Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Notes and Comments

Manual vs. automated erythrocyte assessment for polyploidy detection in neotropical fish: a comparative study

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Over the past decades, the pursuit for a healthier life and the continuous growth of world population made the fisheries consumption to double (9.9 to 20.2 kg per capita) what led the world aquaculture, in 2020, to reached a record of 122.6 million tones, the equivalent to 281.5 billion US dollar FAO (2022). To attend such ascending demand, biotechnological techniques aiming better productivity in fish have been developed and one of the most promising is the triploid induction.

Triploid fish – individuals with three sets of chromosomes - are generally sterile due to altered gamete formation, which offer several advantages to aquaculture (Nascimento et al., 2017). Primarily, sterility diverts energy away from gonads towards somatic growth, increasing fish size and, consequently, overall yields (Nascimento et al., 2017). Moreover, sterility mitigate environments risks in case of environmental escapes (Piferrer et al., 2009).

However, following triploid induction, the confirmation of ploidy becomes essential. Besides several techniques can be employed (ex. Flow cytometry and cytogenetic analysis), the blood smear technique, in particular, is based in the measurement of erythrocyte nuclei, which in polyploid fishes are larger due to higher DNA content. This technique offers the advantages of simplicity, cost-effectiveness, and field applicability (Fukushima et al., 2012). However, manual assessment of erythrocyte measurements remains time-consuming, especially when dealing with a substantial number of cells. Consequently, automatic measurements are an interesting alternative to optimize this process. Therefore, the aim of this study was to evaluate two distinct methodologies – manual and automated – for characterizing erythrocytes among diploid and triploid specimens from four economically significant Neotropical fish species: *Astyanax altiparanae* (Garutti & Britski, 2000), *Brycon amazonicus* (Spix & Agassiz, 1829), *Prochilodus lineatus* (Valenciennes, 1837), and *Pimelodus maculatus* (Lacepède, 1803).

The experiment was conducted at the Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental/ Instituto Chico Mendes de Conservação da Biodiversidade (CEPTA/ICMBio), Pirassununga City, São Paulo State, Brazil. Procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of CEPTA (CEUA//CEPTA#0231.000070/2018-63).

Triploid fishes were induced in accordance with our previously established protocols (Adamov et al., 2017; Bertolini et al., 2020; Nascimento et al., 2021; Yasui et al., 2020). We used 10 diploids and 10 triploids and of *Astyanax altiparanae* (8 months of age), 6 diploids and 11 triploids of *Prochilodus lineatus* (2 years), 12 diploids and 3 triploids of *Brycon amazonicus* (2 years), and 6 diploids and 3 triploids of *Pimelodus maculatus* (1 year). The fish were maintained separated in 1000m^2 earthen ponds and feed twice a day with a commercial diet (4200 kcal kg^{-1} and 45% crude protein).

The animals were then anesthetized using a eugenol solution (1 g.L^{-1}), and blood was collected via caudal puncture using a syringe containing 1 drop of EDTA (5%). Subsequently, smears were prepared by placing a drop of blood on a glass slide and gently dragging it from one side to another (Levy-Pereira et al., 2019). The prepared smears were then stained using the Panótico Rápido kit (only step 1, for staining of the nuclei) and observed under and optical microscope (Nikon, Japan) at a magnification of 400x. Microphotographs were captured using Nis-Ar Elements software (Nikon, Tokyo, Japan), and subsequent both manual and automatic analysis was performed using the open-source software Image J (National Institutes of Health, USA).

For automated measurements, the images were imported into ImageJ, converted to an 8-bit format, and then subjected to automatic thresholding, resulting in grayscale images with a white background and black nucleus. Subsequently, in the "analyze particles" option, we specified the minimum and maximum size (in pixels) for each analyzed particle. This step prevented overlapped cells from being counted as one and ensured the exclusion of small particles (debris). Nuclei extending beyond the edges of the microphotographs were systematically excluded from the analysis (Figure 1). This meticulous procedure allowed for the comprehensive analysis of nearly all nuclei in the images, providing a wealth of data for each fish.

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Received: 25 September 2023 – Accepted: 25 March 2024

For manual analysis, 50 nuclei were randomly selected and measured for each fish after the calibration of the scale (using the "set scale" option). The area, perimeter, and major and minor axes were then measured within the nucleus of each image for each fish and method, and the results were compared between 2n and 3n individuals.

To confirm the results, ploidy was also determined by flow cytometry using the procedure developed by Xavier et al. (2017). Consequently, just confirmed diploid and triploid fishes were used. The paired t-test that was carried out at a level of 5% significance (STASTICA; v. 70; StatSoft).

For *A. altiparanae*, triploid individuals had larger nuclei in terms of area, perimeter, and major axis when manually analyzed. Automatic analysis also showed larger measurements in these aspects for triploid specimens. Among diploid individuals, there were significant differences in these characteristics between manual and automatic analyses. However, for triploid individuals, no differences were found between manual and automatic methods (Table 1).

In *P. lineatus* and *B. amazonicus*, triploid fish had significantly larger erythrocyte nuclei in terms of area, perimeter, major axis, and minor axis, regardless of whether manual or automatic analysis was used. Among diploid and triploid fish, all these characteristics showed differences between manual and automatic methods (Table 1).

In the case of *P. maculatus*, triploid fish displayed larger erythrocyte nuclei measurements in terms of area, perimeter, major axis, and minor axis through manual analysis. Automatic analysis showed increased area and perimeter for triploid specimens. Similar to the other species, measurements of these characteristics were taken for both diploid and triploid fish using both manual and automatic methods (Table 1).

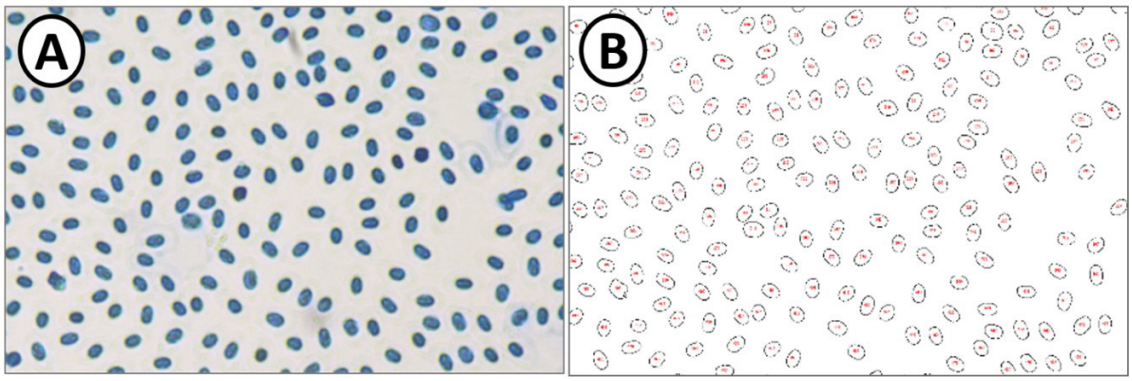

Figure 1. Blood smear of diploid *Astyanax altiparanae,* showcasing the colored nucleus (A) and the results after analysis using ImageJ software (B).

Table 1. Manual and automatic nuclear measurements of area (μm²), perimeter (μm), major axis (μm) and minor axis (μm) of diploid and triploid fish of *Astyanax altiparanae*, *Prochilodus lineatus*, *Brycon amazonicus* and *Pimelodus maculatus.*

Parameter	Drying	*Astyanax altiparanae*		*Prochilodus lineatus*		*Brycon amazonicus*		*Pimelodus maculatus*	
		2n	3n	2n	3n	2n	3n	2n	3n
Area	Manual	11.33 ± 1.02^{Aa}	17.31 ± 1.48^{Ba}	14.00 ± 0.20^{Aa}	19.43 ± 0.34^{Ba}	14.08 ± 0.12^{Aa}	19.79 ± 0.53^{Ba}	15.06 ± 0.32^{Aa}	15.47 ± 0.84^{Ba}
	Automatic	15.10 ± 0.69^{Ab}	19.10 ± 0.80^{Bb}	14.77 ± 0.19^{Ab}	20.37 ± 0.48^{Bb}	14.73 ± 0.18^{Ab}	20.87 ± 0.88^{Bb}	15.59 ± 0.48^{Aa}	21.13 ± 0.31^{Ba}
Perimeter	Manual	13.11 ± 0.59^{Aa}	16.63 ± 0.68^{Ba}	14.29 ± 0.12^{Aa}	17.21 ± 0.14^{Ba}	14.48 ± 0.08^{Aa}	17.57 ± 0.31^{Ba}	14.98 ± 0.15^{Aa}	16.59 ± 0.34^{Ba}
	Automatic	15.43 ± 0.56^{Ab}	17.25 ± 0.41^{Bb}	14.65 ± 0.10^{Ab}	17.61 ± 0.21^{Bb}	14.83 ± 0.10^{Ab}	17.88 ± 0.39^{Bb}	15.15 ± 0.28^{Aa}	17.99 ± 0.18^{Ba}
Major axis	Manual	4.50 ± 0.22^{Aa}	6.04 ± 0.26^{Ba}	4.96 ± 0.05^{Aa}	6.24 ± 0.04^{Ba}	4.97 ± 0.03^{Aa}	6.25 ± 0.12^{Ba}	4.92 ± 0.06^{Aa}	5.61 ± 0.08^{Ba}
	Automatic	5.27 ± 0.16^{Ab}	6.24 ± 0.14^{Bb}	5.11 ± 0.04^{Ab}	6.42 ± 0.07^{Bb}	5.22 ± 0.04^{Ab}	6.46 ± 0.16^{Bb}	5.06 ± 0.10^{Aa}	6.09 ± 0.07^{Aa}
Minor axis	Manual	2.94 ± 0.17^{Aa}	3.54 ± 2.24^{Aa}	3.59 ± 0.03^{Aa}	3.96 ± 0.05^{Ba}	3.57 ± 0.02^{Aa}	4.03 ± 0.03^{Ba}	3.90± 0.06^{Aa}	4.19 ± 0.14^{Ba}
	Automatic	3.64 ± 0.07^{Aa}	3.89 ± 0.08^{Bb}	3.68 ± 0.03^{Ab}	4.03 ± 0.06^{Bb}	3.59 ± 0.02^{Aa}	4.11 ± 0.07^{Ba}	3.92 ± 0.05^{Aa}	4.42 ± 0.06^{Aa}

Data are show as mean ± SE (standard error). Distinct upper-cases and lower cases letters indicate differences between columns and lines, respectively, in each parameter (T-paired test; $P < 0.05$).

Given that most protocols for inducing polyploidy are not 100% effective, mechanisms to confirm ploidy are imperative. In this context, we demonstrate that both manual and automatic measurement of erythrocyte nuclei serve as effective tools for confirming triploidy in *A. altiparanae*, *B.amazonicus*, *P. lineatus*, and *P. maculatus*. Nevertheless, the majority of studies traditionally rely on manual analysis, which is both labor-intensive and time-consuming (Fukushima et al., 2012; Piferrer et al., 2009). In contrast, the automatic method employed in this study proved to be efficient, enabling the measurement of hundreds of erythrocytes within a matter of seconds.

Consequently, in situations where flow cytometry or other techniques are unavailable, the blood smear method could serve as a valuable alternative for identifying polyploid animals. Hence, considering that the species under investigation (*A. altiparanae*, *P. lineatus*, *B. amazonicus*, and *P. maculatus*) hold significance in both research and aquaculture, the assessment of their triploidy by the method described in this study could potentially may facilitate the managing of triploid organisms in the future.

Acknowledgements

The authors are grateful to São Paulo Research Foundation (FAPESP) (2010/17429-1; 2011/11664-1; 2016/12383-0); AES Tietê; National Council for Scientific and Technological Development (CNPq #471140/2012-0) and Coordination of Superior Level Staff Improvement (CAPES; Ph.D. Scholarship).

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