Chromosome Research 1995, 3, 285–290

Post-zygotic modifications and intra- and inter-individual nucleolar organizing region variations in fish: report of a case involving *Leporinus friderici*

Pedro M. Galetti Jr, Carlos A. Mestriner, Paul J. Monaco & Ellen M. Rasch

Received 7 September 1994; received in revised form 5 January 1995
Accepted for publication by M. Schmid 5 January 1995

Silver nitrate staining, a rapid and efficient method, has proven to be excellent for nucleolar organizing region (NOR) studies in fish. Some fish appear to have only two NOR-bearing chromosomes in their karyotype, whereas others probably have several. In the present study we analyzed the NORs of *Leporinus friderici*, a species that, on the basis of previous studies, has been considered as representative of species with NORs carried by a single chromosome pair. The analyses were performed by a combination of three methods, *i.e.* silver nitrate staining, staining with the GC-specific fluorochrome chromomycin A_3, and *in situ* hybridization with digoxigenin-labeled probes. The results showed that, although more frequent and conspicuous in a single chromosome pair, the NORs of this species are present in multiple chromosomes. Intra- and inter-individual variations observed by the three methods strongly suggest the occurrence of post-zygotic modifications involving NORs. NOR identification in fish, almost exclusively performed by the silver nitrate method, is currently being re-evaluated by methods such as chromomycin A_3 staining and *in situ* hybridization, which may provide important information leading to a better understanding of chromosome evolution in these animals.

Key words: fish, fluorescent CMA bands, *in situ* hybridization, NOR polymorphisms

Introduction

Since the late 1970s, nucleolar organizer regions (NORs) have been identified in the karyotype by a rapid and easy technique involving impregnation with silver nitrate (Goodpasture & Bloom 1975, Howell & Black 1980). This method has revealed that most fish studied so far have only a single pair of homologous Ag-NOR-bearing chromosomes (*e.g.* Gold 1984, Moreira Filho *et al.* 1984), while others show Ag-NOR sites distributed among multiple chromosomes (*e.g.* Almeida Toledo *et al.* 1985, Galetti *et al.* 1985). Polymorphisms in these regions, however, are frequent (Foresti *et al.* 1981). In the genus *Leporinus* Ag-NOR sites are characteristically found in only two chromosomes in the complement. Their location in different chromosome pairs in different species may confer a significant cytotaxonomic value on this trait (Galetti *et al.* 1984).

Previous studies on *Leporinus friderici* have identified Ag-NOR sites on the short arm of the second largest chromosome of the complement (Galetti *et al* 1984). These results were later confirmed in specimens from a different population (Galetti *et al.* 1991). However, in this later study, one individual was identified as presenting as many as five chromosomes bearing Ag-positive sites, suggesting a larger number of NORs in the complement. According to the authors, since silver staining only detects rDNA sites that are active in the preceding interphase (Hsu *et al.* 1975, Miller *et al.* 1976) the number of inactive NORs might be underestimated in those fish with only two Ag-NORs.

It has been frequently observed that, regardless of any genetic activity, the NORs of certain lower vertebrates, especially amphibians and fish, can be identified in the karyotype by the use of fluorochromes such as chromomycin A_3 and mitramycin A, which preferentially bind to GC-rich sequences in DNA (*e.g.* Schmid 1980, Mayr *et al.* 1985, Amemiya & Gold, 1986, 1988, Schmid & Guttenbach, 1988). The association of fluorochromes and silver studies has been useful for the detection of polymorphisms at these sites (Phillips *et al.* 1989a,b, Sola *et al.* 1992a,b, Galetti & Rasch 1993a,b). Besides these methods, non-isotopic *in situ* rDNA hybridization, especially with biotin-labeled probes and fluorescence detection (FISH), has several times been

P. M. Galetti Jr (corresponding author) and C. A. Mestriner are at the Department of Genetics and Evolution, Federal University of São Carlos, 13565-905 São Carlos, SP, Brazil. Fax: (+55) 162 74 83 05; E-mail: dpgj@power.ufscar.br. P. J. Monaco and E. M. Rasch are at the Department of Anatomy and Cell Biology, East Tennessee State University, Johnson City, TN 37614, USA.

considered decisive in a more precise identification of NOR sites in the complement, and has been increasingly used in fish studies (Pendás *et al* 1993a,b, 1994).

In the present paper we report a chromosome analysis carried out on *Leporinus friderici* specimens using silver nitrate and chromomycin A_3 staining. C-banding and *in situ* hybridization with digoxigenin-labeled rDNA probes. The combined use of these methods allowed us to detect a major pair of NOR-bearing chromosomes and at least two other pairs showing secondary NORs, which often varied from cell to cell in the same individual.

Materials and methods

We studied the mitotic chromosomes of 10 *Leporinus friderici* specimens (Anostomidae, Characiformes) from two different hydrographic systems, *i.e.* the Candeias River, a tributary of the Madeira River in the Amazon basin, and the Mogi-Guaçu River of the upper Paraná basin. Except for animals 5891 and 5892, the only two obtained from the second collection site, all fish had been previously studied for NORs by the silver nitrate method (Galetti *et al.* 1991).

Chromosome banding.

The NORs were reanalyzed using colloidal silver nitrate emulsion (Howell & Black, 1980). In addition, C-banding analyses were performed using the method described by Sumner (1972), and chromomycin A_3 staining was carried out in combination with the counterstain distamycin A as described by Schweizer (1976) and Schmid (1980), with some adaptations to fish chromosome studies (Galetti & Rasch 1993a).

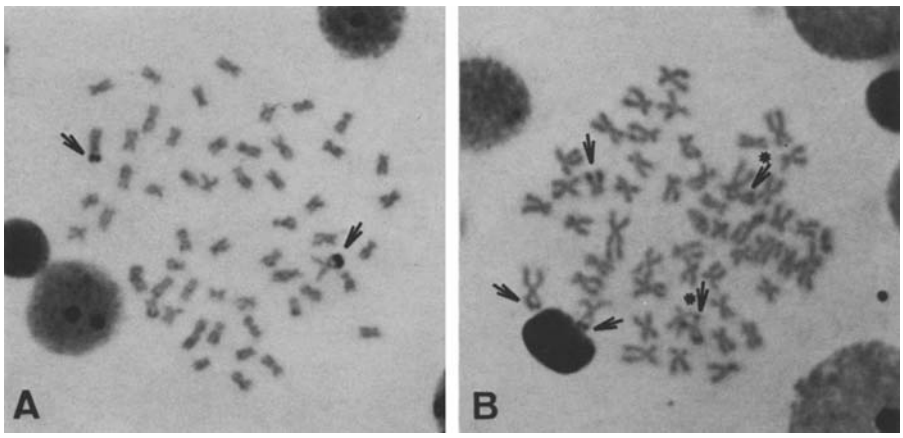

Figure 1. Silver nitrate stained metaphases. **A** The two major NOR-bearing chromosomes corresponding to pair 2 in the complement are indicated. **B** Five Ag-NOR-bearing chromosomes detected in the specimen 4976. The asterisk-marked arrows show Ag-NOR sites in both telomeres of a median sized metacentric (pair 13).

Table 1. NOR-bearing chromosome frequencies of *L. friderici* shown by silver nitrate (Ag-NOR), chromomycin A_3 (CMA) and *in situ* hybridization with digoxigenin-labeled probes (DIG)

	Ag-NOR						CMA						DIG					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
4976	14	16	4	2	6				2	4	6		1	4				
5241	1	16							1	8	1	2						
5243								15	7	2	1		2	1	1		1	
5643		11					2	5	4	1				2				
5644	1	8					3	9		2								
5648	7	9					18	14	5					5	2			1
5649	1	21						1	5					4		5	1	
5682							2									1		1
5891							1	2	2	1			3					
5892							10	10										
Total	24	81	4	2	6	0	36	56	26	18	8	2	6	16	3	6	2	2

In situ hybridization

Hybridization assays were carried out with human rDNA 18S probes labeled with digoxigenin and identified by the immunocytochemical method with anti-digoxigenin–alkaline phosphatase stained with nitro-blue tetrazolium (NBT) as described by the supplier (Boehringer Mannheim, Germany).

Results and Discussion

Analysis of the NORs by silver nitrate staining confirmed previous studies (Galetti *et al.* 1984) indicating that Ag-NORs are present only in the telomere of the short arm of the second metacentric pair (Figure 1A). In specimen 4976, however, at least five chromosomes with conspicuous silver labeling were observed (Table 1, Figure 1B), corroborating results previously reported by Galetti *et al.* (1991). In this specimen, in addition to the Ag-NOR sites on chromosome pair 2 in the complement, other sites were observed in both telomeres of a metacentric pair of intermediate size (pair 13) and in the subterminal portion of the long arm of one of the chromosomes of pair 26, a small submetacentric. Conspicuous associated C-banding-positive blocks were also detected in all of these segments (Figure 2C).

In fact, NOR polymorphism has been observed quite often in fish, and has been demonstrated by cytogenetic (*e.g.* Foresti *et al.* 1981, Gold 1984, Moreira Filho *et al.* 1984, Phillips *et al.* 1989b, Sola *et al.* 1992a,b) and also by molecular methods (Monaco *et al.* 1988, Phillips *et al.* 1989a). Owing to their repetitiveness in the genome of different organisms, these cistrons are highly susceptible to pre- and post-zygotic structural modifications as a result of unequal crossing over, regional duplications, transpositions and other rearrangements. However, when NORs are identified with silver nitrate staining (Ag-NORs), variations due to structural modifications and those of functional origin are indistinguishable, since only truly functional sites are identified by silver nitrate (Hsu *et al.* 1975, Miller *et al.* 1976). In this case, those rDNA cistrons present in the genome but inactive as a result of regulation processes will remain masked (Schmid 1980).

In order to verify if the Ag-NOR polymorphism observed in *Leporinus friderici* is a product of genetic regulation or the result of true structural changes involving this chromosome region, analyses using chromomycin A_3 (CMA) were performed. Approximately 150 cells from different individuals were counted and considerable inter- and intra-individual variation in the pattern of CMA-positive bands was demonstrated

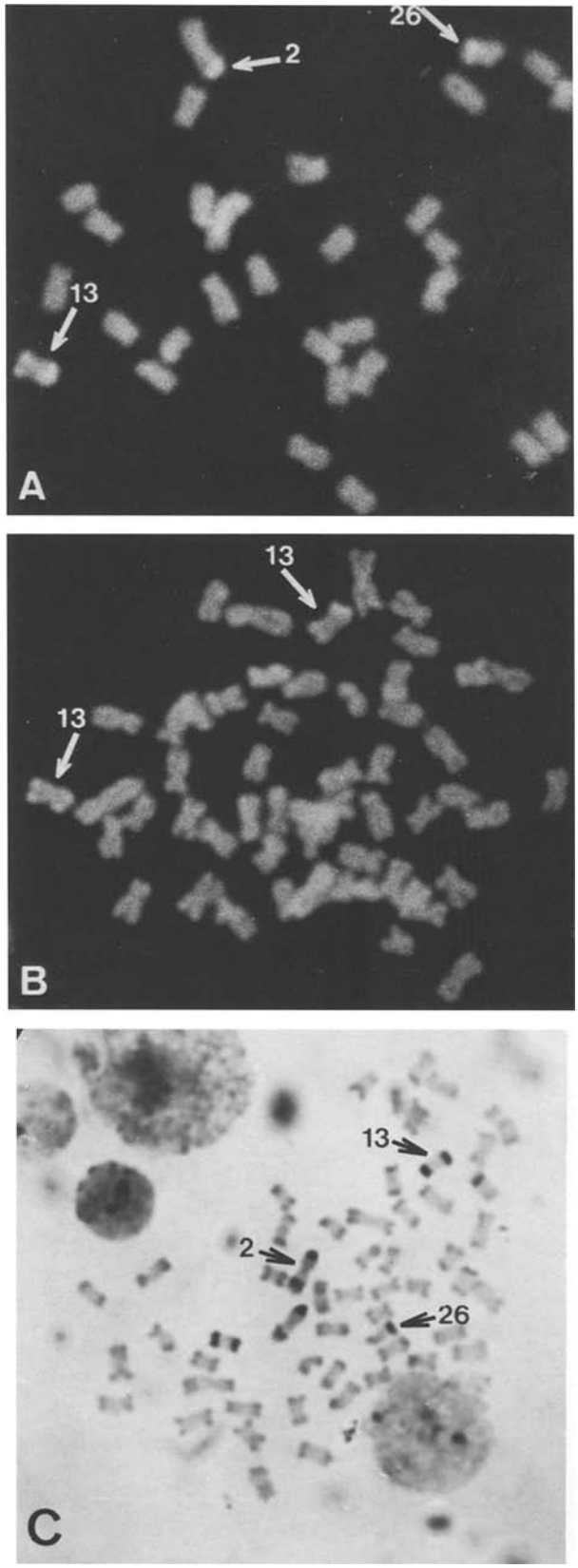

Figure 2. A & B Partial chromomycin A_3-stained metaphases. CMA fluorescent bands in the telomeres of a chromosome 2,13 and 26 (**A**). Two chromosomes 13 with CMA bands in both telomeres (**B**) **C** C-banding metaphase. A chromosome of each pair 2, 13 and 26, bearing conspicuous heterochromatin blocks, is indicated.

(Table 1). Although CMA-positive fluorescent segments were always present in pair 2 in the complement, other CMA-positive bands were also observed (Figure 2A & B & 3). Approximately 50% of the cells analyzed showed 3–6 chromosomes bearing CMA-positive regions, consistent with the three homologous pairs bearing Ag-NOR sites in specimen 4976. In addition to these, some other small CMA-positive bands were sporadically detected, such as that appearing in the pericentromeric region of pair 18 in the complement and in the telomeric region of pairs 4 and 7. (Figure 3).

The same tendency to variation was also observed by analyzing chromosomes subjected to *in situ* hybridization with digoxigenin-labeled rDNA 18S probes that were visualized by NBT staining. Owing to methodological difficulties related to visualizing the usually small NBT deposits, this analysis was limited to a small number of metaphases in each individual, and only those cases in which there was no doubt were considered. Positive signals were identified at least at the sites present in pairs 2, 13 and 26 of the complement (Figure 4, centre). Heteromorphisms, such as a probable duplication in chromosome 2 or deletion of one of the telomeres of one chromosome 13, may occur. Thus, the results of hybridization appear to be consistent with the multiple Ag-NORs observed only in specimen 4976, reinforcing the observation of secondary constrictions in these regions (Figure 4, left) and the presence of CMA-positive bands (Figure 4, right) in the animals analyzed.

However, in contrast to silver staining, the results of both CMA staining and *in situ* hybridization would not be expected to vary within the same individual unless independent structural differences occur from cell to cell. Similar intra-individual variations in the fluorescent CMA bands have been identified recently in some other fish species (Galetti & Rasch 1993a,b, Mestriner 1993) and may be more common than initially suspected. Although methodological artifacts cannot be completely excluded as a source of some of the variability we have observed here, it seems unlikely that both CMA staining and *in situ* hybridization would be subject to the same technical flaws. Thus, there are two main hypothesis to explain these findings. These variations might be exclusively due (1) to heteromorphisms of a GC-rich heterochromatin linked or not linked with NOR sites (Pendás *et al.* 1993a,b) or (2) to *de facto* changes in the set of rDNA cistrons. Our C-band analyses showed quite conspicuous NOR-associated heterochromatin segments in chromosome pairs 2, 13 and 26 in *L. friderici*. However, in many cells these segments were considerably reduced with chromomycin A_3, or could not be identified at all. These findings suggest that the reduction or lack of CMA-positive bands does not seem to be a direct response related to heterochromatin, but may rather be a strong indication of modifications at the level of rDNA cistrons. Furthermore, if variations occur within rDNA cistrons, they should result in heteromorphisms detectable by *in situ* hybridization, as was indeed the case for *L. friderici*. Post-zygotic modifications may be an important mechanism for variations among organisms and, since their discovery (McClintock 1956), transposons have been the best evidence that these changes occur continuously along the genome. The occurrence of these mechanisms modifying fish NORs may reflect the plastic condition of the genome of these animals. Plants, in general, are considered to be organisms of high plasticity from a genetic viewpoint, able to stand large variations in the genome such as polyploidization and aneuploidy. Several plants show NOR variation from cell to cell, suggesting that these cistrons or segments of them may jump along the chromosomes (Schubert & Wobus 1985). As a parallel example, fish as a group may perhaps represent one of the vertebrates of high genetic plasticity, with marked genomic variations. The results of *in situ* hybridization with rDNA probes as used here proved to be highly variable. Although artifacts cannot be ruled out completely and the method used may have been of considerably reduced efficiency, at least part of the variation observed in the hybridization assays is consistent with the idea that the NORs of these fish may be moving along the genome.

The results obtained for *Leporinus friderici* show that studies of NOR identification in fish carried out exclusively using the silver nitrate method deserve re-

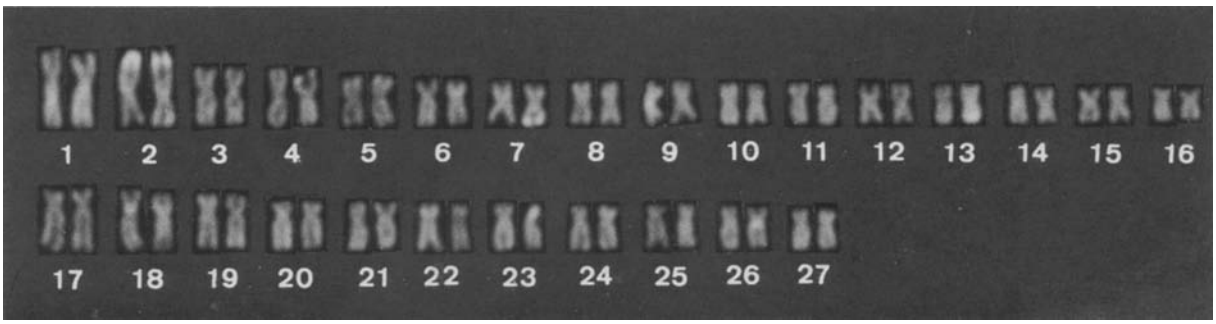

Figure 3. Karyotype of *L. friderici* showing the two major chromosomes (pair 2) bearing CMA fluorescent bands. Other minor CMA bands may be observed in the telomere of chromosomes 4 and 7 as well as in the centromeric region of chromosome 18.

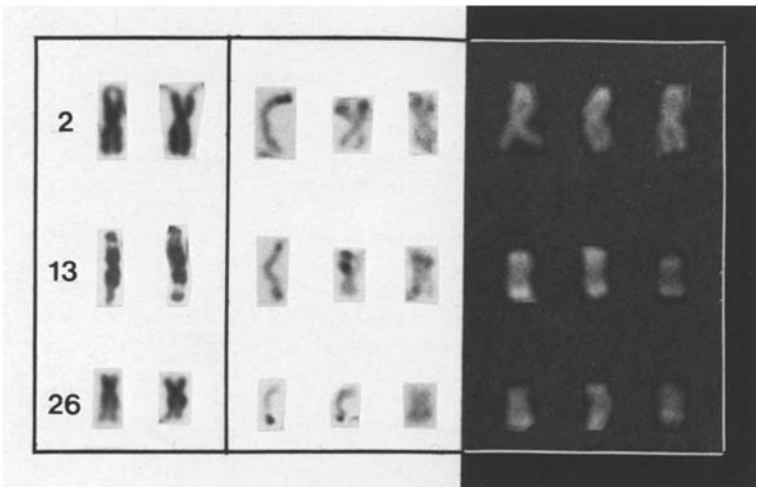

Figure 4. Giemsa-stained chromosome pairs 2, 13 and 26 showing secondary constrictions (left). The same chromosomes from different metaphases observed after *in situ* hybridization with digoxigenin-labeled rDNA 18S probes (center), and after CMA staining (right).

evaluation. The combined use of methods based on different mechanisms of action, as is the case for silver staining, chromomycin A_3 staining and *in situ* hybridization, should make new contributions to the understanding of the distribution of these cistrons in the chromosome complement of fish and to studies on the chromosome evolution of these animals.

Acknowledgements

This work was supported in part by a grant to E.M.R. from the Whitehall Foundation (Palm Springs, Florida, USA). P.M.G.J. was supported by a fellowship from the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (Brazil).

References

Almeida Toledo LF, Foresti F, Toledo Filho SA (1985) Spontaneous triploidy and NOR activity in *Eigenmannia sp* (Pisces, Sternopygidae) from the Amazon basin. *Genetica* **66**: 85–88.

Amemiya CT, Gold JR (1986) Chromomycin A3 stains nucleolus organizer regions of fish chromosomes. *Copeia* **1986**: 226–231.

Amemiya CT, Gold JR (1988) Chromosomal NORs as taxonomic and systematic characters in North American cyprinid fishes. *Genetica* **76**: 81–90.

Foresti F, Almeida Toledo LF, Toledo Filho SA (1981) Polymorphic nature of nucleolus organizer regions in fishes. *Cytogenet Cell Genet* **31**: 137–144.

Galetti Jr PM, Rasch EM (1993a) Chromosome studies in *Poecilia latipunctata* with NOR polymorphism as shown by silver nitrate and chromomycin A3 (Teleostei: Poecillidae). *Ichthyol Explor Freshwaters* **4**: 269–277.

Galetti Jr PM, Rasch EM (1993b) NOR variability in diploid and triploid forms of *Poecilia formosa* as shown by silver nitrate and chromomycin A3 staining. *Brazil J Genet* **16**: 927–938.

Galetti Jr PM, Foresti F, Bertollo LAC, Moreira Filho O (1984) Characterization of eight species of Anostomidae (Cypriniformes) fish on the basis of the nucleolar organizing region. *Caryologia* **37**: 401–406.

Galetti Jr PM, Silva EB da, Cerminaro RT (1985) A multiple NOR system in the fish *Serrasalmus spilopleura* (Serrasalminae, Characidae). *Brazil J Genet* **8**: 479–484.

Galetti Jr PM, Mestriner CA, Venere PC, Foresti F (1991) Heterochromatin and kayotype reorganization in fish of the family Anostomidae (Characiformes). *Cytogenet Cell Genet* **56**: 116–121.

Gold JR (1984) Silver-staining and heteromorphism of chromosomal nucleolus organizer regions in North American cyprinid fishes. *Copeia* **1984**: 133–139.

Goodpasture C, Bloom SE (1975) Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosome* **53**: 37–50.

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36**: 1014–1015.

Hsu TC, Spirito SE, Pardue ML (1975) Distribution of 18S and 28S ribosomal genes in mammalian genomes. *Chromosoma* **53**: 25–36.

McClintock B (1956) Controlling elements and the gene. *Cold Spring Harbor Symp Quant Biol* **21**: 197–216.

Mayr B, Ráb P, Kalat M (1985) Localisation of NORs and counterstain-enhanced fluorescence studies in *Perca fluviatilis* (Pisces, Percidae). *Genetica* **67**: 51–56.

Mestriner CA (1993) Análise das regiões organizadoras de nucléolos e investigação do sistema XX/XY descrito para *Leporinus lacustris* (Pisces, Anostomidae). Masters Thesis, Universidade Federal de São Carlos, 70.

Miller DA, Dev VG, Tantravahi R, Miller OJ (1976) Suppression of human nucleolus organizer activity in mouse–human somatic hybrid cells. *Exp Cell Res* **101**: 235–243.

Monaco PJ, Rasch EM, Musich PR (1988) Polymorphisms in ribosomal DNA of unisexual fish. *Copeia* **1988**: 774–777.

Moreira Filho O, Bertollo LAC, Galetti Jr PM (1984) Structure and variability of nucleolar organizer regions in Parodontidae fish. *Can J Genet Cytol* **26**: 564–568.

Pendás AM, Morán P, García-Vázquez (1993a) Multi-chromosomal location of ribosomal RNA genes and heterochromatin association in brown trout. *Chrom Res* **1**: 63–67.

Pendãs AM, Morán P, García-Vázquez E (1993b) Ribosomal RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. *Cytogenet Cell Genet* **63**: 128–130.

Pendás AM, Morán P, Freije JP, García-Vázquez E (1994) Chromosomal mapping and nucleotide sequence of two

tandem repeats of Atlantic salmon 5S rDNA. *Cytogenet Cell Genet* **67**: 31–36.

Phillips RB, Pleyte KA, Ert LMV, Hartley SE (1989a) Evolution of nucleolar organizer regions and ribosomal RNA genes in *Salvelinus*. *Physiol Ecol Jpn Spec* U1: 429–447.

Phillips RB, Pleyte KA, Ihssen PE (1989b) Patterns of chromosomal nucleolar organizer region (NOR) variation in fishes of the genus *Salvelinus*. *Copeia* 1989: 47–53.

Schmid M (1980) Chromosome banding in amphibia V. Highly differentiated ZZ/ZW sex chromosomes and exceptional genome size in *Pyxicephalus adspersus* (Anura, Ranidae). *Chromosoma* **80**: 69–96.

Schmid M, Guttenbach M (1988) Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. *Chromosoma* **97**: 101–114.

Schubert I, Wobus U (1985) *In situ* hybridization confirms jumping nucleolus organizing regions in *Alium*. *Chromosoma* **92**: 143–148.

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* **58**: 307–324.

Sola L, Iaselli Y, Rossi AR, Rasch EM, Monaco PJ (1992a) Cytogenetics of bisexual/unisexual species of *Poecilia*.III. The karyotype of *Poecilia formosa*, a gynogenetic species of hybrid origin. *Cytogenet Cell Genet* **60**: 236–240.

Sola L, Rossi AR, Iaselli V, Rasch EM, Monaco PJ (1992b) Cytogenetics of bisexual/unisexual species of *Poecilia*.II Analysis of heteorchromatin and nucleolar organizer regions in *Poecilia mexicana mexicana* by C-banding and DAPI, quinacrine, chromomycin A3, and silver staining. *Cytogenet Cell Genet* **60**: 229–235.

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. *Exp Cell Res* **74**: 304–306.