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SOP for genome size estimations from collection specimens

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ABSTRACT

Genome size data is crucial now for comparative studies with the goal of understanding the mechanisms of genomic change and their biological implications related to genome size diversity. But also, now this data is actively sought by the biodiversity genomics community given that is a a valuable proxy for the estimations of the cost and challenge of sequencing and assembling a potential target genome. Given the (sometimes) extreme diversity of genome sizes among the organisms within the same phyla or even genus, it is important to provide genome size estimations for the maximum number of taxa possible, to aid on the accuracy of genome sequencing depth, among other things. Traditionally, genome size estimations for multicellular species are performed using two methods: Feulgen densitometry (now most often based on image analysis) and flow cytometry. In both cases, live/fresh or flash-frozen (e.g., in liquid nitrogen or on dry ice) material of animal tissues are preferred over fixed material because the nuclei remain intact and can be prepared and stained more easily. However, this poses many challenges and limitations for rare taxa and those coming from extreme environments or sites that are very difficult to access. Therefore, estimations from preserved material are now more necessary than ever if we want to accomplish the difficult task of sequencing virtually the whole biodiversity of the planet.

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Modified from a protocol by Mohammed Tawfeeq and Jean-François Flot from Université libre de Bruxelles (ULB).

Biological materials

- Tissue(s) of the sample(s) to be analysed, conserved in 96% ethanol at 25°C or below. A week before the start of the process renew the ethanol.

- We use 3 different standards:

a) 1 *Periplaneta americana* (brain only): before cutting the head off put it in the freezer for 5 seconds. Look for the white area (where the brain is) and press both sides with the forceps to get all the white material out. Scratch the head as well to get more tissue out.
b) 3 individuals of *Lasius niger* (without abdomen part).
c) 1 Axolotl embryo.

You can use other standards, but it is important to know the genome size for those to make comparisons.

Procedure

Step 1. Preparation of the tissue samples:

Under the fume hood:

- Prepare and label microscope slides with name of the specimens with pencil.

Note: the slides need to be completely cleaned by placing a few drops of 96% ethanol and then wipe them up with filter paper to avoid unwanted crystals that will overlap with the nuclei when photographing the slides.

- Using fine scissors cut a piece of the tissue (preferably legs, muscles, or brain) of a minimum size of 0.5 cm long and 0.3 cm wide and using a sterilized razor blade place it directly on the labelled slide.

- Add a few drops of acetic acid 40% to the tissue and then start chopping the tissue directly on the slide using the sterilized razor blade.

- After chopping the tissues into very tiny particles, cover them with drops of acetic acid. The suspension solution that has the cells must look homogenized and not very milky, or have grainy texture that will be visible to the naked eye when visualizing the slide under black background. To homogenise it more, pipette the suspension up and down and add few more drops of acetic acid to avoid cell clumps.

Note: To have well-dispersed cells (not overlapping), stir the suspension for a bit with round movements using the edge of the razor.

- To dry slides, keep them in horizontal position at 25°C for 3 hours until the acetic acid completely dries out.

- When the slides are dry, put few drops of ethanol 96% on them, place them in a dark place (e.g., incubator) for 24 to 48 hours at 25°C. Usually 24 hours are enough.

Note: remember to also incubate the fixative you will use in the next step at 25ºC overnight.

- Prepare the fixative solution (100 ml):

Methanol……85 ml
Formaldehyde……10 ml
Acetic acid……5 ml

Note: when preparing the fixative, cover the mouth of the bottle with Parafilm before putting the tap to avoid evaporation when incubating it overnight at 25ºC.

Step 2: Fixation (the following day):

- Pour the fixative solution into a glass tank (the ones traditionally used for staining).

- Put the slides (remember to include the standards and the samples) into their dedicated places in the slide rack, then place the slide rack into the prepared fixative bath. Add the slide rack very very slowly (remember to do this every time).

- Close the glass tank hermetically with its lid, cover it with two layers: parafilm and aluminium foil, then keep it in dark place for 24 hours at 25 °C.

Note: The prepared solution can be increased accordingly, depending on the size of the glass tank that you have. As a quality control, standards must be included in same chemical bath as the samples per Feulgen run.

Note: Put the HCL 5N and Schiff reagents at 25°C overnight for efficient chemical reaction. This can be done after finishing 2nd step.

Step 3: Hydrolysis and staining:

- **Washing 1**: take the slide rack out of the solution from the previous step, and place into new glass tank filled with distilled water for a duration of 5 minutes x2.

- **Hydrolysis**: place the slide rack in a new glass tank, then fill the latter with HCL 5M until the slides are completely immersed. Firmly cover the tank with its lid, place two layers: parafilm and aluminium foil, then keep it in dark place for 2 hours at 25 °C.

- **Washing 2**: repeat washing of point 1. Take the slide rack out of the solution from the previous step, and place into new glass tank filled with distilled water for a duration of 5 minutes x2.

- **Staining**: place the slide rack in a new glass tank, then fill the latter with Schiff until the slides are completely immersed. Firmly cover the tank with its lid, place two layers: parafilm and aluminium foil, then keep it in dark place for 2 hours at 25 °C. IMPORTANT: read the following step before.

During this time: prepare a **fresh solution** of sodium metabisulfite shortly before the 2 hours are over. To prepare 200 ml of sodium metabisulfite solution, mix the following in a glass bottle:
- 10 ml sodium metabisulfite solution of 100 g/L.
- 188 ml distilled water.
- 2 ml HCl 5M.

Shake vigorously up and down and then laterally.

- **Washing 3**: repeat point 1, but instead of distilled water, here fill the tank with the prepared sodium metabisulfite buffer for a duration of 5 minutes x2.

- **Washing 4**: repeat point 1.

- **Drying the slides**: using a pipette, carefully add a few drops of ethanol 70% on the slides and wait 10 minutes. Add a few drops of ethanol 96% on the slides and wait until it's dry completely. Right after, let the slides rest in the dark for 24 h overnight.

Step 4: Photographing:

- Slide preparation for visualization: use immersion oil to mount the slides and visualize using 100x objectives with immersion oil (1-2 drops).

- Use the same light settings for all pictures, including those of standards, and make sure you take them in the same microscope.

- Perform “white balance” in the standards, and use the same settings across all pictures. You can use ToupView software to ease the picture capture. Then perform image analysis using the software ImageJ.

Step 4.1: Data generation

- Using the software ImageJ. Go to “analyze” and click on “Set Measurements”. There, select “Area” and “Mean gray value”, which will be the two parameters used for estimating volume (area) and Optical Density (mean gray value).

- We now analyze the standards, using “polygon selection” draw the perimeter of the nucleus and then click on analyze.

- Repeat the process by selecting the area nearby the same nucleus to form an enclosed shape similar to donuts shape then click on “Analyze” then hit “Measure, to substract the gray value from the background later.

- We will use the area of the nucleus, the mean gray value of the nucleus, and also the mean gray value of the neighbouring area (but not the area value). Paste them in 3 columns in an excel, to perform correlations later.

- Measure at least 30 nuclei.

- Estimate the optical density (OD), integrated optical density (IOD), and IODC values.

  a. The OD is estimated through the formula:

  OD = gray area of nucleus / gray area of background

  b. The IOD is estimated through the formula:

  IOD = area of the nucleus * OD

  c. The IODC is estimated through the formula:

  IODC = IOD average / IOD standard deviation

- After analysing 30 nuclei of each standard/sample, you must see a linear relationship between 1/OD and the nuclear area size in a scatter plot, otherwise either the image quality is deemed not good, and consider photographing the slides again or you need to re-do the experiment while paying attention to the slide cleanness (crystals and dirt).

- We estimate the C-value of the standards using 3 standards and comparing them to each other.
- Then, we try to initially estimate the genome size (C-value) of the sample using the standards (at least two of the standards) through the formula:

C-value = IOD sample / IOD standard1 * C-value standard1

For example, we estimate the genome size of *P. americana* although it is already known (3.41 pg) by using *L. niger* and axolotls. They both should give same or similar estimation to the actual estimation. The same thing goes for *L.niger* and axolotl.

- Then, we try to initially estimate the genome size (C-value) of the sample using the standards (at least two of the standards) through the formula (Table 1):

C-value = IOD sample / IOD standard1 * C-value standard1

Genome size calculation formula		
Standard 1		
IOD of specimen	**C value of standard**	**IOD of standard**
P. americana	*L niger*	1000873588
1000873588	0.31	
C-value of specimen	0.31	

Table 1: Genome size calculation formula examples.

Lab materials:

- Ethanol 96% and 70%.

- Acetic acid 40 %.
- Methanol 99%.
- Formaldehyde 37% (w/w) in aqueous solution stabilised with 7-8% methanol. Thermo: A16163
- Acetic acid 90%. VWR 2019.295
- HCl 5N.VWR 30018.320
- Distilled water.
- Schiff reagent.
- Sodium metabisulfite. VWR 7920.295
- Plastic based slide staining
  rack (chemical resistant) + staining tank or jar (4 items).
- Razor blades, forceps, scissors, petri dish, pipets, glass becker.
- Slides and cover slides.
- Immersion oil.

Equipments and softwares:

- Incubator, fume hood, digital balance, FHD camera mounted to compound microscope.
- Computer + preinstalled softwares: ToupView (comes ready with the camera) + Image J + R studio.

ToupView: software to be used for camera identification with the computer.Click on the link to download: http://www.touptek.com/download/showdownload.php?lang=en&id=33
ImageJ: Software for image analysis.Click on the link to download: https://imagej.nih.gov/ij/download.html