Theor Appl Genet (1992) 85:68–72

Frequency analyses of active NORs in nuclei of artificially induced triploid fishes

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Received April 5, 1991; Accepted March 10, 1992
Communicated by G. Wenzel

Summary. The correspondence between increased numbers of both chromosomal and nuclear NORs and artificially induced triploidy in three fish species (rainbow trout, *Oncorhynchus mykiss*; common carp, *Cyprinus carpio*; and tench, *Tinca tinca*) has been confirmed by CMA_3 fluorescence and Ag-staining. The frequencies of cell nuclei with one, two and three active NORs, as revealed by Ag-staining, has been analyzed statistically to find the minimum cell number which verifies the increased ploidy level. A minimum sample size of about 80 cells exhibiting three active NORs is sufficient to confirm triploidy in all three species and may be of use for categorising other ploidy-manipulated fish species.

Key words: Active NORs – Artificial triploidy – Rainbow trout – Common carp – Tench – Minimum necessary sample size

Introduction

Phillips et al. (1986) have proposed that it should be possible to determine the ploidy level of artificially induced polyploid fishes by means of the numbers of nucleolar organizer regions (NORs) in interphase nuclei as revealed by the silver staining procedure. This approach implies that the number of NORs corresponds to the ploidy level. However, there are at least two complications to the practical application of this approach. Firstly, the number of NORs need not necessarily correspond to the ploidy level. Evolutionary diploid fish species have sometimes increased the number of their NORs by various chromosomal rearrangements while evolutionary polyploid species often have the number of NORs decreased by a re-diploidization processes. For example, diploid North American leuciscine cyprinids frequently exhibit multiple NOR sites in their complements (Amemiya and Gold 1990) while the evolutionary tetraploid common carp, *Cyprinus carpio*, posesses only two NOR sites (Zan et al. 1986; Mayr et al. 1986a; Sola et al. 1986). A similar situation exists in evolutionary polyploid salmonids where some species such as *Salvelinus* spp., exhibit multiple NOR sites (Phillips et al. 1989; Mayr et al. 1988) while other species, such as rainbow trout, *Oncorhynchus mykiss*, possess only two NORs sites (Mayr et al. 1986a, 1988). Hence, a necessary prerequisite for determination of ploidy level through the use of NORs is a correct description of their numbers and localization in normal, ploidy non-manipulated, individuals. Secondly, the silver staining procedure is known to demonstrate the remnants of the Ag-stainable rRNA-protein complex synthesized by the NOR in the preceding interphase, and so identifies only the active NORs (Fakan and Hernandez-Verdun 1986). The actual number of NOR sites, therefore, need to correspond to the number of NORs revealed by the silver staining procedure, as has been demonstrated by the application of CMA_3 fluorescence which labels the corresponding NOR sites regardless of their activity. In fishes, this correspondence has been confirmed for the chromosomes of all species so far analyzed sequentially by these techniques (for review see Ráb and Mayr 1987). All these factors must be taken into account when using ths approach to ploidy determination. Since the application of silver staining for NOR quantification seems to be the most simple, inexpensive, rapid, and convenient method of ploidy determination of all those currently used for ploidy-manipulated fishes (Flajšhans 1992), we tested this approach in more detail. We selected three model fish

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species in which the correspondence between silver-stained and CMA_3-labeled NORs is well documented namely the rainbow trout (Mayr et al. 1986a, 1988), the common carp (Mayr et al. 1986b; Sola et al. 1986) and the tench (Mayr et al. 1986b) and where, additionally, the technique of ploidy manipulation can be easily applied (Chourrout et al. 1986; Hollebecq et al. 1988; Linhart et al. 1991). In the present paper, the correspondence between the di- and triploid levels, as assessed by karyological analysis and the frequency analysis of silver- and CMA_3-stained NORs in interphase nuclei, is reported, together with a calculation of the minimum number of cells necessary for the determination of ploidy levels.

Materials and methods

Triploid yearlings of the rainbow trout, *Oncorhynchus mykiss*, and triploid larvae and yearlings of the common carp, *Cyprinus carpio*, and the tench, *Tinca tinca*, were used for this study. Triploid rainbow trout were produced by means of heat shock at the Salmonid Research Laboratory at Rutki, Poland, according to Chourrout et al. (1986), while triploid common carp and tench were produced by means of hydrostatic pressure shock and cold shock, respectively, at the Experimental Hatchery of R.I.F.C.H. at Vodňany (Linhart et al. 1991; Flajšhans et al. 1992). Chromosome preparations of yearlings of all three species were made following the method of Ráb and Roth (1988) to confirm triploidy; the quantification of NORs in erythrocyte nuclei of silver-stained blood smears was also used. Chromosome preparations of larvae were made according to Baksi and Means (1988 I), as modified by Flajšhans (1992), for both chromosome analysis and rapid NOR quantification in interphase cells. Briefly, before commencing exogenous feeding larvae were either kept in 0.02% colchicine overnight, than exposed to hypotonic 0.075 M KCl or to re-distilled water for 30 min and fixed in methanol-acetic acid, or all these steps were omitted. In both cases the larvae were smeared onto ethanol-purified microscope slides in a drop of 60% acetic acid at room temperature, followed by fixation with methanol and air-drying. Silver staining was used according to Howell and Black (1980) as modified by Ráb and Roth (1988). CMA_3 staining followed the protocol of Schweizer (1981). The number of individuals analyzed and the cells scored per specimen is given in Tables 1 and 2. The minimum necessary number of cells to determine ploidy level was tested by regression analysis.

Results

The three selected species have NOR sites localized in one chromosome pair only, in the normal diploid state i.e., with two chromosomal and/or interphase NORs per diploid set. In all three species the increase of ploidy level to triploidy results in a corresponding increased number of NOR sites, i.e., three chromosomal and/or interphase NORs as is evident from CMA_3 fluorescence analysis. We exemplify this situation with chromosomes (Fig. 1a) and interphase nuclei (Fig. 2a) of triploid rainbow trout. However, silver staining demonstrated one, two or three

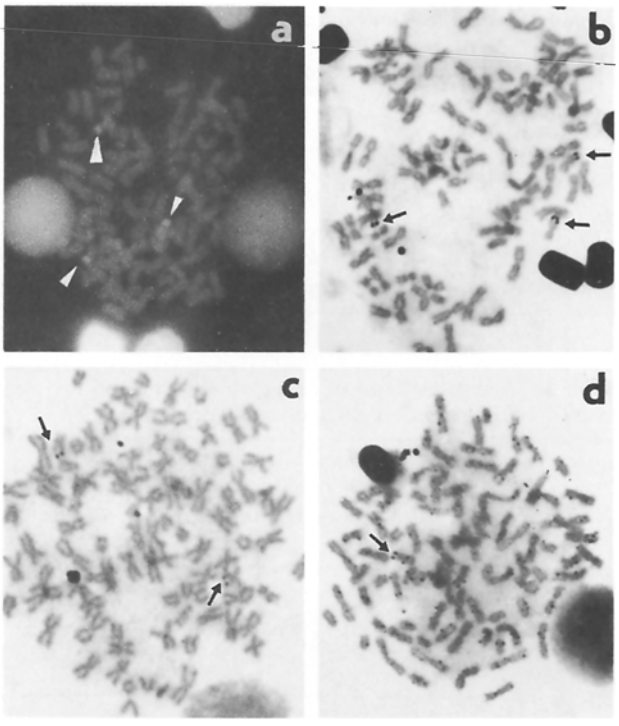

Fig. 1a–d. Metaphase chromosomes from triploid rainbow trout stained with CMA_3 (**a**) and silver (**b, c, d**) for localization of NORs. CMA_3-stained metaphase always exhibits three (**a**) NOR-associated heterochromatin-positive clusters (*arrowheads*), while those stained with silver exhibit three (**b**), two (**c**) or one (**d**) Ag-positive NORs (*arrows*)

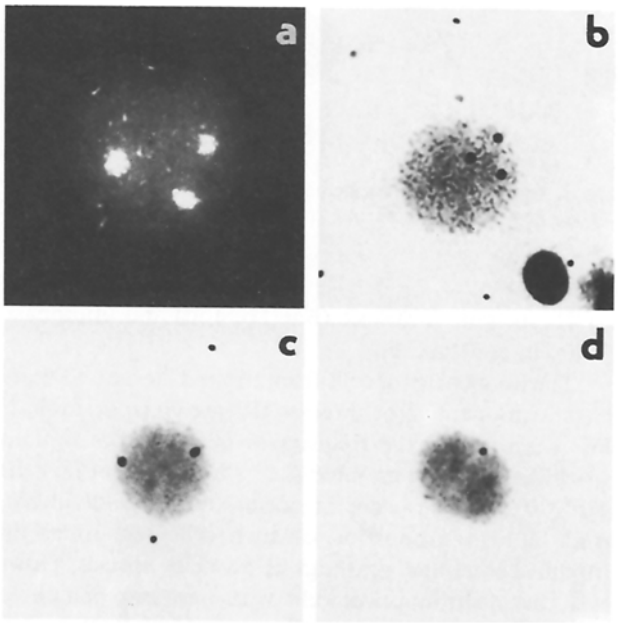

Fig. 2a–d. Interphase nuclei from triploid rainbow trout stained with CMA_3 (**a**) and silver (**b, c, d**). CMA_3-stained nuclei always exhibit three nucleolar-associated clusters while those stained with silver exhibit three (**b**), two (**c**) or one (**d**) Ag-stained nucleoli

Table 1. Average fractions of cells (%) with one, two and three Ag-stained NORs per cell in triploid larvae and yearlings

Species	Growth stage	No. of NORs per cell			No. of cells per individual	No. of individuals scored
		1	2	3		
Cyprinus carpio	Larvae	10.30	38.09	51.61	100	23
	Yearlings	4.50	32.25	63.25	100	20
Tinca tinca	Larvae	8.01	51.79	40.20	100	72
	Yearlings	10.50	45.25	44.25	100	10
Oncorhynchus mykiss	Yearlings	4.60	24.80	70.60	100	20

Table 2. Average fractions of cells (%) with one and two Ag-stained NORs per cell in diploid yearlings

Species	No. of NORs per cell		No. of cells analysed per individual	No. of individuals scored
	1	2		
Cyprinus carpio	22.75	77.25	100	4
Tinca tinca	18.50	81.50	100	4
Oncorhynchus mykiss	28.20	71.80	100	5

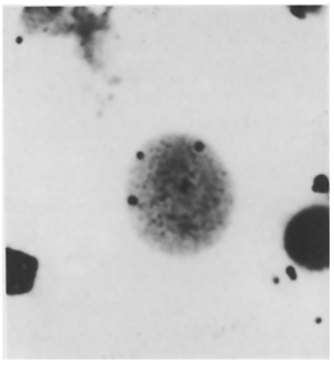

Fig. 3. Interphase nucleus demonstrating a rare case with four Ag-stained nucleoli from one triploid rainbow trout

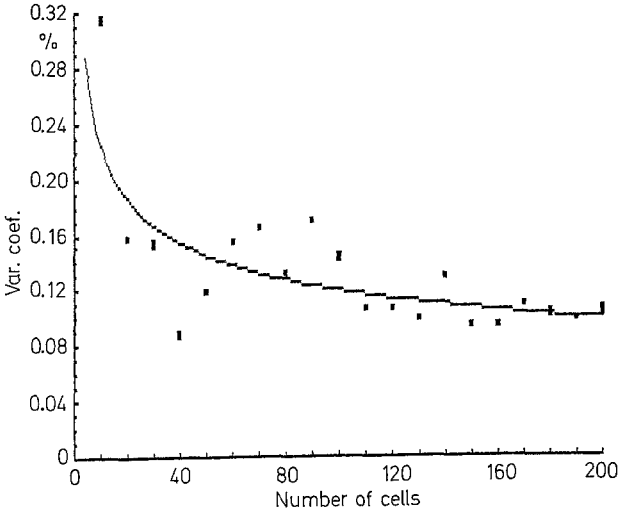

Fig. 4. A test of the minimum cell sample size necessary to confirm the ploidy level of triploid carp larvae by using the variation coefficients of nucleolar frequencies in relation to the number of cells sampled

NORs in both C-mitotic (Fig. 1 b, c, d) and interphase (Fig. 2 b, c, d) nuclei.

The frequencies of cells from triploid larvae and yearlings with one, two or three NORs are given in Table 1. For comparison, the frequencies of cells from diploid yearlings are given in Table 2. It is evident, that no cells with three NORs occur in normal diploid individuals, while a high proportion of such cells was found in triploid larvae and yearlings of all three species. However, the distribution of cells with one, two and three NORs differs both between larvae and yearlings as well as between the species under study.

During the quantification of NORs we detected several cells with four NORs in one triploid yearling of the rainbow trout (Fig. 3). Therefore, we quantified the distribution of all four variants. In more than 1000 cells, we found 7.51% of cells with one NOR, 36.92% with two, 51.11% with three and only 4.45% of cells with four NORs. The occurrence of this last variant proved not to be statistically significant ($P<0.05$).

In order to assess the minimum necessary number of cells per specimen required for the confirmation of ploidy level, the variation coefficient of the distribution frequencies of one, two and three NORs in samples of 10, 20, . . . to 200 cells was plotted against the number of cells and evaluated by regression analysis. Figure 4. shows the distribution of cells with three NORs in triploid carp larvae while Fig. 5 shows the distribution of cells with three NORs in triploid carp yearlings. Nearly the same curves were found for both larvae and yearlings of triploid rainbow trout and tench. It would appear, therefore, that a total of 80 analyzed cells is sufficient for the confirmation of the tested larvae or yearlings as triploid, given that the number of cells with three NORs is about 50% of the total examined and that the variation coefficient of this number is so low.

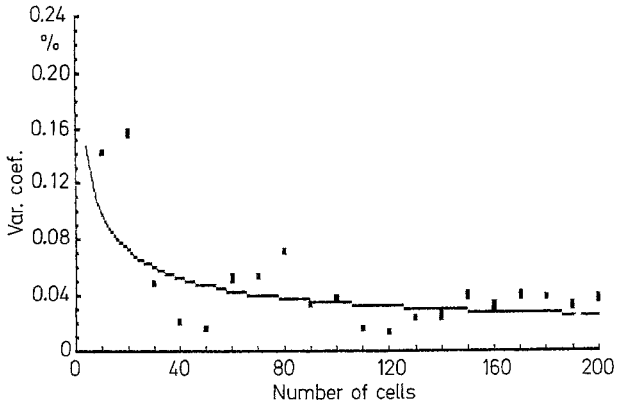

Fig. 5. A test of the minimum cell sample size necessary to confirm the ploidy level of triploid carp yearlings by using the variation coefficients of nucleolar frequencies in relation to the number of cells sampled

Discussion

Ploidy level can be determined by means of direct karyological analysis, by enzyme electrophoretic analysis, or by methods of cell/DNA content analysis such as flow cytometry, microdensitometry, cell size measurement or Coulter Counter Channelyzer Analysis (for detail reviews see Thorgaard 1983; Benfey et al. 1984; Wattendorf 1986; McCarter 1987; Flajšhans 1992). Most of these methods require more or less expensive equipment or else are time-consuming. Most also require blood or tissue samples, which means a delay of several weeks or more before it is possible to obtain a result. It would clearly be advantageous to check the success of a polyploidy inducing treatment with a method which facilitates analysis just after hatching. The application of silver staining for the quantification of NORs provides just such a solution.

In this study, we confirm that an increase in ploidy level brought about by experimental manipulation leads to an increase in the number of NOR sites as revealed both by CMA_3 and silver-staining. Therefore, we confirm the validity of the approach proposed by Phillips et al. (1986) for determining ploidy level by NOR quantification in ploidy-manipulated fishes. Phillips et al. (1986) studied the active NOR distribution in ploidy-manipulated rainbow trout and two other *Oncorhynchus* species. In addition to the rainbow trout we have used two ploidy-manipulated cyprinids: carp and tench. Phillips et al. (1986) found a higher frequency of cells with three NORs in embryos (75%) than in 6-month fingerlings (43%) with an even lower frequency in 1–2 year-old fishes (36%). Clearly our results (Tables 1 and 2) differ from theirs, a difference which could be in part due to the fact that the frequency of NOR activity as revealed by the silver staining of sufficiently large samples may not be simply age dependent. Although we do not propose to discuss this problem in more detail at this time, our unpublished data indicate that factors other than age e.g., tissue-, season-, strain-, or species-specificity may be involved. The existence of a triploid individual with a fraction of cells with four NORs could be explained as a result of polyploid mosaicism or else by chromosomal rearrangements involving the NOR-carrying chromosomes. A similar situation has been noted by Mayr et al. (1986b) in diploid *Tinca tinca* and two other diploid cyprinid species where a statistically insignificant occurrence of cells with three Ag-stained NORs was reported.

Despite these complications we confirm that ploidy determination is possible by means of the quantification of silver-stained NORs in interphase nuclei since a clear statistical relationship exists between ploidy level and the number of NORs. We conclude that a sample of 80 cells is the minimum necessary to statistically confirm triploidy. Moreover, since the value of this minimum sample size was found to be more or less equal for both salmonids (rainbow trout) and cyprinids (carp, tench) we suggest that it may be adequate for use with other ploidy manipulated fish species.

Acknowledgements. Our thanks to Dr. Petr Roth, Institute of Animal Physiology and Genetics, Department of Genetics, C.S.A.S. Liběchov, for valuable help and comments, to Dr. Oldřich Daněk, Research Institute of Fish Culture and Hydrobiology, Vodňany, for computer processing our data, and to Mrs. Karin Kottová and to Mrs. Daniela Odleváková of both Institutes, respectively, for their technical assistance.

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