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MODIFIED METHOD OF METAPHASE PLATES OBTAINING FOR POLYPLOID FISH GENERA *CARASSIUS* AND *COBITIS* KARYOTYPING (ACTINOPTERYGII, CYPRINIFORMES)

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Modified Method of Metaphase Plates Obtaining for Polypoid Fish Genera *Carassius* and *Cobitis* Karyotyping (Actinopterigii, Cypriniformes). Pukhtayevych, P. P. — Modern methods for obtaining metaphase plates from the somatic cells of fish, most effective in the study of polyploid species, were selected and tested. The technique with $CoCl_2$ and colchicine treatment is recommended on the basis of empirical data for representatives of the genera *Carassius* and *Cobitis*. The detailed description of this modified technique and characteristics of metaphase plates of *Carassius auratus* Linnaeus, 1758, *Carassius carassius* Linnaeus, 1758 and triploid form of *Cobitis taenia* Linnaeus, 1758 received under tested method is presented in the paper.

K e y w o r d s: karyology, Osteichthyes, metaphase plate, colchicine.

Модификация методики получения метафазных пластинок при кариотипировании полиплоидных рыб родов *Carassius* и *Cobitis* (Actinopterigii, Cypriniformes). Пухтаевич П. П. — Отобраны и апробированы современные методики получения метафазных пластинок соматических клеток рыб, дающих наибольший эффект при исследовании полиплоидных видов. На основании эмпирических данных в отношении представителей родов *Carassius* и *Cobitis* рекомендуется методика с использованием $CoCl_2$ и колхицина. В статье приведено подробное описание модифицированной методики и полученные в результате её применения метафазные пластинки *Carassius auratus* Linnaeus, 1758, *Carassius carassius* Linnaeus, 1758 и триплоидной формы *Cobitis taenia* Linnaeus, 1758.

К л ю ч е в ы е с л о в а: кариология, костные рыбы, метафазная пластинка, колхицин.

Introduction

Two basic methodical approaches: direct (in vivo) and indirect (in vitro), are currently applied for obtaining mitotic metaphase of animals. The first approach is rather simple, inexpensive and allows quick result. It is associated with the use of colchicine that blocks proliferative cells of kidneys, spleen, gill epithelium, etc. at the metaphase (Kligherman, Bloom, 1977; Hartley, Horne, 1983, and others). Main disadvantage of this approach is a small number of metaphases produced in preparations.

Indirect approach involves prior cultivation of isolated organism's cells on the growth media in vitro (Gold, 1979; Orlov, Bulatova, 1983; Hartley, Horne, 1983; MacGregor, Varley, 1986, and others). This sort of techniques makes it possible to get much better results, as in such a case the stimulators of mitosis, like phytohemagglutinin (PHA) and fetal calf serum (FCS), are applied (Hartley, Horne, 1983). However, this approach requires special equipment, high quality serum and PHA, takes more time than the direct approaches, and is related with a risk of bacterial infection. Besides, changes in the number and morphology of chromosomes may take place in cell cultures, especially in long-term ones (Amemiya et al., 1984).

Application of the stimulators of mitosis in the direct approaches could be regarded as a compromise. That sometimes enables to substantially improve the quality and increase the number of metaphase plates produced in preparations (MacGregor, Varley, 1986; Manilo, 1986).

Contrary to the intensive development of cytogenetic investigations in different groups of the vertebrates during last decade, such studies of fish were not so successful and large-scale (Hartley, Horne,

1985). The reason apparently is relatively large number of small-sized chromosomes in their diploid sets (Gold, 1979).

Attempts to use the tissues of embryos (Simon, 1963), as well as juvenile and mature individuals: like epithelium of scales and gills, anterior kidney, testis, heart tissues (Lakra, Bhondae, 1996; Völker, Kullmann, 2006; Kalous et al., 2010, and others), were carried out as to improve existing techniques for producing of karyological preparations of fish.

Cytogenetic studies of fish are developing intensively during recent decades due to gradual improvements in the techniques for obtaining chromosomal preparations. Mitosis stimulants, such as phytohemagglutinin (PHA), are commonly used for fish injection before colchicine treatment (Lin, 1982; Gold et al., 1990; Baruffaldi et al., 1992). This substance can induce synthesis of the chromosomal DNA and transition of cells that were nonproliferating in norm, into the mitotic cycle of division. Mitogen of American Pokeweed, that derived from the roots of *Phytolacca americana* (Fames et al., 1964) and is similar to the PHA, is applied quite frequently. That mitogen is characterized by low leuco-agglutinative properties, and is particularly highly effective in cases when the PHA does not allow achieving necessary results. Good potential for cytogenetic study of fish has application of phenylhydrazine (Cucchi, Baruffaldi, 1988), horse serum (Ojima, Kurishita, 1980), cobalt chloride (Cucchi, Baruffaldi, 1990), concavalin A (Banerjee, 1987) and yeasts (Oliveira et al., 1988).

At the same time, current techniques for producing karyological preparations of fish are not always sufficiently rewarding and require some improvements, especially regarding polyploid species. The number of chromosome preparations with high quality metaphase plates is influenced by such factors as the method of fishery and period of specimen delivery to the laboratory, experimental conditions, research equipment, season, etc.

Therefore, objective of the study is the selection and modification of most productive and available techniques for obtaining metaphase plates of fish in our conditions that would be relevant for further analysis.

Material and methods

The material for the study were specimens of spined loaches and crucian carps fished out in six different water reservoirs of Zhytomyr oblast, Ukraine (table 1) during June and July, 2013. Examined localities are represented by rivers, forest lakes, canals and marshes.

Most effective one for the study of Osteichthyes genera *Cobitis* and *Carassius* proved to be our modified technique, based on air-drying of slides (Ráb, Roth, 1988; Cucchi, Baruffaldi, 1990; Boroñ, 1994). It is based on usage of cobalt chloride solution, as a stimulator of mitosis, and colchicine solution for working with kidneys.

The technique that has been applied for obtaining metaphase plates is rather multistep, thus it is described successively.

Preliminary treatment. Cobalt chloride at the rate of 1 ml of a $CoCl_2$ / per 100 g body mass (0.1 % solution for crucian carps and 0.03 % solution for spined loach was administered intraperitoneal; then fish were placed into well-aerated tanks for one day. After 24 h, 0.1 % solution of colchicine at the rate of 1 ml / per 100 g body mass was applied. After 1.5–2 h, the following procedures were carried out.

Kidney isolation and preparation of suspension. Widest part of the kidney was removed from fish body, after that it was dissected by microscopic needles with adding of 3–4 drops of hypotonic solution up to formation of homogeneous suspension

Hypotonization and preliminary fixation. Using a syringe, cell suspension was placed into centrifuge tubes with 5 ml of a 0.075 M KCl hypotonic solution. Hypotonic treatment was performed in an incubator at 37 °C for 25–40 min; period of treatment was determined empirically for different species of fish. After hypotonization, 5 drops of freshly prepared cold fixative (3 methanol: 1 acetic acid) were added to the

Table 1. Materials used in karyologycal analysis

Таблица 1. Материал, использованный для кариотипического анализа

Place of collection	Species	Number of specimens	Sex	
			♀	♂
Baranivka District, Virlya Village (canal)	*C. taenia*	11	11	–
Novograd-Volynskyi District, Nova Romanivka village (tributary of the Sluch River)	*C. taenia*	9	9	–
Romaniv District, Hodyha Village (marsh)	C. *carassius*	5	4	1
Romaniv District, Vyla Village (forest lake)	C. *carassius*	15	12	3
Chervonoarmiisk District, Trudove Village (forest lake)	C. *auratus*	13	9	4
Zhytomyr District, Buki Village (lake)	C. *auratus*	7	6	1

tubes and suspension was gently stirred with a Pasteur pipette. Later, 5 ml of fixative were added in 5 min, and then the suspension was stirred once more.

Centrifugation and fixation. Final suspension was centrifuged at 1300 rpm for 10 min. Supernatant then was gently removed and 5 ml of freshly prepared cold fixative was added and stirred. The suspension was left at 4 °C for 25 min, and then was centrifuged. The last procedure was repeated 3 times. Totally, centrifugation was fulfilled 4-times and general period of fixation was at least 2 h.

Slides preparation. After the last fixation, supernatant was removed, a few drops of fixative were added to the cell suspension, all gently stirred and 3–4 drops of the concentrated cell suspension were dropped onto cold, wet slides from a distance of about 10–30 cm. These slides were dried over fire or under an electric lamp, washed in ethanol and dried again. The slides were stained in azure-eosin solution by Romanovsky (1891), for 15 min.

Metaphase plates were analyzed and their photographs were taken under the light microscope Delta Optical Genetic Pro. The slides with most distinct morphology of chromosomes that were used for karyograms and further comparative karyological study were selected for taking pictures. The chromosome forms were defined according to classification by Levan and others (Levan et al., 1964).

Results and discussion

Investigations were based on two diploid species of the genus *Carassius*, which are ancient amfidiploids (Vasiliev, 1985), and triploid representatives of *Cobitis taenia* Linnaeus, 1758. Studied species of the genus *Carassius* were *C. auratus* Linnaeus, 1758 and *C. carassius* Linnaeus, 1758, diagnosed by biochemical gene marking.

Application of proposed modified technique for producing chromosomal preparations resulted in obtaining metaphase plates (fig. 1) suitable for further analysis. Received results are presented in .

About a hundred metaphase plates of each species in the whole were analyzed. Under the execution of all technical requirements of the method, the percent of slides with metaphase plates verged towards 90 % and the number of plates applicable for morphological analysis was 60 %. Such result is quite successful, since provides with adequate amount of material for analysis and allows realization of complete cytogenetic study in diploid-polyploid complex of fish.

Karyotyping is the most reliable method to check the ploidy. This is direct method, based on counting the number of metaphase chromosomes in somatic cells. Due to the fact that kidneys of fish have hematopoietic function and their cells are regularly dividing, this organ is most suitable for kariological study and assessment of ploidy level.

Treatment by a solution of colchicine is the standard procedure for majority of current cytogenetic techniques applied to fish. This chemical can block mitotic division of a cell during the metaphase. Usage of cobalt chloride was revealed to be effective for stimulation of cell division. Cobalt chloride stimulates formation of erythropoietin, leading to increase of proliferative cells in hematopoietic organs (Webb, 1962).

Optimal concentrations of cobalt chloride were applied for producing the karyologic preparations; those concentrations had not resulted in the death of the examined fish during preliminary treatment and had assisted to obtain high quality metaphase plates.

Table 2. Karyotypes of fish species studied

Таблица 2. Кариотипы исследованных видов рыб

Species	Number of specimens	Number of chromosomes	Karyotype			
			m	sm	sta	nf
C. taenia	20	3n = 75	29	33	13	137
C. auratus	20	2n = 100	22	32	46	154
C. carassius	20	2n = 100	20	40	40	160

Note. Abbreviations indicate: nf — number of chromosome arms.

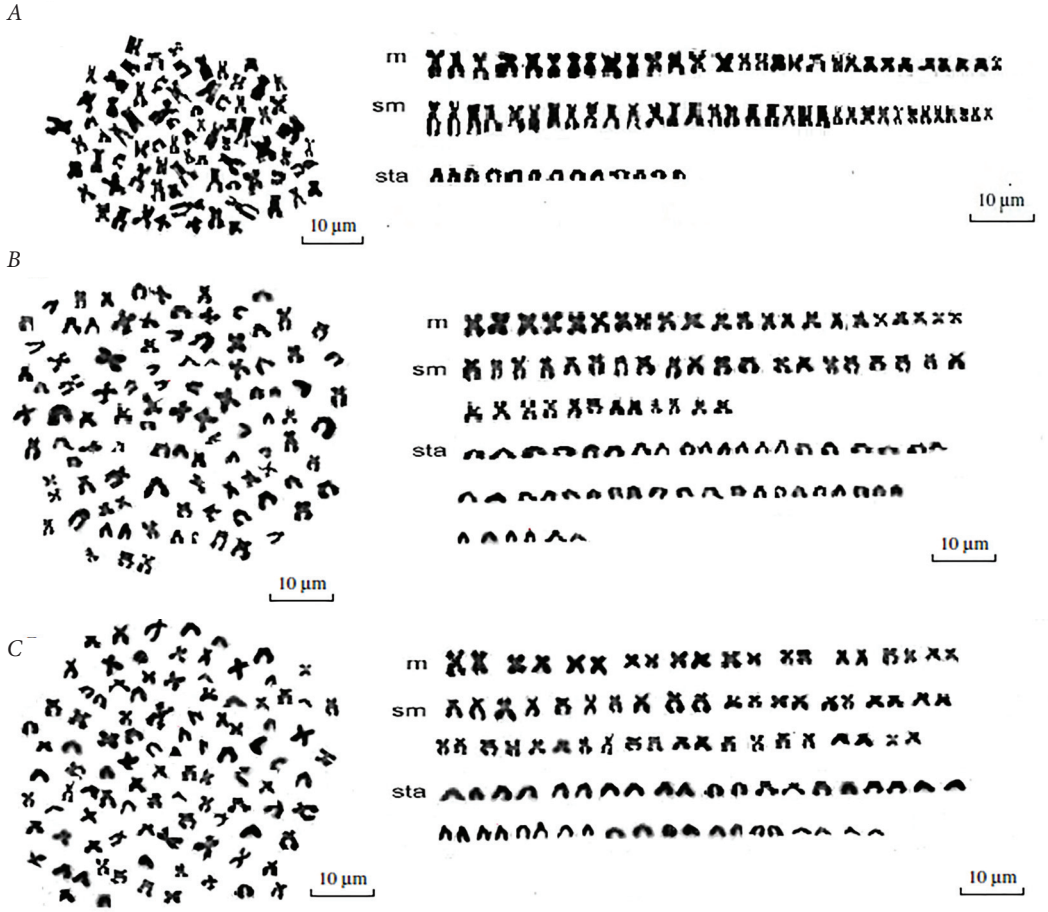

Fig. 1. Mitotic metaphases and karyograms of studied fish species: *A* — *C. taenia*; *B* — *C. auratus*; *C* — *C. carassius*. Abbreviations indicate: m — metacentric; sm — submetacentric; sta — subtelo- and acrocentric chromosomes.

Рис. 1. Митотические метафазы и кариограммы исследованных видов рыб: *A* — *C. taenia*; *B* — *C. auratus*; *C* — *C. carassius*.
Условные обозначения: m — метацентрические; sm — субметацентрические; sta — субтело- и акроцентрические хромосомы.

Conclusions

The applied technique for producing the preparations of mitotic chromosomes was effective for diploid-polyploid complexes of fish. Its application provided an opportunity, in a relatively short period of time, of obtaining metaphase plates that were suitable for further quantitative and qualitative analysis.

These obtained metaphase plates are characterized by sharp boundaries; individual chromosomes are clearly identified and densely arranged within the plate; the number of overlapping chromosomes is minor and that allows karyotyping and assessment of chromosomes' morphological types.

Used technique made it possible for the first time to carry out a cytogenetic examination of fish in Ukraine. Polyploid members of the genera *Carassius* and *Cobitis* were studied as an example. The number of chromosomes was established and

the morphological analysis was carried out. This technique with minor modifications can also be successfully tested on other representatives of freshwater and salt water fish taxa.

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