. 2f and 3f); thus, it disappeared before reaching diakinesis. In diplotene nuclei, the largest bivalent showed four to five chiasmata in the majority of evaluated cells (Figs. 2e and 5c, d), while it usually had two chiasmata in diakinesis (Figs. 2f and 3f).

FISH with telomeric probe and localization of 18S rDNA

FISH with an 18S rDNA probe revealed that the 18S rDNA loci were localized in the pericentromeric region of the short arm of the metacentric chromosome pair 4. Positive signals of ribosomal DNA were visible at the sites of secondary constrictions of mitotically dividing spermatogonia. In pachytene nuclei of spermatocytes, the 18S rDNA-FISH probe hybridized close to the centromere of one NOR-bivalent, as indicated by the association with DAPI highlighted centromeric heterochromatin (Fig. 4d–g). Some homologue chromosomes of meiotic bivalents showed few smaller, inconspicuous, interstitial blocks of heterochromatin. FISH with an 18S rDNA probe, performed after YOYO-1 staining, showed in pachytene spermatocytes a cluster of rDNA genes co-localized with a nucleolus bonded with the NOR bivalent (Fig. 3e).

As a single rDNA cluster was found in the karyotype of *K. abbottinae*, the silver (Ag nitrate) staining was applied in order to reveal the activity of rDNA sites in previous cell division. In principle, this staining indicates proteins associated with active rRNA genes (Howell and Black 1980). In most interphase nuclei, $AgNO_3$ detected the presence of single active nucleolus, and in the majority of meiotic prophase chromosomes, one large compact nucleolus was regularly observed from early leptotene nuclei until early diplotene (Fig. 5). This proved that the rDNA cluster represents a transcriptionally active NOR.

FISH with the sequence $(TTAGGG)_n$ as a DNA probe revealed typical twin fluorescent signals at the ends of all chromosomes of *K. abbottinae* (Fig. 4i, j). Intensive signals were discovered only at the ends of all chromatids in all analysed chromosomes. It is common that the size of the telomeric repeat units in the interstitial region of chromosomes is smaller than their size in the telomeric region. We therefore performed telomeric TSA-FISH on meiotic chromosomes, which could reveal interstitial telomeric sequences (ITS). However, no FISH signals were found in the internal chromosome regions.

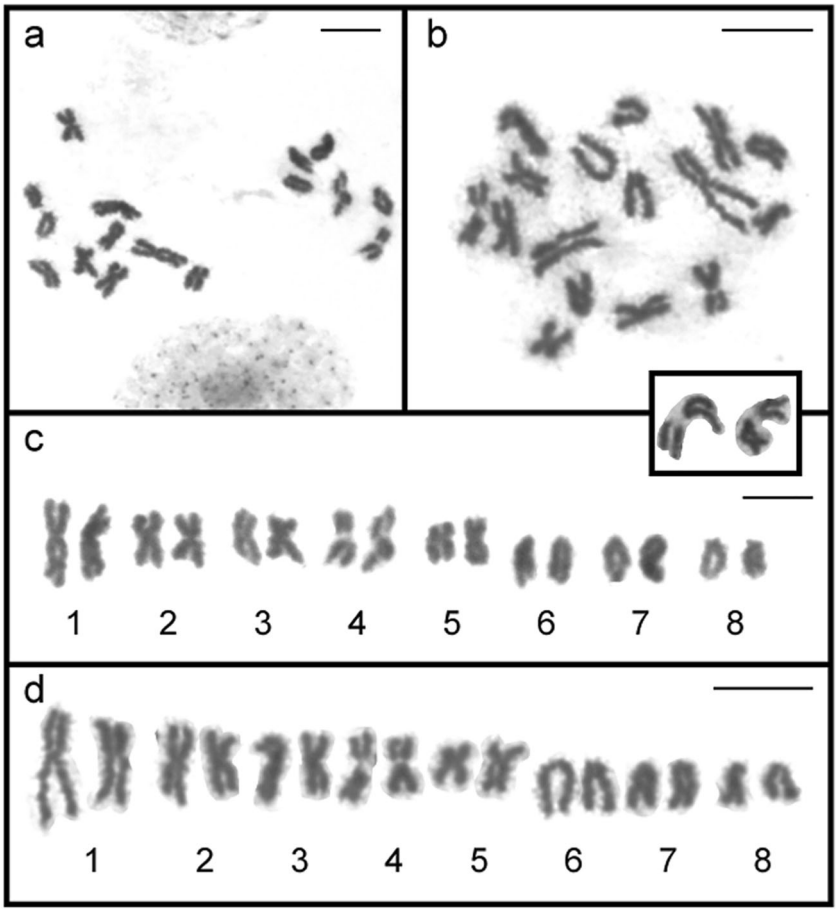

Fig. 1 Giemsa-stained chromosomes of *Khawia abbottinae*. **a**, **b** Mitotic metaphase, $2n = 16$. **c**, **d** Karyotypes derived from mitotic metaphases on **a** and **b**. The inset in the upper right corner shows a detail of chromosomes no. 4 with distinct secondary constriction. Scale bar = 10 μm

Discussion

In this paper, the chromosomes of the tapeworm *K. abbottinae* were described for the first time. Our karyotype analysis revealed that *K. abbottinae* is a diploid species with $2n = 16$ chromosomes, as for the other 4 species of the genus *Khawia* described so far: *K. japonensis* (Grey 1979), *K. rossittensis* (Grey 1979), *K. saurogobii* (Orosová et al. 2010a) and *K. sinensis* (Petkevičiūtė 1998; Mutafova and Nedeva 1999; Orosová and Oros 2012). However, among these species, differences in chromosomal morphology are observed. Karyotypes of *K. saurogobii* and *K. sinensis* consist of three pairs of metacentric and five pairs of telocentric (acrocentric) chromosomes ($n = 3m + 5t$) while that of *K. japonensis* and *K. abbottinae* contain five pairs of metacentric and three pairs of telocentric chromosomes ($n = 5m + 3t$) (Orosová et al. 2010a, this paper). Identical diploid number ($2n = 16$) and only two types of chromosomes shape (metacentric and telocentric) indicate the relatively high similarity of the macro-chromosomal structure in the five species of the genus *Khawia*. Another common feature among all species of *Khawia* spp. is that the first chromosome pair is significantly larger than the rest of the chromosomes and also is always metacentric. Despite obvious karyotype conformity, some interspecific differences in chromosome morphology expressed by the centromere position of individual pairs were observed. As shown in Fig. 6, *K. saurogobii* and *K. sinensis*, both with $n = 3m + 5t$, are evidently dissimilar in the centromere position of the sixth and eighth chromosome pairs. Similarly, *K. japonensis* and *K. abbottinae*, characterized by $n = 5m + 3t$, clearly differ from each other in relation to the centromere position of chromosome pairs 3, 4, 7, and 8. The differences in chromosome morphology without variation on the chromosome number suggest the occurrence of intrachromosomal changes, i.e. pericentric inversions, in the speciation processes of this lineage. Possible causes of the slight differences in the measurements of chromosomes were well discussed in Orosová and Oros (2012).

Comparison of two nuclear ribosomal (ssrDNA and D1-D3 lsrDNA) and two mitochondrial (*nad3* and *cox1*) genes confirmed the paraphyletic status of the genus *Khawia* and suggested that this genus is splitting into three major groups characterized by their fish definitive hosts (Schloz et al. 2011; Xi et al. 2013). The first group conformed that *K. baltica* was transferred to the genus *Caryophyllaeus* (Barčák et al. 2017). Recent phylogenetic analysis supported the close relationship of *K. abbottinae* to the more advanced *K. japonensis*, *K. rossittensis*, and *K. parva* (Xi et al. 2018). The species of

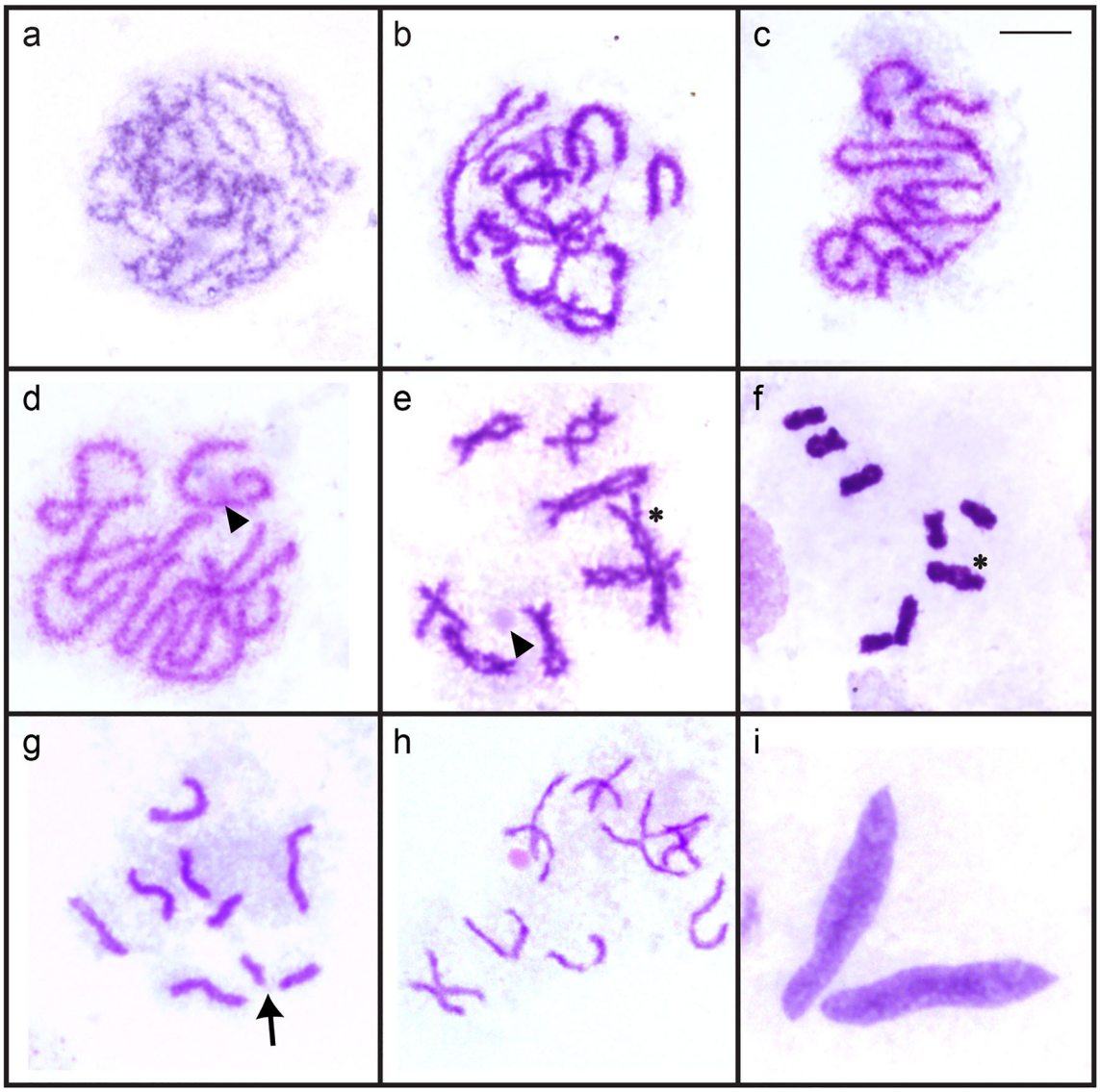

Fig. 2 Meiotic division of spermatocytes of *Khawia abbottinae* stained with Giemsa. **a** Leptotene. **b** Late zygotene. **c**, **d** Pachytene stages with eight bivalents. **e** Early diplotene with five chiasmata in the first bivalent. **f** Diplotene/diakinesis with two chiasmata. **g** First meiotic metaphase. **h** Early anaphase II. **i** Two spermatids. Arrowhead in **d** and arrow in **g** indicate a secondary constriction of the fourth chromosome pair. Asterisks in **e** and **f** indicate the first bivalent. Scale bar = 10 μm

this lineage showed more frequent bi-armed chromosome than mono-armed ones.

The species *K. saurogobii* and *K. sinensis*, which are very similar in terms of molecular features but apparently different morphologically, shared a mutual position in the phylogenetic tree (Scholz et al. 2011; Xi et al. 2018). In both species, the telomeric chromosome structure prevailed over the metacentric one. All the present cytogenetic features, even though they are scarce, show their validity as phylogenetically informative data.

Based on the current phylogenetic tree topology published by Xi et al. (2018, see Fig. 4), three karyotypic clades can be recognized. The first one is formed by *K. sinensis* and *K. saurogobii*, the second one by *K. abbottinae*, *K. japonensis*, and *K. rossittensis*, and the last one by *K. armeniaca*. The congruence of the molecular data to comparative karyological features indicates a high capability of karyotype evolution being reflected in the evolutionary history of the genus and fits into the category of karyotypic orthoselection, according to White (1973). The telocentric character of majority chromosomes of *K. saurogobii* and *K. sinensis* could be regarded as ancestral. In contrast, more numerous bi-armed elements of *K. abbottinae* and *K. japonensis* are supposedly derived. Unfortunately, to date, the karyotype of the member of the last third *Khawia* clade formed by a single *K. armeniaca* species is unknown.

Insufficient karyological data make it difficult to come up with a wide generalization, but we can assume that the

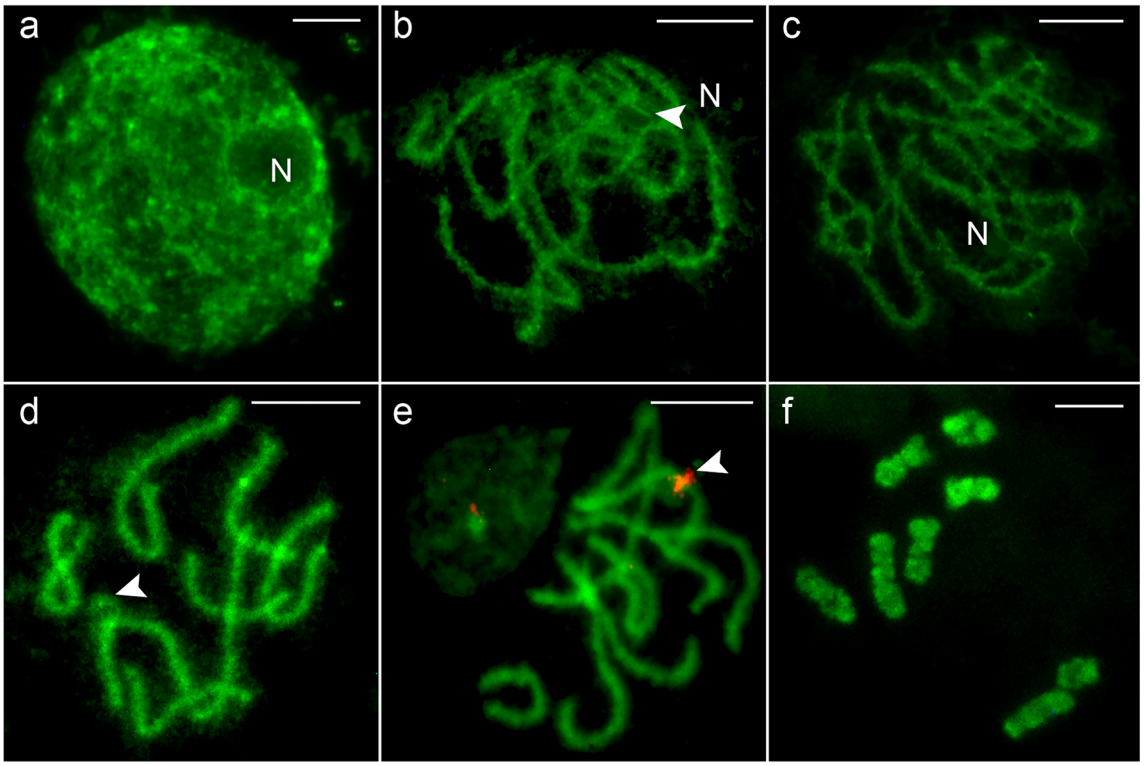

Fig. 3 Visualization of the nucleoli (N) in meiotic cells stained with YOYO-1. **a** Interphase nucleus. **b**, **c** Zygotenes. **d** Pachytene. **e** Pachytene complement (right) showing one YOYO-1-stained nucleolus that co-localize with a cluster of hybridization signal of the 18S rDNA probe and secondary spermatocyte (left). **f** Diplotene/diakinesis stage. Arrowheads indicate the nucleolus. Scale bar = 10 μm

karyotype evolution within the genus *Khawia* is conservative, proceeding without any changes in the chromosome number. In many cases, cestodes are characterized by the conservatism in chromosomes number within the genus (for a review, see Špakulová et al. 2011). The main mechanism that contributed to the cytogenetic divergence of *Khawia* species with $2n = 16$ was apparently pericentric inversions. Pericentric inversions are quite common in the cytogenetic divergence of animal species they have also been reported in many other platyhelminth parasite species (Birstein 1991; Hirai et al. 2000; Petkevičiūtė and Bondarenko 2001; Petkevičiūtė 2003; Bombarová et al. 2007; Stanevičiūtė et al. 2015).

A course of meiotic spermatocyte division coincides well with usual processes known in the majority of other animals (Grelon 2016), and spermatogenesis proceeds as usual, with sperm being formed after two meiotic divisions. Similarly, nucleolus degradation seemed to be the standard and takes place before diakinesis (Pawlovski and Cande 2005). The meiotic bivalent no. 1 displayed a comparatively high number of chiasmata in the diplotene nuclei. Chiasmata are important in gene shuffling between homologous chromosomes (John 1990). The majority of studied cells of *K. abbottinae* showed four to five chiasmata, indicating a higher recombination rate. Chiasma formation has not yet been investigated within

Table 1 Measurements (means ± SD) and classification of chromosomes of *Khawia abbottinae*

Chromosome number	Absolute length (mean ± SD) (μm)	Relative length (mean ± SD) (%)	Centromeric index (mean ± SD)	Classification
1	10.2 ± 1.37	18.03 ± 2.40	45.81 ± 2.76	m
2	8.8 ± 1.07	15.49 ± 1.89	45.33 ± 2.66	m
3	8.0 ± 1.27	14.24 ± 2.06	46.14 ± 1.39	m
4	7.2 ± 1.46	12.73 ± 2.56	40.60 ± 2.60	m
5	6.5 ± 1.46	11.56 ± 2.41	41.55 ± 3.11	m
6	6.2 ± 1.74	11.00 ± 2.84	0	t
7	5.3 ± 1.54	9.23 ± 2.71	0	t
8	4.6 ± 1.37	8.10 ± 2.41	0	t

m metacentric chromosome, *t* telocentric chromosome

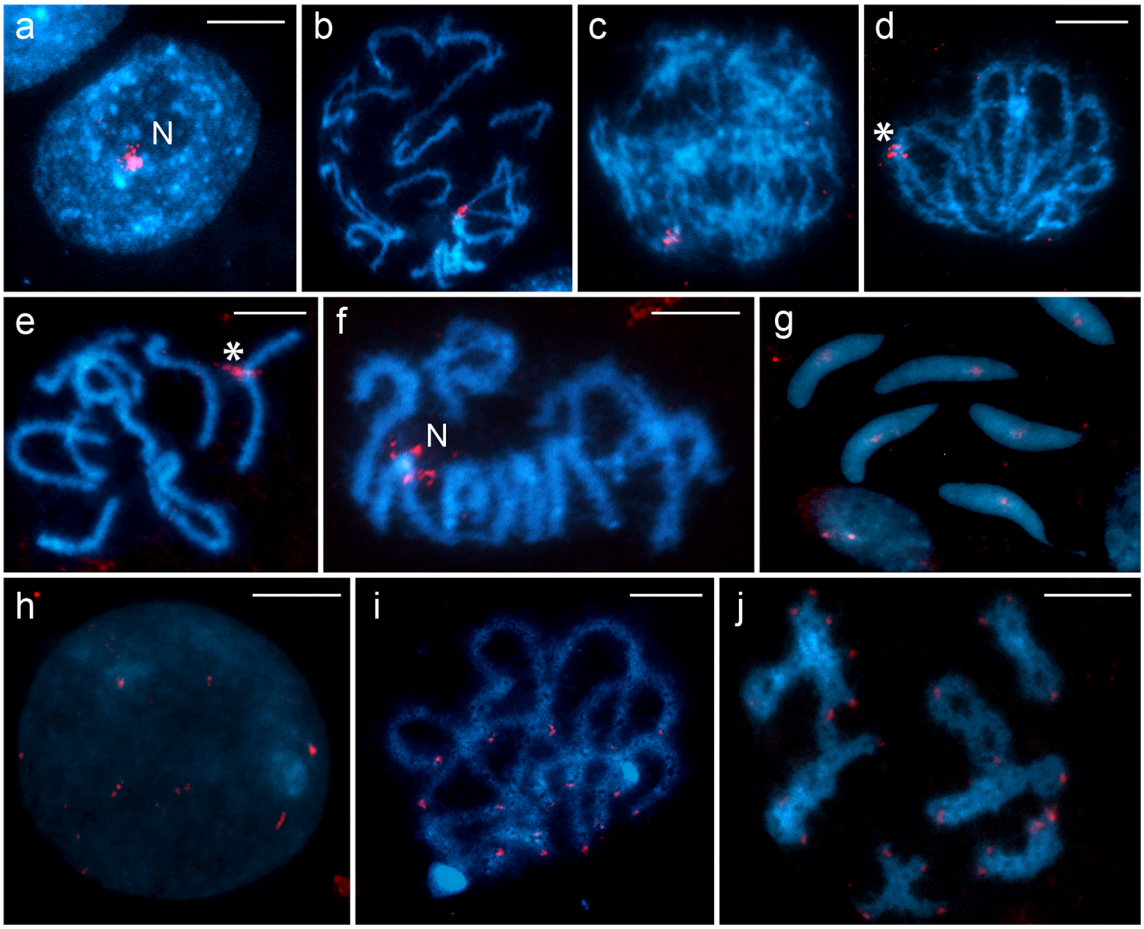

Fig. 4 Chromosomes of *Khawia abbottinae* stained with DAPI (blue) and 18S rDNA (**a**–**g**) and $(TTAGGG)_n$ (**h**–**j**) fluorescence in situ hybridization probes (red). **a** Interphase nucleus with a large nucleolus and one 18S rDNA cluster. **b** Mitotic prometaphase. **c** Zygotene. **d** Pachytene bivalents in a bouquet stage showing a cluster of interstitial rDNA signals associated with DAPI block. **e** Pachytene complement of eight bivalents. Asterisk indicates the fourth bivalent with rDNA cluster. **f** Early diplotene. **g** Spermatids with 18S rDNA signals associated with DAPI-highlighted block. **h** Interphase nucleus with hybridization signals of telomeric probe. **i** Pachytene and **j** diplotene with hybridization signals of telomeric probe at the chromosomal ends of each bivalent. Scale bar = 10 μm

monozoic tapeworms (Caryophyllidea). There are only a few data available; some may be evaluated from previously published images of meiotic division in tapeworms (Orosová et al. 2010b; Bombarová and Špakulová 2015). It is apparent that chiasma distribution within bivalents appears to be determined by morphological features of the chromosomes. The same correlation between the number of chiasmata and chromosome length was reported in the fluke subclass Aspidogastrea (Petkevičiūtė 2001; Bombarová et al. 2015).

All of the *Khawia* species share a common feature, i. e. similar content of heterochromatin distribution, with conspicuous heterochromatin located in the pericentromeric region of almost all chromosomes. A low amount of heterochromatin as well as its presence in the centromeric or pericentromeric regions is a common characteristic in most of Cestoda, Digenea, Aspidogastrea, and Monogenea species (Rausch and Rausch 1990; Mutafova and Gergova 1994; Špakulová and Scholz 1999; Orosová and Oros 2012; Zadesenets et al. 2012; Bombarová et al. 2014; Bombarová and Špakulová 2015; Bombarová et al. 2015). Alternatively, the presence of heterochromatin blocks out of the centromeric region has been reported in the caryophyllidean cestodes *Caryophyllaeides fennica* (Orosová et al. 2010b) and *Atractolytocestus huronensis* (Kráľová-Hromadová et al. 2010; Špakulová et al. 2019). The more or less non-variable pattern of heterochromatin dispersion indicates that the presence of heterochromatin remains consistent over the evolutionary history within cestode lineages.

The telomeric repeat motif $(TTAGGG)_n$ is established as the ancestral sequence for all Metazoa (Meyne et al. 1989; Traut et al. 2007). All the up-to-date studied representatives from the phylum Platyhelminthes have $(TTAGGG)_n$ telomeric repeats, and the same motif was shown to be present in three tapeworm species (Bombarová et al. 2009). In addition to the telomeric pattern at the chromosome ends, ITSs were detected in many species (Schubert 2007), and the existence of such sequences was often related to alteration in chromosome morphology (Meyne et al. 1990). Meiotic pachytene bivalents are

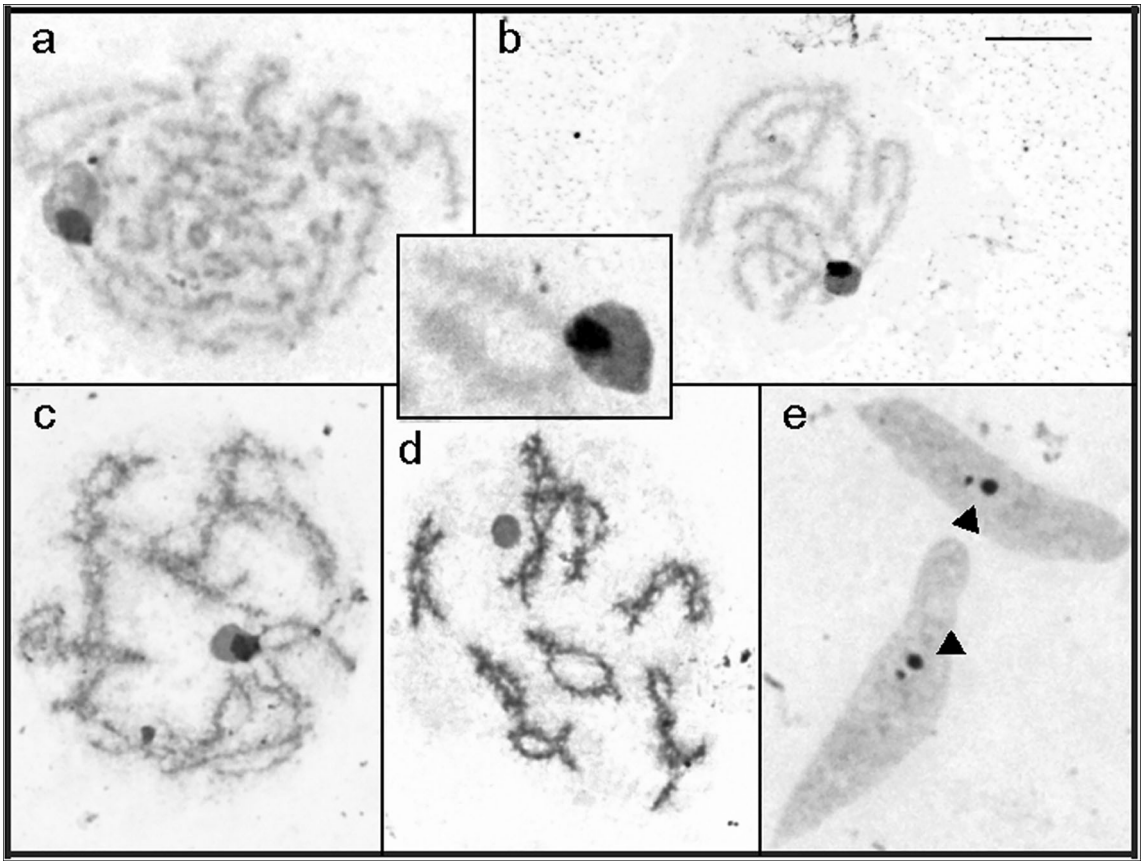

Fig. 5 Silver staining of meiotic cells of *Khawia abbottinae*. **a** Zygotene nuclei with nucleolus on the periphery. **b** Pachytene stage, the inset in the middle shows a detail of chromosome bivalent with one large nucleolus. **c**, **d** Diplotene showing residues of the nucleolus. **e** Spermatids. Arrowheads indicate NOR site. Scale bar = 10 μm

suitable for karyological examination due to their much longer length. This allowed the researcher to "increase the resolution of the analysis along the length of the chromosomes fourfold in comparison with the FISH on normal metaphase chromosomes" (p. 82 Zadesenets et al. 2012). After TSA-FISH, no signals localized in the interstitial position were found in mitotic or meiotic chromosomes of *K. abbottinae*. If the presence of ITS in the species genome shows the large chromosomal rearrangements during karyotype evolution, then we could come to conclusion that the occurrence of such sequences is not very likely in genus *Khawia*, because these tapeworms are characterized by a constant diploid number.

The number and location of NORs vary among related genomes; therefore, comparative evolutionary studies can use the position of 18S rDNA as an appropriate genetic marker, which can be also used for the purpose of genetic identification of related species (Singh et al. 2009; Nguyen et al. 2010; Silva-Neto et al. 2015; Teixeira et al. 2017). The mapping of the number and location of rDNA sites in Cestoda species is until now a scattered task, since only

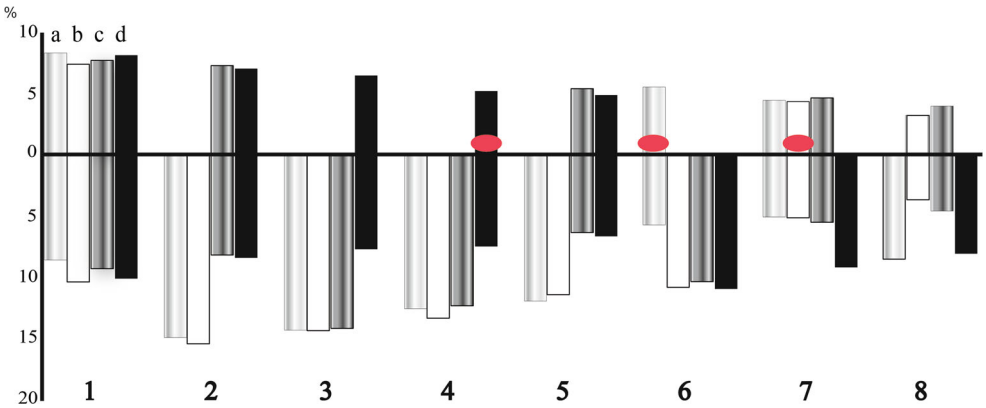

Fig. 6 Comparison of idiograms of chromosomes of **a** *Khawia sinensis* (Orosová and Oros 2012), **b** *K. saurogobii* (Orosová et al. 2010a), **c** *K. japonensis* (Grey 1979), and **d** *K. abbottinae* (present paper), constructed from data on relative length. 1–8, chromosome pair number; red dot, location of 18S rDNA cluster

four lytocestid tapeworm species had been previously analysed. Two loci of rDNA genes per each of three chromosomes of the triplet 2 were revealed in the triploid parthenogenetic species *A. huronensis* (Kráľová-Hromadová et al. 2010; Špakulová et al. 2019). In *C. fennica*, FISH with 18S rDNA probe identified a size variation of the two non-homologous rDNA clusters positioned in the pericentromeric region of two metacentric chromosome pairs 8 and 9 (Orosová et al. 2010b). One cluster of major ribosomal DNA arrays was found in *K. saurogobii* (Orosová et al. 2010a) and *K. sinensis* (Orosová and Oros 2012); however, obvious interspecific differences were observed in rDNA location. In *K. saurogobii*, the 18S rDNA locus was situated on the secondary constriction of the short arm of chromosome 7; in addition, heteromorphism in the size of homologous FISH signals was observed in this species. Moreover, FISH in *K. sinensis* showed NOR in the pericentromeric region on the small metacentric chromosome pair 6. Analogous position of the 18S rDNA genes in the pericentromeric region of the small metacentric chromosome pair 4 in *K. abbottinae* corresponds to that reported in two other *Khawia* species and indicates only a few changes of 18S rDNA loci within this genus. Available data allow us to conclude that there is a certain pattern with the location of NOR near the centromere.

Further, silver staining gives us additional information on the activity of the ribosomal loci. As silver can also bind on chromosomes to parts not corresponding to NORs (Dobigny et al. 2002), NOR activity was confirmed by the presence of a nucleolus connected to the meiotic bivalents at prophase I. In the *K. abbottinae* slides, one active NOR was detected, since only one big nucleolus was regularly observed during meiotic interphase and prophase I divisions of spermatogonia. Generally, one chromosome responsible for nucleolar organization seem to be the common pattern in caryophyllidean species (Orosová et al. 2010a; Orosová et al. 2010b; Orosová and Oros 2012; Bombarová et al. 2015).

Disagreement between morphological and genetic data is the core of the problem of the current taxonomy and systematics of tapeworms (Scholz et al. 2011). In the case where the traditional familial classification is outdated and the morphological and molecular studies are incongruent, cytogenetic data may be helpful as the karyotype is considered to be a part of the phenotype of an organism (Petkevičiūtė et al. 2018). There is a need for additional karyological analyses of further caryophyllidean species. However, phylogenetic value of the karyological analyses would improve if the variation in the chromosomal position of other repeated regions (histones, 5SrDNA, satDNA) is analysed so that they can clarify the chromosomal rearrangements involved in the speciation of this group of parasites.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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