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Heterochromatins and band karyotypes in three species of salmonids

B. Mayr [1], M. Kalat [2] and P. Rab [3]

[1] Institute for Animal Breeding and Genetics, Veterinary University, A-1030 Vienna, Austria
[2] Ludwig Boltzmann Institute for Immuno- and Cytogenetic Research, Veterinary University, A-1030 Vienna, Austria
[3] Czechoslovak Academy of Sciences, Institute for Animal Physiology and Genetics, Department of Genetics, CS-277 21 Libechov, Czechoslovakia

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Summary: The heterochromatins of rainbow trout (*Salmo gairdneri* R.), brown trout (*Salmo trutta fario* L.) and brook trout (*Salvelinus fontinalis* M.) were characterized by sequential chromomycin A_3/distamycin A/DAPI (CDD) and DAPI/actinomycin D (DAPI/AmD) fluorescence. On most biarmed chromosomes, an equilocal localization of prominent DAPI/AmD positive, chromomycin A_3 negative, AT-rich blocks at the centromeres were observed in all three species. Band karyotypes of the three species were established. In rainbow trout, several DAPI/AmD positive heterochromatin blocks behaved positive in a silver-staining method. Mitotic and interphase studies proved the presence of inter-individual NOR variation in brown trout. The NORs of brook trout were localized on chromosomes 5, 10, 14, 15 and 29.

Key words: Heterochromatin – DAPI/actinomycin D – *Salmo gairdneri* R. – *Salmo trutta fario* L. – *Salvelinus fontinalis* M.

Introduction

Recently, Ag-NOR staining and counterstain-enhanced fluorescence methods, including chromomycin A_3 (CMA_3), have been applied to salmonid chromosomes (Phillips and Ihssen 1985; Phillips et al. 1986; Mayr et al. 1986). In these cases, some brilliant CMA_3 positive blocks and bands have been observed at, or close to, the NORs. In our preceding study (Mayr et al. 1986), we investigated the species *Salmo gairdneri* and *Salmo trutta*. In the present paper, we include the salmonid *Salvelinus fontinalis* and a DAPI/AmD fluorescence procedure was applied for the banding patterns. Moreover, a modified distamycin A/DAPI procedure was applied.

Materials and methods

The following specimens were included in our study: *Salmo gairdneri* R., $2n=62$, 3 males, 3 females; *Salmo trutta fario* L., $2n=80$, 2 males, 3 females; and *Salvelinus fontinalis* M., $2n=84$, 4 males, 3 females. Standard procedures for chromosome preparation were used. Silver-NOR staining was performed either as shown by Kodama et al. (1980) or Howell and Black (1980). Subsequent to silver staining and photography of suitable metaphases, the slides were destained and the same metaphases were subjected to the chromomycin A_3/distamycin A/DAPI (CDD) counterstaining method (Schweizer 1981). By photographing selected cells with different filter combinations (Leitz-Diaplan microscope; filter blocks A_3 and E_3), the CDD staining was sequentially recorded. Subsequently, the slides were destained again and the same cells were analyzed by the DAPI/actinomycin D counterstaining method (Schweizer 1976). Additionally, stimulated blood lymphocyte smears were prepared to determine the number of nucleoli (Agstaining) and the number of heterochromatic spots (DAPI/AmD staining).

Results

On the centromeres of many biarmed chromosomes of *S. gairdneri*, prominent DAPI/AmD-positive blocks were detected. The fluorescence intensity of these blocks often varied between the concerned homologous chromosomes of an individual. Especially brilliant DAPI/AmD clusters were found at chromosomes 1, 4, 9, 13, 15, 16 and 22. Figure 1 demonstrates the karyotype of *S. gairdneri* based on a silver, CMA_3 and DAPI/AmD stained metaphase (Fig. 4). The DAPI/AmD positive heterochromatin behaved heterogeneously in the silver-staining procedure of Kodama et al. (1980). In some chromosomes, DAPI/AmD positivity was accompanied

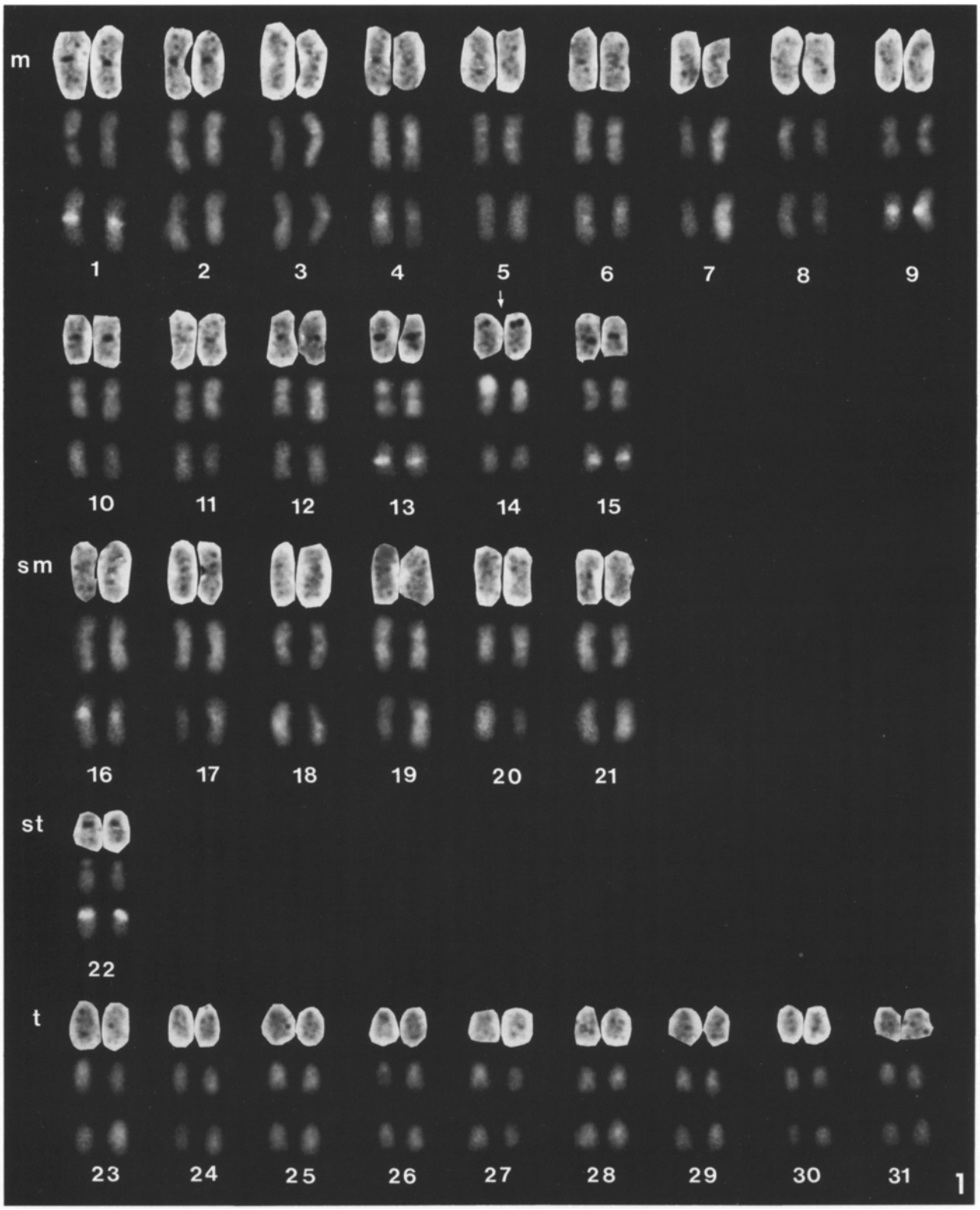
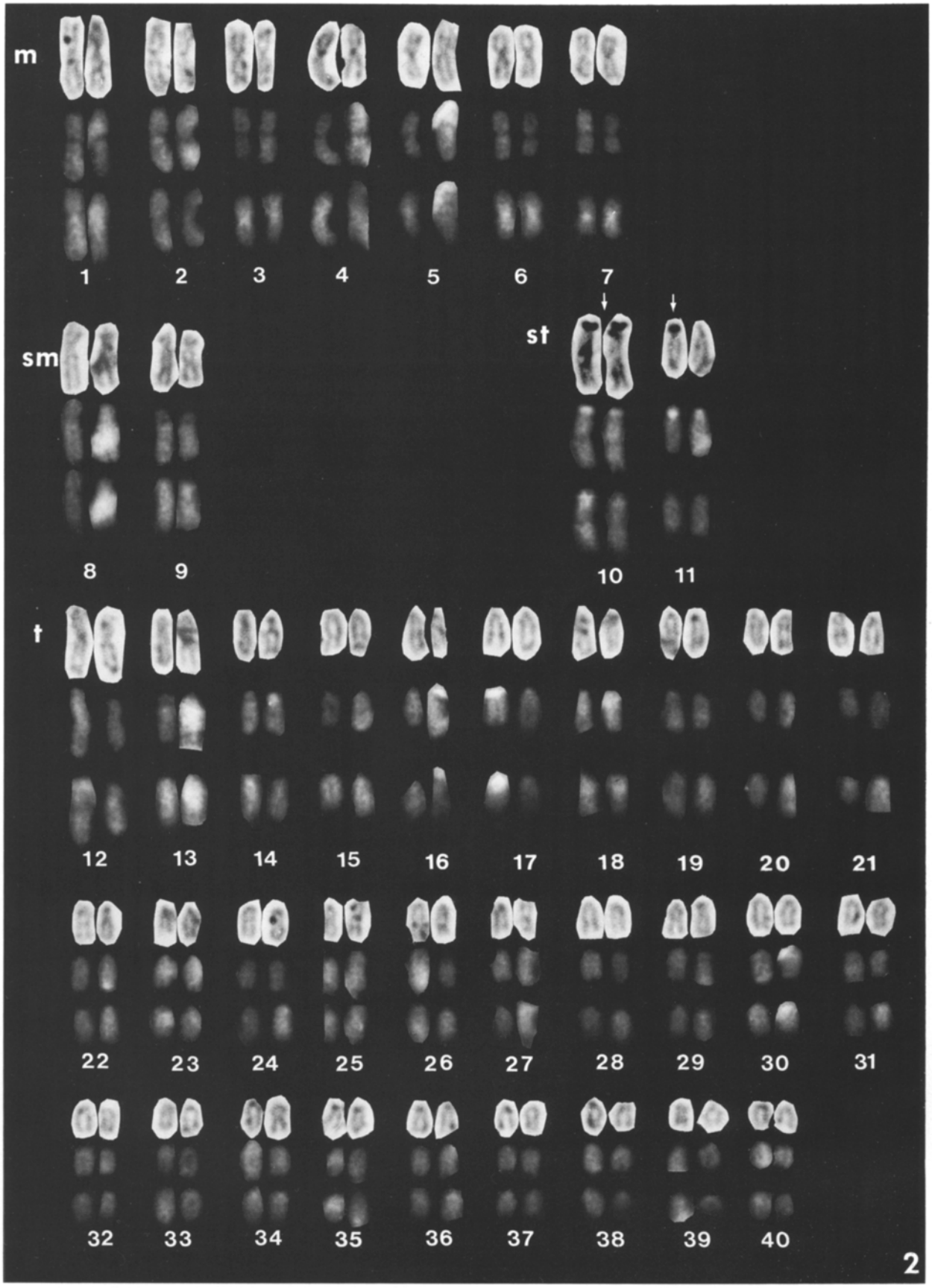


Fig. 1. Sequential Ag-CDD-DAPI/AmD staining metaphase of *Salmo gairdneri*. Ag-staining after Kodama — *upper lines*; CMA_3 — *middle lines*; DAPI/AmD — *lower lines*. Note the two Ag-NORs on no. 14 and the prominent centromeric DAPI/AmD blocks on the biarmed chromosomes nos. 1, 4, 9, 13, 15, 16 and 22

Fig. 2. Sequential Ag-CDD-DAPI/AmD staining metaphase of *Salmo trutta fario*. Ag-staining after Kodama — *upper lines*; CMA_3 — *middle lines*; DAPI/AmD — *lower lines*. Note the two Ag-NORs on no. 10 and one NOR on no. 11 (*arrows*)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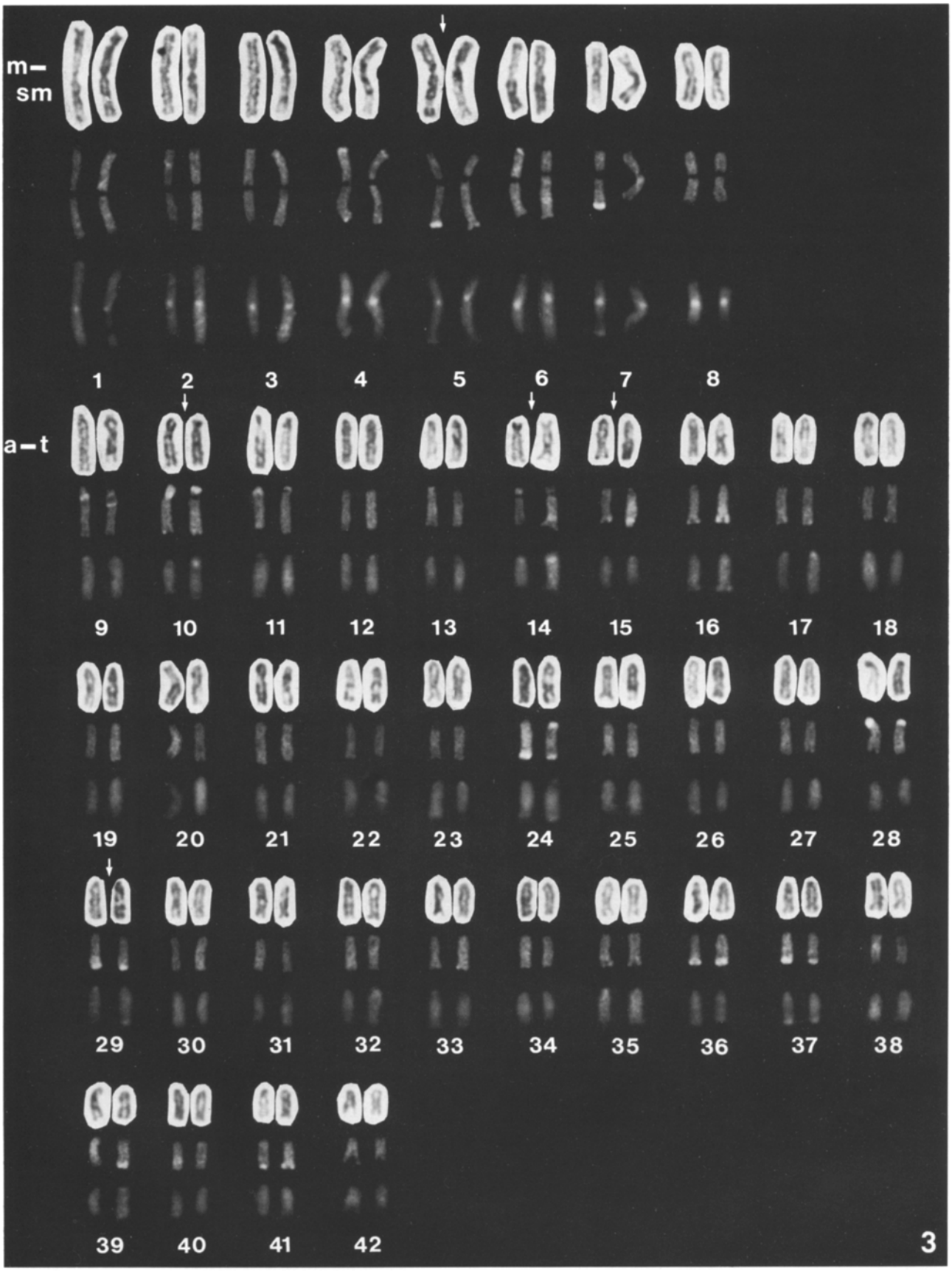
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by positive silver-staining. Striking examples were chromosomes 1, 4, 13, 15 and 22. On the other hand, some prominent DAPI/AmD positive heterochromatin clusters appeared pale in this silver observation. Examples were chromosomes 9 and 16. In our interphase studies in *S. gairdneri,* the DAPI/AmD positive heterochromatic regions became evident as brightly fluorescing spots. As expected from metaphase results, several DAPI/AmD intense chromatin spots were associated with a clear silver positive staining behavior, while others were not (Fig. 10a, b). Interestingly, the positive silver-staining of centromeres as found in the silver method of Kodama et al. (1980) was generally undetectable in the silver method of Howell and Black (1980). In the CMA_3-observation, the DAPI/AmD positive spots appeared very weakly stained or unstained. The CMA_3-brilliant bands flanking Ag-NORs remained unstained by DAPI/AmD (*arrow,* Fig. 1). Our distamycin A/DAPI staining procedure led to some differential visualization of the DAPI/AmD positive bands. However, distamycin A/DAPI bands were generally less intensely fluorescing as compared to DAPI/AmD bands (Fig. 7). It was stringently dependent on optimal microscopic optics and UV-exposure time. For this reason, they remained undetected in our earlier paper (Mayr et al. 1986).

In *S. trutta,* DAPI/AmD positive centromeric spots were observed on several chromosomes. Examples are chromosomes 1, 3, 6 and 7 (karyotypes in Fig. 2 and corresponding metaphase in Fig. 5). In contrast to *S. gairdneri,* no clearly positive Ag-staining behavior was observed at DAPI/AmD positive heterochromatin blocks in any of our applied silver-staining techniques. Our interphase results further confirmed this observation. DAPI/AmD positive spots became evident in the interphases and no unequivocable Ag-spots as corresponding counterparts were detected. Four of the five investigated individuals of *S. trutta* possessed Ag-NORs on chromosome pair no. 10 (Figs. 2 and 8). The fifth screened individual possessed an additional Ag-NOR on the homologue of a second chromosome pair no. 11 (Figs. 2 and 9). In our material, no individual with Ag-NORs on both homologues on no. 11 was present. In this animal (*S. trutta,* no. 3) a considerable percentage (21,6%) of interphases exhibited three nucleoli. Interestingly, even four nucleoli were not infrequent (1.4%) in the interphases of this animal (Table 1). As expected, the nucleoli were larger in cells with one nucleolus than in cells with more nucleoli due to nucleolar assembly.

In *Salvelinus fontinalis,* spectacular DAPI/AmD positive centromeric heterochromatic blocks have been found on the biarmed chromosomes (karyotype in Fig. 3 and corresponding metaphase in Fig. 6). Small centric DAPI/AmD spots were also detected on some telocentric chromosomes. Similary, as in *S. trutta* but in contrast to *S. gairdneri,* the DAPI/AmD blocks did not show up as clearcut silver-staining in our applied silver techniques (Fig. 3). Accordingly, our interphase studies revealed bright DAPI/AmD spots, which did not clearly show up in silver-staining. In many cases, the interphasic DAPI/AmD positive heterochromatin structures had an elongated, decondensed appearance. In the CMA visualization, the centromeric DAPI/AmD positive bands remained unstained and dark. Ag-NORs were detected on chromosomes 5, 10, 14, 15 and 29. The NORs localization in nos. 10, 14 and 29 were satellite stalks of the short arms, while the NOR-localisations in nos. 5 and 15 were the telomeres of the long arm. A high degree of intra-individual Ag-NOR variation was detected in *Salvelinus fontinalis.* The number of nucleoli in the interphases were as follows: one in 10%, two in 20%, three in 25%, four in 20%, five in 15%, six in 7%, seven in 2% and eight in 1%.

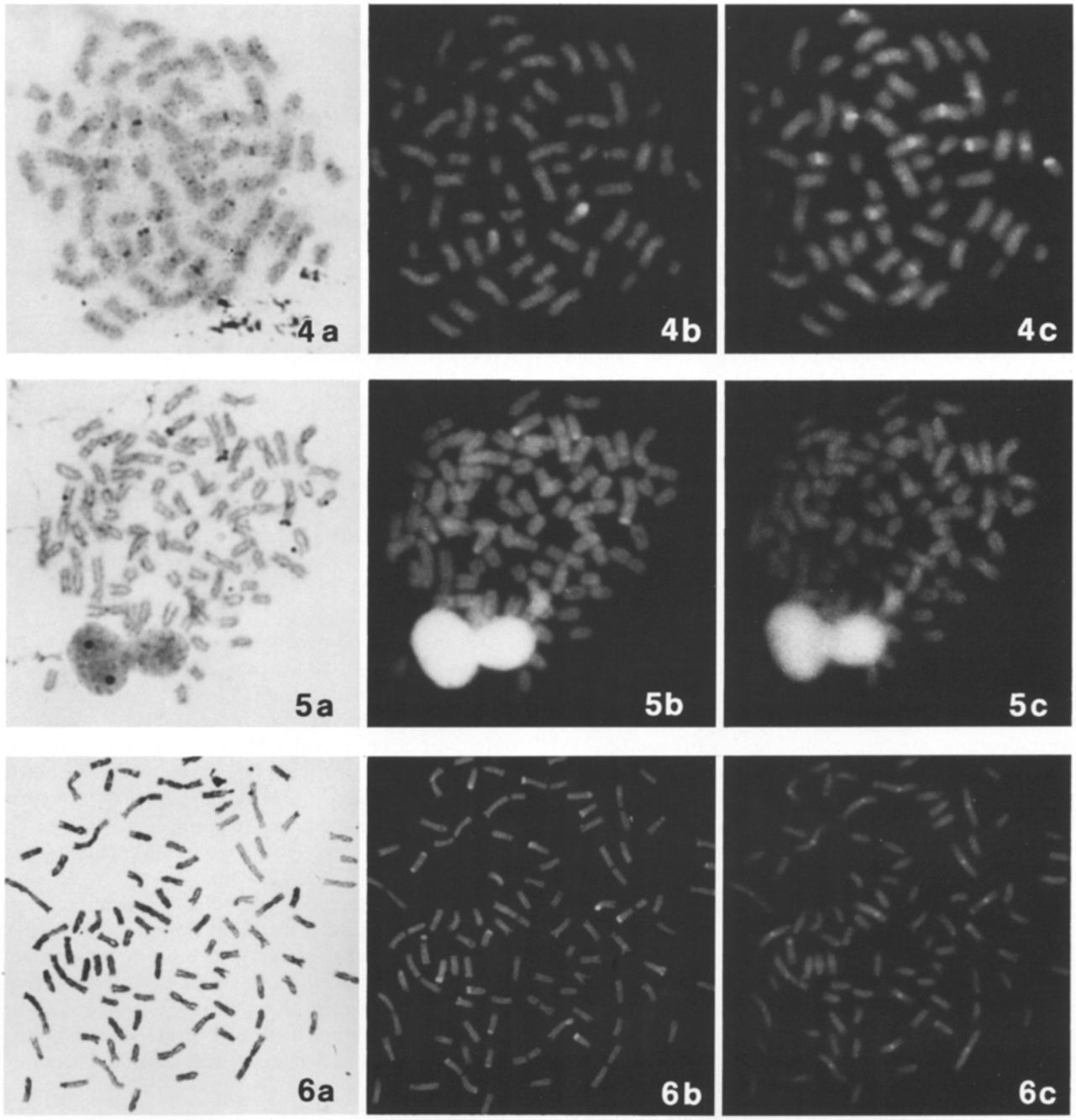
Clear CMA_3 positive blocks were observed associated with most Ag-NORs. Examples are chromosomes 5, 10 and 14. Interestingly, on Ag-NOR chromosome 29 this correspondence was hardly detectable. This chromosome bore a prominent CMA_3-positive region on the telomeres of its long arm, but only a faint one on its NOR-associated satellite stalks. A broad panel of further chromosomes demonstrates imposing CMA-blocks on their telomeres. The most convincing examples are nos. 24, 29, 36, 37, 39 and 41 (Fig. 3).

In regard to the homologisation and identifications, it has to be emphasized that trout chromosomes are fish chromosomes and therefore their banding patterns are considerably less satisfactory as compared to mammalian ones. Nevertheless, many chromosome pairs can be karyotyped easily and with high degrees of certainty. Typical members of this group with easily and certainly

Table 1. Frequency of interphase cells with 1, 2, 3 and 4 nucleoli in five individuals of *Salmo trutta fario* (1000 cells were counted in each individual

Animal no.	% cells with one nucleolus	% cells with two nucleoli	% cells with three nucleoli	% cells with four nucleoli
1	33.8	66.2	–	–
2	44.5	55.7	–	–
3	22.2	54.8	21.6	1.4
4	41.2	58.8	–	–
5	45.9	54.1	–	–

Fig. 3. Sequential Ag-CDD-DAPI/AmD staining metaphase of *Salvelinus fontinalis.* Ag-staining after Kodama — *upper lines*; CMA_3 — *middle lines*; DAPI/AmD — *lower lines.* Not the Ag-NORs on nos. 5, 10, 14, 15 and 29. Clear centromeric DAPI/AmD bands are visible on the biarmed chromosomes



Figs. 4–6. Sequential Ag-CDD/DAPI/AmD stained metaphase of *Salmo gairdneri* (**Fig. 4**), *Salmo trutta* (**Fig. 5**) and *Salvelinus fontinalis* (**Fig. 6**). **a** Ag-staining after Kodama; **b** CMA_3; **c** DAPI/AmD

homologizable chromosomes in the rainbow trout are chromosomes 1, 2, 3, 5, 9, 12–17 and 22. To this group in brown trout belong nos. 1–3, 6–13 and 18. Members of this group in brook trout are nos. 1–11, 14–19, 28, 29, 36, 37 and 41.

On the other hand, difficulties and uncertainties arose in the karyotyping of several pairs of our karyotypes. This holds true in spite of the combined and concerted consideration of DAPI/AmD and chromomycin A_3 banding and silver staining. Following are some typical examples of chromosome groups with tediously and hardly identifiable chromosomes. Chromosomes 7, 8, 11, 18–21 and 26–31 are concerned in rainbow trout. In brown trout are nos. 14–17, 24–27 and 30–36. In brook trout, nos. 20, 21, 25, 27, 32, 35 and 38 are typical examples.

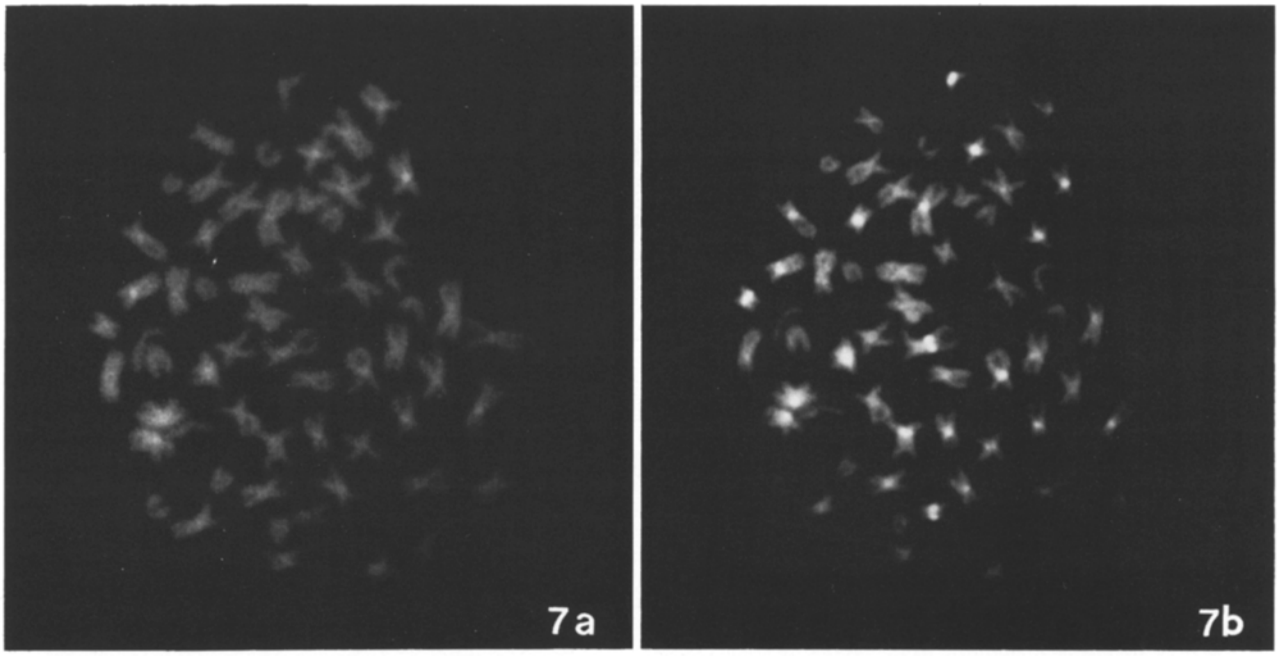
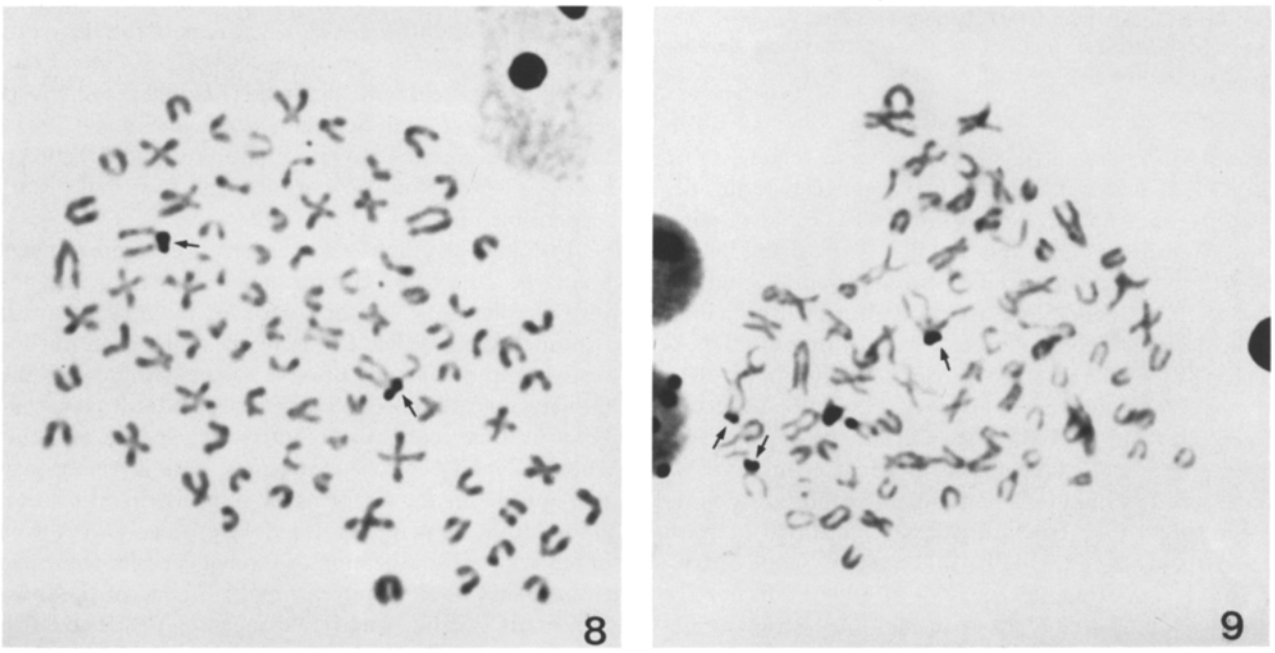


Fig. 7. **a** Distamycin A/DAPI and **b** DAPI/AmD stained metaphase of *Salmo gairdneri*. Note the different intensity of DAPI positive spots in both staining procedures



Figs. 8 and 9. Ag-NORs stained metaphase of two individuals of *Salmo trutta*. Note the two NORs in one fish (*arrows* — **Fig. 8**) and three NORs in another one (*arrows* — **Fig. 9**)

Discussion

Investigations on heterochromatin in Salmonids have been performed mostly by C-banding (Thorgaard 1976; Zenzes and Voiculescu 1975; Lee and Wright 1981; Ueda and Ojima 1983) and Q-banding (Abe and Muramoto 1974; Phillips and Zajicek 1982). The application of DAPI/AmD staining to the chromosomes of three species of salmonidae in the present study allowed for the selective demonstration of certain fluorescent DAPI-positive centromeric heterochromatin. Fluorescent DNA-banding dyes of different specifities has made it

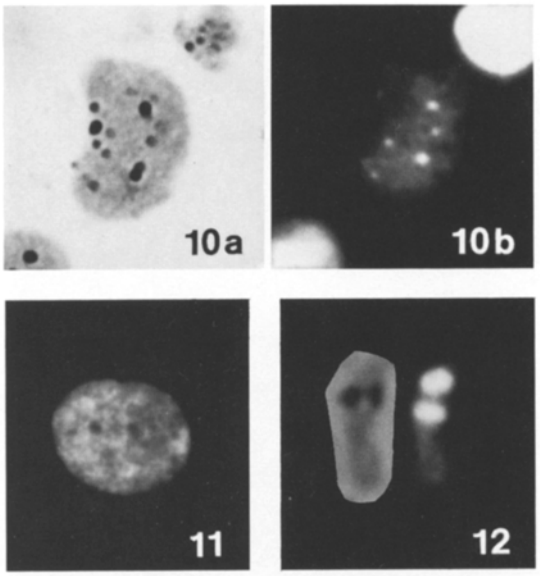


Fig. 10a and b. An interphasic cell of *Salmo gairdneri* **a** Ag-stained after Kodama and **b** DAPI/AmD staining. **b.** Many silver positive dots are visible in addition to the NORs. In some cases they are associated with DAPI/AmD positive staining

Fig. 11. DAPI/AmD stained interphasic cell of *Salmo trutta.* DAPI/AmD positive spots are clearly visible

Fig. 12. Sequential Ag-CDD-staining of *Salmo gairdneri.* The Ag-NORs (*left*) are spatially separated from their flanking CMA_3 – positive clusters (*right*)

possible to characterize heterochromatic regions more precisely. Such dyes fall into two major categories, depending on whether they exhibit AT-specific (Quinacrin, Hoechst 33 258 and DAPI = 4′–6′ diamidino-2-phenylindole) or GC-specific (chromomycin A_3 and mithramycin) binding and fluorescent properties (John et al. 1985). The combination of DAPI with other DNA-binding dyes (like distamycin A or actinomycin D) often gives a differential and/or improved resolution of certain heterochromatin areas. The centromeres of biarmed metacentric and submetacentric chromosomes of all three species exhibited a considerable equilocality in respect to their heterochromatic composition. In most cases they stained DAPI/AmD positive and CMA_3-negative. This staining behavior strongly indicates the presence of AT-rich DNA-sequences. An intriguing observation is the heterogeneous behavior of the DAPI/AmD positive heterochromatic areas in respect to their accompanying silver-staining structures. Thus, in the Ag-technique of Kodama et al. (1980) in *S. gairdneri,* most DAPI/AmD positive centromeres revealed positive Ag-staining, while in *S. trutta* and *Salvelinus fontinalis* no such centromeric Ag-staining was attained. This interesting fact is observed not only in metaphases but also very convincingly in interphases. The nature of this differential silver-staining behavior in DAPI/AmD positive centromeric areas of Salmonidae awaits further biochemical and histochemical approaches. An interpretation of these positive structures in the silver-staining method of Komada et al. (1980) is further complicated by the negative silver response of these regions by the silver-method of Howell and Black (1980).

Clear CMA_3 positive staining is associated with the NORs in Salmonidae (Phillips and Ihssen 1985; Mayr et al. 1986). Especially spectacular are the impressive CMA_3 positive blocks upstream and downstream of the Ag-NORs discretely spaced in our cytological separations in *S. gairdneri* (Fig. 12; Mayr et al. 1986). Another interesting observation was the high inter-individual variability of NORs in *S. trutta.* One of our investigated individuals showed a third Ag-NOR (on one homologue no. 11). In our earlier investigation on *S. trutta,* no such individual (ten individuals were investigated) was encountered (Mayr et al. 1986). However, this NOR variability is compatible with the frequent findings of intraspecific, inter-individual NOR-variation in many vertebrates, e.g., mammals (Mikelsaar et al. 1977; Mayr and Schleger 1981; Stefanova 1983). Thus, in a larger sample of *S. trutta fario,* the finding of individuals with four Ag-NORs will be very probable in future studies. Interestingly, no such individual was found in the material of Phillips and Ihssen (1985). The remarkable degree of intra-individual variation of Ag-NORs in *Salvelinus fontinalis* is consistent with analogous findings in other vertebrates (Mayr and Schleger 1981; Stefanova 1983). Probably different activation status of the functional trait Ag-NOR expression are at least to a major part responsible for.

Fish karyotyping is a somewhat cumbersome undertaking because many chromosomes show poor and not very characteristic banding patterns along their arms. Although these difficulties were encountered in our investigation, our results provide further progress to the ultimate goal of the establishment of standardized karyotypes in three economically interesting species of trout. The availability of such cytogenetic data is a necessary prerequisite for the performance of in situ hybridization studies for gene mapping. It will be especially worthwile in the genomic localization of foreign genes in trangenic trouts. This whole complex represents one of the most important questions and challenges of freshwater fish breeding research.

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