

# Molecular cytogenetic analysis of a triploid population of the human broad tapeworm, *Dibothriocephalus latus* (Diphyllobothriidea)

## Research Article

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### Abstract

The large-sized tapeworm *Dibothriocephalus latus* is known as the broad or fish-borne cestode of mammals that is capable to infect humans and cause diphyllobothriosis. Recently, molecular data on *D. latus* has been accumulating in the literature and a complete genome sequence has been published; however, little is known about the karyotype and chromosome architecture. In this study, an in-depth karyological analysis of 2 *D. latus* specimens was carried out. The plerocercoids originated from a perch caught in subalpine Lake Iseo (Italy) and the tapeworms were reared in hamsters. Both specimens contained cells with a highly variable number of chromosomes ranging from 18 to 27. Nevertheless, the largest portion of mitotic figures (47%) showed a number corresponding to the triploid set,  $3n = 27$ . Accordingly, the karyotype of the analyzed specimens consisted of 9 triplets of metacentric chromosomes. Fluorescence *in situ* hybridization (FISH) with the 18S rDNA probe clearly demonstrated the presence of 3 clusters of hybridization signals on the triplet of chromosome 7, thus confirming the triploid status of the specimens. FISH with a telomeric (TTAGGG)<sub>n</sub> probe confined hybridization signals exclusively to the terminal chromosomal regions, supporting the earlier findings that this repetitive motif is a conserved feature of tapeworm telomeres.

### Introduction

Tapeworms (Cestoda) of the order Diphyllobothriidea are a unique group that occurs in a wide host spectrum, including all groups of tetrapodes (amphibians, reptiles, birds and mammals), with an almost cosmopolitan distribution in fresh and sea water, but also in the terrestrial environment (Kuchta *et al.*, 2008). Systematics and phylogenetic relationships of the Diphyllobothriidea are not definitely resolved, because many taxa have not yet been molecularly analyzed (Waeschenbach *et al.*, 2017). A total of 70 species of 18 genera and 3 families are currently recognized as valid in this order (Kuchta and Scholz, 2017). Diphyllobothriideans also include several important human parasites, such as some species of the genera *Dibothriocephalus*, *Diplogonoporus* and *Spirometra* (Kuchta *et al.*, 2015; Waeschenbach *et al.*, 2017). Human disease caused by *Dibothriocephalus* (formerly *Diphyllobothrium*) tapeworms, commonly referred to as ‘broad tapeworms,’ is the most important fish-borne zoonosis (Scholz *et al.*, 2009), with up to 20 million people infected worldwide (Chai *et al.*, 2005).

*Dibothriocephalus latus* is considered the principal and most frequent human fish-borne cestode in Europe (Scholz and Kuchta, 2016). Recent records and surveys in Europe indicate that this broad tapeworm is now less common in the former historical endemic areas, i.e. Scandinavian countries, but its frequency persists or even has an increasing tendency in the area of Alpine lakes (Switzerland, eastern France and northern Italy) (Kuchta *et al.*, 2015; Gustinelli *et al.*, 2016; Radačovská *et al.*, 2019). Moreover, plerocercoids of *D. latus* have recently been reported from rainbow trout (*Oncorhynchus mykiss*) from Argentina (Kuchta *et al.*, 2019).

The life cycle of *D. latus* is complex and requires 3 hosts to complete, including planktonic crustaceans serving as the first intermediate host. Freshwater fish: mainly perch (*Perca fluviatilis*), pike (*Esox lucius*) and burbot (*Lota lota*) serve as second intermediate hosts, and fish-eating mammals (including humans) represent its definitive host (Kuchta *et al.*, 2015).

Although the zoonotic cestode *D. latus* has been attracting scientists for a long time and biological information, including the complete genome sequence (International Helminth Genomes Consortium, 2019), has accumulated in the literature, the karyotype and chromosome characteristics remain unknown. A few published karyological studies of members of the family Diphyllobothriidae have shown that the haploid (i.e. basal) chromosome number (*n*) is identical in all studied species,  $n = 9$  (Wolcott, 1959; Wikgren and Gustafsson, 1965; Sasada, 1978; Petkevičiūtė, 1992; Petkevičiūtė, 1996; Okino *et al.*, 2017). Most of them have

2 sets of chromosomes with diploid number  $2n = 18$ , but 2 species, *Spirometra erinaceieuropaei* and *S. mansonioides*, have 3 sets of chromosomes and are described as triploids, with  $3n = 27$  (Sasada, 1978; Liu and He, 1988; Okino et al., 2017). Karyotypes of all studied species are highly symmetrical in relation to chromosome morphology, consisting only of bi-armed small- to medium-sized chromosomes (Špakulová et al., 2011). An exception to this rule is *Schistocephalus solidus* that has nearly half of the chromosome pairs mono-armed (Petkevičiūtė, 1996). All published karyological analyses were restricted to classical staining techniques without the use of more advanced cytogenetic techniques, such as fluorescence *in situ* hybridization (FISH). In addition, no information is yet available on spermatogenesis in these fish-borne zoonotic cestode species.

To fill this gap in cytogenetic knowledge, we present a detailed analysis of the *D. latus* karyotype. A particular interest was paid to variable chromosome numbers observed in our preliminary research, suggesting possible polyploidization of the karyotype. First, the numerical variation was critically reexamined in the chromosome set of *D. latus*, including a detailed study of mitotic chromosome morphology. Then FISH was applied with the 18S rDNA probe to determine the number and location of major ribosomal DNA clusters, which are suitable and readily available molecular markers of mitotic as well as meiotic chromosome sets. FISH was also performed with a  $(TTAGGG)_n$  probe to find out if this ancestral telomeric motif of Metazoa is conserved at the end of *D. latus* chromosomes. The eventual presence of interstitial telomeric sequences (ITSs) was examined by FISH with tyramide signal amplification (TSA-FISH) with high detection sensitivity (Carabajal Paladino et al., 2014).

## Material and methods

### Origin of plerocercoid larvae

Plerocercoids of *D. latus* were collected from 132 European perch (*P. fluviatilis*) originating from Lake Iseo (Italy) in May 2018. Lake Iseo is the fourth largest of the glacial lakes in the subalpine region in the north of the country (45°43'N, 10°05'E). The fish were collected by professional fishermen using a gill net. The perch were immediately placed on ice and transported to the laboratory within 16 h, where their musculature was examined for parasites by parasitological necropsy (detailed filleting of the whole musculature). All obtained plerocercoids of *D. latus* were examined under a stereomicroscope for morphological characteristics and placed in 0.9% NaCl solution before hamster infection.

According to their morphology, all plerocercoids were identified as *D. latus* larvae. Ten plerocercoids were fixed in 96% ethanol for subsequent molecular genotyping. Genomic DNA was extracted using the QIAamp® DNA mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction and then used as a template for polymerase chain reaction (PCR). PCR amplification of the *D. latus* cytochrome c oxidase subunit 1 (*cox1*, 437 bp) was performed according to Wicht et al. (2010). Multiplex PCR of the *COI* gene confirmed that the plerocercoid larvae belong to *D. latus*.

### Infection of experimental animals

The hamster, *Mesocricetus auratus*, which was used as an experimental host in this study, has not been confirmed to serve as the definitive host for *D. latus* under natural conditions; however, the animals were successfully infected in several previous studies (Andersen, 1972, 1978; Andersen and Halvorsen, 1978; Dick and Poole, 1985). All 24 experimental hamsters were maintained in the animal holding facilities of the Faculty of Agrobiography,

Food and Natural Resources, Czech University of Life Science Prague (CZU), and kept under the following standard conditions: humidity of  $55 \pm 60\%$ , temperature of  $22 \pm 2^\circ\text{C}$ , photoperiod of 12 h light : 12 h dark. The hamsters were given standard rodent food and water *ad libitum*. Infection was performed in full consciousness of the animals by a single oral administration of 2 plerocercoids (per host); care was taken to ensure that each hamster swallowed both plerocercoids.

### *Dibothriocephalus latus* specimens

Host animals were anaesthetized with isoflurane (i.e. halogenated ether) and sacrificed by injecting the commercial preparation T-61 at designated time (20–22 days postinfection). The small intestine was removed from freshly killed hosts. Only 2 out of 24 infected hamsters were positive. Two alive tapeworms of *D. latus* (430- and 452-mm long) were isolated, rinsed several times in saline solution, and processed immediately for cytogenetic analysis.

### Chromosome preparations

For cytogenetic analysis, whole live specimens were treated with 0.025% colchicine solution in saline for 1 h to arrest mitosis in dividing cells at the metaphase stage, and placed in hypotonic solution (0.075 M KCl) for 4 h for swelling at room temperature. During this treatment, the worms were torn along their entire length (each proglottid) using an insulin syringe. The torn worms were placed in freshly prepared cold fixative solution (methanol: acetic acid = 3:1) for 30 min (with 2 replacements of the fixative) and then stored at  $-20^\circ\text{C}$  until further use. Chromosome preparations were obtained by the spreading technique (Orosová and Špakulová, 2018). Briefly, glass slides were incubated in acid ethanol for 1 h. A small piece of fixed parenchyma with testes was placed on a dry-cleaned slide in a drop of 60% acetic acid and macerated using tungsten needles, or alternatively, a larger piece (1 proglottid) was transferred to an Eppendorf tube with 60% acetic acid and homogenized. Then, a few drops of macerated or homogenized suspension were applied to the slide and spread using a heating plate at  $45^\circ\text{C}$ . Finally, the preparations were passed through an ethanol series (70, 80, and 100%, 1 min each) and stored at  $-20^\circ\text{C}$  until further use. Before each staining, slides were removed from the freezer, passed through an ethanol series, and air-dried.

In addition, 57 *D. latus* plerocercoids obtained from 109 European perch were also cytogenetically processed. A total of 92 chromosome slides were prepared and screened. However, all these preparations showed no dividing cells.

### Karyological analysis

Slides were stained with 5% Giemsa (Merck, New Jersey, USA) solution in phosphate buffer (pH 6.8) for 25 min and rinsed with distilled water. Morphometric measurements of metaphase chromosomes were carried out on captured images. The chromosomes were arranged according to the centromere position and a karyotype was assembled. Chromosome numbers were counted in 272 mitotic cells from 2 individuals. Absolute length (AL, chromosome long  $q$  ( $L$ ) + short  $p$  ( $S$ ) arm length in  $\mu\text{m}$ ), relative length ( $=\text{AL} \times 100\% / \text{half-length of all chromosomes in metaphase spread}$ ) and the centromeric index [ $=S/(L + S)$ ] were measured and calculated in 15 best mitotic spreads with clearly distinguishable chromosomes. The mean and standard deviation were calculated using a Microsoft Excel spreadsheet. Chromosomes were classified according to Levan's nomenclature method (Levan et al., 1964).

### Silver nitrate staining

Silver nitrate ( $\text{AgNO}_3$ ) staining was performed following the standard protocol commonly used for cestodes (Ráb and Roth, 1988). Briefly, 6 drops of 50%  $\text{AgNO}_3$  solution and 3 drops of 2% gelatine in 1% formic acid were mixed on the surface of the chromosome spread preparation and covered with a coverslip. Then the slide was incubated on a heating plate at 45°C until the preparation turned light brown. Slides were rinsed with flowing distilled water and air-dried.

### DAPI staining

Chromosome preparations were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) as described in Traut *et al.* (1999). Briefly, slides were rinsed in phosphate-buffered saline (1×PBS) containing 1% Triton X-100 for 5 min and stained with DAPI ( $0.5 \mu\text{g mL}^{-1}$  in PBS containing 1% Triton X-100) for 15 min in the dark, washed in 1% Kodak-PhotoFlo in 1×PBS for 3 min, and then briefly washed in 1% Kodak-PhotoFlo in milliQ water, all at room temperature. Finally, the slides were mounted in an antifade based on 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich).

### Probe preparation

The 18S rDNA probe, about 1500 bp long, was generated by PCR from *D. latus* genomic DNA extracted using the QIAamp® DNA mini Kit. PCR was performed using 2 primers, 18S-WormA forward (5'- GCGAATGGCTCATTAATCAG-3') and 18S-WormB reverse (5'- CTTGTTACGACTTTTACTTCC-3'), as described by Littlewood and Olson (2001). Telomeric probe ( $(\text{T}^n\text{TAGGG})_n$ ) was generated by non-template PCR as described previously by Sahara *et al.* (1999). Probes were labeled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) using the improved nick translation procedure described by Kato *et al.* (2006) with some modifications. The 20  $\mu\text{L}$  reaction contained 1  $\mu\text{g}$  of unlabeled 18S rDNA probe, 50  $\mu\text{M}$  of dATP, dCTP and dGTP, 10  $\mu\text{M}$  of dTTP, 20  $\mu\text{M}$  of labelled nucleotides, 1× nick translation buffer (50 mM of Tris-HCl, pH 7.5; 5 mM of  $\text{MgCl}_2$ ; 0.005% of bovine serum albumin, BSA), 10 mM of  $\beta$ -mercaptoethanol, 0.005 of U DNase I and 20 U of DNA polymerase I (both ThermoFisher, Waltham, MA, USA). The reaction time was carried out for 50 min at 15°C for 18S rDNA probe and 1 h at 15°C for telomeric probe.

### FISH with biotin-labeled probes

Fluorescence *in situ* hybridization was performed according to the standard protocol used for insects (Sahara *et al.*, 1999) with slight modifications for cestodes (Orosová and Špakulová, 2018). Preparations were treated with proteinase K (20  $\text{mg mL}^{-1}$ ) in 1×PBS for 5 min at 37°C, washed twice in 1×PBS for 5 min each, and then digested by 100  $\mu\text{g mL}^{-1}$  RNase A in saline sodium citrate (2×SSC) for 1 h at 37°C and washed twice in 2×SSC for 5 min each. Then the slides were incubated in 5×Denhardt's solution for 30 min at 37°C. Chromosomal DNA was denatured in 70% formamide in 2×SSC for 3 min and 30 s at 68°C. The probe cocktail for each slide (10  $\mu\text{L}$ ; 50% deionized formamide, 10% dextran sulfate, 2×SSC) contained ~50 ng of biotinylated 18S rDNA probe and 25  $\mu\text{g}$  of sonicated salmon sperm DNA (Sigma-Aldrich). 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

**Table 1.** Chromosome numbers in mitotic cells of *Dibothriocephalus latus*

Chromosome number	Analyzed mitotic metaphases	
	No.	%
18	16	5.9
20	10	3.7
22	19	7.0
24	35	12.9
26	65	23.9
27	127	46.7
Total metaphases	272	

min, 37°C). Hybridization signals were amplified and visualized by 3-step detection, Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA), biotinylated anti-streptavidin (Vector Labs. Inc., Burlingame, CA, USA) and Cy3-conjugated streptavidin. The preparations were counterstained with  $0.5 \mu\text{g mL}^{-1}$  DAPI in a DABCO-based antifade.

### FISH with tyramide signal amplification

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  $\mu\text{g mL}^{-1}$  RNase A for 1 h at 37°C and blocked with 5×Denhardt's solution for 30 min at 37°C. Chromosomes were denatured in a probe mix containing 10–30 ng of the labeled 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-horseradish peroxidase conjugate (PerkinElmer, Waltham, MA, USA) diluted by 1:1000 concentration 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  $0.5 \mu\text{g mL}^{-1}$  of DAPI.

### Microscopy and image processing

The stained preparations were observed using 2 fluorescence microscopes, Leica DM 4000 B equipped with a digital camera DFC 450 C and Olympus B51 equipped with a DP70 CCD camera, and photographed separately for each fluorescent dye. Black-and-white fluorescent images were pseudocolored (light blue for DAPI, red for Cy3) and merged using Adobe Photoshop, version 7.0.

## Results

### Basic mitotic karyotype

Analysis of dividing mitotic cells from 2 adult worms showed high variation in the number of chromosomes. Table 1 shows the range of chromosome counts of the 272 mitotic metaphases evaluated (135 from the first and 137 from the second tapeworm specimen) with clearly distinguishable chromosomes. Both specimens showed 6 cell types that differed in chromosome number, ranging from 18 to 27 chromosomes. The most frequently

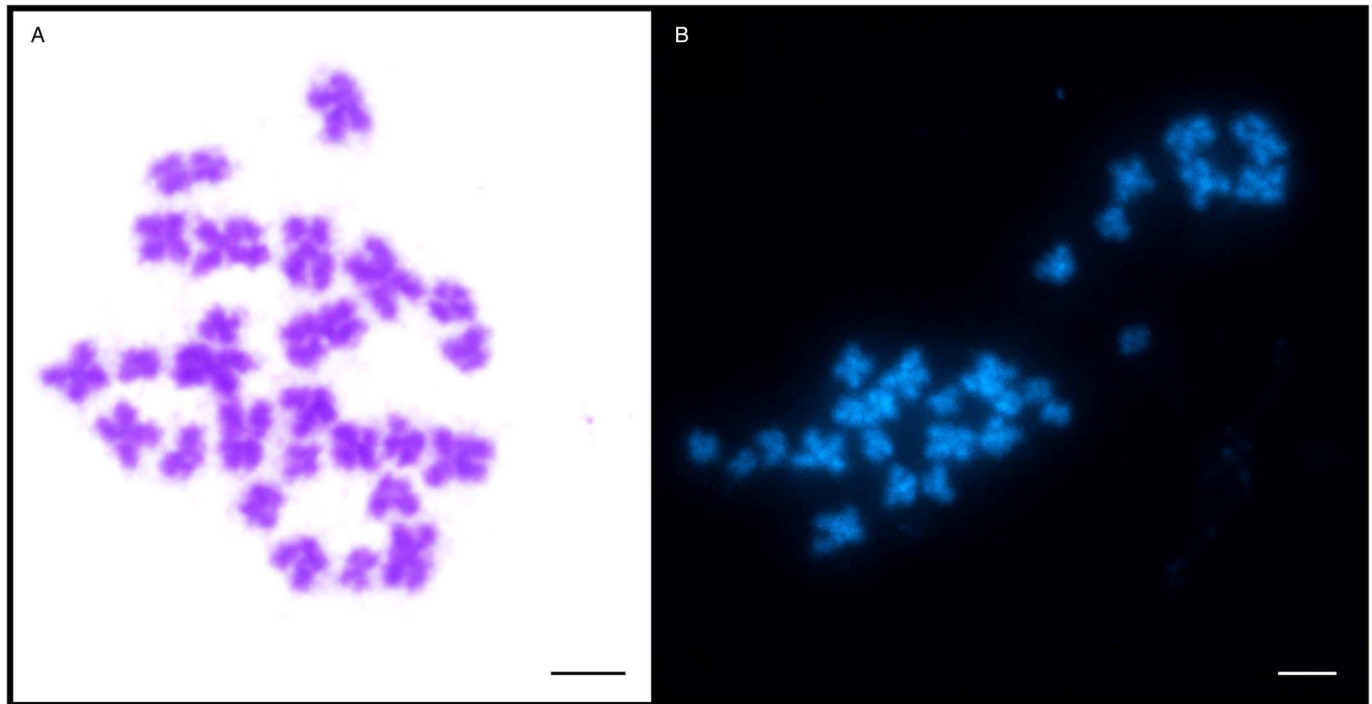


Fig. 1. Metaphase chromosomes of *Dibothriocephalus latus*. (A) Giemsa and (B) DAPI staining ( $3n = 27$ ; scale bar =  $10\ \mu\text{m}$ ).

detected chromosome count in mitotic cells from testes, ovary and vitellaria of adult worms was 27 (46.7%, Fig. 1), which is the number corresponding to the triploid set,  $3n = 27$ . The remaining mitotic metaphases evaluated were therefore aneuploid without 1–9 chromosomes (Table 1). No significant differences in the length of the 3 chromosomes of each triplet were determined by *post hoc* test. Similarly, no significant differences in the karyotype structure were found between the 2 evaluated adult worms. The evident predominance of the  $3n = 27$  cytotype in both *D. latus* specimens clearly indicated the triploid nature of this Alpine lake population (Fig. 2).

Twenty mitotic metaphases from 2 individuals were karyotyped and the data obtained are summarized in Table 2. The chromosomes were relatively small; the largest and the smallest chromosomes measured  $5.63$  and  $2.82\ \mu\text{m}$ , respectively. The standard karyotype is symmetric; it is composed of 27 metacentric chromosomes divided into 9 triplets of homologous chromosomes of gradually decreasing size (Fig. 2A). Triplets nos. 1–4 were quite easily distinguishable by length, while triplets 5–9 were more difficult to distinguish due to their high similarity in length. No secondary constriction was seen on the Giemsa-stained chromosomes. The karyotype formula can be summarized as  $3n = 27$ ;  $n = 9\ \text{m}$  and the fundamental number of arms is  $\text{FN} = 54$ . The total length of the haploid complement reached  $36.53\ \mu\text{m}$  on average.

#### Number, activity and chromosomal location of rDNA clusters and telomeric FISH

FISH with 18S rDNA probe showed clear hybridization signals on both chromatids of 3 homologous chromosomes in mitotically dividing cells in all chromosome number variants (all cytotypes) (Fig. 2B, Fig. 3) and also in interphase nuclei (Fig. 3A). These signals were approximately having the same intensity in all 3 chromosomes. Based on the results, we concluded that *D. latus* has a single locus for rRNA genes (i.e. the nucleolar organizer region, NOR) per haploid genome, which is located interstitially,

postcentromeric on the short arms of the small metacentric chromosome No. 7 (7p cen). In DAPI-counterstained FISH preparations, the constitutive heterochromatin pattern showed only weak markings in the centromeric regions of all metaphase chromosomes (Fig. 2B).

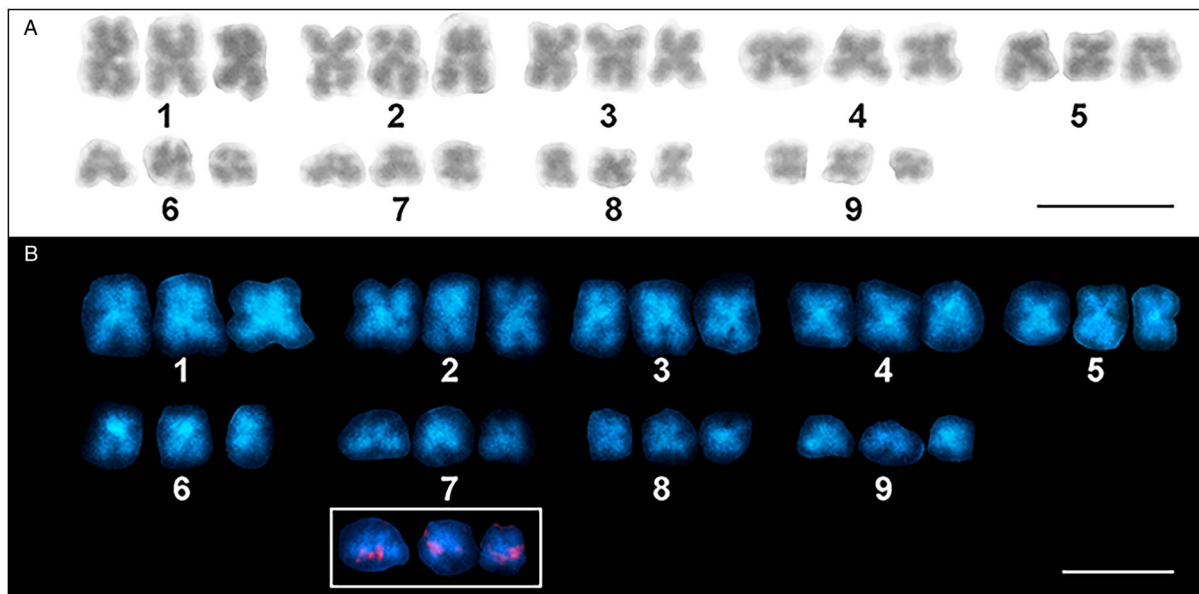
FISH with telomeric (TTAGGG)<sub>n</sub> probe showed typical twin hybridization signals at the ends of both chromatids in all chromosomes (Fig. 4). Interstitial clusters of telomeric repeats (ITS) were not detected in *D. latus* chromosomes, although we used a more sensitive TSA-FISH method with this probe on long pachytene chromosomes (Fig. 4B and D).

AgNO<sub>3</sub> staining (Ag-banding) of NOR-associated protein was used to demonstrate the activity of the rRNA genes. A variable number of 1–3 Ag-positive NORs were observed in pachytene nuclei of *D. latus* (Fig. 5) with 3 active nucleoli as the most common variant. A maximum of 2 nucleoli were observed in the interphase nuclei (Fig. 5C).

#### The course of meiosis

During the slide preparation from the fixed material, we processed the whole worm, piece by piece from scolex to the last proglottid. However, we did not record all stages of meiotic division. When the worm was divided into 3 parts, numerous mitotic metaphases were observed in the first proximal third of the strobila, while few mitotic metaphases and selected stages of spermatocyte division (early and late pachytene nuclei) were found in the middle part of the body. In the last third of the strobila, mitotic chromosomes were not observed and meiotic figures (pachytene and late metaphase II nuclei) occurred only very sporadically; this part of strobila often contained proglottids full of eggs.

As shown by 18S rDNA FISH (Fig. 6), both univalent and bivalent of the triplet No. 7 might be present during meiotic prophase. Three homologues of triplet No.7, carrying ribosomal genes, did not pair and remained as 3 univalents in most spermatocyte prophase (Fig. 6E–G) or formed 1 bivalent plus 1 univalent (Fig. 6C and D). The subsequent stages of meiosis I

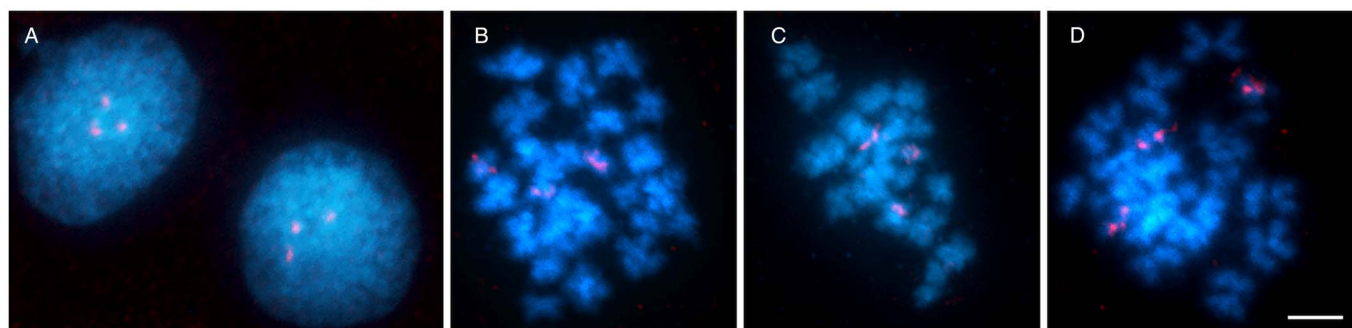


**Fig. 2.** Karyotype derived from mitotic metaphase cells of *Dibothriocephalus latus* ( $3n=27m$ ). (A) Giemsa staining. (B) DAPI staining showing AT-rich bands. The small inset shows a detail of chromosomes of the triplet No. 7 with distinct 18S rDNA [nucleolar organizer region (NOR)] loci located on each individual chromosome (scale bar =  $10\mu\text{m}$ ).

**Table 2.** Measurement (mean  $\pm$  s.d.) and classification of chromosomes of *Dibothriocephalus latus*

Chromosome number	Absolute length <sup>a</sup> (mean $\pm$ s.d.) ( $\mu\text{m}$ )	Relative length (mean $\pm$ s.d.) (%)	Centromeric index (mean $\pm$ s.d.)	Classification
1	$5.63 \pm 0.70$	$15.41 \pm 1.91$	$47.60 \pm 1.25$	M
2	$5.13 \pm 0.63$	$14.04 \pm 1.73$	$46.71 \pm 1.40$	M
3	$4.84 \pm 0.60$	$13.25 \pm 1.63$	$47.45 \pm 0.93$	M
4	$4.29 \pm 0.49$	$11.73 \pm 1.34$	$47.60 \pm 1.27$	M
5	$3.88 \pm 0.44$	$10.63 \pm 1.20$	$45.97 \pm 2.28$	M
6	$3.48 \pm 0.41$	$9.54 \pm 1.13$	$46.47 \pm 1.50$	M
7	$3.32 \pm 0.37$	$9.09 \pm 1.02$	$46.26 \pm 2.16$	M
8	$3.14 \pm 0.39$	$8.59 \pm 1.08$	$47.33 \pm 1.61$	M
9	$2.82 \pm 0.28$	$7.73 \pm 0.76$	$47.61 \pm 1.64$	M

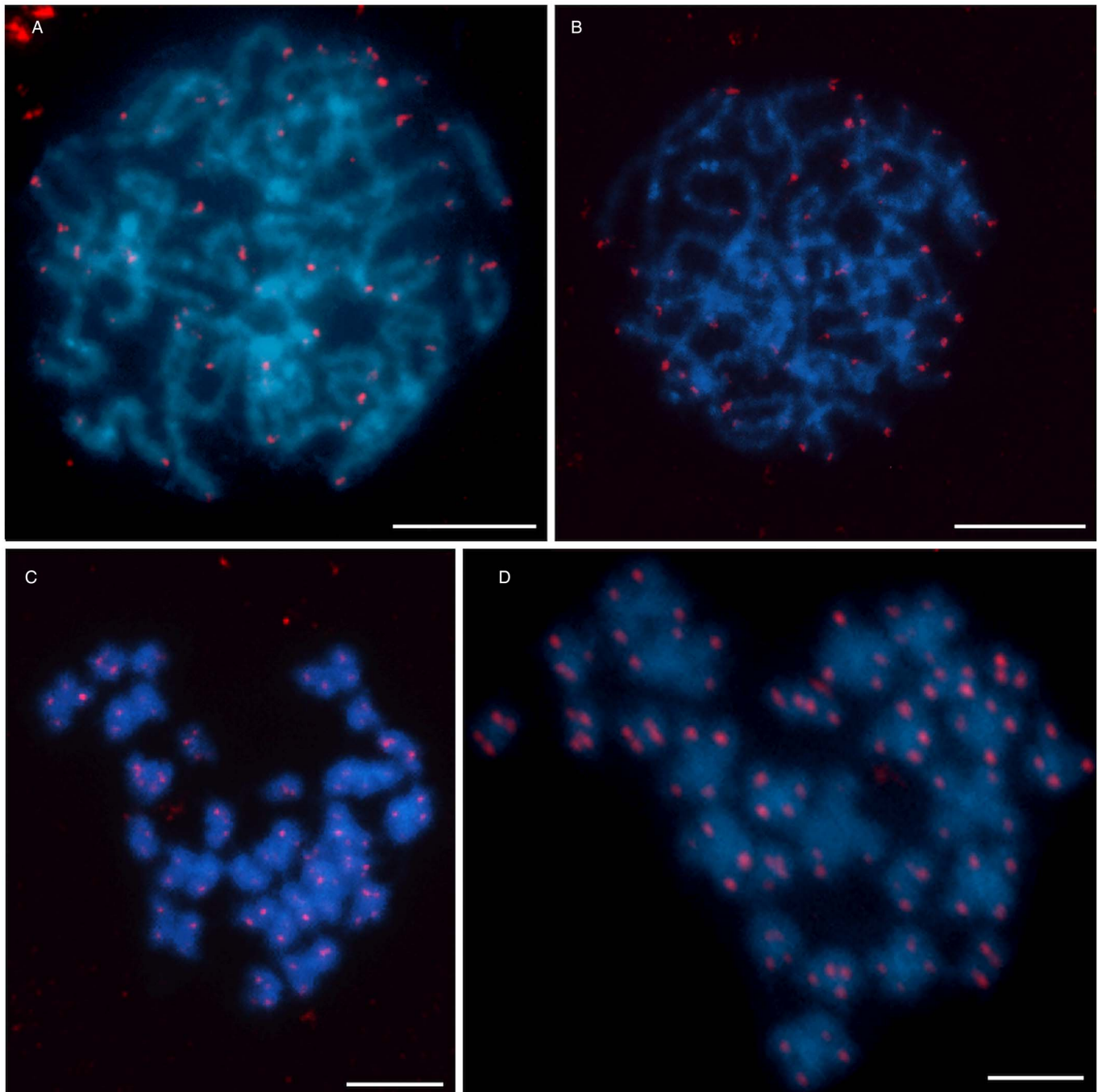
<sup>a</sup>Mean absolute lengths were calculated from 20 mitotic metaphases. Abbreviations: s.d., standard deviation; M, metacentric chromosome.



**Fig. 3.** Chromosomes of *Dibothriocephalus latus* stained with DAPI (blue) and 18S rDNA fluorescence *in situ* hybridization probe (red). (A) An interphase nucleus with 3 18S rDNA clusters. (B, C) Mitotic metaphases showing 3 chromosomes with hybridization signals. (D) Early mitotic anaphase showing early segregation of chromatids to opposite poles (scale bar =  $10\mu\text{m}$ ).

(metaphase I and anaphase I) were not found on the preparations. However, there are 2 lines of evidence that meiosis II occurred, though rarely. First, we observed metaphase II nuclei with unbalanced chromosome numbers (Fig. 6H) due to failure of

chromosome segregation during meiosis I. Second, we found early stages of spermiogenesis (developing spermatozoa) on slides from both specimens examined (Fig. 6I). However, fully developed sperm were missing.



**Fig. 4.** Distribution of telomeric sequence (TTAGGG)<sub>n</sub> after FISH in *Dibothriocephalus latus* showing an exclusively telomeric pattern. (A) Pachytene nucleus and (C) mitotic metaphase after FISH with biotin-labeled telomeric probe. (B) Pachytene nucleus and (D) mitotic metaphase after TSA-FISH (scale bar = 10 μm). Abbreviations: FISH, fluorescence *in situ* hybridization; TSA-FISH, FISH with tyramide signal amplification.

## Discussion

### *Chromosome numbers, karyotype structure and interspecific chromosome variations*

Currently, there is very limited knowledge about the karyotypes of diphylobothriidean species. Karyological data have so far been obtained for only 7 species out of 58 valid taxa of the family Diphylobothriidae (Špakulová *et al.*, 2011; Scholz *et al.*, 2019). These data showed that the basic chromosome number is  $x = 9$  with a predominance of diploid species ( $2n = 2x = 18$ ), except for 2 triploids with  $3n = 3x = 27$  (Sasada, 1978; Liu and He, 1988; Okino *et al.*, 2017). Published karyotypes are composed exclusively of metacentric and submetacentric chromosomes; the obvious exception is *S. solidus*, which is diploid and has 5

metacentric and 4 acrocentric chromosome pairs (Petkevičiūtė, 1996).

As found in this study, *D. latus* from the isolated Alpine Lake Iseo shares the basic set of chromosomes ( $x = 9$ ) with other diphylobothriidean species, as well as the overall gross morphology of chromosomes (i.e. all metacentric). Based on our detailed analysis of karyotype architecture and morphometric data, we can conclude that *D. latus* tapeworms from the isolated Alpine Lake Iseo population are triploids ( $3n = 3x = 27$  m). In addition to the triploid number of 27, our study revealed a surprisingly high level of aneuploidy with 5 different chromosome numbers, namely 18, 20, 22, 24 and 26.

A former cytogenetic study on 3 congeneric diphylobothriidean species from Finland, including our model species *D. latus* (syn.

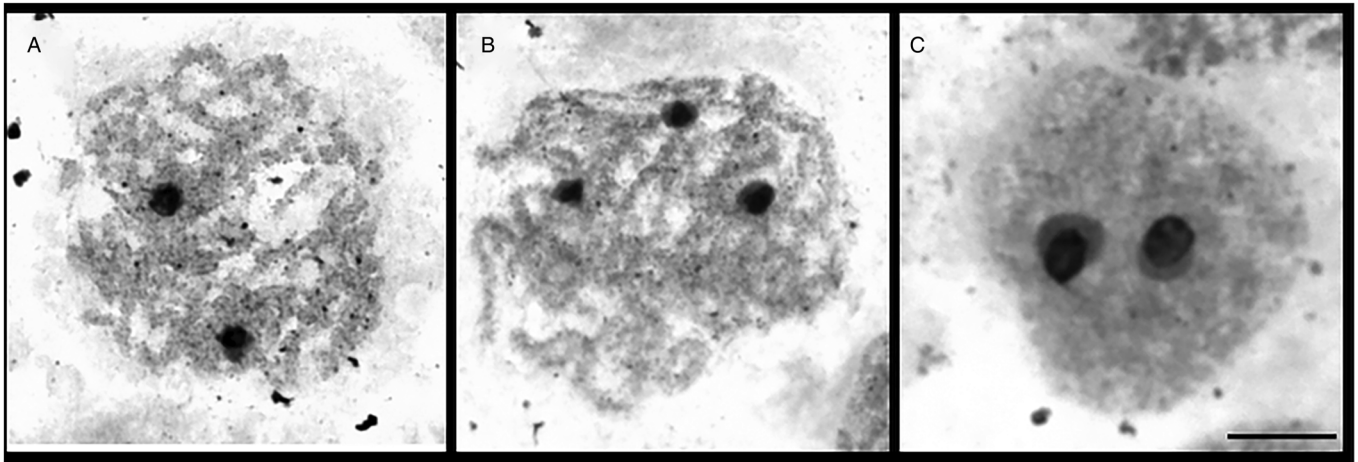


Fig. 5. Visualization of nucleoli (N) in meiotic spermatocytes of *Diphyllobothrium latum* after  $\text{AgNO}_3$  staining (scale bar =  $10\ \mu\text{m}$ ).

*Diphyllobothrium latum*), *D. ditremus* (syn. *Diphyllobothrium osmeri*) and *D. dendriticus* (syn. *Diphyllobothrium dendriticum*), revealed similar variations in chromosome numbers from 8 to 28, although to a lesser extent compared to this work (Wikgren and Gustafsson, 1965). All 3 analyzed species then belonged to the genus *Diphyllobothrium* at that time and were characterized as diploids. The authors attributed these variations to either preparation artifacts or the occurrence of somatic aneuploidy. Regarding chromosome morphology, they did not find any differences between the congeners. Haploid complements included 7 metacentric and 2 submetacentric chromosomes, which is slightly different from our Italian triploid population of *D. latum* with 9 metacentric chromosomes. However, it should be noted that chromosome morphometries in those works have been documented on squashed or section slides without indicating centromeric indexes and should therefore be treated with caution. Petkevičiūtė (1992) reported the same chromosome number and ploidy level ( $2n = 18$ ) and only minor morphological differences between chromosomes for 2 species of this family, *D. ditremus* (syn. *Diphyllobothrium ditremum*) from a geographically distant population in Chukotka, Russia and *Ligula intestinalis* from a population in Lithuania. Apart from ploidy in the population of *D. latum* from Italy, a comparison of the chromosome sets of *D. ditremus*, *D. dendriticus*, *L. intestinalis* and *D. latum* population from Finland indicates only small karyotype diversity, with no apparent structural and numerical variations. As the fundamental number of arms is also identical in these 4 species, small interspecific variations in chromosome morphology can be best explained by intrachromosomal changes, i.e. small deletions or duplications. These cytogenetic similarities also reflect well their close phylogenetic relationships (see Fig. 3 in Waeschenbach *et al.*, 2017 and Fig. 7 in this study). In the current phylogenetic tree, *S. solidus* was placed in 'earlier diverging position together with *Spirometra* spp.' (p. 837 in Waeschenbach *et al.*, 2017). This is in line with known karyological data, as *S. solidus* has a less symmetrical complement, with nearly half of the chromosome pairs being acrocentric (Petkevičiūtė, 1996), and therefore can be considered as evolutionary older species in this group. Its karyotype may represent an ancestral condition in Diphylobothriidea compared to other more derived chromosome forms and taking into account that pericentric inversion contributed to the karyotype divergence of some species (Petkevičiūtė, 1996). However, traditional karyological methods alone are not sufficient to elucidate the evolutionary history of these tapeworms, which points to the need of molecular cytogenetic

markers. Moreover, mapping of cytogenetic markers can also determine a level of ploidy by identification of homologous chromosome.

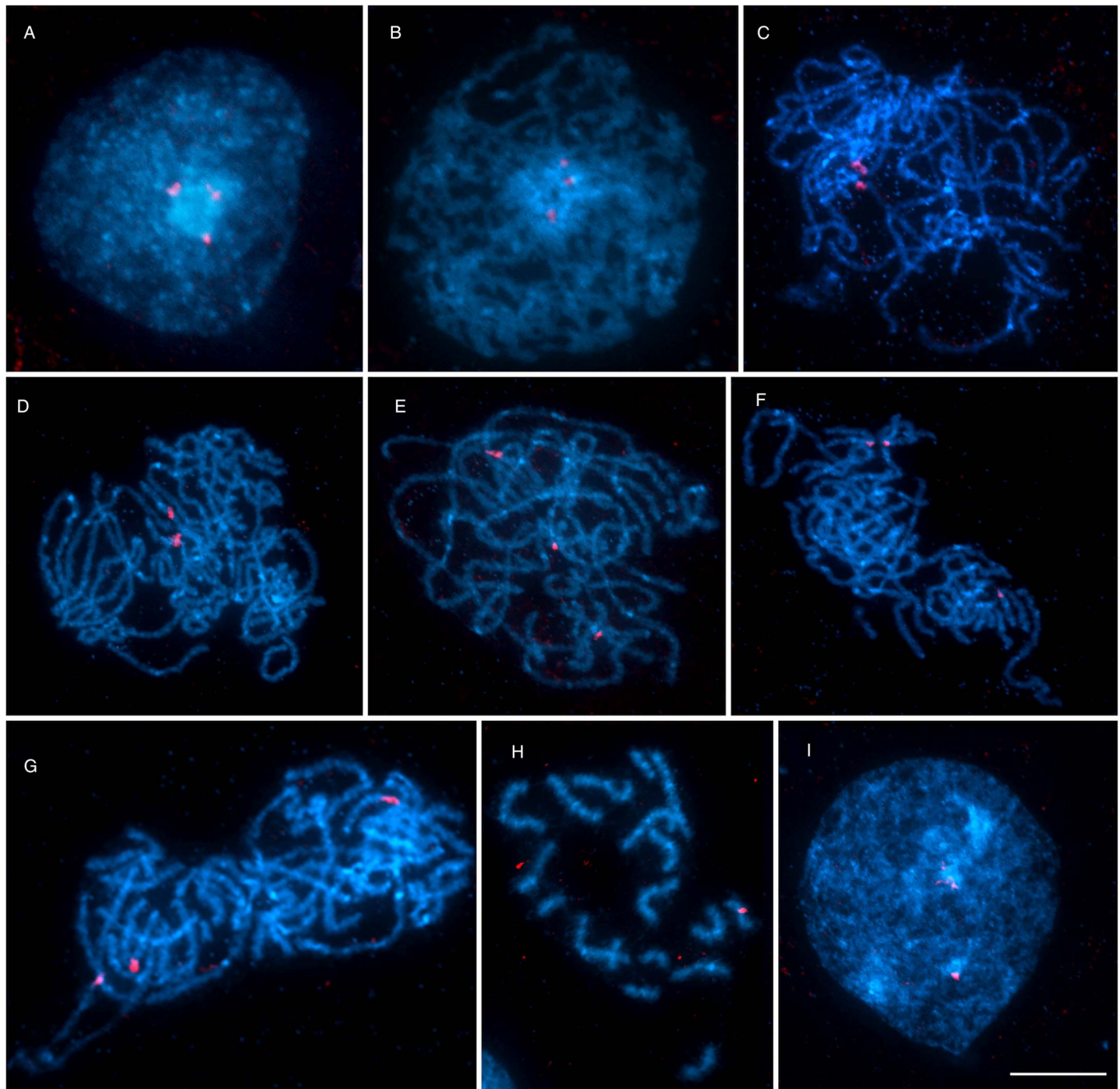
#### Chromosomal location of the 18s ribosomal RNA and telomere constitution

The Italian *D. latum* population is the first investigated for the chromosomal location of ribosomal RNA genes in the order Diphylobothriidea. Using FISH with 18S rDNA probe, we identified a single major rDNA site on all 3 chromosomes of triplet No. 7, clearly demonstrating the triploid nature of the studied population. Although data on rDNA in related diphylobothriideans are not available, 18S rDNA in *D. latum* mapped to a pericentromeric position on small metacentric chromosomes, as in all other previously analyzed tapeworm species (Orosová *et al.*, 2019). Our results are thus in line with the hypothesis of a generally constant position of rDNA loci in different families of Cestoda (Orosová *et al.*, 2019).

The detection of vertebrate telomere repeat motif  $(\text{TTAGGG})_n$  at all chromosome end in *D. latum* population is consistent with results obtained in mitotic and meiotic chromosomes of other tapeworm species. Exclusively telomeric signals were reported in 4 tapeworm species, namely *Caryophyllaeus laticeps*, *Caryophyllaeides fennica* and *Khawia abbottinae* (Caryophylliidea), and *Nippotaenia mogurndae* (Nippotaeniidea) (Bombarová *et al.*, 2009; Orosová *et al.*, 2019). Only the karyotype of *Atractolytocestus huronensis* (Caryophylliidea) exhibited also hybridization signals for interstitial telomeric sequences (ITSs), in addition to the telomeric signals, but always only in 1 chromosome of 3 of 5 triplets (Špakulová *et al.*, 2019). Our findings together with results obtained so far indicate that this hexanucleotide repetitive sequence is a conserved feature of cestode telomeres. The absence of ITS in *D. latum* karyotype suggests that this species has undergone no structural arrangements of chromosomes during its evolution.

#### The course of meiosis

The course of the meiotic division of spermatogonial cells of *D. latum* was aberrant. We did not observe all stages of spermatogenesis, which could be to some extent associated with the colchicine treatment, as it blocks the segregation of chromosomes or chromatids so that cell division is interrupted in metaphase. However, the main cause of abnormal meiosis is undoubtedly triploidy. During the first meiotic prophase, in pachytene nuclei,



**Fig. 6.** The course of meiotic division in *Dibothriocephalus latus* after FISH with 18S rDNA probe (red) counterstained with DAPI (blue). (A) Interphase nucleus with a large nucleolus and 3 18S rDNA clusters. (B) Leptotene. (C–G) Pachytene nuclei showing irregular pairing of spermatocyte chromosomes carrying rDNA genes; (C, D) pachytene with NOR-bearing triplet No. 7 in form of 1 univalent and 1 bivalent or (E–G) 3 univalents. (H) Metaphase II. (I) Secondary spermatocyte, the early stage of spermiogenesis (scale bar = 10  $\mu\text{m}$ ). Abbreviations: FISH, fluorescence *in situ* hybridization; NOR, nucleolar organizer region

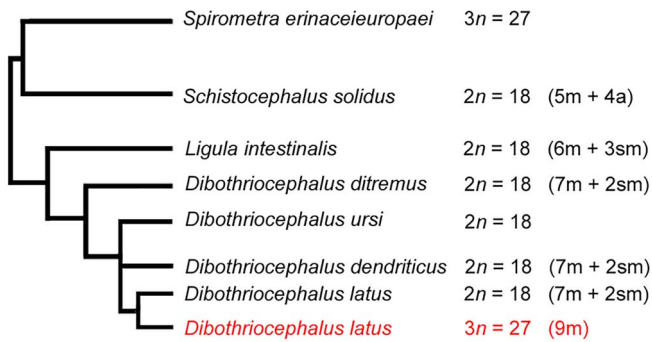
2 different arrangements of 3 homologous chromosomes No. 7 were easily identified in FISH preparations by 18S rDNA marker. We observed either a bivalent plus univalent or 3 unpaired univalents, but never a trivalent configuration that is probably unstable (see Marec, 1996). Similar pairing behavior can also be expected in the other chromosome triplets. Interestingly, we did not observe any diplotene/diakinesis nuclei, although in the case of bivalent configurations, crossing-over could occur to some extent (Shaffer *et al.*, 1971). We assume that chromosomes segregated randomly during anaphase I and also segregation of chromatids during anaphase II was irregular, resulting in aneuploid gametes with variable chromosome numbers. Similar random segregation of multivalent chromosome associations and the production of aneuploid gametes were described in many taxa (Comai, 2005; Chinone *et al.*, 2014). Regarding the meiotic division of triploid

tapeworm species, there are very few comparable data in the literature. Abnormal spermatogenesis and production of aneuploid gametes by random chromosome segregation were also observed in 2 triploid caryophyllidean species, *A. huronensis* and *Glaridacris catostomi* (Jones and Mackiewicz, 1969; Grey and Mackiewicz, 1980; Bruňanská *et al.*, 2009; Špakulová *et al.*, 2019). As with *D. latus*, no mature functional sperm was detected.

#### Origin of polyploidy

Polyploidy is a well-tolerated widespread phenomenon in the eukaryotic world and plays an important role in the evolution and development of species (Storchova and Pellman, 2004). The most interesting result of the present study is the revealed difference in the ploidy ( $2n$  vs  $3n$ ) of 2 geographically distant





**Fig. 7.** Summary of available data on the diploid/triploid chromosome number ( $2n/3n$ ) and chromosome morphology in karyologically studied diphylobothriidean species along with their latest phylogenetic relationships based on Waeschenbach *et al.* (2017).

populations of the same species *D. latus*. Such interpopulation differences are not uncommon and have been observed in many plants, and, to a lesser extent, occurs in animals (Park *et al.*, 2000; Giuliano-Caetano *et al.*, 2001; Gassner *et al.*, 2014; Winterfeld *et al.*, 2019) and flatworms and oligochaetes as well (Fujino and Ishii, 1982; Rhee *et al.*, 1987; Agatsuma *et al.*, 1994; Shen *et al.*, 2011; Zadesenets *et al.*, 2016). In fact, diploid/triploid populations are known even among the basal cestode orders (Grey, 1979; Grey and Mackiewicz, 1980; Petkevičiūtė and Kuperman, 1992; Špakulová *et al.*, 2011). Triploidy is frequently associated with parthenogenesis (Otto and Whitton, 2000), a phenomenon also known in parasitic flatworms. For instance, sexual worms tend to be diploid, whereas parthenogenetic worms are triploid and tetraploid (Benazzi, 1982). Among tapeworms, examples of such close association are *A. huronensis* ( $3n = 24$ ) (Jones and Mackiewicz, 1969) and *G. catostomi* ( $3n = 30$ ) (Grey and Mackiewicz, 1980). The difference between them is that *A. huronensis* is an exclusively triploid parthenogenetic species without an obvious diploid ancestor while only 1 geographically isolated triploid population was found in the generally diploid *G. catostomi*. The latter type has been found in several other parasitic plathyhelminths, including representatives of Trematoda (e.g. *Paragonimus westermani*, Blair *et al.*, 2016).

Apparently, polyploidization events could arise by chance several times across a wide range of taxa. There are 2 different ways for parthenogens to amplify their genomes; first by autopolyploidy (additional haploid chromosome complement comes from the same species) or second by allopolyploidy (due to interspecific hybridization) (Otto, 2007). In the case of *A. huronensis*, Špakulová *et al.* (2019) hypothesized that ancestral interspecific hybridization with the diploid congener *Atractolytocestus tenuicollis* could be the cause of its triploidy. The origin of the polyploid nature of the *G. catostomi* population is a matter of speculation. Geographic parthenogenesis is a common phenomenon denoting 'large variety of patterns where sexual and related asexual forms differ in their geographic distribution' (p. 1 in Tilquin and Kokko, 2016). Grey and Mackiewicz (1980) mentioned a possible link between parthenogenesis and triploidy in *G. catostomi* population isolated due to the separation of their fish host (suckers) in the dam, while the other 5 populations were diploid.

Hermaphroditic tapeworms can reproduce by cross-fertilization as well as by selfing (Christen and Milinski, 2003). *Dibothriocephalus latus* is usually found as a single plerocercoid in the musculature of the second intermediate fish host or as a single worm in the intestine of the final host (Gustinelli *et al.*, 2016; Radačovská *et al.*, 2019). This model has also been confirmed for the *D. latus* from the Lake Iseo, considering that a single plerocercoid from 1 fish develops into 1 adult worm in the definitive host and thus causes a low level of genetic

polymorphism in this *D. latus* population (Bazsalovicsová *et al.*, 2018). This suggests self-fertilization as a likely way of reproduction of this tapeworm. However, sexual reproduction involves the fusion of an egg and sperm. Since functional mature sperm were not observed in our work and some steps of meiosis were apparently skipped in this species, parthenogenetic reproduction is evident. Parthenogens do not need to find a partner to mate and reproduce (Schiffer *et al.*, 2019). Reproduction without male sperm suppresses the ability to gain genetic variability in a species, as the process of meiotic recombination is responsible for maintaining genetic heterozygosity. It means that parthenogenetic reproduction in *D. latus* also leads to the loss of genetic variability and to an increase of homozygosity; microsatellite analysis of the same Italian *D. latus* population revealed a high level of monomorphic (72 out of 78) loci but not complete loss of heterozygosity (Bazsalovicsová *et al.*, 2018). It is very likely that crossing-over, in univalent/bivalent arrangements (discussed above), where 2 chromosomes pair, occurs between sister chromatids and a small amount of variations are maintained in the genome (Lutes *et al.*, 2010).

The karyotyped triploid *D. latus* population has  $3n = 27$  chromosomes, i.e. 3 haploid sets of chromosomes. No significant morphometric differences in chromosomes within the individual triplets were seen. Therefore, it is probable that the isolated *D. latus* population from Iseo Lake became parthenogenetic through autopolyploidy rather than by interspecific hybridization and that all 3 complements come from the same ancestral species. Parthenogenesis, or more specifically parthenogenetic races, is often associated with the occupancy of habitats that are at higher altitudes or latitudes and tend to be characterized by anthropogenic pollution compared with their sexual relatives (Gassner *et al.*, 2014). Northern Italy is known for its heavily industrialized cities that surround several natural lakes, including Lake Iseo. Moreover, this lake is also polluted by trash and untreated sewage system; it means that the lake receives urban and industrial effluent (Gustinelli *et al.*, 2016).

In summary, we assume that most likely all the facts discussed (intensity of infection – missing sexual partner, geographical isolation and to some extent also polluted environment) gave rise to the Italian triploid parthenogenetic population of *D. latus* and hypothetically, this phenomenon is not historically distant. Nevertheless, the question of the origin of triploidy and parthenogenesis remains open until further in-depth analysis of this unusual karyotype is performed and further studies on samples from different populations are available.

**Author contribution.** M.O. and F.M. conceived the study; M.O. and A.R. performed the field-collection, M.O. and Mi.O. examined the fishes, fixed the parasites and infected the experimental animals. Z.C. was responsible for laboratory animals and their welfare; M.O., Mi.O., A.M. and I.P. performed experiments and data analysis. M.O. and F.M. prepared a draft of the manuscript. All authors discussed the results and commented on the manuscript. All authors approved the manuscript.

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**Conflict of interest.** The authors declare there are no conflicts of interest.

**Ethical standards.** The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

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