Genetica 69: 111–118 (1986).
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NORs and counterstain-enhanced fluorescence studies in Cyprinidae of different ploidy level

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Abstract

The ribosomal RNA gene expression in the genomes of evolutionary diploid (*Scardinius erythrophthalmus, Leucaspius delineatus, Tinca tinca)* and polyploid species *(Cyprinus carpio, Carassius carassius, Carassius auratus gibelio, Carassius auratus auratus)* of Cyprinidae has been investigated by means of a silver nitrate technique. The diploid species investigated exhibited only one pair of chromosomes with nucleolus organizers (NOR). Higher numbers of rRNA-expressing chromosomal sites in several evolutionary polyploid species (Carassins) gave evidence against a complete functional diploidization, at least with regard to the NOR bearing chromosomes in these species. The NORs displayed a heterochromatic brilliant chromomycin A_3 fluorescence. No distamycin-A/DAPI-bright heterochromatic blocks were detected in the genomes of the Cyprinidae.

Introduction

Ohno *et al.* (1967) provided evidence suggesting that there are diploid-tetraploid relationships caused by an early polyploidization event among members of the fish family Cyprinidae. Their results were based on studies of the chromosome numbers and DNA values of the species. Up to now tetraploidy has been reported in the following cyprinid genera:
Aeroscheilus (Suzuki & Taki 1981), *Aulopyge* (Berberovic *et al.*, 1973), *Barbus* (Sofradzija & Berbenik, 1973; Cataudella *et al.*, 1977), *Carassius* (Ojima *et al.*, 1972; Zan & Song, 1980), *Cyprinus* (Zan & Song, 1980), *Tor* and *Schizotharaeichthys* (Khuda-Bukhsh, 1982; Rishi *et al.*, 1983). In different subspecies of *C. auratus* individuals with 100, about 150 and about 200 chromosomes are known (Ueda & Ojima, 1978).

Evolutionary tetraploid cyprinid species are thought to be engaged in the process of diploidization at the level of gene expression (Klose *et al.*, 1969). This process is believed to be far advanced in cyprinids (Klose *et al.*, 1969; Schmidtke & Engel, 1975; Schmidtke *et al.*, 1976; Schmidtke *et al.*, 1979).

Our investigations are aimed at the comparative cytogenetic determination of the number and localisation of Ag-NOR-positive (18S or 28S ribosomal RNA producing) chromosomes of some cyprinid species of different levels. Furthermore we investigated the genomes of the same species by counterstain-enhanced fluorescence methods.

Material and methods

The diploid group comprised *Tinca tinca* (2n = 48, 3 males and 2 females); *Scardinius erythrophthalmus* (2n = 50; 2 males, 2 females); *Leucaspius delineatus* (2n = 50; 2 males, 3 females). All these animals originated from the Elbe and from the Danube river. Additionally, specimens of the evolutionary polyploid species *Cyprinus carpio* L. (2n = 100) were analyzed. These 3 males and 7 females originated from Czechoslovakian and Aus-

trian commercial fish breeding ponds. The specimens of *Carassius carassius* (2n = 100, 3 males, 1 female) originated from the Elbe. Specimens of *Carassius auratus* were also studied. They were of 2 different origins: (a) artificially reared *C. auratus auratus* (2n = 100); 4 males, 1 female) and (b) 5 wild females of *C. auratus gibelio* (2n = 160) from the Danube river. The hexaploid individuals (2n = 160) of *C. auratus gibelio* are all females, propagated unisexually by gynogenesis (Kobayashi *et al.*, 1970).

Standard procedures for chromosome preparation were used as described elsewhere (Rab, 1981). Silver-NOR-staining was performed after Kodama *et al.* (1980). Subsequent to the silver staining and photography, the preparations were destained and the same metaphases were subjected to the chromomycin A_3/distamycin A/DAPI (CDD) counterstaining method (Schweizer, 1980). By photographing selected cells with different filter combinations (e.g. the sequential use of Leitz filter blocks A_3 and E_3), the chromomycin A_3 and the distamycin A/DAPI staining were sequentially recorded. Subsequently, the slides were destained again and the

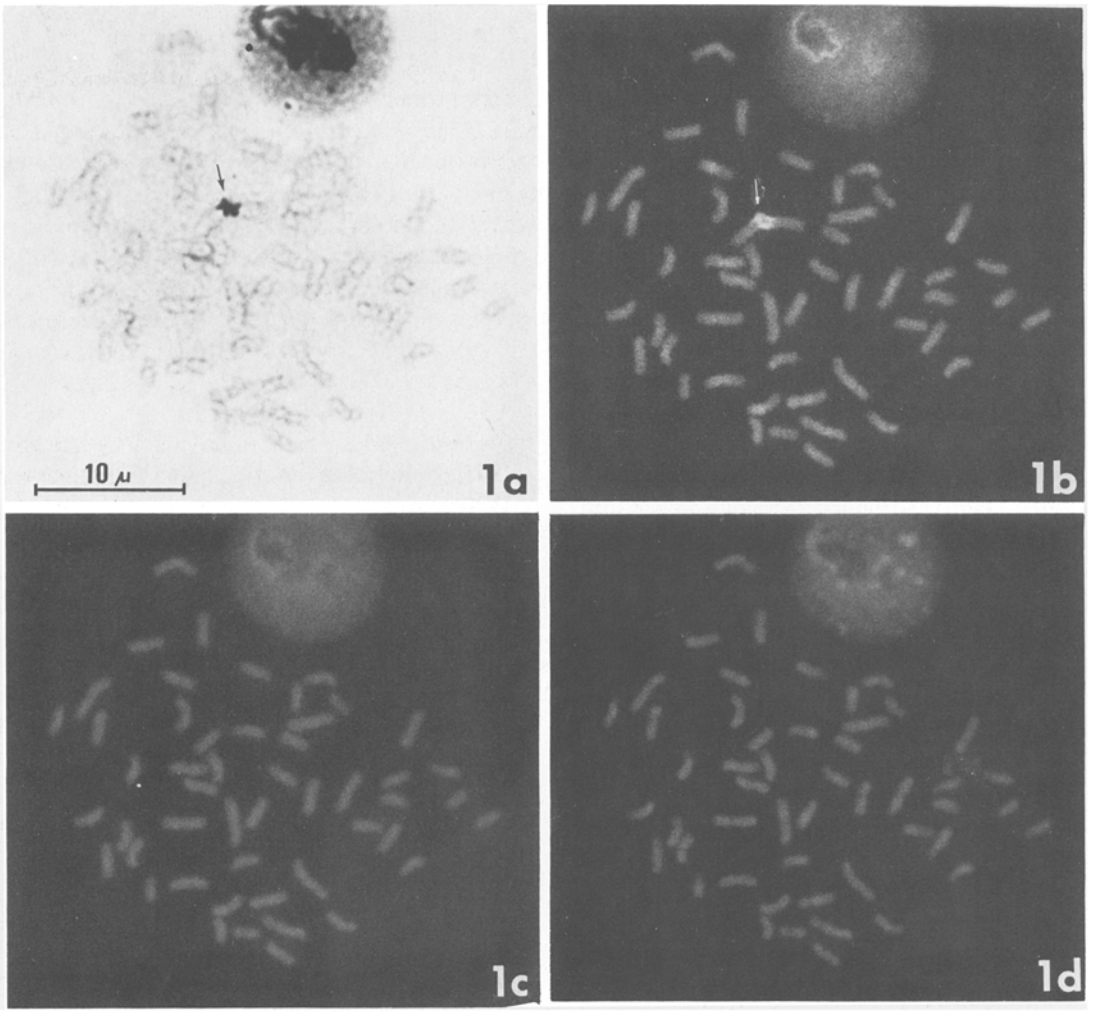


Fig 1. Sequential Ag-CDD staining of a lymphocyte metaphase of *Leucaspius delineatus:* (a) Ag-NOR-staining, note the two Ag-NORs on the associated two homologous chromosomes no. 24 (arrow); – (b) Chromomycin A_3 staining, note brilliant chromomycin A_3 fluorescence associated with the NORs regions (arrow); – (c) Distamycin A/DAPI, note lack of differentially stained fluorescence blocks; – (d) DAPI/Actinomycin D staining.

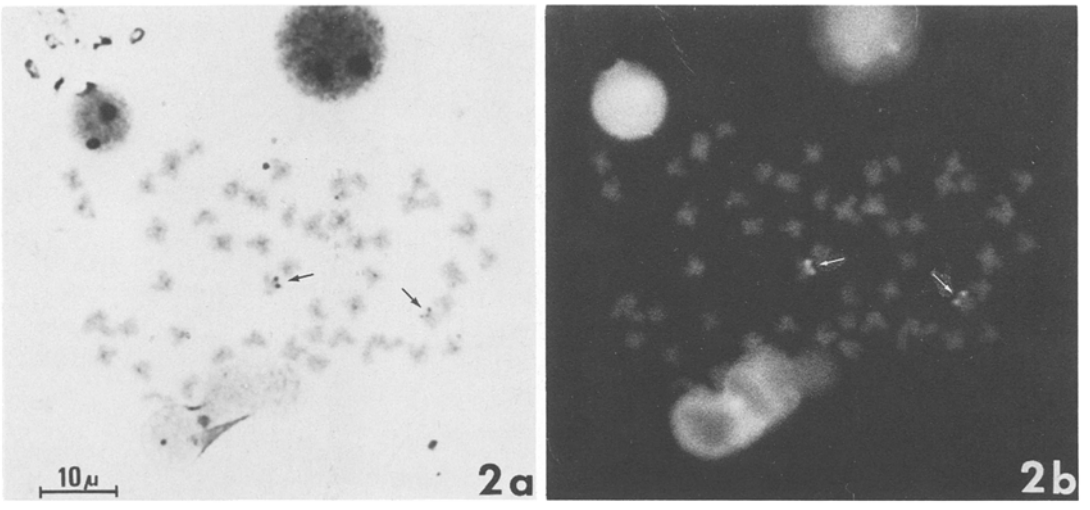


Fig. 2. Sequential Ag-CDD staining of a lymphocyte metaphase of *Scardinius erythrophthalmus:* (a) Ag-NOR staining; note the Ag-NOR regions (arrows); – (b) chromomycin A_3 staining, arrows indicate NOR region-associated chromomycin-A_3-positive heterochromatic block.

same cells were analyzed by the DAPI/actinomycin D counterstaining method (Schweizer, 1976). Additional studies were performed on interphase nuclei in order to determine the number of nucleoli present.

Results

Our investigations clearly indicated the presence of two NOR chromosomes in the metaphases of the diploid species *T. tinca, L. delineatus* and *S. erythrophthalmus.* The localisation of Ag-NORs was on satellites of small acrocentric chromosomes in the leuciscine species: Chromosome pair no. 24 in *L. delineatus* and chromosome pair no. 23 in *S. erythrophthalmus.*

In *T. tinca,* NORs were localised on secondary constrictions of chromosome pair no. 3. Figs. 1 and 2 demonstrate Ag-NORs of *L. delineatus* and *S. erythrophthalmus* in sequentially Ag and CDD stained metaphases. Figure 3 demonstrates an Ag-NOR stained karyotype of *T. tinca.* The nucleolus organizing regions are often seen in conventional Giemsa preparations as unstained or pale staining regions and are often involved in associative figures. Figures 4 and 5 demonstrate karyotypes prepared from Giemsa stained metaphases of the diploid species of the subfamily *Leuciscinae* Nucleolar chromosomes were readily identifiable in spite of the unsatisfactory band resolution along the chromosome arms. The chromosomal localisation of the Ag-NORs in *Carassius* and the carp are demonstrated in Figures 6 to 8. At present a reliable and unequivocal identification of the NOR chromosomes in a karyotype is impossible, because in these species chromosomes appear very condensed and no band resolution was achieved. Interphases exhibiting three nucleoli were seen infrequently in these diploid species (See Table 1).

In the metaphases of evolutionary tetraploid species of carp (2n = 100) we consistently found two Ag-NORs and no significantly higher numers of nucleoli in the interphase cells (see Table 1).

Quite a different situation was highlighted in the evolutionary tetraploid *C. carassius auratus auratus* (2n = 100) and *carassius* (2n = 100) as well as in the evolutionary nearly hexaploid *Carassius auratus gibelio* (2n = 160).

In the *C. auratus auratus* and *C. carassius* metaphases, three Ag-NORs were found. The frequencies of interphases exhibiting three nucleoli were rather high in these species (20.4% and 21.7% respectively). Even more extreme was the situation in our *C. auratus gibelio.* Here we found four Ag-NORs in the metaphases and the frequencies of interphase nuclei with 3 (28.4%) or 4 (11.3%) nucleoli were rather high.

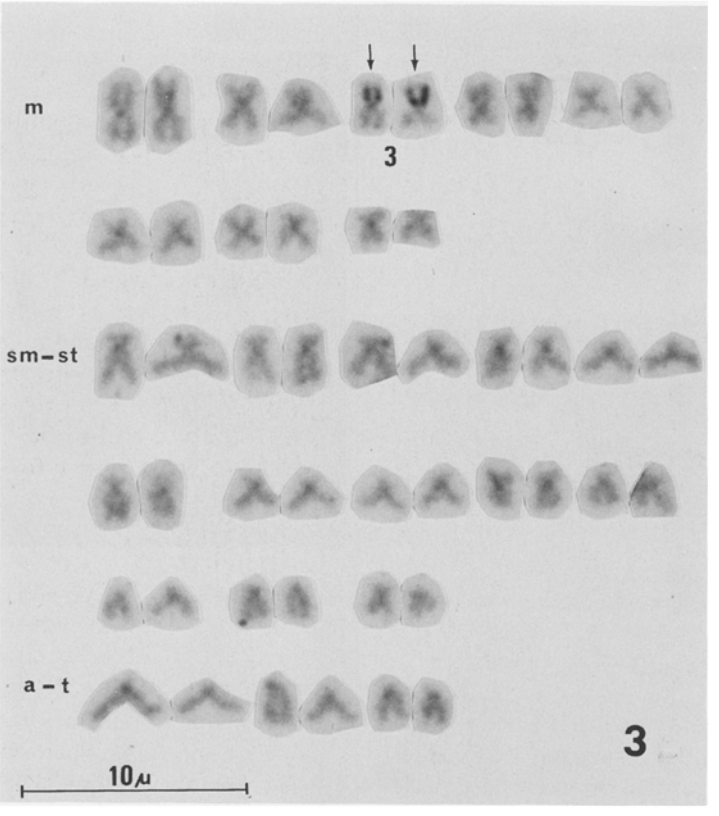
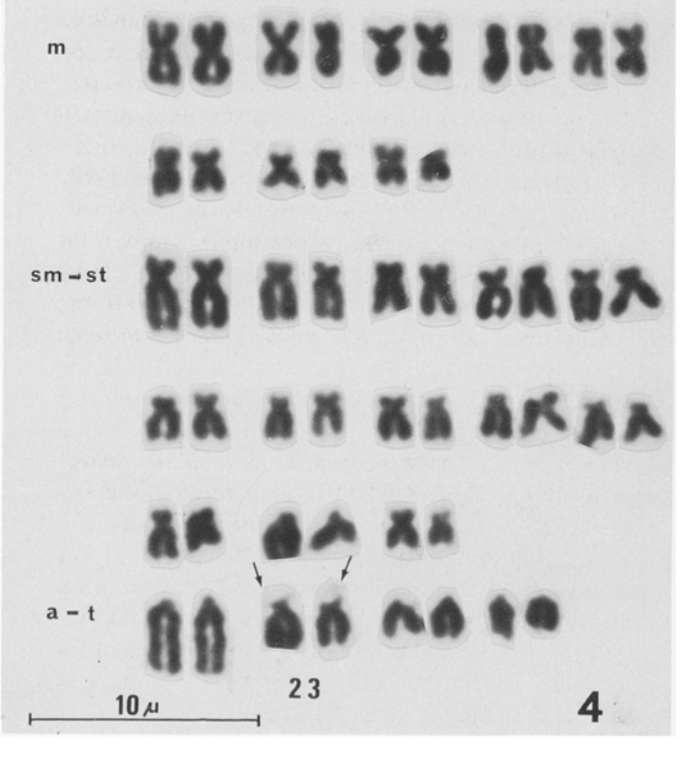


Fig 3. Ag-NOR stained karyotype of *Tinca tinca*. Note NORs on secondary constriction of chromosomes no. 3.



Chromomycin/Distamycin/DAPI staining revealed a rather uniform chromomycin A_3 fluorescence along the length of the metaphase chromosomes without satisfactorily resolvable bands in all the species examined. However, the nucleolus organizer regions displayed a brilliant chromomycin A_3 fluorescence (see e.g. Fig. 1b). The nucleoli in interphase nuclei were also contrasted by a brilliant chromomycin A_3 fluorescence. In all the investigated species, the distamycin in A/DAPI-analyses of the metaphases did not selectively highlight heterochromatic regions and the fluorescence pattern along the chromosome is rather uniform (Fig. 1c). DAPI/actinomycin D staining also did not provide selectively fluorescing prominent heterochromatic markers; moreover, no clear banding patterns were

Fig. 4. Giemsa stained karyotype of *Scardinius erythrophthalmus*. Note satellite stalks on acrocentric NOR chromosome pair no. 23 (arrows).

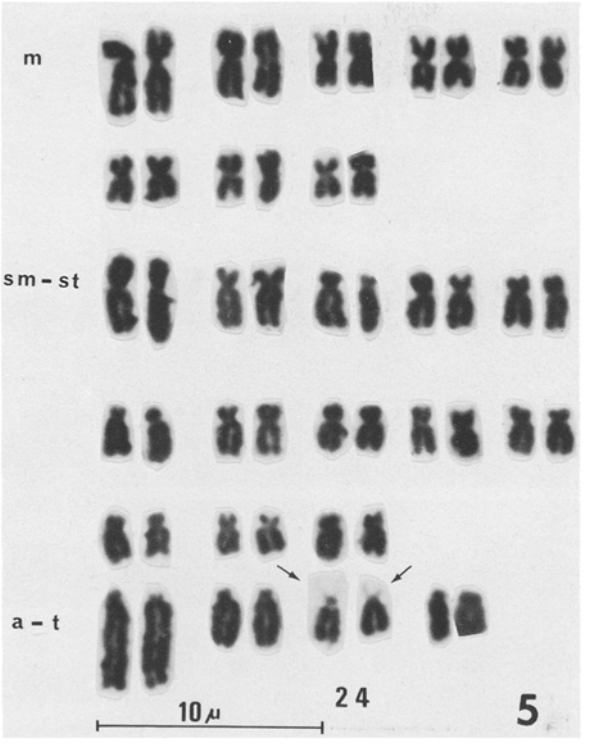


Fig. 5. Giemsa stained karyotype of *Leucaspius delineatus*. Arrows indicate satellite stalks of acrocentric NOR-chromosome pair no. 24.

observed along the highly condensed metaphase chromosomes by this procedure (Fig. 1d). Nevertheless, CDD and DAPI/actinomycin-D-induced banding patterns were readily observable along the chromosome arms in the less condensed prophases and prometaphases. The DAPI/actinomycin D fluorescence of the nucleolar organizer constrictions was extremely weak and less intensive than the fluorescence of the non-nucleolar chromosomal regions.

Discussion

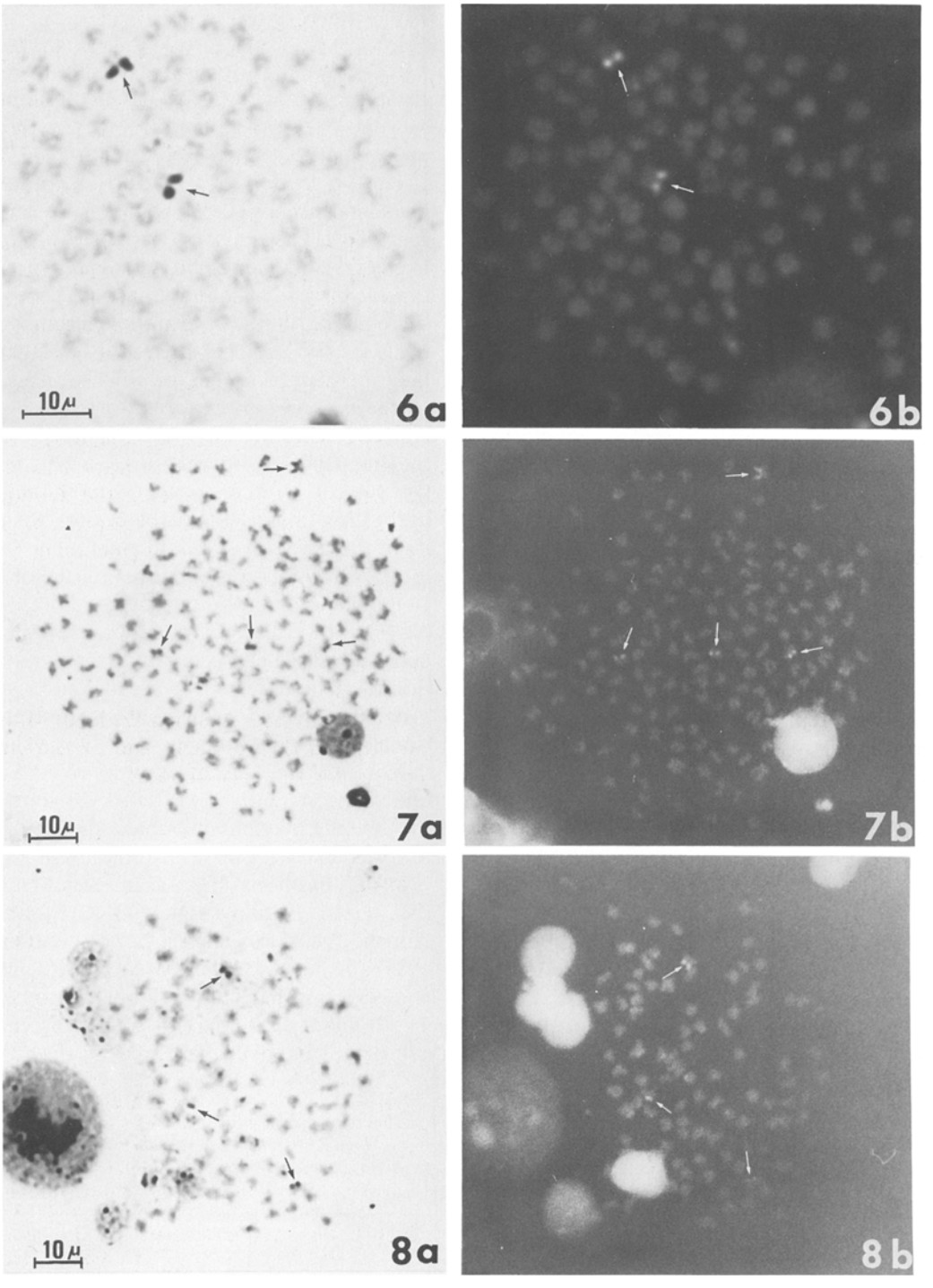
Although we consistently found only one pair of chromosomes with Ag-NORs in the diploid species, more than two nucleoli were seen in the interphase nuclei at a low frequency.

These exceptions are probably attributable to the incidental occurrence of polyploid cells in diploid species. However, the existence of a second nucleolus-organizing chromosome pair cannot be excluded with absolute certainty. Theroretically such an Ag-NOR on another chromosome pair could be too small to be clearly visualized under the experimental design for our metaphase studies. In the evolutionary tetraploid carp also only two Ag-NOR chromosomes are regularly visible and the frequency of interphase cells with more than two nucleoli is not distinctly higher in the carp than in the diploid species. For this reason, the probability of the existence of a third and fourth NOR chromosome is rather low. However, partial or complete functional inactivation of a third and a fourth NOR in the evolutionary tetraploid species of carp could be a further mechanism to prevent the detection of metaphase NORs, because silver staining is generally believed to demonstrate only transcriptionally active rRNA genes.

However, the lack of chromomycin A_3 positive (and concomitantly Ag-NOR-negative) clusters in the genome may possibly make the latter explanation less likely. Positive Chromomycin A_3 staining of NORs has been observed in several plant genera (Schweizer, 1976; Deumling & Greilhuber, 1982; Appels, 1982), in some species of Anura (Schmid, 1982), in a grasshopper species (Schweizer *et al.*, 1983) and in a fish species (Mayr *et al.*, 1985). The brilliant chromomycin A_3 fluorescence of the 'ribosomal bands' is favoured by the rather high

Table I. Frequency of interphase cells with 1, 2, 3 and 4 nucleoli in seven species of Cyprinidae (1000 cells were counted in each species).

	% of cells with one nucleolus	% of cells with two nucleoli	% of cells with three nucleoli	% of cells with four nucleoli
Leucaspius delineatus	57.0	41.5	1.5	-
Scardinius erythrophthalmus	68.7	28.2	3.1	-
Tinca tinca	26.0	71.5	2.5	-
Cyprinus carpio	40.5	57.0	2.5	-
Carassius carassius	32.9	39.6	21.7	5.8
Carassius auratus auratus	37.1	42.5	20.4	-
Carassius auratus gibelio	23.0	37.3	28.4	11.3



Figs. 6–8. Fig. 6. Sequential Ag-CDD staining of a lymphocyte metaphase of *Cyprinus carpio:* (a) Ag-NOR-staining (arrows indicate silver NORs); – (b) Chromomycin A_3 staining. *Fig. 7.* Sequential Ag-CDD staining of a lymphocyte metaphase of *Carassius auratus gibelio:* (a) Ag-NOR-staining; – (b) Chromomycin A_3 staining. *Fig. 8.* Sequential Ag-CDD staining of a lymphocyte metaphase of *Carassius carassius:* (a) Ag-NOR-staining; – (b) Chromomycin A_3 staining.

GC-content of rDNA (e.g. Sinclair & Brown, 1971) because chromomycin A_3 is thought to selectively bind to GC-rich DNA (Schweizer, 1981). The negative staining of the chromomycin-A_3 positive ribosomal bands by distamycin A/DAPI and DAPI/actinomycin D is in good accordance with this explanation, because DAPI is known to be AT-specific (Schweizer, 1981). Of course, it is unknown whether the chromomycin-A_3-stained GC-rich sequences are ribosomal cistrons themselves or sequences associated with them.

If we accept the presence of only one Ag-NOR chromosome pair in the carp genome, this does not necessarily justify any statement for either a ribosomal DNA gene loss or inactivation. For instance, unequal crossing overs and exchanges could have taken place between NOR-regions resulting in addition of rDNA to the NOR of one originally present chromosome and loss of rDNA in the other chromosome. In any case, the biochemical results of twice the amount of rDNA cistrons in the carp (Schmidtke & Engel, 1976), as compared to diploid species appear to be in line with our cytogenetic findings.

The individuals of the evolutionary tetraploid *C. auratus auratus* demonstrated three Ag-NORs in their metaphases; in contrast, individuals of the hexaploid *C. auratus gibelio* were characterized by four Ag-NOR chromosomes per metaphase. These results clearly suggest that these particular nucleolus organizing regions are still present in an active state, i.e. they have not been lost or silenced. Nevertheless, this does not mean, that no NOR-loss or inactivation has taken place in these species. Theoretically, the twofold ($4n = 100$ vs 50 in tetraploids) and nearly threefold ($6n = 160$ in hexapoids) chromosome number would make possible Ag-NOR numbers of 4 (2×2) or 6 (3×2) conceivable.

These speculative maximal NOR numbers have never been reached in our chromosome study. As in the carp, the possibility of unequal crossing cannot be ruled out as an explanation. As in the diploid species and in the carp, the silver NOR clusters encountered in our Carassins were regularly accompanied by chromomycin A_3 positive clusters. No evidence for additional prominent chromomycin A_3 positive, Ag-NOR negative clusters were found. Again, such a finding could have led, with restrictions, to the cautious speculation of inactivated rDNA genes.

Earlier comparative investigations on evolutionary tetraploid ($2n = 100$) species of Cyprinidae comprising *Cyprinus carpio* (Takai & Ojima, 1982), *C. auratus auratus, C. auratus* ssp., *C. auratus buergeri, C. auratus grandoculis* and *C. auratus cuvieri* (Takai & Ojima, 1982; Ojima & Yamano, 1980) consistently led to the detection of only two Ag-NORs associated with the 12th largest chromosome pair. In contrast, hexaploid ($2n = 150$) and unisexual gynogenetic *C.a. langsdorfii* bear Ag-NORs in a submetacentric chromosome pair and several 'tiny' chromosomes (Ojima & Yamano, 1980). Our results provide information about a new possibility for the easy depiction of marker chromosomes in diploid and polyploid fish species. They provide new knowledge about relationships of nucleolus organizer region and GC-rich heterochromatic markers in Cyprinidae. However, the development of potent reliable banding techniques for producing banded karyotypes in fishes including Cyprinidae would be very desirable.

Acknowledgements

We thank the Austrian Federal Ministries of Health and Environment, and of Agriculture and Foresty for financial support.

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Received 4.6.1985 Accepted 15.12.1985.