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RESEARCH PAPER

Methodology for intravital mitotic chromosome preparation from regenerated tissue derived from the tail tips of tadpoles

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Abstract. We propose a modified and updated protocol to obtain mitotic chromosomes from the regenerated tissue of *Pelophylax* tadpole tail tips. Chromosomal preparations from regenerated tissue results in high-quality and clean slides suitable for further staining and study. Tadpoles remain alive, undergo minimum suffering, and can be grown to adulthood for further investigation. The method could be used for other groups of Anura and modified for other species with the ability to regenerate their tissues.

Key words: karyoanalysis, *Pelophylax*, polyploidy, regeneration

Introduction

Metaphase chromosome data are of great importance for studying different groups of amphibians, including the hybridogenic complex of water frogs in the genus *Pelophylax*. The *Pelophylax esculentus* complex comprises diploid and triploid hybrid *P. esculentus* (Linnaeus, 1758) and their parental species, *Pelophylax lessonae* (Camerano, 1882) and *Pelophylax ridibundus* (Pallas, 1771). Hybrid frogs coexist and crossbreed with one or both parental species (Plötner 2005). Water frogs from the *P. esculentus* complex are of interest for cytogenetic studies of the phenomenon of hemiclonal inheritance, and are of also great interest regarding the composition and sustainability of their population systems. In natural population systems, some offspring are not viable due to a specific set of genotypes inherited from the parents (Günther & Plötner 1988, Plötner 2005, Shabanov et al. 2020). Selective death of specific genotypes leads to differences in the composition of the same generation at different life stages (Biriuk et al. 2016a). However, investigation of the phenomenon of selective death requires mass cytogenetic studies that can be conducted using two different approaches: 1) carrying out a series of studies with numerous samples from the same generation at different stages of development; 2) intravital (i.e. performed on a living individual) identification of genotype of the same individuals, which permits their fate to be tracked. While the second approach is more informative, its implementation requires methods that allows non-invasive chromosomal preparations of tadpoles. Gaining clean and qualitative chromosome preparations is vital for subsequent treatment with dyes and microscopy. For example, for fluorescent *in situ* hybridization (FISH) that allows *P. lessonae* and *P. ridibundus* genotypes to be distinguished (Ragghianti et al. 1995). Since there are no clear morphological features that allow larvae of different *Pelophylax* species to

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be distinguished, or between hybrid tadpoles of different ploidy, FISH remains the most reliable method for studying the genome compositions of tadpoles from different population systems.

Methods to obtain chromosomes vary in cost, complexity, and time needed for each step. The most common tissue used for chromosome preparations of water frogs is the intestine, though the testes (for males), bone marrow (for adults and tadpoles), and gills (for tadpoles) can also be used (Biriuk et al. 2016b). The squash method was the first used for *Pelophylax* chromosome preparations (Wickbom 1945). Subsequently, preparations were made by dropping cell suspensions on to heated slides (Sumner et al. 1973). Methods were further modified with prolonged centrifugation and the addition of supplementary steps and reagents (Macgregor & Varley 1988, Ogielska 2009). The essential problem with all these techniques is the necessity to sacrifice animals. Therefore, methods to obtain chromosomal preparations that do not require the animal to be killed are needed. Chromosome preparations from cultured cells may not always be suitable due to their cost and complexity.

Intravital methods to obtain chromosomes and nucleoli have been used for the Caudata (i.e. Fankhauser 1938, Ting 1950), but these methods have two serious disadvantages that complicate their current use. Both were using outdated reagents and solutions (such as Bouin's fluid that contains formaldehyde) with preparations made with the squash method, which require quite sophisticated skills and often results in poor-quality results. Using tail tips was shown for Anuran tadpoles (i.e. Bogart 1968, Menzies & Tippett 1976, George & Lennartz 1980). However, in first two cases tail tips of growing tadpoles were used instead of regenerated tissue and avoidance of killing the subjects was not the goal. Menzies & Tippett (1976) use the word "regenerate" but in fact they cut the tail tip and let it proliferate for a couple of hours before adding colchicine. Our previous studies have shown that using a similar method for wild-caught *Pelophylax* tadpoles at late stages of development (after stage 30 according to Gösner 1960), results in the absence of both nuclei and chromosomal plates (Sherstiuk et al. 2016). Treatment in colchicine dissolved in 0.07 M KCl solution results in multiple nuclei without chromosomal plates suitable for counting (Lukan et al. 2017). George & Lennartz (1980) used actual regeneration in *Xenopus laevis*, but did not use colchicine and did not obtain chromosomal plates, since they were interested only in studying nucleoli.

In this paper the updated and modified intravital method with a step-by-step protocol for obtaining mitotic chromosomes from *Pelophylax* tadpoles that takes advantage of their ability to regenerate is presented.

Material and Methods

Female *P. esculentus* from the Lower Dobrytskyi Pond (NNP "Homilshansky lisy", Kharkiv region, Ukraine, 49°55′ N, 36°30′ E) and male *P. ridibundus* from Pisochne Pond (Volyn region, Ukraine, 51°56′ N, 23°91′ E) were used for artificial crossing in December 2018. In natural population systems hybrid *P. esculentus* usually coexist with one or both parental species (*P. lessonae* and *P. ridibundus*) and can crossbreed with each other. Therefore, the artificial crossing that was performed corresponded with a process that occurs in the wild.

Pelophylax species used for crossings are not listed in the IUCN Red List of threatened species or the Red Data Book for Ukraine. All procedures for gamete and tissue collection were performed in a way that minimized distress to the animals in accordance with guidelines for the use of live amphibians and reptiles in field and laboratory research of the Herpetological Animal Care and Use Committee (HACC) and Ukrainian law for the protection of animals from cruelty. Karyological work with amphibians was approved by the committee on bioethics of V. N. Karazin Kharkiv National University (minutes №4, 21. 04. 2016).

To stimulate gamete production, the male frog was injected with 2 µl of surfagon, a synthetic analogue of luteinizing hormone (concentration 5 µg/ml, "Lanshim", Ukraine), two hours before crossing, and the female was injected with 2.5 µl of surfagon 24 hours before crossing (Bobrova et al. 2014). Fertilization was carried out in plastic Petri dishes filled with fresh water. A few days after fertilization, embryos were transferred to separate plastic tanks (0.5-1 litre of freshwater per tadpole) which were aerated using a compressor at room temperature (20-25 °C). Water was changed once every day or every other day. The tadpoles were fed with TetraMin (Tetra®, Germany) flakes daily. The stages of tadpole development were identified following Gösner (1960).

Six randomly selected tadpoles at stages from 28 (hind limb bud development) to 33 (with paddle-

Tissue type	Intestine	Gills	Regenerated tail tip
Tissue collection	1. Dissect anesthetized tadpole from ventral side 2. Cut the whole intestine 3. Clean it from digestion products	1. Dissect anesthetized tadpole from ventral side 2. Cut the gills	1. Cut the tail tip 2. Wait for regeneration for 1 week 3. Cut the regenerated tissue
Colchicine treatment	12-14 h (incubate in colchicine solution the whole tadpole before the tissue collection)	12-14 h (incubate in colchicine solution the whole tadpole before the tissue collection)	4 h (incubate the cut regenerated tail tip)
Incubation in hypotonic solution	20 min	20 min	20 min
Carnoy's fixation	30 min	30 min then change	30 min then change
Centrifugation	250 g for 5 min	no	no

70% Acetic acid fixation

Putting 3 drops onto the slide heated to 60°C

Drying at 60°C for 1.5-2 h

Fig. 1. Schematic protocol of chromosome preparations optimized for each type of tissue.

shaped feet) were anaesthetised with 2% lidocaine (local anaesthetic that blocks sodium channels to minimize tadpole suffering) and had 1/3 of their tails removed. Regeneration required restoration of a sufficient amount of tissue for analysis, which took about one week (some regenerated tissue can be obtained in less than a week, but not enough for preparation of more than one slide).

Proposed protocol

The method proposed here uses regenerated tail tissue (Fig. 1). The regenerated tail tips were cut (Fig. 2) and placed in 0.4% colchicine for one to six hours to stop formation of the mitotic spindle. Tissue was then transferred to 0.07 M KCl for 20 min and fixed with Carnoy's solution (three parts of ethanol and one part of glacial acetic acid).

Small pieces (about 2 mm length) of the regenerated tail were homogenized in 100 µl of 70% glacial acetic acid. The remaining tissue can be stored in Carnoy's solution at 4 °C for a long period (months/years) or at room temperature for a shorter duration (days/weeks), but it is important

Fig. 2. Tadpole with regenerated tail tip (arrow) placed on wet fabric.

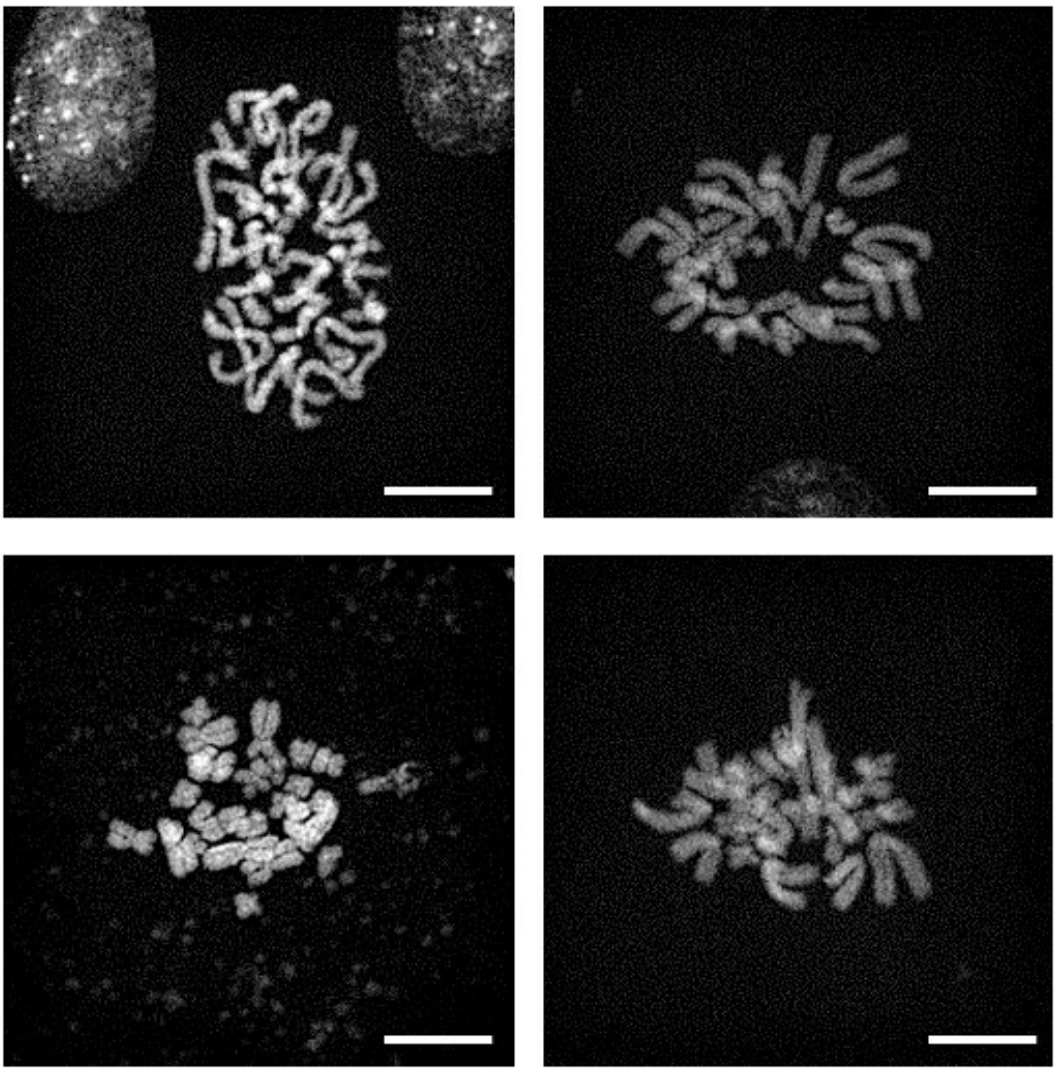

Fig. 3. Mitotic chromosomal plates from regenerated tissue of tadpole tail tips. Stained with DAPI. Scale bar – 10 μm.

to prevent the tissue from drying out by adding fresh Carnoy's solution to the tube.

After homogenization, 50 μl of cell suspensions were placed on a slide heated to 60 °C. Heating is not mandatory but it hastens drying and prevents formation of acetic acid crystals. The slides were left at 60 °C for two hours to prepare them for staining.

To compare with a proven method, the six tadpoles were placed in a 0.4% colchicine solution for 12-14 hours and then sacrificed using anaesthesia with 2% lidocaine for one minute. Gills and intestine were dissected, incubated in 0.07 M KCl, and stored in Carnoy's solution with three changes and centrifuging (250 g for 5 min) every 30 min (Sumner et al. 1973, Biriuk et al. 2016b with modifications). Staining was performed according to the combined method of Ragghianti et al. (1995) and Ogielska et al. (2004) with some modifications. Slides were treated with 0.005% pepsin at 37 °C for three minutes, washed in 1 × PBS (phosphate buffered saline), 2% PFA (paraformaldehyde) and again in 1 × PBS for 10 min at each stage, dehydrated in ethanol, air-dried and stained with DAPI (4',6-diamidin-2-fenylindol) (125 μg/ml) diluted in 1 × PBS. Slides were photographed with × 40 objective under a Leica DM 2000 microscope equipped with a standard fluorescence filter set ("DAPI 390" filter cube, excitation 395/25), with Leica DFC3000 G camera and LASX software. Several chromosomal plates were counted manually for each slide.

Results

After one-three hours of colchicine treatment no chromosomal plates were found. Numerical chromosomal plates and nuclei were observed after four and five hours of colchicine treatment (Fig. 3 and Fig. 4C). The mean number of chromosomal plates per one drop of cell suspension was

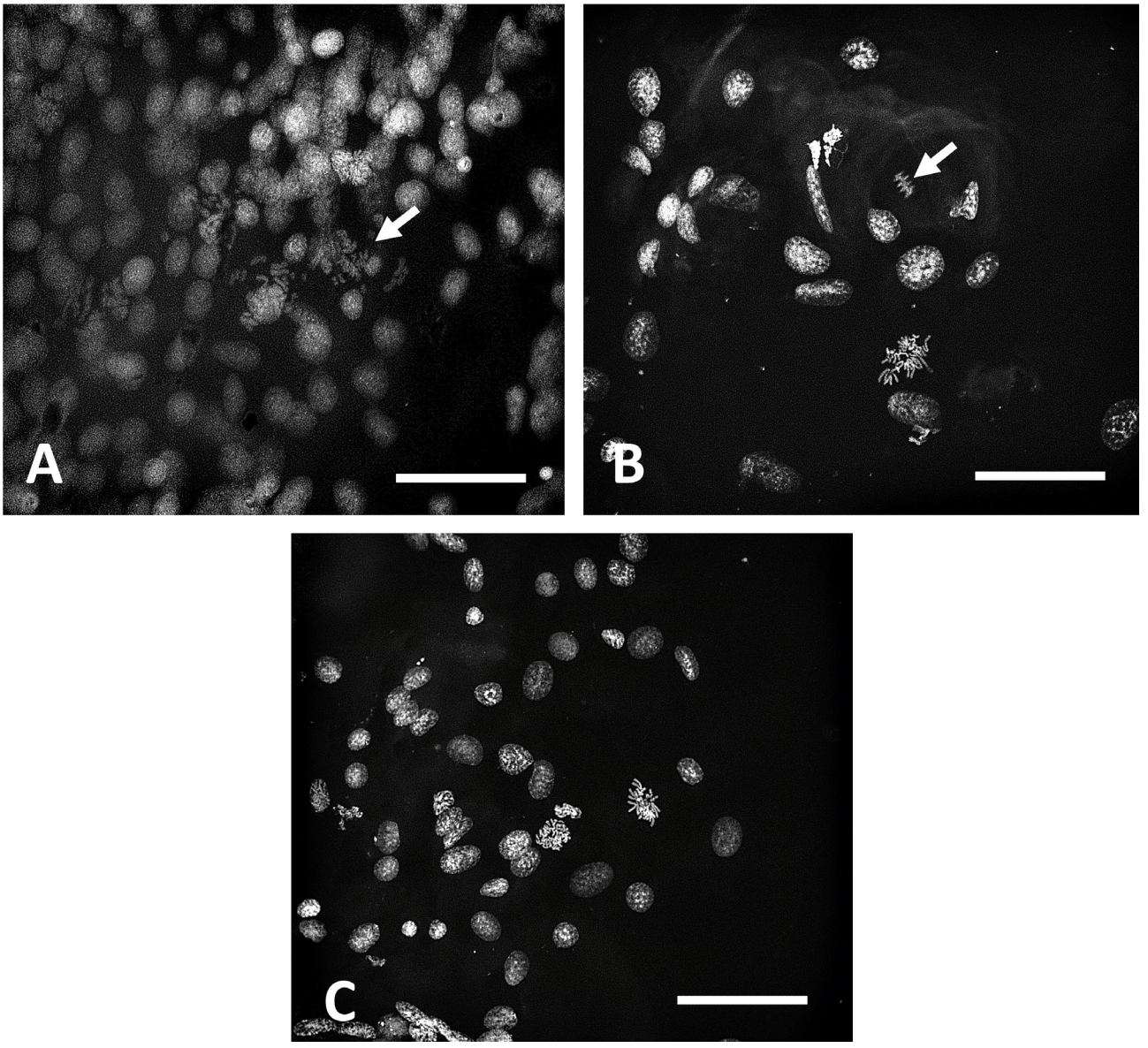

Fig. 4. Preparations of different tissues. A – intestine, arrow indicates chromosomal plate overlaid by nuclei, mean # of chromosomal plates in intestine was 5.6 ± 3.6 (mean ± SD); B – gills, arrow indicates algae polluting the slide, mean # of chromosomal plates in gills was 9.8 ± 2.5 (mean ± SD); C – regenerated tail, mean # of chromosomal plates in tail was 10.0 ± 5.2 (mean ± SD). Stained with DAPI. Scale bar – 100 µm.

10.0 ± 5.2 (mean ± SD). Therefore, the recommended time for performing colchicine treatment is four hours. The usefulness of two-week regeneration was also analysed, but no chromosomes were found on slides. Comparison of regenerated tissue with gills and intestine preparations are shown in Fig. 4.

Discussion

Different types of tissues can be used for chromosomal preparations of tadpoles, but each has advantages and disadvantages.

Intestine epithelium can be easily extracted and results in a large amount of tissue. However, using the intestine has some disadvantages: 1) animals must be sacrificed; 2) preparations are often polluted with green algae and waste products of digestion. Using the gills of tadpoles results in clean preparations and distinct mitotic chromosomal plates but still requires sacrifice of the animal. The small size of the gills and their compacted tissue also makes it difficult to cut and macerate.

The modified method proposed in this study offers the opportunity to avoid killing the animals and their ability to regenerate tissue is used to obtain clean proliferating tissue. The regenerating tissue is almost transparent and does not contain melanophores or intra-epidermal pigments, which are usually present in the tail tips of tadpoles.

This tissue provides clean preparations with no polluting elements. After cutting the regenerated tissue, tadpoles can regenerate their tails again and can be grown to maturity and used for further studies. If tadpoles are obtained from nature they can be released back into the wild, with appropriate measures to prevent the introduction of disease. However, the release of tadpoles obtained from laboratory crossings is illegal in many countries and highly irresponsible because of the risk of genetic pollution.

The only two disadvantages of the proposed method are the time needed for tissue regeneration and the unfeasibility of identifying the sex of tadpoles immediately without waiting for metamorphosis and artificial overwintering. Working with colchicine also requires caution and implementation of appropriate protective measures (wearing gloves and a face mask), since this compound is toxic in high concentrations. All the other reagents are safe and readily accessible.

Although the suggested intravital method has been proposed for the study of the unusual hybridogenic water frog complex, it could be used for other groups of Anura and modified for other species that possess the ability to regenerate their tissues.

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Author Contributions

E. Pustovalova and A. Fedorova designed the experiment, D. Shabanov conceived the study. E. Pustovalova performed tissue collection and obtained chromosome preparations, A. Fedorova took photos and performed data analysis, E. Pustovalova and A. Fedorova wrote the manuscript with comments provided by D. Shabanov.

Data Availability Statement

The data that support the findings of this study are available in the Figshare Digital Repository: "Methodology for intravital mitotic chromosome preparation from regenerated tissue of tadpoles' tail tips" at http://doi.org/10.6084/m9.figshare.19397822.

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