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Cytophotometric Determination of Genome Size in Two Species of *Cyclops* of Lake Baikal (*Crustacea*: *Copepoda*, *Cyclopoida*) in Ontogenetic Development

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Abstract—The genome size of *Cyclops* in cells at early stages of cleavage (up to the fifth division) and in somatic cells was estimated by static digital Feulgen cytophotometry in order to study quantitative changes in DNA content during chromatin diminution. Described here cytophotometric method was approbated on five different digital-imaging systems in blood cells of four vertebrate species. In all cases, we observed a direct correlation between the data obtained with known from the literature on genome size and high reproducibility, which will allow these systems to be used in future work. We also optimized the conditions for DNA hydrolysis of both blood smears and for two species of *Cyclops* from the Moscow population as 30 min in 5 N HCl at 24°C. Here, we first revealed chromatin diminution in two endemic Baikal species of Cyclopoida: *Acanthocyclops incolotaenia* and *Diacyclops galbinus*. We estimated the extent of chromatin diminution in *Diacyclops galbinus* as 95.5–96.2%. Cytometric analysis of the third species, *Mesocyclops leuckarti*, did not reveal obvious chromatin diminution.

Keywords: *Cyclops*, chromatin diminution, DNA cytophotometry

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INTRODUCTION

Chromatin diminution (CD) is the process of elimination of a part of genetic material at early stages of embryogenesis; unlike heterochromatization or post-transcriptional silencing, it is a very drastic and irreversible mechanism of inactivation of genetic material in eukaryotic genomes. The CD process has been described both in a group of protozoa (ciliates) and in invertebrates (cyclops, nematodes, dipterous insects). In several cases, it is also present in chordates, such as in hagfish (Cyclostomata) and in marsupials (some species of bandicoot). In ciliates, elimination of some of the chromosomes is connected with the formation of macronucleus and in dipterous insects with determination of sex and genomic imprinting, while in cyclops and nematodes it is linked with differentiation of somatic cells and cells of the embryonic pathway (Grishanin et al., 2006).

The study of CD in ciliates has shown that the entire set of characters of this process can also be present in phylogenetically distant ciliates. Thus, the process of genome reorganization in *Chilodonella uncinata* (class Phyllopharyngea), as well as in Spirotrichea, is characterized by the formation of "giant" highly polytenized chromosomes before elimination and, as a result, by formation of so-called "gene-sized chromosomes." The time of divergence between these classes is estimated to be at least 580 million years ago, and, apparently, the process of elimination arose twice within ciliates (Katz and Kovner, 2010). Therefore, studying the distribution of the CD process across phylogenetic lineages is of significant interest.

Cyclops, like some other crustaceans undergoing CD, have a complex program of the germ line versus soma differentiation, including selective elimination of the entire chromosomes, chromosome breakage at certain sites, and subsequent healing of chromosomal fragments into the entire chromosome, as well as elimination of excised chromosomal fragments (Grishanin et al., 2006). CD in *Cyclops* was first observed in 1894 (Häcker, 1894) and later was comprehensively analyzed at the cytological level for three species of *Cyclops* (Beermann, 1977). Further studies revealed chromatin elimination in 19 out of 35 examined

[1] **Abbreviations**: CD—chromatin diminution, CCD—charge-coupled device.

Cyclops species (Beermann, 1977; Einsle, 1996; Dorward and Wyngaard, 1997; Wyngaard and Rasch, 2000; Grishanin et al., 2004; Rasch and Wyngaard, 2006a, 2006b; Grishanin, 2008). Most species that have CD were found among the genus Cyclops and, according to Uli Einsle (Einsle, 1996), the features of chromatin diminution in this genus can also be considered a criterion for identification of the species.

To evaluate the distribution of chromatin diminution in nature among representatives of the suborder Cyclopoida, we have developed a new realization of digital cytophotometric method in the Feulgen-stained nuclei (Omelyanchuk et al., 2010). This method allows measuring the DNA amount in nuclei before and after the chromatin diminution even at a low amount of the available biological material for studies. Its advantages also include the possibility of performing cytophotometric measurements without the use of special expensive equipment, with application of any transmitted light microscope equipped with digital camera. In this work, we paid the main attention to *Cyclops* inhabiting Lake Baikal, the fauna of which contains a significant diversity of species. As a result of our studies, we were the first to establish the presence of CD in two Baikal species of *Cyclops*: *Acantocyclops incolotaenia* and *Diacyclops galbinus*.

MATERIALS AND METHODS

Collection of biological material. Females of *Acantocyclops incolotaenia, Diacyclops galbinus*, and *Mesocyclops leuckarti* with egg sacs containing embryos were collected in August 2009–2010 in the coastal area of the Lake Baikal at the depth of 7–10 m by colleges from Limnological Institute of the Siberian Branch of the Russian Academy of Sciences (Irkutsk) in the region of Bolshie Koty. The *M. leuckarti* individuals were additionally collected in small lakes located near the B. Kotinka River (lakes 1 and 3).

Fixation of tissue and preparation of cytological slides. Captured females carrying egg sacs were fixed on ice in ethanol/acetic acid (3 : 1) for 20 min. The fixative then was washed out twice in 96% ethanol for 10 min. The fixed material was transferred into 70% ethanol and kept at 6°C before use.

To perform cytophotometrical measurements, the *Cyclops* female egg sacs were washed out of ethanol in distilled water twice for 5 min. Then, in a drop of 45% acetic acid, the embryos were separated from each other, gently covered with coverslips, which were removed after being frozen in liquid nitrogen. The preparations were washed in 96% ethanol twice for 10 min. To produce cytological preparations of *Cyclops* somatic cells, swimming legs were used, cells of which contain a diploid amount of DNA (Stich, 1962; Rasch and Wyngaard, 2006b).

As reference standards, we used nuclei of differentiated blood cells of several organisms: *Gallus domesticus, Danio rerio, Homo sapiens*, and *Rana arvalis,* in which the DNA content amounts were 1.25, 1.68~1.80, 3.5, 4.65~7.17 pg, respectively (http://www.genomesize.com/). Blood smears were fixed in the ethanol–acetic acid mixture (3 : 1) for 20 min on ice, washed out twice in 96% ethanol, and air-dried.

Slides of *Cyclops* embryo sacs were stained with DAPI (0.5 μg/mL) or with Hoechst 33258 (0.25 μg/mL) to determine the stage of cleavage in embryo and the presence or absence of mitotically dividing cells with the use of fluorescent microscopy. Further cytophotometric analysis of the DNA levels did not reveal any notable effect of the fluorescent stain on binding of fuchsine with DNA.

Feulgen staining. Fuchsine (Dia-M, Russia) was used for Feulgen staining. Each staining procedure included 30–40 cytological slides and from one (*Gallus domesticus*) to four blood smears used as a reference standards. DNA was hydrolyzed in 5 N HCl for 30 min at room temperature (24°C); the preparations were stained with Schiff's reagent (pH 2.2–2.4) for 2 h and washing in sulfuric waters for 40 min (eight changes for 5 min). The sulfuric waters in each experiment were prepared for 30–60 min before use. The preparations were stained by the standard protocol (Rasch, 2004).

To determine the optimal time of hydrolysis, four time points were used: 15, 20, 30, and 60 min. In the experiment, blood smears of three standard objects were used—*G. domesticus, D. rerio*, and *H. sapiens*—as well as early and late embryos of two species of *Cyclops*: *C. kolensis* and *C. insignis* (Moscow population).

Cytophotometry. In this work, the method of cytophotometric determination of the DNA content was used in nuclei stained by Schiff's reagent by applying digital microphotography (Omelyanchuk et al., 2010). The measurements were performed on four microscopes in the transmitted light mode: an Axiovert-200 inverted microscope (Carl Zeiss, Germany), a DM-4000 fluorescent microscope (Leica, Germany), and DIALUX 20 EB (Leitz, Germany) and an AxioLab (Carl Zeiss, Germany) light microscopes. Measurements on the Axiovert-200 microscope were performed with the use of an Achroplan objective of 20× with an aperture of 0.40 and with the use of an AxioCam MRc camera in the 8-bit dynamic range, as well as with an A-plan 40× objective with an aperture of 0.65 and an AxioCam ICm1 camera in the 12-bit dynamic range. Measurements on the DM-4000 microscope were performed with the use of an objective of 40× with an aperture of 0.65 and a Leica DFC420 camera with a 12-bit dynamic range, while on the DIALUX 20 EB microscope they were carried out with the use of an objective of 40× with an aperture of 0.65 and a CANON EOS 500D camera in the 14-bit dynamic range. Finally, the AxioLab microscope was supplied by an MRCm1 12-bit camera (objective 40× and aperture 0.6). Digital images were obtained in

monochromatic light by using an interference green filter with a wavelength of 551 ± 10 nm.

A rectangular image containing an image of the nucleus was excised with the aid of the ImageJ open-source program. Digital processing of the obtained image was performed by a program of our design (in the Mathcad-14 software medium, which has procedures for inputting digital images) as follows.

(1) From the intensity of image pixels of the nucleus image, we subtracted the intensities of the pixels of the image of nontransparent ink fragments of sizes somewhat exceeding the nucleus image and obtained image I1 (taking into account flashes of optics and preparation of nontransparent ink fragments as has been described in detail by Omelyanchuk et al. (2010)).

(2) The subimage was isolated of the transparent area on the I1 image located near the nucleus and used to determine intensity of the light. The intensity of each I1 pixel was normalized per the mean intensity of the incident light. The digital matrix obtained in this way consists of digits reflecting the light transmission factor (τ) by areas of nucleus.

(3) In accordance with Bouger–Lambert–Beer's law, optical density D of each elementary scanned area was calculated by the formula $D = -\log \tau$.

(4) In the method of scanning, the DNA content in the nucleus is considered proportional to the sum of optical densities of elementary scanned areas (Agroskin and Papayan, 1997, p. 160, formula V.1). Proceeding from this, we used the sum of logarithms of the whole matrix of transmission factors taken with the reverse sign as the measure of the DNA content in the nucleus. A small number of pixels of the image I1, in which $\tau < 0$ or $\tau > 1$, were ignored in the DNA content calculations, as we considered these cases to be random charge fluctuations on the photomatrix elements. In general, the approach that we developed, like the early methods applied in Feulgen cytometry, does not the use special procedures of isolation of nucleus boundaries.

Statistical processing of data. For each experiment, samples (arbitrary units) were obtained both for nuclei of all standard objects and for the *Cyclops* cell nuclei. For each sample, we calculated the arithmetic mean and the standard deviation (MS Excel). The calibration curve of the DNA content in blood cell nuclei of reference standards was constructed using the arithmetic mean and the standard error of the mean $SE_{\overline{X}}$ for experimental data. To compare five different optical systems, regression and correlation coefficients were calculated.

To estimate DNA content (in picograms) of embryo cell nuclei in *Cyclops*, we used data on the DNA amount in nuclei of *Gallus domesticus* erythrocytes—1C = 1.25 pg (http://www.genomesize.com/)—as the spread of values obtained for this standard was the lowest.

RESULTS AND DISCUSSION

Processing of method digital cytophotometry (quality of calibration of dependences for different optical systems). To process the method of cytophotometrical measurement of the DNA content in nuclei stained with Schiff's reagent, calibration curves were constructed for each system of obtaining of images. Figure 1 shows calibration curves for experiments performed on Axiovert-200 (Figs. 1a, 1b), DM-4000 (Fig. 1c), DIALUX 20 EB (Fig. 1d), and AxioLab (Fig. 1e) microscopes. Since the proposed implementation of the method of digital cytometry differs from earlier ones, we considered it important to understand to what degree the obtained results depend on the optics of the microscope and properties of the digital camera. Thus, the Axiovert-200 microscope was supplied by an 8-bit camera (AxioCam MRc) or 12-bit camera (AxioCam ICm1); the DM-4000 microscope, by a 12-bit camera; the DIALUX 20 EB microscope, by a 14-bit camera (Canon EOS 500D); and the AxioLab microscope, by a 12-bit camera (AxioCam ICm1). To elaborate our method, we carried out 11 experiments with the use of four standard objects.

In using a camera with a large dynamic range (Fig. 1b), experimental data on the DNA content in nuclei of reference standards were shown to confirm with the literature data more precisely. However, use of a camera with 8-bit image digitization also allows obtaining data with rather high precision (Fig. 1a), and all five systems of obtaining digital images can be used.

For each calibration curve, coefficients were calculated of regression and correlation, as well as the error of the regression coefficient. Estimation of the significance of differences in regression coefficients in experiments performed on one microscope in all four cases did not reveal essential differences between them. Figure 1 presents five graphs for each system of obtaining digital images.

In measuring the ratio of the standard error value to the value of the arithmetic mean of DNA content (arbitrary units) for *G. domesticus*, it was shown that, in all 11 experiments, the coefficient of variation was within the diapason from 0.003 to 0.02. In the same interval, values were present of the ratio of the regression-coefficient errors to the regression coefficient itself. Thus, relative errors in determining the mean DNA content for different error standards are grounds for using one of them (in our case, *G. domesticus*) for calculation of the absolute DNA amount in other studied species. From this fact, it follows that the optic systems and chemical staining procedures used make it possible to determine the DNA content in nuclei within the diapason of 1–7 pg DNA with equal precision.

Determination of optimal time of DNA hydrolysis. In the course of the procedure of Feulgen staining, the

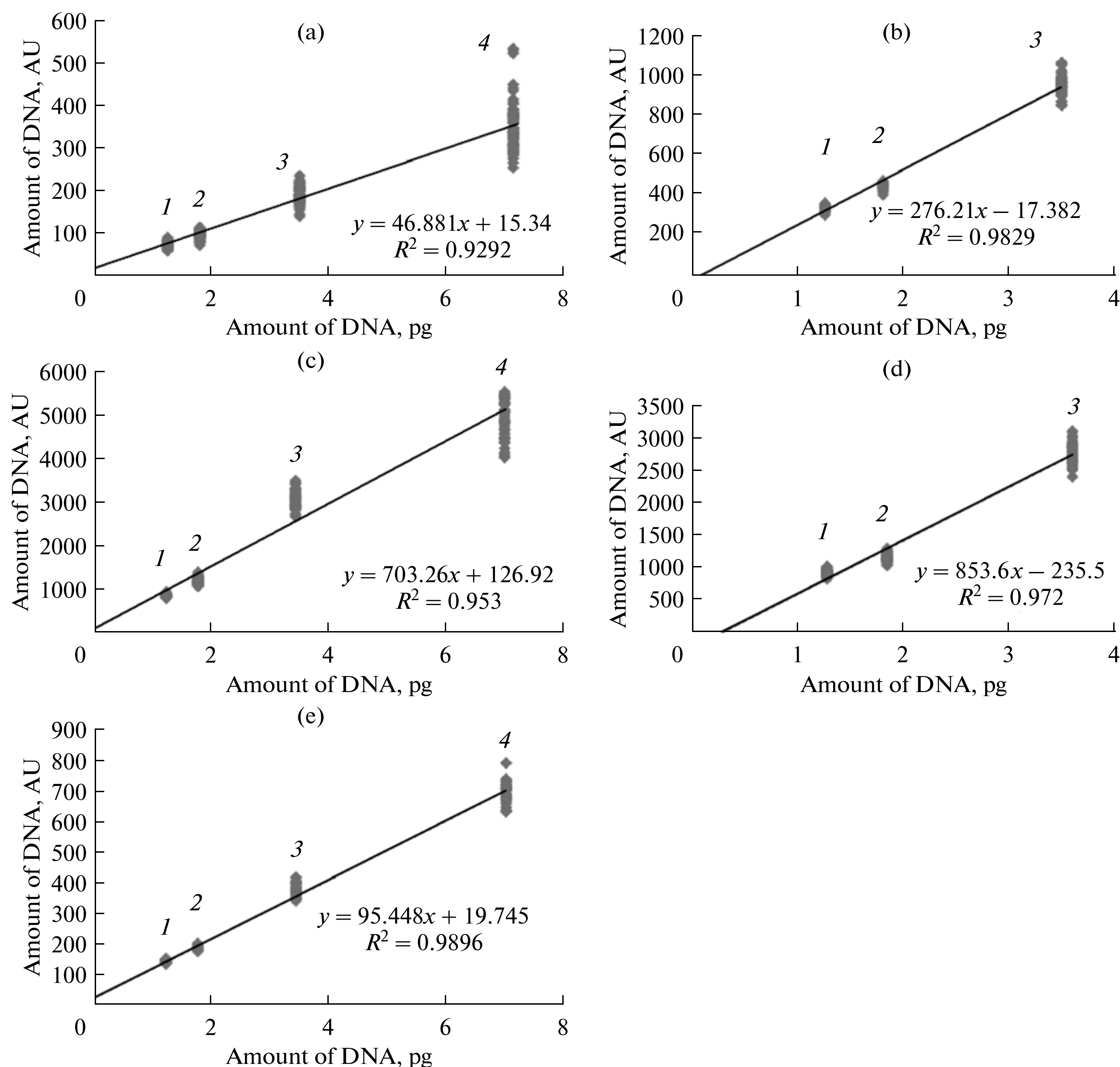

Fig. 1. Calibration curve constructed with DNA content in blood cell nuclei of reference standards.
Abscissa—amount of DNA according to the literature data (pg) (http://www.genomesize.com); ordinate—DNA content measured in experiments (AU). Columns indicate ranges of values: $\bar{x} \pm SE_{\bar{X}}$. (a) Axiovert-200 microscope (AxioCam MRc camera), (b) Axiovert-200 microscope (AxioCam ICm1 camera), (c) DM-4000 microscope (DFC-420 camera), (d) DIALUX 20 EB microscope (Canon EOS 500D camera), and (e) AxioLab microscope (AxioCam ICm1 camera). (*1*) *Gallus domesticus*, (*2*) *Danio rerio*, (*3*) *Homo sapiens*, and (*4*) *Rana arvalis*. The linear regression equation and value of the reliability of the approximation (R^2) are shown on the charts (MS Excel).

optimal DNA hydrolysis time (30 min) was established for three reference standards: *G. domesticus*, *D. rerio,* and *H. sapiens*, as well as for two the best-studied *Cyclops* species: *C. kolensis* and *C. insignis* (Figs. 2a–2e). In agreement with the obtained data for all the here-studied biological objects, we performed DNA hydrolysis for 30 min (for Feulgen staining) in 5 N HCl at room temperature. It is noteworthy, that Rasch and Wyngaard (2006a) have revealed that the copepod nuclei hydrolysis time in alcohol–acetic acid fixative should not exceed 15–20 min. The increase of the optimal hydrolysis time to 30 min in our experiments may be due to the initial treatment of objects, i.e., to the prolonged fixation of individuals in the acetate fixative: 1–4 min in the experiments of Rasch and Wyngaard (2006a) and 20 min in our experiments.

Analysis of DNA diminution in *Cyclops*. In our studies, the presence of chromatin diminution in two Baikal Cyclops species—*Acantocyclops incolotaenia* and *Diacyclops galbinus*—was established for the first

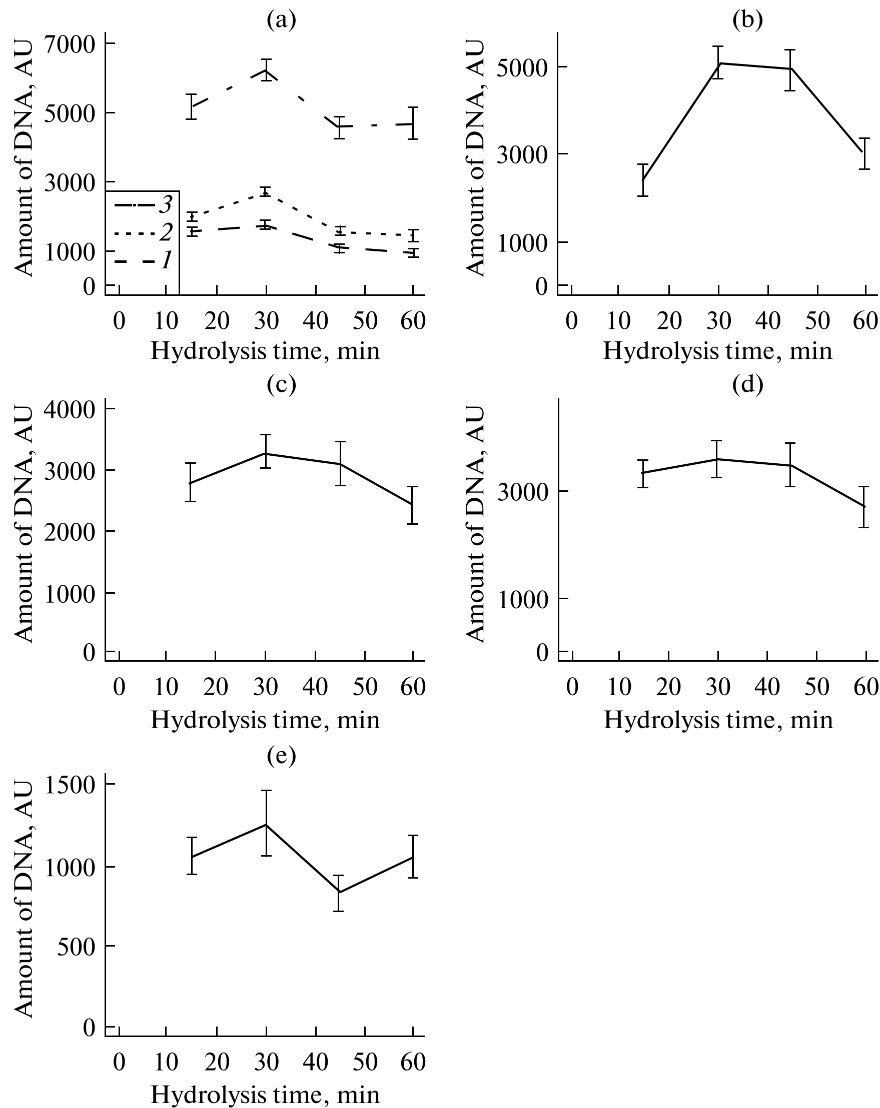

Fig. 2. Graphic dependence of the amount of Feulgen dye bound to the nucleus and hydrolysis time in 5 N HCl at room temperature (24°C).

x-axis—hydrolysis time (min); *y*-axis—DNA content measured in experiments (AU). (a) (*1*) *G. domesticus*, (*2*) *D. rerio*, and (*3*) *H. sapiens*; (b) *C. insignis* embryos at early cleavage stages; (c) *C. insignis* embryos at later stages of cleavage; (d) *C. kolensis* embryos before CD; and (e) *C. kolensis* embryos after CD. On the graphs, vertical bars indicate standard deviation (DIALUX 20EB).

time. Figure 3 presents photographs of nuclei of the *Cyclops A. incolotaenia* at the stage of chromatin diminution (Fig. 3a) and at the stage of late embryonic development (Fig. 3b). In Fig. 3a, Feulgen-positive granules of eliminated DNA can be observed, which serves as evidence of CD in this species. Figure 3b shows *A. incolotaenia* embryos at the late stage of embryonic development, probably after diminution. In the center of each embryo, there are two or three large nuclei not undergoing diminution; they are surrounded by smaller nuclei, in which some of the DNA has already been eliminated.

According to the results of cytophotometrical analysis, in *D. galbinus* the percent of eliminated DNA amounts to around 95.5–96.2%, whereas the size of the pre- and postdiminution genome differs significantly and amounts to 8.27 ± 1.01 pg and 0.36 ± 0.08 pg, respectively (table).

As a result of analysis of the DNA content in the *M. leuckarti* germ and somatic cells, we observed an amount of DNA in somatic cells that was reduced by 7–10% compared with the germ ones (table). Such a low level of differences allows it to be suggested that there is an absence of CD in this species. Nevertheless, we cannot rule out as well the presence of a small DNA elimination. The good reproducibility of results for the cyclops collected in different habitats is a good corroboration of this (table). At the same time, the DNA

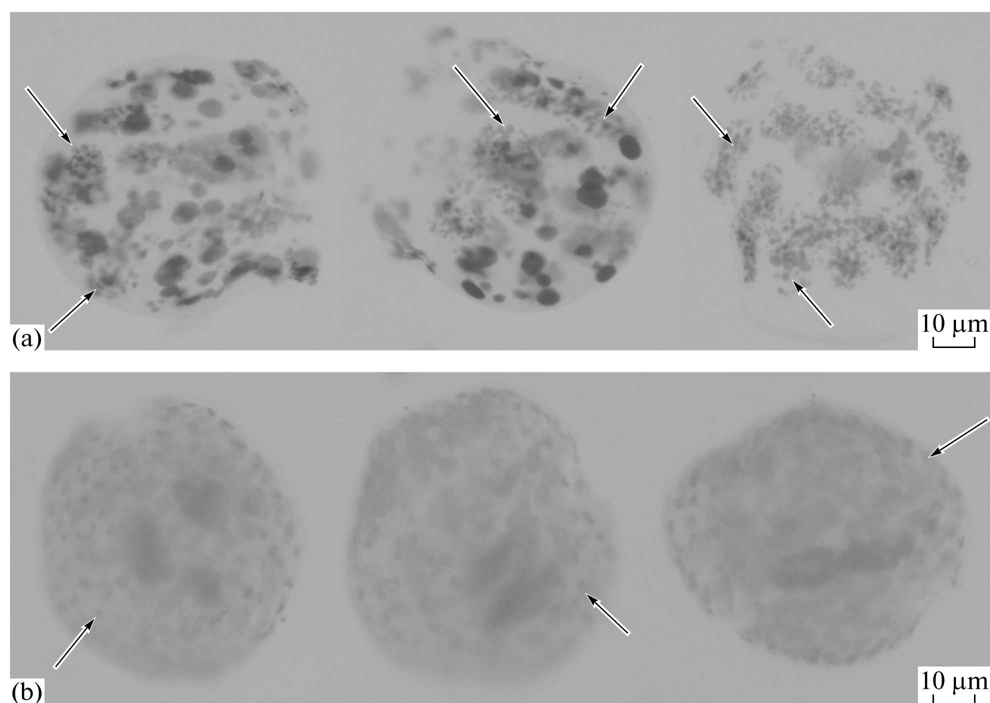

Fig. 3. Embryos of *A. incolotaenia* (Feulgen staining).
(a) CD in *A. incolotaenia* embryos; (b) *A. incolotaenia* embryos at later stages of cleavage, after CD. Arrows indicate granules of (a) discarded DNA and (b) presomatic cells after CD. Ob. 40×.

content in this species in somatic cells (2C) amounts to 1.56–1.60 pg and is almost twice the value (0.76 pg) established earlier (Wyngaard and Rasch, 2000; Rasch, Wyngaard, 2006b). This discrepancy may be due to population differences in the *Cyclops* used for analysis: Rasch and Wyngaard used *M. leuckarti* of the European population (Germany). Elucidation of this problem requires carrying out additional comparative analysis.

The obtained data indicate the absence of a certain "critical" DNA mass necessary for realization of CD in the somatic cell genome. Thus, in diploid postdiminutional cells, the DNA content amounts to 0.7 pg (*D. galbinus*), whereas in species with no CD these values are equal to 1.6 pg in *M. leuckarti* and 6.8 pg in *C. insignis* (Semeshin et al., 2011). It is to be noted that cytometric analysis of 11 *Cyclops* species of the Genus *Mesocyclops* (Rasch and Wyngaard, 2006b) revealed a significant interspecies variability in the DNA content from 0.54 pg in *M. thermocyclopoides* to 3.0 pg in *M. edax* in nuclei of somatic cells (2C). CD was observed in three species of this genus (in total, four species were studied on the subject of CD). It is to be emphasized that the quantitative DNA content in the genome of germ cells (2C) in these species also is in a wide range: from 8.43 pg in *M. longisetus* to 15.8 pg in *M. edax* (Rasch and Wyngaard, 2006b).

The data obtained during measurement of the DNA amount in nuclei of the germ cell pathway (oocytes, spermatocytes) or of early embryonic cells, as well as in nuclei of somatic cells, show that postdiminution genomes (of somatic cells) of different species of *Cyclops* resemble each other and genomes of the species with missing CD in DNA content much more. It is interesting to note that the maximum size of the somatic genome among *Cyclops* was revealed in *Cyclops insignis* and amounted to 3.4 pg (Semeshin et al., 2011). This value even exceeds the sizes of prediminution genomes of several species: *C. strenuus, C. furcifer*, and *C. divulsus* (Beermann, 1977; Wyngaard, 2006a).

All obtained here data suggest that CD (as a mechanism of "junk DNA removal" on early stages of organism development) allows rapid accumulation of redundant DNA in germ line cells and the details of this mechanism might be species specific. This fact is confirmed by studies dealing with prediminution DNA or eliminated DNA parts in *Cyclops*. Thus, the prediminution genome was shown to contain more repeated sequences (Degtyarev et al., 2004), ribosomal genes (Zagoskin et al., 2010), and tandemly repeated sequences from 2–9 bp (Drouin, 2006). Moreover, in the genome of germ cells *Cyclops kolensis*, intermicrosatellite sequences were found that had been removed completely from the genome of somatic cells (Zagoskin et al., 2008). Further data accumulation on CD in *Cyclops* is needed to shed light on problem of genome size paradox, and our methodical elab-

Amount of DNA (pg) in cells before and after chromatin diminution

Cyclops species (population, microscope used)	Prediminution development		Postdiminution development		Eliminated DNA, %;
	Anaphase, 2C	Metaphase, 4C	Late embryos, 2C; 4C	Swimming legs, female, 2C;	
Diacyclops galbinus, (Baikal, AxioLab)	16.53 σ = 2.02 *n* = 33 (16–32 cells)	31.6 σ = 3.22 *n* = 19 (metaphase, 16–32 cells) 31.76 σ = 2.00 *n* = 11 (prophase nuclei, 4–8 cells)	0.71 (anaphase) σ = 0.15 *n* = 15; –	0.63 σ = 0.09 *n* = 36	95.51–96.19%
M. leuckarti, (Baikal, AxioLab)	1.73 σ = 0.13 *n* = 4 (16–32 cells)	3.62 σ = 0.26 *n* = 5 (prophase nuclei, 16–32 cells)	1.55 σ = 0.14 *n* = 73; 3.32 σ = 0.36 *n* = 39 (64 and more cells)	1.60 σ = 0.15 *n* = 50	
M. leuckarti, (lake 1, AxioLab)	1.72 σ = 0.11 *n* = 34 (16 cells)	3.72 σ = 0.19 *n* = 5 (16 cells)	1.51 σ = 0.08 *n* = 8; 3.38 σ = 0.11 *n* = 3	1.62 σ = 0.07 *n* = 47	
M. leuckarti, (lake 3, AxioLab)	1.74 σ = 0.09 *n* = 22 (4 cells)	3.85 σ = 0.24 *n* = 3 (4 cells)	1.60 σ = 0.14 *n* = 7 3.54 σ = 0.22 *n* = 4 (64–128 cells)	1.56 σ = 0.31 *n* = 30	

Note: Arithmetic mean, σ (standard deviations), and number of measurements (*n*) are given.

orations on the application of digital microphotometry may be quite useful.

Thus, the method used for measurement of the amount of DNA allows the presence or the absence of chromatin diminution in the studied *Cyclops* species to be established. The method provides similar results in the case of using various optical systems of recording images (at any rate, for the tasks that were solved in the work). An increase in the dynamic range of the CCD camera allows reducing the dispersion of the obtained data. The methodical study performed in the work of the process of Feulgen staining of nuclei has shown the level of DNA hydrolysis used in our work to be acceptable and optimal for many species. Careful statistical processing of data has shown that, at least in the diapason of 1–17 pg, the nuclear DNA content can be measured with equal relative error. The measurement that were performed have allowed the range to be expanded of *Cyclops* species studied with respect to absence of chromatin diminution and establishing the presence of diminution in two *Cyclops* species inhabiting Lake Baikal to be established for the first time.

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