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MITOTIC AND MEIOTIC CHROMOSOMES OF THE GREAT RAMSHORN SNAIL *PLANORBARIUS CORNEUS* (LINNAEUS, 1758) (GASTROPODA, PLANORBIDAE) FROM LAKE KORTOWSKIE

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A b s t r a c t

An analysis of meiotic and mitotic chromosomes of *P. corneus* inhabiting Lake Kortowskie was made in order to verify the use of different tissues and colchicine treatments, the hypotonization time and two methods of chromosome slide preparation. In total, 30 chromosomal slides of six individuals were analyzed. The well spread chromosomes were introduced onto the slides by dropping a cell suspension of the mantle epithelium, foot and intestine of each individual, directly injected with colchicine, after 20 min of hypotonization. The karyotype was composed of 2n=36 biarmed chromosomes, thirty metacentrics with the rest being submetacentrics, NF=72. In the slides of the gonads the meiotic chromosomes in spermatogenesis were observed as being in prophase I (leptoten, zygoten, and diakinesis) and in telophase I. In diakinesis 18 bivalents were formed. No disturbances were observed during meiosis. The spermatozoa were typical of aquatic molluscs; consisting of a spherical head, a short midpiece and a long tail.

CHROMOSOMY MITOTYCZNE I MEJOTYCZNE ZATOCZKA ROGOWEGO *PLANORBARIUS CORNEUS* (LINNAEUS, 1758) (GASTROPODA, PLANORBIDAE) Z JEZIORA KORTOWSKIEGO

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Abstrakt

Analiza chromosomów mitotycznych i mejotycznych zatoczka rogowego *P. corneus* z populacji w Jeziorze Kortowskim pozwoliła na określenie warunków wykonywania preparatów chromosomowych. Łącznie analizowano 30 preparatów chromosomowych wykonanych z sześciu osobników. Najlepiej rozproszone chromosomy uzyskano na preparatach wykonanych metodą nakrapiania utrwalonej zawiesiny komórek na szkiełko. Komórki te pochodziły z nabłonka płaszcza, jelita oraz nogi każdego z osobników, które poddano iniekcji kolchicyną. Najkorzystniejszy czas hypotonizacji wynosił 20 minut. Kariotyp składał się z 2n=36 chromosomów dwuramiennych, 30 metacentrycznych i sześciu submetacentrycznych, NF=72. W preparatach z gonad przeprowadzono obserwację chromosomów w różnych stadiach spermatogenezy: profazy I (leptoten, zygoten, diakineza) oraz w telofazie I. W profazie mejozy I chromosomy tworzyły 18 biwalentów. Proces mejozy przebiegał prawidłowo. Plemniki były typowe dla mięczaków wodnych; składały się z kulistej główki, krótkiej wstawki i długiej wici.

Introduction

Molluscs are represented by about 130,000 species. Knowledge of their cytogenetic features has grown with the development of research techniques. A review of the number of chromosomes in metazoans (HARVEY 1920, after NAKAMURA 1986) included only 44 mollusc species. In the years 1930–1969, papers were published describing the karyotypes of 622 species and sub-species of molluscs, and contained the chromosome number of taxa representing the following classes: gastropods Gastropoda, bivalves Bivalvia, chitons Polyplacophora, cephalopods Cephalopoda and scaphopods Scaphopoda. Although most of the data concerned Gastropoda; the karyotype of more than 300 species of snail have been described, including about 20 species analyzed using banding chromosome patterns (NAKAMURA 1986).

Karyotypes of snails are mainly composed of biarmed chromosomes, meta- and submetacentric (THIRIOT-QUIEVREUX 1994, 2003). Gastropods traditionally classified as Pulmonata contain about 36 thousand terrestrial and aquatic species (JÖRGER et al. 2010) and they are relatively well recognized karyologically. This is an informal group of snails and slugs characterized by their ability to breathe air, by virtue of having a pallial lung instead of a gill, or gills. The chromosome number and karyotype of about 70 species is known (less than 0.5% of all species have been described). Chromosomal measurements and chromosome banding patterns using AgNOR staining, C and G banding techniques of 34 species have been described (NAKAMURA 1986, THIRIOT-QUIEVREUX 2003, VITTURI et al. 2005).

The Pulmonata species of the family Planorbidae proved to be interesting due to the large morphological diversity and still unresolved phylogenetic relationships. Within this family about 40 genera and about 160 species are recognized (MEIER-BROOK 2002, ALBRECHT et al. 2007), including 20 species

that occur in Poland (BOGDANOWICZ et al. 2008). The genus *Planorbarius* contains two species, *P. corneus* (Linnaeus, 1758) and *P. metidjensis* (Forbes, 1838). However, because of the high morphological diversity within *P. corneus* sensu lato, between 5 and 8 sympatric species have been recognized as being distributed throughout Ukraine, with *P. corneus, P. banaticus, P. purple, P. grandis* and *P. stenostoma* commonly occurring (STADNICHENKO 1990, after GARBAR and GARBAR 2007). Comparative cytogenetic studies concerning the structure of karyotypes and the structure of chromosomes stained by banding techniques, for example, can provide the diagnostic data for these species.

The great ramshorn snail *P. corneus* is widely distributed in Europe and Northern Asia. It commonly occurs in Poland in standing and running freshwater reservoirs that are strongly overgrown. It is a polytypic species, which means that it is divided into subspecies: *P. corneus arabatzis* (Reischütz, Reischütz & Fischer 2008), *P. corneus grandis* (Dunker, 1850) and *P. corneus corneus* (species nominative) (Linnaeus, 1758) (SEDDON and VAN DAMME 2011).

Although several previous studies have described the number of chromosomes (BURCH 1961, BOTTKE 1982) and recently also the structural karyotypes of several species of *P. corneus* sensu lato (GARBAR and GARBAR 2007), the morphological plasticity of this species was the inspiration to undertake the research by the authors of this report. Chromosomal studies of gastropods, including *P. corneus* are difficult and complicated due to a relatively low number of metaphase samples suitable for analysis being observed (GARBAR and GARBAR 2007).

Aquatic organisms, including freshwater gastropods, are the first to suffer from the effects of environmental pollution that contaminates water bodies. Animals inhabiting them are exposed to a progressive degradation of their living environment that may lead to changes in their functional morphology, including the level of genomes and chromosomes. One of the effects of the pollution of aquatic environments may be a disturbance in the process of meiosis for living organisms, such as gastropods (BARSIENE 1994).

The aim of this present study was the analysis of meiotic and mitotic chromosomes of *P. corneus* individuals inhabiting Lake Kortowskie. This was preceded by a verification of the possibility of applying the techniques of snail chromosomal preparations as described in the literature.

Material and Methods

The study was performed by using six individuals of *P. corneus* (Fig. 1) collected from Lake Kortowskie. Chromosomal preparations were performed

using a modified technique described in the available literature (GILL and CAIN 1980, YARAYABHAND et al. 1998, VITTURI et al. 2004, GARBAR and GARBAR 2007, LEITAO et al. 2009) and our own experience (WOŹNICKI and BOROŃ 2003, BOROŃ et al. 2004). After collection from the environment and during the subsequent research, snails were kept in a well-aerated aquarium.

Fig. 1. Great ramshorn snail *Planorbarius corneus*

To inhibit cell division at the metaphase stage of mitosis, snails were subjected to colchicine. For this purpose, the snails were divided into two groups. Three individuals were injected directly with 0.05% colchicine solution in the amount of 0.1–0.3 ml/animal, and then left in a small aquarium containing about 600 ml of water, for about 20 hours. The remaining three individuals were placed in a 0.02% aquatic solution of colchicine in a small aquarium containing about 600 ml of water, in a dark place for about 20 hours. After a specified time, the individuals from both groups were processed using the same procedure. Snails were sacrificed by placing them in an aquatic solution of 2-phenoxyethanol. After that, they were dissected and the fragments of the following tissues were collected: the epithelial cells of the mantle edge, pallial lung, foot, intestine and gonad. Tissues were subjected to two ways of preparation:

a) small pieces of tissue (3–4 x 3–4 mm) were placed in 0.075M KCl hypotonisation solution for 20 or for 30 minutes, at room temperature,

b) small pieces of tissue were ground in a glass homogenizer and the obtained cell suspension was subjected to hypotonisation in a 0.075M KCl solution for 20 or for 30 minutes, at room temperature.

After this time, the tissue (a) or suspension cells (b) were fixed in a solution of methanol and glacial acetic acid at a ratio of 3:1. In the case of tissue

fragments (a) fixation consisted of changing the fixative solution three times at intervals of 10–15 minutes. Whereas, the cell suspensions (a) were centrifuged three times for 10 min at 1000 rpm/min and after each centrifugation the fixative solution was exchanged with freshly prepared solution.

Chromosomal preparations were performed using the following techniques adequate for the tissue samples (1) and the cell solutions (2):

1. The tissues samples were placed on a microscope slide moistened with a fixative and then they were turned using preparative needles and forceps so that the surface of the tissue touching the slide left the exterior cells exposed to hypotonisation and fixation.

2. The 'splash' technique was used as follows:

a) cell suspensions were dropped with a pipette onto microscope slides from a height of about 30 cm,

b) a small volume of the cell suspensions were fixed in a mixture of a fixative composed of 55% methanol and 45% acetic acid.

After that, the cell suspension was dropped with a pipette onto a microscope slide but in a different way. A drop of cell suspension was placed on a microscope slide with a pipette and after a few seconds it was withdrawn back into the pipette. In this way we observed the cells arranged concentrically in rings formed after the evaporation of the fixative.

The chromosome slides were dried at room temperature for 24 hours, and then were stained with 5% Giemsa solution for 15 min. After that, they were rinsed in running water and then twice in distilled water and air dried at room temperature.

Analysis of chromosomal preparations and photographic documentation were performed using an Olympus BX51 light microscope equipped with a camera and MultiScan Karyotype software. Chromosomes were classified into morphological categories such as metacentric (Ms) and submetacentric (SMs) according to the method proposed by LEVAN et al. (1964).

Results

The best samples for observation and chromosomal spread were obtained by using the dropping cell suspension technique derived successively from the mantle edge (Fig. 2), the foot and the intestine of individuals which were directly injected with colchicine solution. The shorter hypotonisation time of 20 minutes was better than the longer time of 30 minutes. On the other hand, more metaphase plates were observed using the longer hypotonisation time, but most of them contained chromosomes that were rather clustered and not well spread out, which were not suitable for counting.

Finally, we found four metaphase plates with relatively well spread chromosomes for counting. The karyotype of *P. corneus* inhabiting Lake Kortowskie was composed of 2n = 36 biarmed elements; 30 metacentrics and 6 submetacentrics (30Ms + 6SMs), and the number of chromosome arms was NF=72 (Fig. 2).

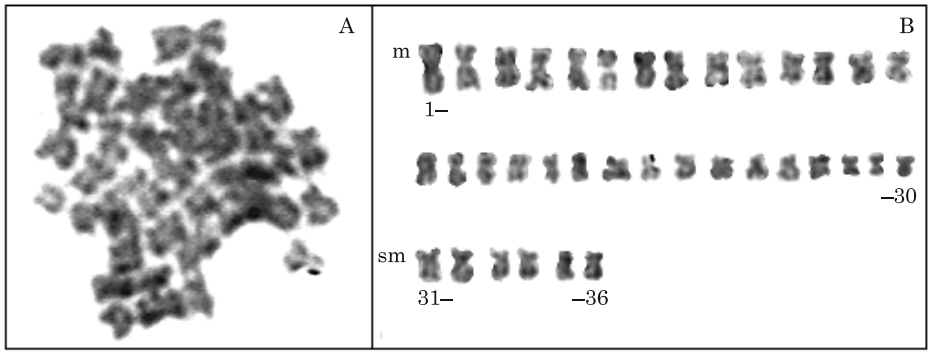

Fig. 2. Metaphase plate (A) and karyotype (B) of *P. corneus* from the cell of mantle edge. Chromosomes; M – metacentric; SM – submetacentric. Magnification 1000x

In the chromosomal slides of the gonads, obtained both by the dropping cell suspension technique and by turning a piece of tissue on the microscope slide, the different stages of meiotic chromosomes in spermatogenesis were observed as follows: prophase I (leptoten, zygoten, chromosomes in an early diakinesis with visible centromeric constrictions and bivalents visible during diakinesis) (Fig. 3 A – G), and the chromosomes during the telophase I stage (Fig. 3 G). In the prophase of meiosis I, the chromosomes formed 18 bivalents, and their number confirmed the diploid number of 2n = 36 chromosomes as a characteristic of this species. In the meiotic chromosomal slides, spermatozoa were also observed, which consisted of a spherical head, a short midpiece and a long tail (*flagellum*) (Fig. 3 H, I).

Discussion

The preparation of chromosomal slides of *P. corneus*

The chromosomes may be obtained from any cells that are actively dividing. Usually in molluscs, they can be obtained from the cells of various tissues, e.g., the mantle epithelium, the kidney, the gonads, tissues of the embryo as

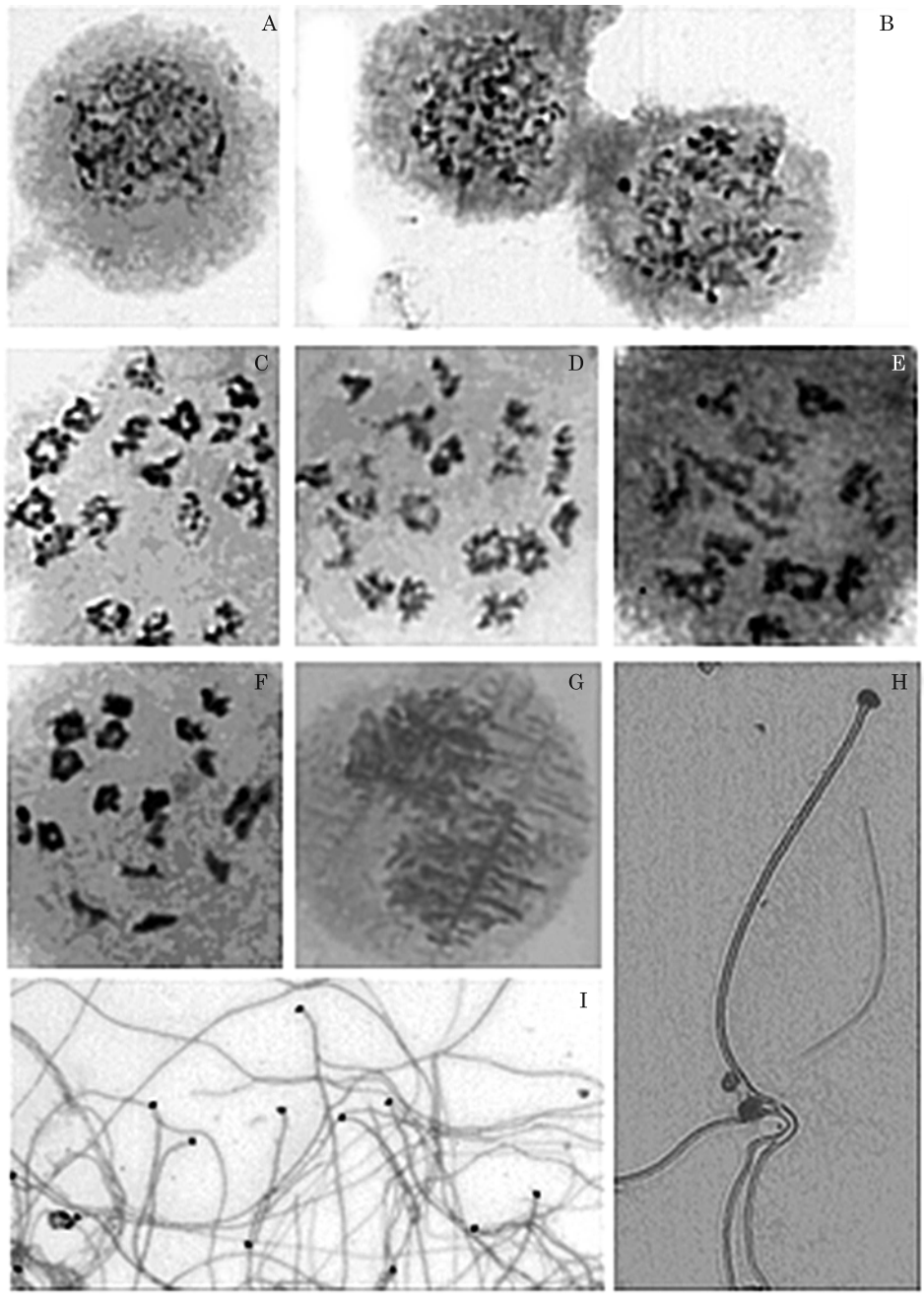

Fig. 3. Meiotic chromosomes in spermatogenesis. Prophase I: leptoten (A), zygoten (B), chromosomes in an early diakinesis (C-D) with visible centromeric constrictions, diakinesis (E-F) with visible bivalents. Telophase I (G). Spermatozoa (H), magnification 400x. Spermatozoa (I), magnification 200x

well as others (NAKAMURA 1986). We had prepared chromosomal slides from five different tissues and most metaphase plates were observed in cells derived from the mantle edge, the intestine and the foot. A good source of cells is also the gonad used mainly for the observation of meiotic chromosomes (GARBAR and GARBAR 2007), which were used in the studies presented here.

Most of the cytogenetic studies of molluscs, including gastropods, refer to the number of chromosomes. There is much less data relating to the karyotype structure of these animals, and even less is known about the structure of the chromosomes. The relatively poor state of knowledge of molluscs is related to the small size of the chromosomes (~ 10 mm) and the lack of research techniques that allow a large number of metaphase plates to be obtained (NAKAMURA 1986).

An analysis of the available literature indicates that in karyological studies *P. corneus* achieved a relatively low number of metaphase plates, which is similar to what was observed in the present paper. In the chromosomal slides prepared from 5 to 35 individuals, there were between 1 and 13 metaphase plates analyzed (GARBAR and GARBAR 2007). In the present study, a relatively large number of 30 chromosomal slides made from the cells of different tissues (the epithelial edge of the mantle, the pallial lung, the foot, the intestine and the gonad) of six individuals were analyzed. Despite the fact that a relatively high number of metaphase plates were observed, only four of them were suitable for a determination of the karyotype.

The best metaphase cells were obtained from the mantle edge, the gonad and the foot after 20 minutes of hypotonisation, and perhaps that time could be shorter. Getting a low number of metaphase plates may be a result of a lack of colchicine activity which could not reach all the tissues. What is important is the mode of how the colchicine is introduced into the snail body; colchicine injection directly into the body was a better method of obtaining proliferating cells than incubating the whole animal in an aquatic colchicine solution.

Mitotic and meiotic chromosomes of *P. corneus*

In snails, cytogenetic observations of both mitotic and meiotic chromosomes are important (NAKAMURA 1986) as described in the presented study. Although in several metaphase plates the chromosomes were clearly visible, which allowed the determination of their characteristic diploid 2n = 36, and four of them were sufficient enough spread to arrange a karyotype. That being said, the results obtained can be regarded as good since the cytogenetic data of *P. corneus* published so far clearly show that it is rather difficult to obtain mitotic metaphase chromosomes of this species (MAKSIMOVA 1995, after GAR-

BAR and GARBAR 2007). The experimentally determined conditions presented in this study for making the chromosome preparations of this species seem to be highly recommendable.

Chromosomes of *P. corneus* in prophase of the first meiotic division, and chromosomes in the metaphase stage of the second meiotic division were for the first time described by MAKSIMOVA (1995), after GARBAR and GARBAR (2007). The number of chromosomes in the haploid set ranged from n = 15 to n = 20, with a predominance of n = 18. According to the author of this cited paper, the variation of this number might be due to the presence of additional chromosomes, but it has not been confirmed by GARBAR and GARBAR (2007) and similarly by the results under this study.

The karyotype of *P. corneus* obtained in this present work contained 2n = 36 chromosomes, and has been previously described in detail by GARBAR and GARBAR (2007). The relative length of chromosomes ranged from 8.42 (1 pair) to 3.69% (18 pair), whereas their total length TCL (total complement length) was 156.56 ± 5.91 microns. The karyotype was arranged according to the size of the chromosomes containing six submetacentric chromosomes (pair numbers: 2, 14 and 17) and thirty metacentric chromosomes (other pairs) and can be presented using the following formula: 2n = 30M + 6SM. The number of chromosome arms was FN = 72. This same karyotype was found in the individuals collected from Lake Kortowskie.

A comparative meiotic chromosome analysis of four morphologically distinct species of the genus *Planorbarius*, viz. *P. corneus, P. banaticus, P. purpura* i *P. grandis* from Ukraine showed no differences in the karyotypes of these species. The karyotype pattern of all species was the same: 2n = 36; 30M + 6SM and FN = 72. This karyotype did not differ significantly in terms of the total and the relative length of the chromosomes and the centromeric index (GARBAR and GARBAR 2007).

In the chromosomal slides made from the gonads, the relatively rare mitotic divisions of the oogonia or spermatogonia were observed, while it is relatively easy to obtain and visualize the snails' meiotic chromosomes forming bivalents (BURCH and PATTERSON 1965, after NAKAMURA 1986). The observed number of bivalents during meiosis, amounting to 18, confirmed the number of mitotic chromosomes. The disturbances in the process of meiosis of gastropods living in the polluted aquatic environments have been detected (BARSIENE 1994). However the observed different stages of meiotic chromosomes in spermatogenesis of investigated individuals indicated the normal process of meiosis.

The documented appearance of sperm which is characterized by a simplified construction should be emphasized. Spermatozoa of the great ramshorn snail are typical for animals using external fertilization, these aquatic animals

use so-called „primitive" sperm. Generally, „primitive" sperm have a head, a short midpiece and a long tail (FRANZÉN 1970). In the current literature, data on sperm morphology among the various groups of molluscs has been used in systematics and phylogeny (DROZDOV et al. 2012).

The results presented here do not reveal any differences between the karyotype of the great ramshorn snail from Lake Kortowskie and the karyotypes formerly reported in the published literature, but only confirmed data on the karyotype of this species. However, the results contributed new data on meiotic chromosomes, and the spermatozoa of this species. Insightful observation of meiosis may in the long-run perspective allow the recording of disturbances in this process among snails, caused by water pollution.

Conclusions

The great ramshorn snail is characterized by a karyotype containing 2n = 36 biarmed chromosomes, *viz.* 30 metacentric and 6 submetacentrics, NF = 72. During the prophase stage of meiosis I, 18 bivalents are observed in this species. The meiosis process in the gonads of *P. corneus* inhabiting Lake Kortowskie did not show any disturbances. The sperm of this species consists of a spherical head, a short midpiece and a long tail and are typical of aquatic molluscs.

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