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Cytologic ploidy determination in fish – an example of two salmonid species

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ABSTRACT

The method of ploidy level determination depends on the equipment, expertise of the lab or farm staff, and on the material studied (fish species, specimens' age and size, etc.). The combination of some simple techniques can be very helpful for fast, reliable and inexpensive assessment of effects of genome manipulations. Among indirect methods the nucleoli quantification seems to be the most convenient one, except that it is limited to species possessing one locus of nucleolar organizer regions (NORs) (rainbow trout as an example). Species with multilocus NORs (like brook trout) need another method, for instance sizing of erythrocytes nuclei. These techniques can be useful for identification of products resulting from new approaches in biotechnology applied to fish culture, as polyploidisation or hybridisation, for characteristics of transformation levels of organisms found in the wild or those produced due to experimental treatments.

INTRODUCTION

Salmonid fish are of great interest in aquaculture in Poland. The most popular is rainbow trout (*Oncorhynchus mykiss*) (over 30% of all fish production), followed by Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*). The biggest advantage of brook trout is that it has proved to be almost completely resistant to viral haemorrhagic septicaemia virus (VHSV) (Dorson et al. 1991), which has plagued trout farming in continental Europe since 1950 (Wolf 1988) and in Poland since 1998. The major problem in intensive brook trout production is the early sexual maturation of this species, which causes many physiological and behavioural drawbacks, responsible for significant losses in the production cycle. This problem can be overcome (like in other fish species) by producing non-maturing fishes through triploidisation.

Artificially-induced triploidy of rainbow trout is popular in aquaculture (Benfey 1996), but other species are also subjected to genome manipulations (Arai 2001; Foresti 2000; Pandian and Koteeswaran 1998). Due to the lack of morphological characteristics that could enable the identification of diploid and polyploid (triploid and tetraploid) fishes, other methods had to be applied (Maxime 2008). The goal of this review is to summarize the information concerning inexpensive and feasible methods for ploidy validation in fish, with two species of salmonids: rainbow trout and brook trout, serving as an example.

The most reliable methods are those which can measure directly the amount of DNA (Harrella et al. 1998). Flow cytometry, spectrofluorometry or microdensitometry are the simplest and quickest techniques of DNA amount validation (Hardie et al. 2002). The major drawback of electronic ploidy determination methods is the cost of the equipment required for analysis.

DIRECT METHOD

Karyotyping

The chromosome counting or karyotyping (the analysis of chromosome number and morphology) is one of the direct methods which enable direct recognition of ploidy level, because the number of chromosomes indicates the amount of DNA molecules. Chromosome preparations can be made from head kidney and gill epithelium, having sacrificed the fish, or from blood cell culture (Ozouf-Costaz and Foresti 1992). Fish from the genera *Oncorhynchus* and *Salvelinus* have different chromosome numbers and morphological characteristics of chromosomes (karyotype). Chromosome studies of rainbow trout natural populations and domesticated strains have revealed a Robertsonian-type polymorphism in

chromosome number, consisting of between 58 and 64 chromosomes (Colihueque et al. 2001; Ocalewicz 2002). This type of variation is due to centric fusion of two nonhomologous chromosomes, which produces a single chromosome, or else two chromosomes are produced by centric fission of a single chromosome. Because of Robertsonian polymorphism, triploid individuals can have 3n=87-96 and tetraploid ones possess 4n=116-128. Chromosome preparations of brook trout show diploid chromosome numbers 2n=84 and 3n=126 (Woznicki and Kuzminski 2002). Chromosome studying is quite a laborious method. It is used only in experiments with a relatively small number of fish, especially in order to calibrate an indirect method or in the event of aneuploidy.

INDIRECT METHODS

Nucleoli quantification

Phillips et al. (1986) and Flajshans et al. (1992) developed a method that makes it possible to identify specimens of different ploidy level. They exploited the fact that in some fish species the number of nucleoli in interphase cells reflects the ploidy level of the organism.

The nucleolus organizer regions (NORs) contain multicopy clusters of the 18S, 5.8S and 28S ribosomal RNA genes (rDNA), responsible for organizing the nucleolus, which is the organelle that synthesises rRNA for ribosomal subunits and therefore is essential for all protein synthesis (Reeder 1990). The NORs can be detected with the silver (Ag-NOR) staining technique (Howell and Black 1980). Silver binds to NOR proteins such as the RNA polymerase I subunit conjugated to rRNA. This enables visualization of all nucleoli and NORs, which were active during the previous interphase. Nucleoli can be visualized in interphase cells from blood or epithelium (fins and gills). Cell preparations are fixed in methanol and stained with silver nitrate as described by Howell and Black (1980). Nucleoli and NOR sites in chromosomes are clearly visible as brown-black dots in a yellow nucleus (Figure 1, 2).

Polymorphism related to transcriptional inactivation of nucleolus organizer regions (NORs) have long been described in many organisms (Castro et al. 1996; Fujiwara et al. 1998; Mellink et al. 1992). This means that some NOR sites can be inactive during transcription and not visible after silver staining. These NORs do not form nucleoli, so the number of nucleoli is variable among cells (Ueda et al. 1988). This variation is not a problem in species possessing only one NOR-bearing chromosome pair, like rainbow trout. In diploid organisms which have NOR-connected genes in only one locus (one pair of chromosomes), one or maximum two nucleoli can be organized (Figure 1A). In triploid fish there are one, two or maximum three nucleoli per cell (Figure 1C), whereas in tetraploids four nucleoli at most can be found per cell (Figure 1E). Kucharczyk et al. (1997) showed that analysis of no more than 40 interphase cells per individual enables positive identification of haploid, diploid and triploid specimens of bream (*Abramis brama*). Babiak et al. (1998) applied this method to examine rainbow trout subjected to mitotic heat shock manipulation, and the authors revealed up to 18.7% of tetraploids among examined fish.

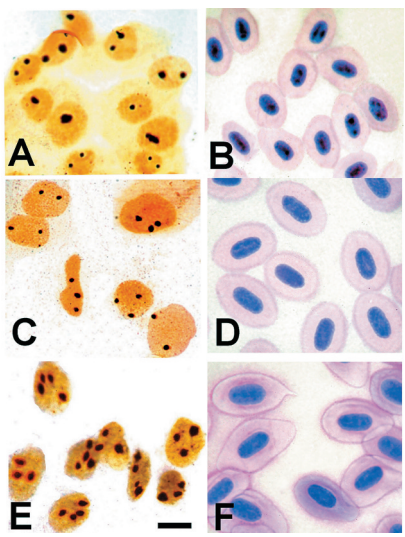

Figure 1. Silver-stained nucleoli (black dots) in fin epithelial cells (left column) and giemsa-stained erythrocytes (right column) of diploid (A and B), triploid (C and D) and tetraploid (E and F) rainbow trout, *Oncorhynchus mykiss*. Bar equals 10μm.

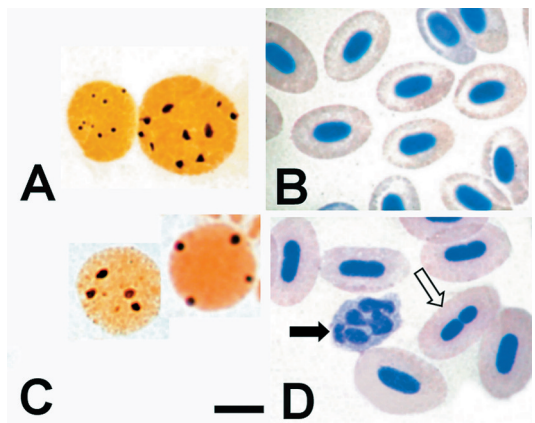

Figure 2. Silver-stained nucleoli (black dots) in fin epithelial cells (left column) and giemsa-stained erythrocytes (right column) of diploid (A and B) and triploid (C and D) brook trout, *Salvelinus fontinalis*. Black arrow shows hypersegmented granulocyte, white arrow shows divided erythrocyte nucleus. Bar equals 10μm.

Table 1. Number of NOR bearing chromosome pairs in aquaculture important fish species.

Species	Number of NOR bearing chromosome pairs	References
Rainbow trout (*Oncorhynchus mykiss*)	1	Phillips et al. 1986
Brook trout (*Salvelinus fontinalis*)	5	Phillips and Ihssen 1985
Atlantic salmon (*Salmo salar*)	1	Woznicki and Jankun 1994
Brown trout (*Salmo trutta*)	1-5	Woznicki et al. 2000
Common carp (*Cyprinus carpio*)	1	Anjum and Jankun 1998
Crucian carp (*Carassius auratus*)	2	Boroń 1994b
Pleuronectidae (*Scophthalmus maximus*, *Platichthys flesus, Solea solea*)	1	Pardo et al. 2001
North African catfish (*Clarias gariepinus*)	1	Ozouf-Costaz et al. 1990
Pacu (*Piaractus mesopotamicus*)	3	Foresti et al. 1994
Black pacu (*Colossoma macropomum*)	2	Foresti et al. 1994
Nile tilapia (*Oreochromis niloticus*)	3	Foresti et al. 1993

Analysis of the number of nucleoli per nucleus was used successfully in the case of rainbow trout (*Oncorhynchus mykiss*) (Flajshans et al. 1992; Kuzminski et al., unpublished data; Phillips et al. 1986) and chinook salmon (*Oncorhynchus tshawytscha*) (Phillips et al. 1986).

However, the technique does not allow reliable identification of fish of higher degree of ploidy (for example in a mixture of 3n, 4n and 6n); in such cases it is only possible to distinguish diploid individuals from polyploid ones. Although one NOR-bearing chromosome pair (i.e. simple NORs) is present in most analyzed fish groups, multichromosomal locations, i.e. multiple NORs, have been described in a number of species (e.g. Galetti and Rasch 1993, Schmid et al. 1995). Among aquaculture important fish species there are both systems of NORs (Table 1). The analysis is much more complicated in species possessing more than one NOR locus. Brook trout has five NOR-bearing chromosome pairs (Phillips and Ihssen 1985), so theoretically 10 nucleoli can be created if all sites are active. Frequently, only one or two nucleoli are observed in cells of this species, because most of sites are inactive or several NOR sites are creating one nucleolus together (NOR association) (Figure 2A, C). Species possessing multilocus rDNA location are characterized by high variation level of active NOR sites in chromosomes and organised nucleoli due to transcriptional inactivation of NOR genes. For this reason, the method relying on counts of nucleoli is useless for ploidy level determination in such species.

Erythrocytes nucleus sizing

An important physiological consequence of induced triploidy is the resultant increase in cell size and decrease in cell number observed for a variety of cell types (Benfey 1999). Many authors (Benfey and Sutterlin 1984; Benfey et al. 1984; Boroń 1994a, b; Chourrout et al. 1986) have documented that the examination and measurement of the erythrocyte size is an easy method to distinguish the triploid and diploid forms of different fish species. The cell and nucleus minor axes were found to be poor predictors of ploidy (Benfey et al. 1984). The increase in erythrocyte cell and nucleus volume associated with triploidy is mainly a result of an increase in their major axis (Benfey and Sutterlin 1984).

Woznicki and Kuzminski (2002) used fish of known ploidy in order to check if measurements of the major axis of erythrocyte nuclei from blood smear reflect the difference between diploids and triploids in brook trout (Figure 2B, D). Triploid and diploid brook trout significantly differed in the length of erythrocyte nuclei (Woznicki and Kuzminski 2002). Measuring erythrocyte nuclei can be helpful in effective estimation of the polyploidization process in fish (Figure 1) (Chourrout et al. 1986; Ocalewicz et al. 2007). The method was used in an experiment concerning efficiency of meiotic pressure shock in brook trout (Kuzminski et al., unpublished data). Such parameters as the pressure, the moment of applying the shock and its prolonged duration were checked in different combinations. Two groups of fish differing in the major axis of erythrocyte nuclei were clearly visible. Among

200 studied fish only two had intermediate length of nuclei in red blood cells. These individuals needed another method to recognize ploidy level such as the chromosome number study and their ploidy was revealed as 2n.

Other cytological parameters

Other cytological parameters can be also useful in ploidy level determination. Wlasow et al. (2004) described "ploidy specific pattern" of nuclei in erythrocytes and granulocytes in brook trout. Erythrocytes of triploid brook trout were elongated. Some of them exhibited abnormal nuclear changes. The nuclei were divided into two rather equal parts (Figure 2), the status that has been defined as nuclei segmentation (Yokote 1982). The number of erythrocytes with nuclear segmentation was significantly higher ($P<0.05$) in triploids than in diploids (Wlasow et al. 2004). Segmentation of erythrocyte nuclei in triploid and diploid brook trout was demonstrated by Benfey (1999) in his review, but no information was made available that would statistically confirm the difference in proportion of this phenomenon in both groups of fish. Wlasow et al. (2004) also showed a significantly higher average number of multi-lobed nuclear granulocytes in triploid than diploid brook trout (Figure 2). Most diploid fish (90%) had granulocytes with three or four lobes, whereas triploids possessed granulocytes with four to six lobes. However, the above cytological parameters can play only a subsidiary role in ploidy validation.

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