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Silver staining of nucleolar organizing regions for ploidy confirmation in Snowtrout *Schizothorax richardsonii* (Gray, 1832)

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ABSTRACT

In aquaculture practices, ploidy manipulation is a common tool for genetic improvement in fishes, with benefits such as increased growth rates, carcass production, and meat quality. Sterile fish is also useful for reducing environmental risk from escaped farm fish and conserving native Ichthyofaunal diversity. To verify the ploidy, a simple and reliable method using silver-stained cell preparations for Nucleolar organizing regions was used for the snow trout, *Schizothorax richardsonii*, in the field condition. For silver staining of cell smears, tissue samples were taken from sacfry , liver, gills, and fin clips. The Nucleolar organizer region (NOR) dots were counted for each specimen. According to the findings, triploid individuals had 1, 2 or 3 nucleoli per cell, whereas diploid individuals had 1 or 2. Alevin cells (60%) contained more nucleoli per cell than fin clip cells (56%), gill cells (34%), and liver cells (16%). However, no cells with three nucleoli were ever found in diploids, indicating that ploidy conformity in this fish species. Based on our findings, silver staining provides a low-cost and simple method for confirming ploidy in snow trout, and it would be ideal for breeding and hatchery operations in the field.

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Ploidy, Sac fry , NORs

1. Introduction

Snow trout belong to the subfamily Schizothoracinae and are categorized as vulnerable by the IUCN in India (2012). It is an economically significant fish of the order Cypriniformes and family Cyprinidae with high food value (Bahuguna and Rayal, 2020). Unlike salmonids, the Snow trout, a minnow is unique in its representation of Himalayan coldwater fisheries(Regmi, 2019). *S. richardsonii* is a major constituent of the Kumaun and Garhwal Himalaya fishery, accounting for more than 50% of the total catch in rivers and streams (Agarwal, 1996). Snow trout are unique to the Himalayas and can be found in streams and lakes that receive snow melt water from the mountains. This fish is known as “Asela” in the middle Himalayas and is commercially important as a food fish in the Himalayan region. Even though this species contributes significantly to the natural composition, it is not considered a potential species for coldwater aquaculture due to its poor growth in captivity.

Chromosome manipulation has been extensively used in salmonids such as Rainbow trout, Atlantic salmon, pink salmon, Coho salmon, brown trout, and arctic char to achieve genetic containment and uninterrupted post-pubertal somatic growth(Shelton *et al.,*1986; Benfey, 1988; Arai and Wilkins, 1987; Crozier and Moffett, 1989; Quillet *et al.,*1991; Gillet *et al.,*2005; Weber *et al.,*2014). Triploidy induction in fish has been found to be effective for population management, enhancing growth in juveniles, and extending survival and improving growth in mature fish (Bazaz *et al.,* 2020). Triploidy induction is the production of individuals with three sets of chromosomes and can be created in fish by blocking the second meiotic division followed by the extrusion of the second polar body by shocking eggs quickly after fertilisation (Asim *et al.,*2020). Triploid fish features are known to positively impact fish output and profitability (Berrill *et al.,* 2012). Although diploid and triploid fish are physically identical throughout their lives, they differ cytologically. As a result, numerous direct and indirect methods exist for determining a fish’s ploidy (Maxime 2008; Tiwary *et al.,* 2004). Among these are nuclear and cellular size measurements (Alcantar-Vazquez 2016; Thomas and Morrison 1995).

The nucleolus organizer regions (NORs) are ribosomal DNA (rDNA) repetitive gene segments formed of unit clusters of 18S, 5.8S, and 28S that code for ribosomal RNA. The nucleolus can be referred as a ribosome factory. Under the effect of RNA polymerase, the NORs are transcribed to rRNA (Crocker, 1990). Silver staining methods easily illustrate the location of NORs by demonstrating the argyrophilic proteins (AgNORs) associated with NORs (Cromie *et al.,*1988). The AgNOR technique is employed for this purpose and the proteins that provide the silver staining are known as AgNOR proteins (Yekeler *et al.,*1993). Because of the argyrophilic character of their non-histone acidic proteins, the silver (Ag-NOR) staining approach can be used to detect the NORs (Thippeswamy *et al.,*2015). Counting nucleoli is a straightforward, low-cost approach for determining ploidy in several fish species. The nucleoli in interphase cells show the level of ploidy in most fish species. As a result, the majority of the fish studied had only one chromosome with a Nucleolar organizer region for each haploid genome, this strategy could be used to a wide range of fish species (Phillips *et al.,* 1986). Harrell *et al.* (1998) determined that nuclear sizing (particle size analysis) is the simplest and quickest way to determine ploidy in fish after comparing triploid induction validation techniques.

2. Materials and Methods

The study was conducted at ICAR-Directorate of Coldwater Fisheries Research (DCFR), Bhimtal (Latitude 29^0 21’N, Longitude 79^0 34’E, 1370 masl) located in Kumaun region of Uttarakhand State (India). Fish that were mature (3 to 4 year age group) were employed for the breeding process. 98 to 100% triploidy induction was accomplished using the right pressure shock and accurate zygote age, which was confirmed by karyotype analysis. The sac fry, gill tissue, fin

clips and liver cells were collected from triploid and diploid snow trout populations. Ten specimens were used for each type of tissue to create 20 slides (80 slides for diploids and triploids).

Each tissue sample was cut into 1 mm pieces and put into a glass tissue grinder. A glass tissue grinder was filled with 1ml of fresh, chilled Carnoy's solution, and it was gently mixed. Grinded tissue was placed in a 2.5 mL vial and Carnoy's solution raised the volume to 2 mL. The cleaned slide was heated to 40 to 50 °C and a 100µL cell solution was put and dried. Dried slide was used for silver staining by using silver nitrate (Howell and Black, 1980). The filter paper removed the silver residual precipitation (Kim et al. 2017). For the staining, two solutions SolI and Sol II, were prepared. Gelatine (0.5g), formic acid (0.25mL) and distilled water(25mL) were used to make Sol I. Silver nitrate (4g) and distilled water(8mL) was used to make the Sol. II. To avoid the light reaction, both solutions were stored in an amber glass bottle wrapped with aluminium foil and kept in a dark box.50µL of Sol I and 100µLof Sol II were put on the slide and combined to stain tissue cells in dried slides. These slides were kept in the dark for 30 min. at 25 °C. Gently wash the slides and observe for AgNOR dots. The AgNOR dots were counted in the microscope at 100X (oil immersion).In each slide, 50 cells (40 from the four corners and 10 from the centre) were randomly chosen (100 per specimen). AgNOR dots were carefully counted after each cell was individually focused.False silver staining was eliminated via filtration to obtain an accurate result (Kim et al. 2017). Modifications to the current study's processing procedures produced the greatest findings. A two-way ANOVA (Graph pad prism 8.0.2) was used to analyze the data for each tissue.

3. Results

According to the findings, all four selected tissues (Sac fry, fin clip, gill, and liver) have only one or two nucleoli per cell in a normal diploid state. However, in Sac fry, fin clip cells, gill cells, and liver cells, increasing the ploidy level to triploidy results in an increase in the number of NORs sites, i.e., 1, 2, or 3 nucleoli per cell (fig. A, B). It is clear that no cell with three NORs can be found in normal diploid tissue, whereas a high proportion of such cells can be found in triploid tissue. The frequency of three NORs was higher in triploid fish Sac fry, fin clips, gill cells, and liver cells than in diploid tissue (Table1).

Table 1. Mean of tissue cells with one, two and three nucleoli per cell in rainbow trout

	Nucleoli per cell			Total individual*	Total slides
Ploidy	1	2	3		
Sac fry					
Diploid (Mean±SD)	49.8±6.2 (43-56)	48.6±6.3	0	10	20
Triploid (Mean±SD)	33.8±3.2	42.1±3.7	59.9±6.8	10	20
Gills cells					
Diploid (Mean±SD)	51.8±6.3	46.7±6.2	0	10	20
Triploid (Mean±SD)	23.6±3.5	41.4±6.2	54.2±5.8	10	20
Fin clips cells					
Diploid (Mean±SD)	57.3±6.7	42.8±6.7	0	10	20
Triploid (Mean±SD)	21.1±3.1	29.6±3.8	35.6±4.1	10	20
Liver cells					
Diploid (Mean±SD)	57.3±6.5	42.7±6.6	0	10	20
Triploid (Mean±SD)	8.9±1.9	10.5±2.2	16.5±2.9	10	

This is the range show that minimum and maiximum number of cell show NOR. If this is unappropriate then it could be removerd from table this is extra not necessary.

We quantified that in a triploid state Sac fry (60%) had a higher percentage of cells with three nucleoli per cell than fin clip cells (56%), gill cells (34%), and liver cells (16%). However, no cells with three nucleoli were ever found in diploids (Fig: 1, 2). which make conformity of ploidy in this fish species.

4. Discussion

Based on our findings, silver staining provides a low-cost and simple method for confirming ploidy in snow trout, and it would be highly suitable in field situations for breeding and hatchery operations. Cyprinid fishes have been the subject of substantial cytogenetic research, with a focus on evolutionary factors. This family has seen chromosomal polyploidization throughout evolution. *Simphodus roissali* (Lopez *et al.,* 1988) and the genus *Blennius* (Gracia*et al.,*

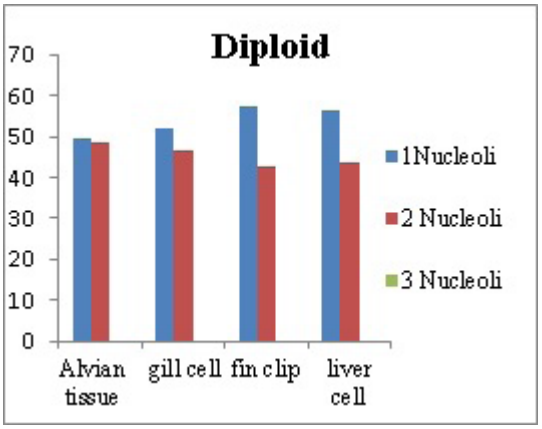

Fig. 1. Nucleoli per cell in Diploid Fish

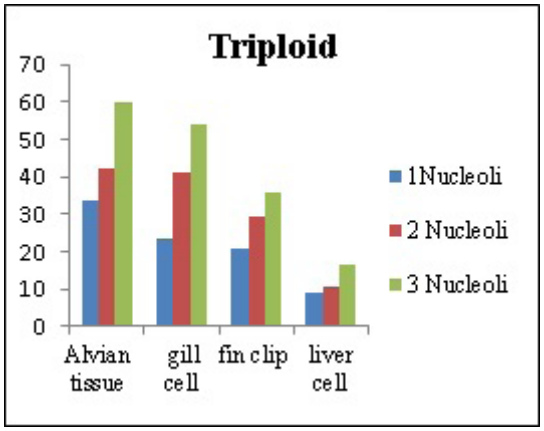

Fig. 2. Nucleoli per cell in Triploid Fish

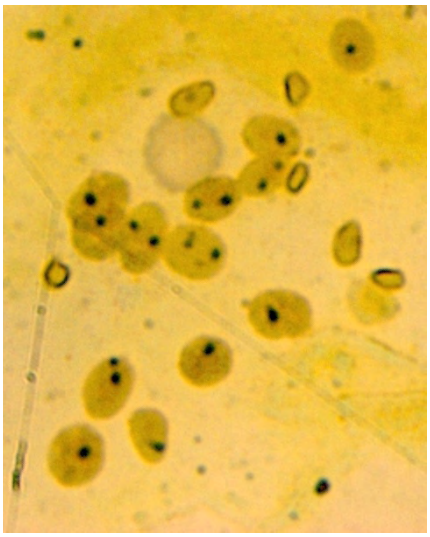

Fig. 3. Diploid snow trout sample comprising one and two interphase NORs in cell

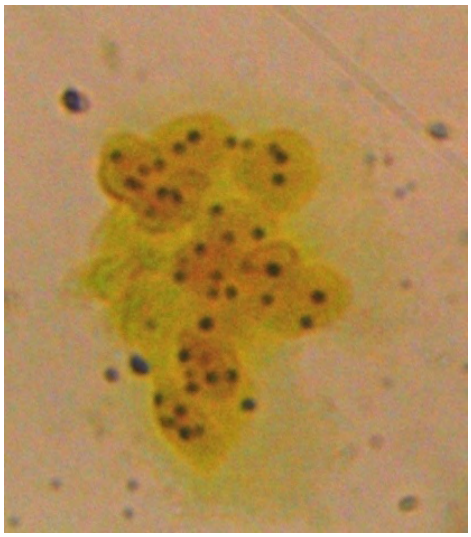

Fig. 4. Triploid snow trout sample comprising one, two and three interphase NORs in cell

1987) both did not have comparable NOR regions that were stained with silver in earlier cases on cyprinids (Gold 1984). According to Phillips *et al*. (1986) embryonic cells that proliferate quickly have a maximal number of nucleoli, making triploidy detection in the early stages of fish development conceivable by silver staining. The results of the current study are consistent with those of earlier research that employed silver staining to calculate ploidy by counting NORs.

5. Conclusion

According to the findings of this study, silver staining is an acceptable ploidy identification method in snow trout not only for technically inept farms but also for fish research personnel. This method is not sophisticated and easily detects triploidy even in dead fishes. This procedure is useful for all stages of the fish's existence, with or without sacrifice, but better results may be obtained in the early developmental stage. We hope this silver staining procedure may be valuable to those who want to raise an all-female or triploid brood of snow trout or other fish species.

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Ethical standards

We declare that the experiments were conducted per the institutional animals ethics committee guidelines.

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