



Comparison of rDNA sequences from colchicine treated and untreated sporocysts of *Phyllodistomum folium* and *Bucephalus polymorphus* (Digenea)[☆]

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Abstract

The most frequently used antimetabolic agent in cytogenetic studies is colchicine. We investigated whether the initial treatment of trematodes for karyological analysis with colchicine would have mutagenic or degradational effect on rDNA sequences. *Dreissena polymorpha* is the intermediate host of *Phyllodistomum folium* and *Bucephalus polymorphus*, and the sporocyst stage of these trematode species develop, respectively, in the gills and gonads of this mussel. Sporocysts of *P. folium* and *B. polymorphus* were obtained from *D. polymorpha* collected from waterbodies in Belarus and in Lithuania. 5.8S and 28S rDNA genes, ITS1 and ITS2 of *P. folium* and *B. polymorphus* were sequenced and compared, and no nucleotide sequence differences between colchicine treated and untreated trematodes were found. Based on these results, we conclude that colchicine treatment for 3–5 h has no mutagenic or degradational effect on rDNA sequences. During the course of this investigation, two genetically different *P. folium* samples were noted in Belarus.

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Abbreviations: PCR, polymerase chain reaction; ITS, internal transcribed spacer; CTAB-DMSO, 1.5% cetyltrimethylammonium bromide and 20% dimethyl sulfoxide in deionized water.

[☆] **Note:** Nucleotide sequence data reported in this paper are available in the GenBank databases under the accession numbers AF533015, AY281127, AY278565, AY281128, AY282496, AY282497, AY288822–AY288832, AY289238–AY289248.

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In recent years, molecular systematic studies combining DNA nucleotide sequences with karyological data have been published in increasing numbers [1–4]. This paper examines whether both types of data can be obtained from the same specimen. In particular, we investigated whether the initial treatment of trematodes for karyological analysis with an anti-mitotic agent would have mutagenic or degradational effect on rDNA sequences.

Ribosomal DNA genes and ITS sequences, which are localized between ribosomal genes are widely used on all trematode taxonomic levels because they consist of regions displaying different degrees of variability. Karyotype, in general, is the complete set of chromosomes of a cell in metaphase. During metaphase, the chromosomes are well condensed and aligned on the metaphase plate, making it an ideal time to examine the chromosomes in a cytological preparation that flattens the nuclei. The arresting of cell division in metaphase by an antimitotic agent is a unique feature of chromosome plate preparations. The most frequently used antimitotic agent in cytogenetic studies is colchicine, an alkaloid isolated from Autumn crocus (*Colchicum autumnale*). This compound suppresses microtubule assembly by binding to the tubulin heterodimer (but not to tubulin), resulting in the cessation of mitosis at metaphase. Colchicine is also widely used as a medication for treatment of various diseases, including several kinds of cancer [5,6]. Despite the prevalence of colchicine usage, no information is available regarding the possible mutagenic or degradational impact of colchicine on DNA nucleotide sequence.

A project to characterize trematode parasites of zebra mussels, *Dreissena polymorpha*, by cytogenetic and molecular methods provided an opportunity to compare ribosomal DNA sequences of trematodes such as *Phyllodistomum folium* and *Bucephalus polymorphus* from colchicine-treated and untreated mussel tissue. Digenetic trematodes are among the most common and abundant of the helminth parasites. The adults are parasitic in all classes of vertebrates, especially fish. Their development requires at least two hosts. *D. polymorpha* is the intermediate host of *P. folium* and *B. poly-*

morphus, and the sporocyst stage of these trematode species develop, respectively, in the gills and gonads of this mussel. Trematode sporocysts are essentially germinal sacs containing germinal cells, which have descended from original ovum. Sporocysts of *P. folium* and *B. polymorphus* were obtained from *D. polymorpha* collected from waterbodies in Belarus (autumn 2000) and in Lithuania (summer 2001) (Table 1).

In each of the Belarussian samples, half of the mussels collected were immediately dissected to identify individuals infected with either *P. folium* or *B. polymorphus*. Pieces of sporocyst-infected tissue were removed from these infected individuals, fixed in CTAB-DMSO (1.5% cetyltrimethylammonium bromide and 20% dimethyl sulfoxide in deionized water), and stored at room temperature. Following standard karyological procedures, the other mussels in each sample were held in 0.005% colchicine in well water for 3–5 h at room temperature, dissected, and sporocyst-infected tissue then fixed in CTAB-DMSO and stored at room temperature. The mussel samples collected in Lithuania (Table 1) were dissected and, in contrast, sporocyst-infected tissues were fixed in 70% ethanol and stored in a freezer (–20 °C).

In July 2002, genomic nucleic acids of the sporocysts were extracted using either DNA/RNA Isolation Kits (Amersham Life Sciences, Inc., Cleveland, OH) or FastDNA extraction kits (Qbiogene Inc., Carlsbad, CA) and were stored at –80 °C in purified water. In this study we used oligonucleotide primers for ITS1, ITS2 and 28S DNA sequences, which are popular for phylogenetic analysis. Main parts of DNA sequences were amplified with published primers. To amplify the 5' end of the 28S gene: Dig12 (AAGCATATCAC-TAAGCGG), forward direction [7]; L0 (GCT-ATCCTGAG(AG)GAAACTTCG), reverse [7]. To amplify the ITS1, 5.8S and ITS2 regions: L5 (TTCACCTCGCCATTACT), reverse direction [8]; GA1 (AGAACATCGACATCTTGAAC), forward [9]; its5Trem (GGAAGTAAAAGTCGTAACAA-GG), forward [10]; its4Trem (TCCTCCGCTTATTGATATGC), reverse [10]. Additionally, newly designed primers were used to check and cover flaws in DNA sequences, which were got with published primers (Fig. 1). To amplify ribosomal

Table 1
Samples of *P. folium* and *B. polymorphus* from which DNA was analyzed

Trema-tode ^a	# ^b	Waterbody sampled ^c	Sampling date (dd-mm-yy) Colchicine treated ^d	GenBank accession number Pairs of primers used to get sequences	
				ITS1-ITS2	28S
P	1	Lukomskoe Lake	13-10-00 –	AY281127 its5Trem-its4Trem, L5-GA1	AY278565 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0, AY281128
P	2	Lukomskoe Lake	13-10-00 –		Digl2-L0, Digl2-PhyllintR, Digl2-HELintR,
P	3	Lepelskoe Lake	9-11-00 –	AY288822 its5Trem-its4Trem	AY282497 Digl2-L0, PhyllintF-L0
P	4	Lepelskoe Lake	9-11-00 –		AY282496 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0,
P	5	Lepelskoe Lake	13-11-00 –	AY288831 its5Trem-its4Trem, L5-GA1	AY288823 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0
P	6	Lepelskoe Lake	13-11-00 –	AY288832 its5Trem-its4Trem, L5-GA1	AY288824 Digl2-L0
P	7	Lukomskoe Lake	12-10-00 +		AY288825 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0
P	8	Lukomskoe Lake	12-10-00 +		AY288826 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0, Digl2-HELintR
P	9	Lukomskoe Lake	12-10-00 +	872/AF533015 its5Trem-its4Trem, L5-GA1	AF533015 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0, HELintF-L0
P	10	Lepelskoe Lake	14-11-00 +		AY288827 Digl2-L0, Digl2-PhyllintR,
P	11	Lepelskoe Lake	14-11-00 +		AY288828 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0, Digl2-HELintR, HELintF-L0
P	12 ^d	Elektrenai Reservoir	15-06-01 –		AY288829 Digl2-L0, Digl2-PhyllintR, PhyllintF-L0
P	13 ^d	Elektrenai Reservoir	26-08-01		AY288830 Digl2-L0

Table 1 (Continued)

Trema-tode ^a	# ^b	Waterbody sampled ^c	Sampling date (dd-mm-yy) Colchicine treated ^d	GenBank accession number	
				Pairs of primers used to get sequences	
				ITS1-ITS2	28S
B	1B	Lukomskoe Lake	13-10-00 –		AY289246 Digl2-L0
B	2B	Lukomskoe Lake	13-10-00 –	AY289240 its5Trem-its4Trem, L5-GA1	AY289248 Digl2-L0, BucintF-L0 Digl2-HELintR HELintF-L0
B	3B	Lepelskoe Lake	13-11-00 –	AY289238 its5Trem-its4Trem, L5-GA1	AY289238 Digl2-L0, Digl2-BucintR, HELintF-L0
B	4B	Lepelskoe Lake	13-11-00 –	AY289239 its5Trem-its4Trem, L5-GA1	AY289242 Digl2-L0, Digl2- BucintR, BucintF-L0
B	5B	Lepelskoe Lake	13-11-00 –		AY289245 Digl2-L0, Digl2-BucintR, BucintF-L0
B	6B	Lepelskoe Lake	10-11-00 +		AY289244 Digl2-L0, BucintF- L0, Digl2- HELintR, HELintF-L0
B	7B	Lepelskoe Lake	14-11-00 +	AY289241 its5Trem-its4Trem, L5-GA1	AY289243 Digl2-L0, Digl2- BucintR, BucintF-L0
B	8B	Lepelskoe Lake	14-11-00 +		AY289247 Digl2-L0, Digl2- BucintR, BucintF- L0, Digl2- HELintR, HELintF-L0

^a P = *Phyllodistomum folium*; B = *Bucephalus polymorphus*.

^b # = the number of a sample.

^c Lukomskoe Lake and Lepelskoe Lake are located ca. 120 km northeast of Minsk, Belarus; Elektrenai Reservoir is located 50 km west of Vilnius, Lithuania.

^d Samples from Elektrenai Reservoir were fixed in 70% ethanol; all other samples were fixed in CTAB-DMSO (1.5% cetyltrimethylammonium bromide and 20% dimethyl sulfoxide in deionized water).

DNA fragments localized at the 5' end of 28S gene: PhyllintF (TGCGCCTCGGTTGTTTAT), forward direction; PhyllintR (ATAAACAACCGA-GGCGCA), reverse; BucintF (TTGCCAGTGC-ACTTTCTC), forward; BucintR (GAGAAAGTG-CACTGGCAA), reverse; HELintF (AGTAACA-TGTGCGCGAGT), forward; HELintR (ACTCG-CGCACATGTTACT), reverse.

DNA fragments were amplified via standard polymerase chain reaction (PCR) using Taq DNA

polymerase (PE Applied Biosystems, Foster City, CA) under the following conditions: 36 cycles of 94 °C, 30 s; 53 °C, 1 min; 72 °C, 2 min. All PCR reactions included negative controls to detect possible contamination. Double-stranded PCR products were visualized on 1–2% agarose gels, purified using GeneClean III DNA Purification Kits (Bio101, Vista, CA), directly sequenced with Big Dye Version 3 Cycle Sequencing Ready Reaction with AmpliTaqFS DNA Polymerase (PE Applied

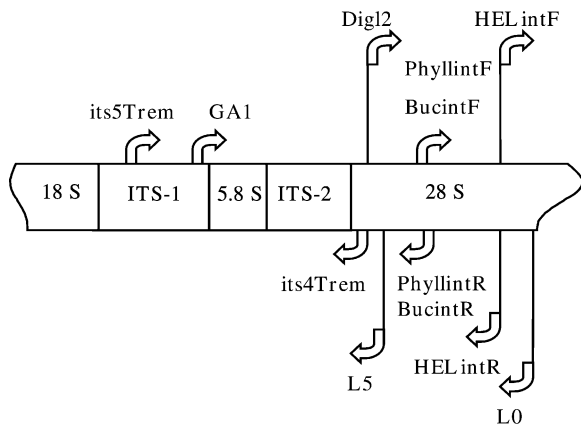


Fig. 1. rDNA primers localization and direction.

Biosystems, Foster City, CA), and fractionated by polymer capillary electrophoresis on either a Prism 3700 DNA Analyzer or a Prism 3100 Genetic Analyzer (PD Applied Biosystems, Foster City, CA).

Sequence confirmation was accomplished by comparing complimentary DNA strands. Editing of nucleotide sequences, contig assembly, and manual alignment of consensus sequences were performed using the software program Sequencher 4.0.5 (Gene Codes Corp., Ann Arbor, MI) for PC. Complete nucleotide sequences are available in GenBank under accession numbers listed in Table 1.

Ribosomal DNA of thirteen samples of *P. folium* and eight samples of *B. polymorphus* were sequenced (Table 1). Of these, five samples of *P. folium* and three samples of *B. polymorphus* were previously treated with colchicine. Despite differences in storage conditions, sampling dates, and localities (Table 1), we found no nucleotide sequence differences between colchicine-treated and untreated samples: the treated and untreated samples from Elektrenai Reservoir, Lukomskoe, and Lepelskoe lakes had identical 28S and ITS1-ITS2 sequences.

We found two genetically different *P. folium* samples in Lepelskoe Lake. rDNA nucleotide sequences from the #11 (colchicine treated, only 28S), #5, and #6 (no colchicine treatment) samples were identical, but these sequences differed

from the other *P. folium* samples (i.e. #1–4, #7–10, #12–13), which were invariant among themselves. For ease of reference, we call this latter collection of samples ‘Group 1.’ Longest, full overlapping sequences from each group were used for detailed comparison. The ITS1 fragment (458 nucleotides) of sample #5 was longer by five nucleotides than the #9 sequence shared among Group 1 samples, and had 10 variable nucleotide sites when compared to #9 (longest Group 1) sequence. The 5.8S gene was invariant throughout all *P. folium* samples. The ITS2 fragment (240 nucleotides) of sample #5 had 3 nucleotide site differences as compared to #9 (Group 1) sequence. The 28S fragment (1288 nucleotides) of sample #11 had seven nucleotide site differences as compared to #9, #12 (Group 1) sequences, and apparently had one insertion and one deletion as compared to #9, #12 (Group 1) sequences.

Based on these results, we conclude that colchicine treatment for 3–5 h has no mutagenic or degradational effect on rDNA sequences. Thus, it appears that a tissue sample prepared for karyological analysis using colchicine treatment can be used for rDNA sequencing via PCR. This conclusion is of considerable importance since it means that both karyological and molecular data can be derived from the same specimen. In addition, our data suggest that the effectiveness of CTAB-DMSO as an rDNA fixative does not depend on cold-temperature storage since samples stored without refrigeration for over one year had the same nucleotide sequences as samples fixed in ethanol and held at -70°C .

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References

- [1] Fernandes-Matioli FMC, Almeida-Toledo LF. A molecular phylogenetic analysis in *Gymnotus* species with inferences on chromosome evolution. *Caryologia* 2001;54:23–30.
- [2] Harvey SC, Masabanda J, Carrasco LAP, Bromage NR, Penman DJ, Griffin DK. Molecular-cytogenetic analysis reveals sequence differences between the sex chromosomes of *Oreochromis niloticus*: evidence for an early stage of sex-chromosome differentiation. *Cytogenet Genome Res* 2002;79:76–80.
- [3] Morgan-Richards M, Trewick SA, Wallis GP. Chromosome races with Pliocene origins: evidence from mtDNA. *Heredity* 2001;86:303–12.
- [4] Odierna G, Andreone F, Aprea G, Arribas O, Capriglione T, Vences M. Cytological and molecular analysis in the rare discoglossid species, *Alytes muletensis* (Sanchez and Androver, 1977) and its bearing on archaebatrachian. *Chromosome Res* 2000;82:435–42.
- [5] Jordan A, Hadfield JA, Lawrence NJ, McGown AT. Tubulin as a target for anticancer drugs: agents which interact with the mitotic spindle. *Med Res Rev* 1998;18:259–96.
- [6] Mukherjee AK, Basu S, Sarkar N, Ghosh AC. Advances in cancer therapy with plant based natural products. *Curr Med Chem* 2001;8:1467–86.
- [7] Tkach V, Pawlowski J, Mariaux J. Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial 1srDNA sequences. *Int J Parasitol* 2000;30:83–93.
- [8] Jousson O, Bartoli P, Pawlowski J. Molecular identification of developmental stages in Opecoelidae (Digenea). *Int J Parasitol* 1999;29:1853–8.
- [9] Cribb TH, Anderson GR, Adlard RD, Bray RA. A DNA-based demonstration of a three-host life-cycle for the Bivesiculidae (Platyhelminthes: Digenea). *Int J Parasitol* 1999;28:1791–5.
- [10] Dvorak J, Vanacova S, Hampl V, Flegr J, Horak P. Comparison of European *Trichobilharzia* species based on ITS1 and ITS2 sequences. *Parasitology* 2002;124:307–13.