Methodological Guide

OPEN ACCESS

Tapeworm chromosomes: their value in systematics with instructions for cytogenetic study

Martina Orosová and Marta Špakulová

Institute of Parasitology, Slovak Academy of Sciences, Košice, Slovakia

Abstract: The history and value of cytogenetic features for addressing questions of the evolution and systematics of tapeworms (Cestoda) are briefly reviewed along with instructions for collecting karyological data. As a supplement to worm morphology, chromosome number and morphology have been helpful in determining the systematic status of some genera in the Diphylobothriidae and species in the Bothriocephallidea. In addition, many new techniques for chromosome analysis have been recently applied in morphological and molecular studies of invertebrates, including tapeworms. Methods of molecular karyology, fluorescence *in situ* hybridisation, and chromosomal location of satellite DNA, microsatellites or histone genes may also provide useful data to inference of taxonomic relationships and for revealing trends or general lines of chromosome evolution. However, as karyological data are available only for few tapeworms, they are seldom an integral part of evolutionary and taxonomic studies of cestodes. A primary reason for this lack of karyological data may lie in general difficulties in working with tapeworm chromosomes. To address these problems, herein we present a well-tested, step-by-step illustrated guide on the fixation of tapeworm material and preparation of their chromosomes for cytogenetic studies. The technique requires standard glassware, few reagents and simple equipment such as needles; it can also be used on other neodermatan flatworms.

Keywords: Cestoda, cytogenetics, karyotype, methodological manual

Chromosomes as physical carriers of genetic information in eukaryotes were first observed in detail in the second half of the 19th century (see Gartler 2006 for historical context). Gradual improvement of techniques of microscopy and cytogenetics has led to accumulation of karyological data and has shown that karyotypes vary widely in many animal groups (Dobigny et al. 2004). They may differ in the number, size, shape and composition of DNA, proteins and partially RNA, and all of these features are subject to evolutionary changes. Indicating evolutionary distance between organisms of different systematic categories, chromosomes have undoubtedly taxonomic importance – alone or together with other modern character-based approaches (Schubert 2007). Attractiveness of cytogenetics lies in the fact that it includes several levels of biological organisation from molecules to chromosome morphology, and thus may provide unique information about specific features of an individual or a population, such as the existence of aneuploidy or polyploidy, presence of supernumerary chromosomes and/or different chromosomal rearrangements (White 1973, Camacho et al. 2000, Nguyen et al. 2010, Petkevičiūtė et al. 2014).

All this is also valid for parasitic flatworms (Neodermata) including their likely most recently evolved group Cestoda, characterised by lacking the gut. Although much

has been learned on tapeworm phylogeny and systematics over recent years using molecular and morphological approaches (Olson and Tkach 2005, Waeschenbach et al. 2007, Olson et al. 2008, Caira et al. 2014, Hahn et al. 2014, Egger et al. 2015, Caira and Jensen 2017, Waeschenbach and Littlewood 2017), relationships between many groups, especially the earliest evolved (‘lower’) cestodes, have not yet been fully clarified. New phylogenetic characters are needed (Littlewood 2008, Lefebvre et al. 2009) and karyology may represent a valuable source of information.

However, there is a considerable gap in the current knowledge of cytogenetics of most parasitic animals, despite the fact that chromosome structure and gene location may provide additional characters of evolutionary relevance also in flatworms (Špakulová et al. 2011). Only 115 species of at least 4,810 nominal species of cestodes, representatives of only 9 of 19 extant orders, have been studied karyologically so far (Špakulová et al. 2011, Orosová and Oros 2012, Bombarová and Špakulová 2015, Caira and Jensen 2017). Thus, chromosomal data are known for only about 2% of tapeworm species.

Early cytogenetic studies were focused exclusively on haploid or diploid numbers, later also on chromosome morphology, which was described in 74 species. The result is a relatively scarce dataset of cytogenetic information on

tapeworms compared to available data in other invertebrate groups, which is most likely due to methodological difficulties in obtaining good cytological preparations. Mitotic chromosomes of tapeworms are typically obtained from dividing spermatogonia and oogonia located in reproductive organs, which are, however, bound within dense parenchyma, making them difficult to isolate and process. Other problems arise from the fact that these organisms are endoparasitic and a host dissection is required to obtain them.

Examination of karyotypes and chromosomal structure can reveal inter- and intraspecific variation that may not be obvious at the level of the gross-morphology or by molecular genomics (Camacho et al. 2000, Dobigny et al. 2004, Bombarová et al. 2015). Chromosome data could be helpful in assessing taxonomic interrelationships among allied species; it has been well illustrated within the cestode order Diphyllbothriidea. Petkevičiūtė (1992) indicated that karyotypes of *Diphyllbothrium ditremum* (Creplin, 1825) and *Ligula intestinalis* (Linnaeus, 1758) are similar in their ranges of chromosome size and morphology. Regardless of being placed in separate families (e.g. Dubinina 1980), their chromosomes showed a considerable degree of conservation and Petkevičiūtė (1992) thus questioned systematic classification of the parasites. Indeed, Bray et al. (1994) supported their affiliation to a single family, Diphyllbothriidae, based on worm morphology. Molecular data also supported a single family classification (Waeschenbach et al. 2017).

Also a karyological analysis of four species of *Bothriocephalus* Rudolphi, 1808, belonging to the order Bothriocephalidea, illustrated how chromosome morphology can provide information on phylogenetic relationships within a genus. *Bothriocephalus scorpii* (Müller, 1776) has a reduced chromosome number of $2n = 12$ and more symmetric complement composed exclusively of biarmed chromosomes in comparison with three members of the genus characterised by 14 chromosomes, namely *Schyzocotyle acheilognathi* (Yamaguti, 1934), *B. claviceps* (Goeze, 1782), and *B. gregarius* Renaud, Gabrion et Pasteur, 1983 (see Bazitov 1978, Nedeva and Mutafova 1988, Petkevičiūtė 2003). According to Petkevičiūtė (2003), the derived karyotype of *B. scorpii* likely arose from a 14-element karyotype of *B. gregarius* by centric fusion of two small acrocentric chromosomes, revealed in its karyotype. This hypothesis has been indirectly supported by recent molecular phylogenetic analyses (Brabec et al. 2006, 2015) indicating that *B. scorpii* really belongs to among more recently evolved species of *Bothriocephalus* species. However, knowledge on tapeworm chromosomes is still very fragmentary and only extensive application of new cytogenetic data in concert with the genomic analyses may help elucidate karyotype evolution within the groups.

Another advantageous use of karyotype data is to facilitate the species differentiation and identification of morphologically similar cestodes (sibling species). For instance, a couple of hardly morphologically distinguishable species of *Eubothrium* Nybelin, 1922 (Bothriocephalidea) can be easily differentiated according to the shape of the smallest chromosome pair No. 8 which is metacentric in

E. crassum (Bloch, 1779) but acrocentric in *E. salvelini* (Schrank, 1790) (see Petkevičiūtė and Bondarenko 2001). It is possible to find similar examples in other groups of parasitic flatworms as well.

Naturally, more advanced methodologies (banding methods, fluorescence *in situ* hybridisation with rDNA or telomeric probes, etc.), which visualise specialised cytogenetic features, represent additional research tools or techniques. These techniques have been applied only to six tapeworm species (for a review, see Orosova and Oros 2012).

In the present paper, we provide detailed protocols as a simple step-by-step guide on how to fix samples of tapeworms in the field and how to prepare them for cytogenetic studies. The methodology described herein has been used in our previous studies and is based on long-term cytogenetic research on parasites (Bombarová et al. 2005, 2009, 2015, Košková et al. 2010, Král'ová-Hromadová et al. 2010, Orosova et al. 2010a,b, Reblánová et al. 2011, Špakulová et al. 2011, Orosova and Oros 2012). We hope that this practical guide will encourage cestodologists to fix material for karyology as well during their field expeditions and stimulate greater in-depth research into the cytogenetics of cestodes, one of the groups of invertebrates most adapted to parasitism.

METHODOLOGICAL GUIDE

Examination of fresh hosts

Samples for cytogenetic analysis should be obtained only from hosts immediately after their death as **only living worms** can be used for karyological studies. Killed hosts can be alternatively kept for a few hours in a refrigerator or on ice (but never frozen). Cestodes should be processed as soon as possible after dissection because chromosomes and DNA start to decompose very fast, even if the hosts or worms are kept in refrigerator. Cestodes of warm-blooded hosts disintegrate more quickly than those of cold-blooded hosts. Adult individuals containing testes and ovaries are preferred because of the higher probability of obtaining dividing cells, but larval stages or immature tapeworms can also be used.

Materials and chemicals used in the field

Materials and tools

- dissecting tools: forceps, scissors, fine tweezers, brush, needles or insulin syringe (single use insulin syringe with integrated needle) for tearing the worm's tegument during hypotonic treatment
- several plastic vials for preparation of solution
- glass/plastic Petri dishes or watch glasses
- glass/plastic Pasteur pipettes
- stopwatch (or your hand watch)
- plastic vials or Eppendorf tubes for storage

Chemicals

- colchicine (0.1% in double distilled water) in glass bottle, usually 25 ml

Fig. 1. Fixation of tapeworms or other small flatworms for cytogenetic studies and chromosome preparation techniques. Schematic drawing of monozoic (non-proglottised) tapeworm and polyzoic (proglottised) tapeworm. **A** – dissect the host, gently clean and remove the tapeworm; treat the worm with 0.025% colchicine for 60 minutes; **B** – treat the worm with hypotonic solution and gently tear the tegument; **C** – transfer the tissues to freshly prepared fixative (methanol: acetic acid 3 : 1) with fine forceps; **D** – transfer the fixed material to Eppendorf tube filled with newly prepared fixative; **E** – cut one small tissue piece and transfer it to a drop of 60% acetic acid on a slide and **F–H** – tear the tissue to very fine pieces; **I** – put the slide on a heating plate at 45 °C and spread the drop of cell suspension; **J** – direction of moving the drop over the slide is indicated.

- methanol p.a. (min. 99.5%), in glass bottle, usually 500 ml
- acetic acid (99%) or glacial acetic acid in glass bottle, usually 200 ml
- distilled water, usually 2,000 ml
- 0.075M KCl in distilled water (0.5592 g KCl/100 ml distilled H₂O, diluted in the laboratory)
- 9 g of NaCl in small plastic bag (pre-prepared for dilution in 1,000 ml of distilled water to make 0.9% saline solution)

Description of the method in the field

Before processing, prepare 0.025% colchicine in the saline solution as follows: mix one part of the 0.1% col-

chicine solution and three parts of the saline solution (e.g. 1 ml 0.1% colchicine plus 3 ml of saline).

Tapeworms should be removed carefully from the host by soft (entomological) tweezers, dissecting needles or brush and processed immediately after their gentle cleaning (in Petri dish with saline solution). If they cannot be processed immediately, which is by far the best option, they can be kept in a small covered Petri dish with saline for a maximum of one hour in cold place (fish parasites) or at normal day temperature (optimum 22–26 °C; bird and mammalian parasites).

Cytogenetic studies require a sufficient number of properly identified parasites; both insufficient number of specimens and uncertain species identification may represent

Fig. 2. Examples of how to tear the tegument with two syringe needles. A–C – monozoic (non-proglottised) tapeworms (*Khawia sinensis* Hsü, 1935); D, E – polyzoic (proglottised) tapeworms *Dipylidium* sp. (D) and *Hymenolepis diminuta* (Rudolphi, 1819) (E).

a serious obstacle. The karyotype should be examined from at least three different individuals to detect possible intra-population polymorphism. It is desirable, if possible, to leave a portion of the cestode strobila for morphological or molecular analysis for correct species identification.

A common problem in preparation of good quality chromosome slides from fixed tapeworms is to find dividing cells in each worm specimen. As mentioned above, adult worms should be preferably used for chromosome preparation. It is very important to obtain a sufficient number of cell divisions from specimens which are not too young but also not too old. Immature tapeworms (or parts of the strobila containing immature proglottids) and larval stages can only be used to observe mitotic chromosomes. Mature proglottids or mature monozoic cestodes that contain fully developed testes and the ovary producing mature oocytes are most suitable for observing meiotic chromosomes. Numerous dividing mitotic cells can be also observed in developing embryos (preoncospherical stage). Fully gravid proglottids with ripe eggs are not suitable for karyological studies.

Pre-fixation options

Two options of fixation of live tapeworms are recommended.

Option 1. If number of specimens is > 5 worms, suitable for cytogenetical studies is available, divide them into two groups (A) and (B).

Group A. Treat some specimens with colchicine (0.025% solution in saline) to increase the number of met-

aphases (Step 1); after colchicine treatment, continue with hypotonic treatment (Step 2).

Group B. Expose the remaining specimens directly to hypotonic treatment (Step 2).

Option 2. If the number of specimens is low (≤ 5 worms), process all of them with 0.025% colchicine solution in saline (Step 1) and continue with hypotonic treatment (Step 2).

Procedure

Step 1. Colchicine treatment

- Place whole alive worms into a small Petri dish with 0.025% colchicine in saline for 60 min to arrest cell division at metaphase (a longer treatment may result in chromosomes being too condensed) (Fig. 1A).
- Caution: Colchicine is a hazardous chemical and the handling, storage and disposal instructions must be observed.

Step 2. Hypotonic treatment

- Transfer entire worm or its part (best part of the parasite containing testes, ovary and vitelline follicles) into 0.075M KCl in Petri dish (whole worm should be immersed in the liquid) (Fig. 1B). The rest of the specimen may be transferred into 96% ethanol for DNA analyses (see Note 1).
- Tear the tegument gently throughout the worm (predominantly mature proglottids) using needles (Figs. 1B, 2) (see Note 2).

- Let the worm in the hypotonic solution for 2 h (time of treatment depends on the size of the worm, i.e. the larger the worm, the longer it should be kept in the solution).

Step 3. Fixation

- Transfer the worm to **freshly** prepared **cold** fixative (methanol: acetic acid 3 : 1) in Petri dish (Fig. 1C) (see Note 3).
- Leave the worm stand for 15 min and repeat the same fixation once more with a new cold fixative.

Step 4. Transport and storage

- Transfer fixed worm to an Eppendorf tube filled with an excess of cold fixative and store them in a cold place (best in freezer) until further use for chromosome preparations (Fig. 1D) (see Note 4). Each vial should be identified with protocol number and can be simply kept in the freezer for several years (see Note 5).

Chromosomes

Preparation of high quality metaphase spreads is essential and critical step for achieving good results in karyotyping and gene localisation. Metaphase spreads should consist of well spread chromosomes, the cytoplasm should not be visible and chromosomes should appear dark grey under phase contrast. If the quality of chromosome preparation is not adequate, staining reaction and hybridisation and detection of DNA probes will be negatively affected.

Below are methods that have been proven to be suitable for the preparation of high-quality chromosome in cestodes. These techniques represent modifications of the procedures described by Traut et al. (1999) and Sahara et al. (1999) for insect chromosomes.

Preparation of chromosome slides in the laboratory

Reagents and equipment

- glass microscopic slides
- acetic acid (99%) or glacial acetic acid
- methanol p.a. (min. 99.5%)
- HCl (36%)
- fixative (3 : 1 mixture of methanol and acetic acid)
- 60% acetic acid (stored in refrigerator)
- ice
- two tungsten needles or two insulin syringes (single use insulin syringe with integrated needle)
- Pasteur pipettes
- Kimwipes
- heating plate (preheated to 45 °C)
- coplin jar with 1% acid ethanol (1 ml of concentrated HCl and 99 ml of 96% ethanol)
- three coplin jars with 70%, 80% and 96% ethanol p.a. (room temperature)

Hot plate technique

For this technique worms after fixation (Step 3) or fixed worms kept in the freezer (Step 4) can be used. Use the slides with a low auto-fluorescence, which also do not exhibit any background fluorescence caused by insufficiently

clean or dirty surface (e.g. Superfrost slides, Menzel-Gläser, Braunschweig, Germany). Incubate the slides for at least 1 h in acid ethanol in coplin jar before use or pre-clear them with ethanol (99.5%). Dry the glass slides using cleaning paper (e.g. Kimwipes). Small pieces of tissue that should contain testes and ovary should be used for chromosome preparation.

Step 5. Maceration

- Place worm or its tissue pieces into a small Petri dish with freshly prepared fixative.
- Cut a small tissue piece (circa 0.5 mm long) from the worm in Petri dish using two fine dissection needles (Fig. 1E) and transfer it on a pre-cleaned glass slide into a drop (15 µl) of cold (stored in refrigerator) 60% acetic acid (Fig. 1F) (see Note 6).
- Tear a small part of tissue into very fine pieces (as small as possible) under dissecting microscope with two tungsten needles or insulin syringes to prepare a cell suspension (Fig. 1G,H).
- Remove all remnants of the tissue that cannot be torn.

Step 6. Spreading

- Put the slide with the drop of acetic acid containing the cell suspension on a heating plate preheated to the temperature of 45 °C and spread the drop very slowly over the slide with the lower bend of the hooked needle (Fig. 1I,J) until the acetic acid has almost all evaporated. Discard the rest of the drop (see Note 7).

Step 7. Dehydration

- Pass the slides through an ethanol series to remove residual water (use three coplin jars with 70%, 80% and 96% ethanol; 1 min each).
- Place the slides in vertical position until dry.

Step 8. Checking quality of chromosome spreads

- Check the quality of chromosome spreads under a phase contrast microscope (see Note 8).

Step 9. Storing

- Finally, chromosome preparations can be stored in slide boxes at -20 °C for a few months or at -70 °C for up to several years before further use (see Note 9).

Notes

1. After colchicine treatment a part of the strobila may be fixed in 96% ethanol for subsequent DNA analysis. The colchicine treatment has no mutagenic or degradational effect on rDNA sequences (Stunžėnas et al. 2004).
2. Proper/adequate tearing of the tegument, mainly of adult tapeworms with thick tegument, and the subsequent hypotonic treatment to swell the nuclei and to release the cells from the testes and ovaries are **key steps** to achieve good preparations. From our experience, the insufficiently torn tegument may considerably affect chromosome spreading.
3. Modified fixative can be also used for fixing the tissues: (a) one part acetic acid and three parts ethanol;

Fig. 3. Species-specific differences in karyotypes of congeneric species (A) *Khawia sinensis* Hsü, 1935 and (B) *Khawia saurogobii* Xi, Oros, Wang, Wu, Gao et Nie, 2009 (Caryophyllidea) as revealed by fluorescence *in situ* hybridisation with 18S rDNA probe (red signals) and chromosome counterstained with DAPI (blue colour). Mitotic metaphases of both species consist of $2n = 16$ chromosomes; arrows indicate rDNA signals on pair No. 6 in *K. sinensis* while on pair No. 7 in *K. saurogobii*; arrowheads indicate pair No. 6 that is metacentric in *K. sinensis* and acrocentric in *K. saurogobii*; asterisks indicate pair No. 8 that is acrocentric in *K. sinensis* while metacentric in *K. saurogobii*.

- (b) Carnoy's solution – fixative composed of ethanol, chloroform and glacial acetic acid (6 : 3 : 1).
4. The procedure of fixation of samples for karyological study is very simple and can be done by any researcher or technician during field conditions. The only small disadvantage in field conditions could be storage of fixed specimens, because optimal preservation of samples is keeping them in the freezer. If this facility is not available, we recommend keeping fixed specimens in the coldest place available, but only for a couple of days. Material collected by this method can be easily sent in plastic vials to specialised cytogenetic laboratories.
5. In case the fixed samples are stored in freezer more than five months, it is advisable to gently replace the old fixative with a fresh solution. The storage period is thus extended.
6. When acetic acid on the slide starts to evaporate during your work (it makes visible white spots), add next 10 µl of acetic acid; it is helpful to keep stock 60% acetic acid on ice during the preparation of chromosome slides.
7. Place the slide in vertical position on filter paper to remove the residual drop or use the edge of Kimwipe to dry it out.
8. This step is especially important if you select slides of high quality for costly and time-consuming fluorescence *in situ* hybridisation (FISH).
9. After removing slides from the freezer and before any subsequent staining or processing, pass the slide immediately through an ethanol series (three coplin jars with 70%, 80% and 96%; 30 s each) to remove ice crystals and water and let them air-dry. It is good to keep one special coplin jar with 70% ethanol at -20 °C and use this precooled 70% ethanol after removing slides from deep freezer to prevent any damage to chromosomes.

DISCUSSION

In general, there are only a few common karyological patterns observed within tapeworms (for details, see Špakulová et al. 2011). Despite the relatively small number of species examined, a wide range of chromosomal numbers was revealed, and several different types of chromosome rearrangements were found in related species. The question that arises is to what extent karyotype features could help us to resolve problematic relationships within the Cestoda. Congruence between chromosomal and molecular data from tapeworm species shows that chromosomal changes can be valuable phylogenetic characters and have phylogenetic significance (Petkevičiūtė 1992, 2002, 2003, Petkevičiūtė and Bondarenko 2001, Petkevičiūtė et al. 2006).

Undoubtedly, our knowledge of diploid number, karyotype and metrical data of metaphase chromosomes is not sufficient to date. The karyology of tapeworms lags behind many free-living invertebrate groups (namely Arthropoda) or even other parasitic flatworms like Trematoda, both in the frequency of analyses and the application of advanced molecular cytogenetic markers facilitating intimate chromosome analysis (e.g. Hirai and LoVerde 1995, Camacho et al. 2000, Hirai 2014, Dalíková et al. 2017). Methods of molecular karyology such as those based on the FISH, which could give us information on important evolutionary features, have so far been applied only in six tapeworm species.

Bombarová et al. (2009) used the FISH with telomeric probe and proved the presence of TTAGGG repeats in three species of cestodes. The localisation of ribosomal genes (18S rDNA) on chromosomes has only been reported in four caryophyllidean species (for review, see Orosová and Oros 2012). In caryophyllideans *Khawia sinensis* Hsü, 1935 and *K. saurogobii*, rDNA-FISH analysis revealed single ribosomal locus located in the pericentromeric region of metacentric pair No. 6 of the first species while on

Fig. 4. Comparison of ideograms of chromosomes of *Khawia sinensis* Hsü, 1935 (black) and *Khawia saurogobii* Xi, Oros, Wang, Wu, Gao et Nie, 2009 (grey) constructed from data on relative length (red dot location of 18S rDNA cluster).

the pair No. 7 of the second congener (Fig. 3). Both species present the same diploid number $2n = 16$ but showed differences in the morphology of chromosome pairs Nos. 6 and 8. In order to better visualise the differences, the ideograms were constructed (Fig. 4).

Hence, much more basic information regarding chromosomal number and morphology coupled with simple banding procedure and light microscopy analysis is needed for several other groups of congeneric species from different cestode families. Moreover, it will be essential for molecular cytogenetic research on tapeworms to apply the FISH procedure wherever possible. Future studies on the chromosomal location of satellite DNA, microsatellites or histone genes may also provide useful markers to infer taxonomic relationships and to reveal trends or general lines of chromosome evolution. It means that a considerable amount of sampled taxa properly fixed for karyology is highly desirable.

We encourage cestodologists to focus on fixing specimens for karyology worldwide in order to increase the information on chromosomal patterns of tapeworm species. It should be emphasised that the proposed methods of fixation for cytogenetic analyses are dependent on only a few more reagents (colchicine, methanol, acetic acid) and simple equipment (needles or insulin syringe) when compared to the classical methods (see Justine et al. 2012) used for collecting parasites in the field.

Acknowledgements. We wish to thank Tomáš Scholz (Institute of Parasitology, Biology Centre CAS, České Budějovice, Czech Republic) and three anonymous reviewers for critical and constructive comments. This study was supported by the Slovak Research and Development Agency (no. APVV-15-0004), SAS-MOST Joint Research Project (no. 2016/7), Grant Agency VEGA (no. 2/0159/16), and as implementation of the project Centre of Excellence for Parasitology (ITMS: 26220120022) supported by the Research and Development Operational Programme funded by the European Regional Development Fund (rate 0.3).

REFERENCES

- BAZITOV A.A. 1978: [Spermatogenesis of the tapeworm *Bothriocephalus scorpii*.] *Ekologia Moria* 2: 87–91. (In Russian.)
- BOMBAROVÁ M., ŠPAKULOVÁ M. 2015: New chromosome characteristics of the monozoic tapeworm *Caryophyllaeus laticeps* (Cestoda, Caryophyllidae). *Helminthologia* 52: 336–340.
- BOMBAROVÁ M., ŠPAKULOVÁ M., KELLO M., NGUYEN P., BAZALOVICSOVA E., KRÁČOVÁ-HROMADOVÁ I. 2015: Cytogenetics of *Aspidogaster limacoides* (Trematoda, Aspidogastrea): karyotype, spermatocyte division, and genome size. *Parasitol. Res.* 114: 1473–1483.
- BOMBAROVÁ M., ŠPAKULOVÁ M., OROS M. 2005: A karyotype of *Nippotaenia mogurndae*: the first cytogenetic data within the order Nippotaeniidea (Cestoda). *Helminthologia* 42: 27–30.
- BOMBAROVÁ M., VÍTKOVÁ M., ŠPAKULOVÁ M., KOUBKOVÁ B. 2009: Telomere analysis of plathyhelminths and acanthocephalans by FISH and Southern hybridization. *Genome* 52: 897–903.
- BRABEC J., KUČHTA R., SCHOLZ T., 2006: Paraphyly of the Pseudophyllidae (Platyhelminthes: Cestoda): circumscription of monophyletic clades based on phylogenetic analysis of ribosomal RNA. *Int. J. Parasitol.* 36: 1135–1541.
- BRABEC J., WAESCHENBACH A., SCHOLZ T., LITTLEWOOD D.T.J., KUČHTA R. 2015: Molecular phylogeny of the Bothriocephalidae (Cestoda): molecular data challenge morphological classification. *Int. J. Parasitol.* 45: 761–771.
- BRAY R.A., JONES A., ANDERSEN K.I. 1994: Order Pseudophyllidae Carus, 1963. In: A. Khalil, A. Jones and R.A. Bray (Eds.), *Keys to the Cestode Parasites of Vertebrates*, CAB International, Wallingford, pp. 205–247.
- CAIRA J.N., JENSEN K. (Eds.) 2017: Planetary Biodiversity Inventory (2008–2017): Tapeworms from Vertebrate Bowels of the Earth. The University of Kansas, Natural History Museum, Lawrence, Special Pub. No. 25, 463 pp.
- CAIRA J.N., JENSEN K., WAESCHENBACH A., OLSON P.D., LITTLEWOOD D.T.J. 2014: Orders out of chaos – molecular phylogenetics reveals the complexity of shark and stingray tapeworm relationships. *Int. J. Parasitol.* 44: 55–73.

- CAMACHO J.P.M., SHARBEL T.F., BEUKEBOOM L.W. 2000: B-chromosome evolution. *Phil. Trans. R. Soc. Lond. B* 355: 163–178.
- DALÍKOVÁ M., ZRZAVÁ M., HLADOVÁ I., NGUYEN P., ŠONSKÝ I., FLEGROVÁ M., KUBÍČKOVÁ S., VOLENÍKOVÁ A., KAWAHARA A.Y., PETERS R.S., MAREC F. 2017: New insights into the evolution of the W chromosome in Lepidoptera. *J. Hered.* 108: 709–719.
- DOBIGNY G., DUCROZ J.F., ROBINSON T.J., VOLOBOUEV V. 2004: Cytogenetics and cladistics. *Syst. Biol.* 53: 470–484.
- DUBININA M.N. 1980: Tapeworms (Cestoda, Ligulidae) of the Fauna of the USSR. Amerind Publishing, New Delhi, 320 pp.
- EGGER B., LAPRAZ F., TOMICZEK B., MÜLLER S., DESSIMOZ C., GIRSTMAIR J., ŠKUNCA J., RAWLINSON K.A., CAMERON C.B., BELI E., TODARO M.A., GAMMOUDI M., NOREÑA C., TELFORD M.J. 2015: A transcriptomic-phylogenomic analysis of the evolutionary relationships of flatworms. *Curr. Biol.* 25: 1347–1353.
- GARTLER S.M. 2006: The chromosome number in humans: a brief history. *Nat. Rev. Genet.* 7: 655–660.
- HAHN C., FROMM B., BACHMANN L. 2014: Comparative genomics of flatworms (Platyhelminthes) reveals shared genomic features of ecto- and endoparasitic Neodermata. *Genome Biol. Evol.* 6: 1105–1117.
- HIRAI H. 2014: Chromosomal differentiation of schistosomes: what is the message? *Front. Genet.* 5: 1–5.
- HIRAI H., LOVERDE P.T. 1995: FISH techniques for constructing physical maps on schistosome chromosomes by FISH. *Parasitol. Today* 11: 310–314.
- JUSTINE J.-L., BRIAND M.J., BRAY R.A. 2012: A quick and simple method, usable in the field, for collecting parasites in suitable condition for both morphological and molecular studies. *Parasitol. Res.* 111: 341–351.
- KOŠKOVÁ E., ŠPAKULOVÁ M., KOUBKOVÁ B., REBLÁNOVÁ M., OROSOVÁ M. 2010: Comparative karyological analysis of four diplozoid species (Monogenea, Diplozoidae), gill parasites of cyprinid fishes. *Parasitol. Res.* 108: 935–941.
- KRÁČOVÁ-HROMADOVÁ I., ŠTEFKA J., ŠPAKULOVÁ M., OROSOVÁ M., BOMBAROVÁ M., HANZELOVÁ V., BAZSALOVICSOVÁ E., SCHOLZ T. 2010: Intraindividual ITS1 and ITS2 ribosomal sequence variation linked with multiple rDNA loci: a case of triploid *Atractolytocestus huronensis*, the monozoic cestode of common carp. *Int. J. Parasitol.* 40: 175–181.
- LEFEBVRE F., GEORGIEV B.B., BRAY R., LITTLEWOOD D.T.J. 2009: Developing a dedicated cestode life cycle database: lessons from the hymenolepidids. *Helminthologia* 46: 21–27.
- LITTLEWOOD D.T.J. 2008: Platyhelminth systematics and the emergence of new characters. *Parasite* 15: 333–341.
- NEDEVA I., MUTAFOVA T. 1988: [To the morphology of *Bothriocephalus acheilognathi* Yamaguti, 1934 (fam. Bothriocephalidae).] *Khelminthologiya* 26: 39–46. (In Bulgarian.)
- NGUYEN P., SAHARA K., YOSHIDO A., MAREC F. 2010: Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). *Genetica* 138: 343–354.
- OLSON P.D., SCHOLZ T., PODDUBNAYA L.G., LITTLEWOOD D.T.J. 2008: On the derived position of *Archigetes* and the early evolution of the tapeworms (Platyhelminthes: Cestoda). *J. Parasitol.* 94: 898–904.
- OLSON P.D., TKACH V.V. 2005: Advances and trends in the molecular systematics of the parasitic Platyhelminthes. *Adv. Parasitol.* 60: 165–243.
- OROSOVÁ M., KRÁČOVÁ-HROMADOVÁ I., BAZSALOVICSOVÁ E., ŠPAKULOVÁ M. 2010a: Karyotype, chromosomal characteristics of multiple rDNA clusters and intragenomic variability of ribosomal ITS2 in *Caryophyllaeides fennica* (Cestoda). *Parasitol. Int.* 59: 351–357.
- OROSOVÁ M., MAREC F., OROS M., XI B.W., SCHOLZ T. 2010b: A chromosome study and 18S rDNA *in situ* hybridization of *Khawia saurogobii* (Cestoda: Caryophyllidae). *Parasitol. Res.* 106: 587–593.
- OROSOVÁ M., OROS M. 2012: Classical and molecular cytogenetics of *Khawia sinensis* (Cestoda: Caryophyllidae), invasive parasite of carp, *Cyprinus carpio*. *Parasitol. Res.* 110: 1937–1944.
- PETKEVIČIŪTĖ R. 1992: Comparative cytogenetics of *Diphyllbothrium ditremum* (Creplin, 1925) and *Ligula intestinalis* (Linnaeus, 1758) (Cestoda: Pseudophyllidae). *Syst. Parasitol.* 23: 167–173.
- PETKEVIČIŪTĖ R. 2002: [Knowledge of the chromosome set of cestodes.] In: A.K. Galkin and H.V. Dubinina (Eds.), *The Problems of Cestodology II. Zoological Institute, Russian Academy of Sciences, St. Petersburg*, pp. 206–220. (In Russian.)
- PETKEVIČIŪTĖ R. 2003: Comparative karyological analysis of three species of *Bothriocephalus* Rudolphi 1808 (Cestoda: Pseudophyllidae). *Parasitol. Res.* 5: 358–363.
- PETKEVIČIŪTĖ R., BINKIENE R., KOMISAROVAS J. 2006: Diversity of Dilepididae (Cestoda: Cyclophyllidae) revealed by cytogenetic analysis. *J. Helminthol.* 80: 59–63.
- PETKEVIČIŪTĖ R., BONDARENKO S.K. 2001: Comparative karyological studies on the species of *Eubothrium* Nybelin, 1922 (Cestoda: Pseudophyllidae). *Syst. Parasitol.* 50: 127–134.
- PETKEVIČIŪTĖ R., STUŽĖNAS V., STANEVIČIŪTĖ G. 2014: Differentiation of European freshwater bucephalids (Digenea: Bucephalidae) based on karyotypes and DNA sequences. *Syst. Parasitol.* 87: 199–212.
- REBLÁNOVÁ M., ŠPAKULOVÁ M., OROSOVÁ M., KRÁČOVÁ-HROMADOVÁ I., BAZSALOVICSOVÁ E., RAJSKÝ D. 2011: A comparative study of karyotypes and chromosomal location of rDNA genes in important liver flukes *Fasciola hepatica* and *Fascioloides magna* (Trematoda: Fasciolidae). *Parasitol. Res.* 109: 1021–1028.
- SAHARA K., MAREC F., TRAUT W. 1999: TTAGG telomeric repeats in chromosomes of some insects and other arthropods. *Chromosome Res.* 7: 449–460.
- SCHUBERT I. 2007: Chromosome evolution. *Curr. Opin. Plant. Biol.* 10: 109–115.
- ŠPAKULOVÁ M., OROSOVÁ M., MACKIEWICZ J.S. 2011: Cytogenetics and chromosomes of the tapeworms (Platyhelminthes, Cestoda). *Adv. Parasitol.* 74: 177–230.
- STUŽĖNAS V., CRYAN J.R., MOLLOY D.P. 2004: Comparison of rDNA sequences from colchicine treated and untreated sporocysts of *Phyllodistomum folium* and *Bucephalus polymorphus* (Digenea). *Parasitol. Int.* 53: 223–228.
- TRAUT W., SAHARA K., OTTO T.D., MAREC F. 1999: Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. *Chromosoma* 108: 173–180.
- WAESCHENBACH A., BRABEC J., SCHOLZ T., LITTLEWOOD D., KUCHTA R. 2017: The catholic taste of broad tapeworms – multiple routes to human infection. *Int. J. Parasitol.* 47: 831–843.
- WAESCHENBACH A., LITTLEWOOD D.T.J. 2017: A molecular framework for the Cestoda. In: J.N. Caira and K. Jensen (Eds.), *Planetary Biodiversity Inventory (2008–2017): Tapeworms from Vertebrate Bowels of the Earth*. The University of Kansas, Natural History Museum, Lawrence, pp. 431–451.
- WAESCHENBACH A., WEBSTER B.L., BRAY R.A., LITTLEWOOD D.T.J. 2007: Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. *Mol. Phylogenet. Evol.* 45: 311–325.
- WHITE M.J.D. 1973: *Animal Cytology and Evolution*. Cambridge University Press, Cambridge, 468 pp.

Received 24 October 2017

Accepted 13 January 2018

Published online 23 February 2018

Cite this article as: Orosová M., Špakulová M. 2018: Tapeworm chromosomes: their value in systematics with instructions for cytogenetic study. *Folia Parasitol.* 65: 001.