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A population analysis of Robertsonian and Ag-NOR polymorphisms in brown trout (*Salmo trutta*)

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Abstract An analysis of Robertsonian polymorphism and variation in the number of active NORs has been carried out in several populations of brown trout (*Salmo trutta*) from Northwestern Spain. The karyotype of this species appears to be soundly established, and essentially no variation has been found in chromosome number. Interindividual and interpopulation variation in arm number was detected, with figures ranging between 100 and 102 among individuals, and between 100.10 and 100.80 among populations. This variation in arm number is solely attributable to the polymorphism of the short arm of the main NOR-bearing pair 11, which can appear from acrocentric to metacentric in different individuals. Most populations analyzed showed the standard distribution of active NORs previously observed in this species. The Miño drainage basin, and specially the Chamoso population, showed a multichromosomal distribution of active NORs, with several new locations, always telomeric. In most cases no concordance was observed between previously detected rDNA sites in *S. trutta* and the new Ag-NOR locations. This fact suggests a transposition mechanism rather than an activation of silent rDNA sites to explain this multichromosomal NOR pattern.

Key words *Salmo trutta* · Population analysis
Robertsonian polymorphism · Ag-NOR pattern

Introduction

Chromosome polymorphims of different types have been described in salmonids (Thorgaard 1983; Phillips et al. 1985; Hartley 1988). Robertsonian translocations, resulting from centric fusions and dissociations, and polymorphisms in nucleolus-organizer regions (NORs), both in size and number, have been widely reported in the karyotypes of salmonid fish (Hartley 1987). Although variation in chromosome number has been soundly documented in several salmonid species with chromosome numbers around 60, the studies carried out in brown trout *(Salmo trutta)* show a more controversial situation (Capanna et al. 1973; Zenzes and Voiculescu 1975; Hartley and Horne 1984; Martínez et al. 1991; Karakousis et al. 1992).

Intraspecific polymorphism in nucleolus-organizer regions, related to the size and number of active NORs, has also been documented in *S. trutta* (Mayr et al. 1986, 1988; Martínez et al. 1991). NOR regions are mainly located on pair number 11 in this species, a third Ag-NOR being ocasionally revealed on a subtelocentric pair (Mayr et al. 1988; Martínez et al. 1991). The existence of some additional CMA3-positive bands, which define NOR regions in fish regardless of their activation status (Phillips et al. 1986; Amemiya and Gold 1986) has been observed in several subtelocentric pairs in brown trout (Mayr et al. 1988; Martínez et al. 1991). Similarly, new rDNA clusters have been identified by in-situ hybridization in this species (Pendás et al. 1993). These facts suggest the existence of new Ag-NORs which could be detected in larger samples of brown trout.

The characterization of genetic polymorphisms at the population level is the first step to better understand their evolutionary role and to obtain some clues about the mechanism responsible for their origin. In order to characterize Robertsonian and Ag-NOR polymorphisms in *S. trutta*, we have analyzed the basic karyotypic features and NOR expression pattern in several populations of this species from Northwestern Spain.

Materials and methods

Samples

One-hundred-and-sixty-nine specimens of *Salmo trutta* collected from nine natural populations (in both running and still water) of Galicia (Northwestern Spain), and two hatcheries commonly used

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for stocking in this area, were analyzed in this study (see Table 1). Hatchery stocks were established around 20 years ago with individuals imported from Germany. Previous isozyme studies revealed the great genetic divergence between the stocking hatcheries and the natural populations employed in this work (D=0.046) (Martínez et al. 1993 a). Similarly, a mixed composition of the non-flowing water populations (Incio and Sobrado), due to genetic introgression from stocking practices, was observed. River populations, by contrast, have not been affected by stocking.

Cell cultures and chromosome counting

Plates were obtained from blood and kidney cultures according to the size of the fish (Sánchez et al. 1990; Martínez et al. 1993 b).

Around 2000 metaphases from 169 fishes were analyzed. The best five plates per individual were used to establish the basic karyotypic features. Plates were stained in 10% Giemsa for 5 min. The NF value was estimated by computing the number of uniarmed and biarmed chromosomes according to Levan et al. (1964).

Ag-staining

This technique was performed according to Howell and Black (1980) with slight modifications. After treatment with $AgNO_3$, the slides were briefly washed in a sodium thiosulphate solution (5% w/v), then in distilled water, and finally stained in 2–3% Giemsa for 5 min.

Results

Chromosome number and arm number

One-hundred-and-sixty-nine individuals of *S. trutta* were used for analyzing the basic karyotypic features in this study. All except one specimen, sampled in the Umia river (U.19), showed the same standard karyotype with a modal number of 2n=80 and a fundamental number ranging from NF=100 to 102. Chromosome counts below 80, found in several plates of different individuals, were always in accordance with a decrease in arm number, which probably excludes the possibility of Robertsonian polymorphism. Only one exceptional individual, U.19, showed a diploid number of 2n=79 retaining a NF=100 in all the plates analyzed. An additional metacentric chromosome, much longer than is usual in this species, could be detected in this specimen, while only one member of two different subtelocentric pairs, 12 and probably 14, were observed in all plates of this individual (Fig. 1). This fact strongly suggests that this metacentric chromosome is the consequence of a centric fusion of two subtelocentrics.

Variation in arm number (NF) was always due to the polymorphism shown by the main NOR-bearing chromosome (pair 11), which can range from acrocentric to metacentric according to the size of its short arm. This variation was observed both in natural populations as well as in hatchery stocks. Important variation was detected, however, in the mean NF between the populations analyzed (Table 1), which demonstrates the existence of differences in the mean length of the short arm of the NOR-bearing chromosome 11. The mean NF figures ranged between 100.10 in the Armenteira river, where most chromosomes were acrocentric/subtelocentric, and 100.80 in the Sobrado lagoon or 100.78 in the Navia river, where a significant number of biarmed no. 11 chromosomes were observed.

Ag-NOR distribution

Close to 1700 plates of 87 individuals taken from six populations were examined for Ag-NORs (Table 1). All populations except Incio and Chamoso exhibited the classical Ag-NOR pattern of this species with the main NORs located in the short arm of both homologs of pair 11, and ocasionally a third NOR in pair 14 (Martínez et al. 1991). The relative presence of this third Ag-NOR was variable among the populations studied, figures ranging between 17.6% and 41.7% in the Navia river and the Veral stock, respectively (Table 1).

Fishes from the Miño drainage basin (Table 1), but especially individuals from the Chamoso river (84%) and to a minor extent from Incio reservoir (15%), showed a non-standard, clearly multichromosomal, NOR location (Table 2; Fig. 2). An important variation was detected in these populations in the number and type of Ag-NOR bearing chromosomes, not only interindividually but also among the cells of a particular specimen. From one to six Ag-NORs were detected in one specimen, with several others exhibiting one to five Ag-NORs (Table 2). The maximum number of nucleoli was even higher in the aforementioned individual (seven nucleoli), which probably indicates the possible existence of more Ag-NORs not detected in our analysis.

Although the classical NOR pattern was observed in some individuals from the Miño basin, several new Ag-NORs were found, mainly in the Chamoso population (Fig. 3). At least nine NOR-bearing chromosomes not described to date were unequivocally identified: three meta-submetacentric chromosomes from different pairs, two of them with telomeric Ag-dots on their short and long arms respectively, and another one with the whole short arm or telomeres Ag-stained (Fig. 3 d–g, respectively); three subtelocentric chromosomes (different from pair 14 previously described), two of them with NORs on their short arms, and another one with Ag-dots in a telomeric position on its long arm (Fig. 3 h, j, k, respectively); and finally, three different acrocentric chromosomes with telomeric NORs (Fig. 3 l–n). Remarkably all the new NORs detected are essentially telomeric.

Another interesting observation in these populations is an atypical NOR expression pattern in which inactivity of one member of pair 11 occurs in most individuals (Fig. 2, Table 2). This is accompanied by the active presence of one member of a sutelocentric pair, probably the 14th, in nearly all individuals analyzed (Table 2). Interestingly, this chromosome shows the presence of NOR duplications, which also accounts for the assumption of a more relevant role by this chromosome in the expression of rDNA genes. In some specially long chromosomes of pair 11 the activation of only one cluster in the main NOR region within specific individuals could also be observed, always in the same position in all metaphases analyzed (Fig. 3 b, c).

As pointed out before, a marked NOR variation was also detected at the intraindividual level in the Miño basin. This fact is detailed in Table 2, where some specimens with 2–5, 1–5 or 1–6 Ag-NORs are shown. Although a broad intra-

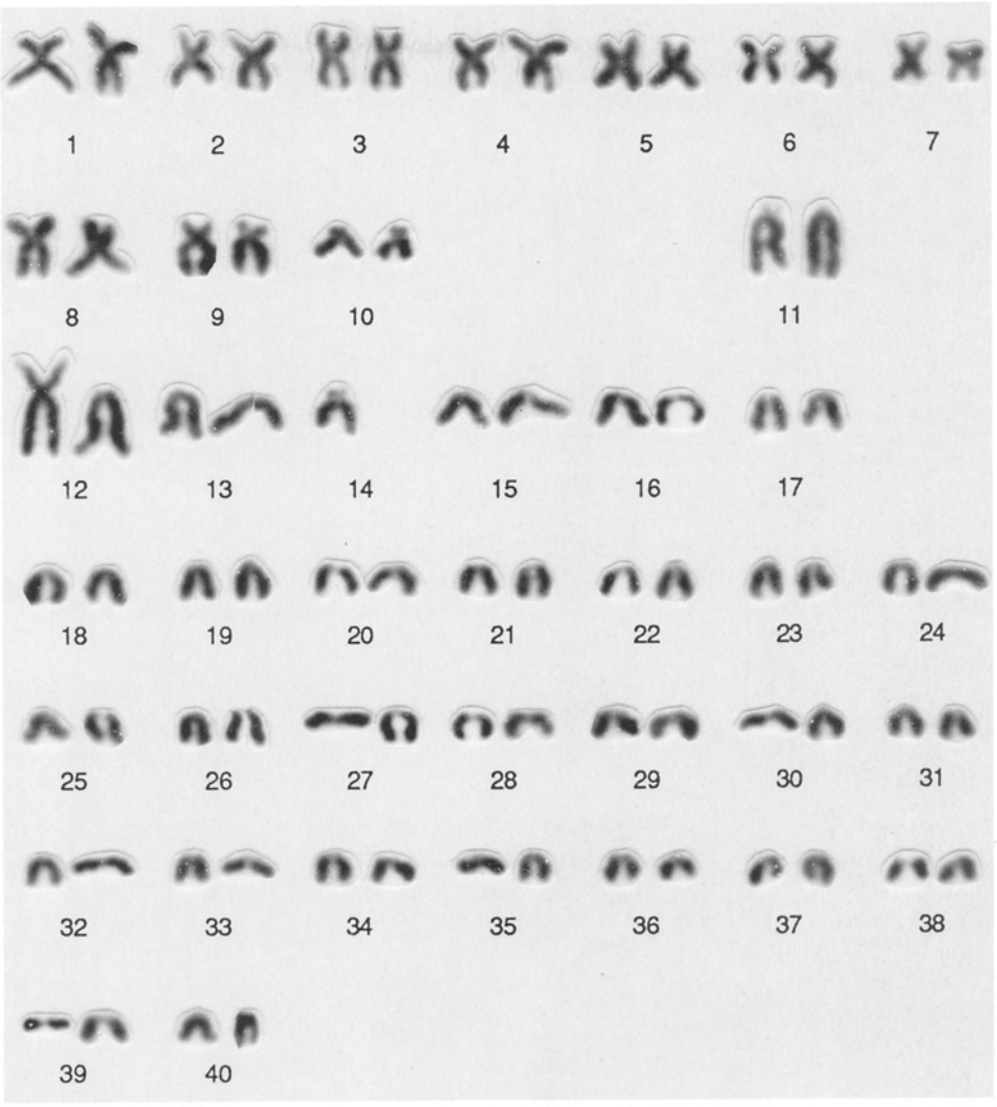

Fig. 1 Standard karyotype with Giemsa staining of the atypical individual U-19 (2n=79). Note the long metacentric chromosome classified as a member of pair 12, which represents a Robertsonian fusion between a member of pair 12 and probably a member of pair 14

Table 1 Main characteristics of the 11 populations of brown trout analyzed in this study. The number of individuals used for the basic karyotype features and Ag-staining are also indicated together with some additional data (mean NF, % 3rd Ag-NOR)

Population	River basin	Origin	No.of fish for chr. counting	Mean NF±SE	No.of fish for Ag-NORs	% 3rd Ag-NOR
Navia	Navia	River	19	100.78±0.15	17	17.6
Tambre	Tambre	River	22	100.48±0.11	14	21.4
Cabalar	Tambre	River	12	100.58±0.15	9	33.3
Sobrado	Tambre	Lagoon	10	100.80±0.13	–	–
Armenteira	Umia	River	10	100.10±0.10	–	–
Chain	Umia	River	4	100.50±0.29	–	–
Umia	Umia	River	10	100.20±0.13	–	–
Incio	Miño	Reservoir	27	100.22±0.10	16	[a]
Chamoso	Miño	River	19	100.21±0.12	19	[a]
Carballedo	–	Hatchery	10	100.30±0.15	–	–
Veral	–	Hatchery	26	100.46±0.10	12	41.7
			169		87	

[a] Atypical Ag-NOR pattern

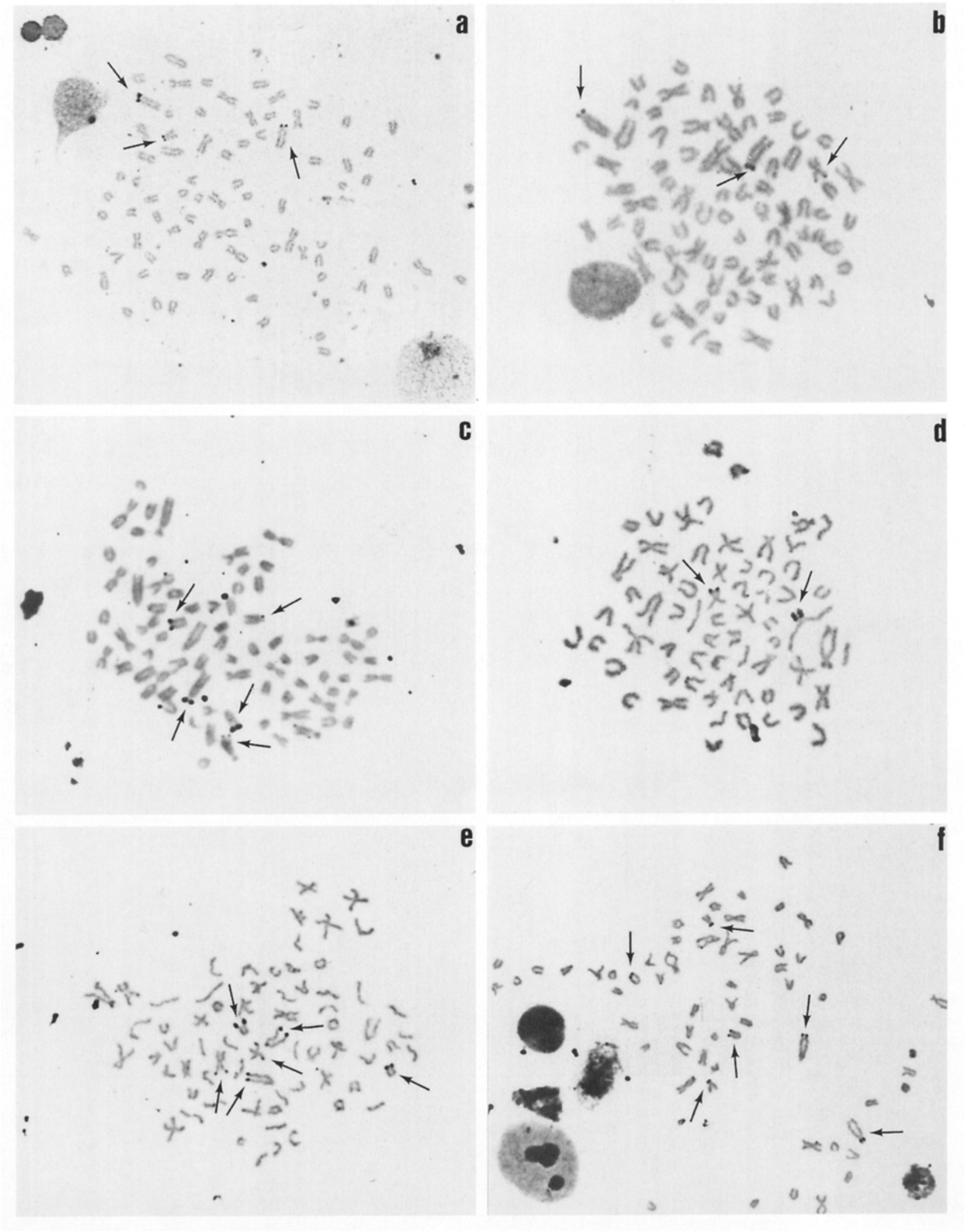

Fig. 2 a–f Different Ag-stained plates from several individuals of *S. trutta*. **a** Standard Ag-NOR pattern; **b–f** Atypical Ag-NOR patterns showing the new multichromosomal location in several individuals from the Incio (**b**) and Chamoso populations (**c, d, e, f**). Two plates from the same specimen, M-17, showing a different Ag-NOR pattern, are presented (**e, f**)

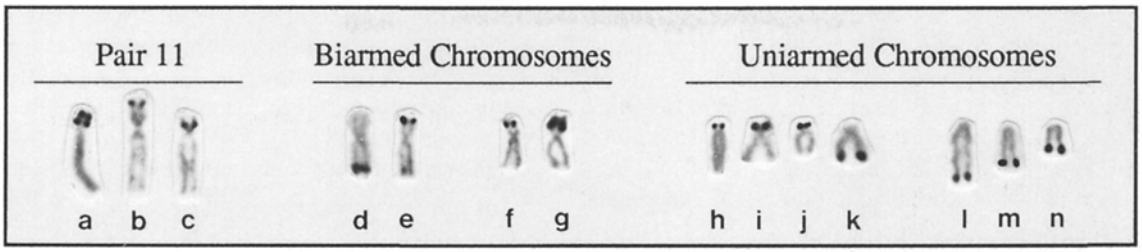

Fig. 3 Ag-NOR bearing chromosomes detected in *S. trutta* in this study. Chromosomes are arranged in groups corresponding to the main NOR-bearing pair 11 (*a–c*), and the biarmed (*d–g*) and uniarmed (*h–n*) chromosomes. Notice the activation of specific NOR clusters in both members of pair 11 from individual M-17 (*b, c*).

Table 2 Chromosome location and intraindividual variation in the number of Ag-NORs of the individuals which exhibited an atypical Ag-NOR pattern in the Incio (I) and Chamoso (M) populations, both pertaining to the Miño basin. 11 and 11′: homologs of pair 11; S: short arm of a subtelocentric chromosome; S*: telomere of the long arm of a subtelocentric chromosome; Sd: NOR duplication on short arm of a subtelocentric chromosome; Ml: telomere of the long arm of a biarmed chromosome; Ms: telomere of the short arm of a biarmed chromosome; A: telomere of an acrocentric chromosome

Fish	Modal Ag-NORs	Additional Ag-NORs	Max. no. of Nucleoli	Cells with different number of Ag-NORs						
				1	2	3	4	5	6	Total
I.11	11, 11′	Ms	3	19	15	1	–	–	–	35
I.12	11, 11′	Ms	3	2	17	9	–	–	–	28
I.15	11, 11′, Ml	A	4	6	10	11	5	–	–	32
I.25	11, S	S	3	2	5	2	–	–	–	9
M.2	11, Ms	11′, S*	4	3	24	4	2	–	–	33
M.6	11, S, S*, Ml	A, A, 11′	5	–	4	10	8	3	–	25
M.7	11, S, Ms	A	4	5	7	13	4	–	–	29
M.8	11, S, Ml	11′	4	2	34	20	3	–	–	59
M.9	11, 11′, A	S, A	5	–	15	17	5	1	–	38
M.11	11, Sd	11′, S	4	41	38	18	4	–	–	101
M.12	11, S	11′, S	4	1	5	3	–	–	–	9
M.13	11, Sd, A	S, Ms, A	4	29	39	37	13	4	–	122
M.14	11, S, S	Ms	3	7	38	14	3	–	–	62
M.15	11, Ms, S	S, S, A	4	5	32	28	16	2	–	83
M.16	11, 11′, Ms	Ml, A	4	–	11	37	14	–	–	62
M.17	11, 12, Ml, A, A, S	11′, Ms, S, S*	7	3	16	29	39	22	5	114
M.18	11, S, Ml	3	3	1	3	1	–	–	–	5
M.20	11, S, Ml, A	A, S, 11′	4	1	12	9	3	1	–	26
M.21	11, S, A	3	3	2	3	1	–	–	–	6
M.22	11, 11′, S	Ml	4	–	1	4	2	–	–	7
				129	329	268	121	33	5	885

individual variation can be observed, a modal expresion pattern with some NORs preferently activated were detected in most individuals analyzed. In general, an adequate correspondence between the maximum number of nucleoli and the maximum number of Ag-NORs is evident.

Discussion

Variation in chromosome number and arm number (NF)

Robertsonian polymorphisms appear not to be unusual among salmonid fish (Hartley 1987). The tetraploid origin of this group coupled with its subsequent diploidization has been suggested as the basis of this type of variation (Ohno et al. 1969; Gold 1979). However, this polymorphism, which is well established for some salmonids with chromosome numbers around 60, is less frequent and controversial in those species with karyotypes containing around 80 chromosomes.

The karyotypic studies carried out in brown trout (*S. trutta*) provide a good reflection of this situation. Although there is a wide consensus around the modal chromosome number in this species (2n=80), some authors have found no evidence for Robertsonian translocations (Capanna et al. 1973; Al Sabti 1985; Martínez et al. 1991), while others claim the existence of this type of variation (Zenzes and Voiculescu 1975; Morán et al. 1989; Karakousis et al. 1992). On the other hand, arm number has been controversial in the brown trout, with figures ranging from 96 (Svärdson 1945) to 104 (Raicu and Taisescu 1977).

In the present work we have studied in depth the basic karyotypic features in a large sample of *S. trutta* taken from

several natural populations of different characteristics (flowing and non-flowing waters) in Northwestern Spain, and two hatchery stocks commonly used for stocking in this area. The results obtained confirm our previous observation of the karyotypic constancy in chromosome number (2n=80) of this species (Martínez et al. 1991). Only one individual out of 169 analyzed undoubtedly showed a karyotype with 2n=79, a consequence of a Robertsonian fusion. This specimen was from the Umia river, where ten individuals were karyotyped. Additionally, this population showed the lowest genetic diversity (H=0.012) amongst those analyzed in a previous isozyme study (Martínez et al. 1993a). These data reinforce the hypothesis of a single mutational event.

The wide polymorphism in arm number found in our study (NF=100–102) is solely attributable to the variation in the short arm of the main NOR-bearing chromosome 11 which ranges from acrocentric to metacentric in different individuals. Remarkable differences in the mean NF between populations were detected, which probably account for the variation in the number of active rDNA genes of the main NOR pair, since the whole short arm is labelled in this species when silver stained (Martínez et al. 1993 b). This technique has proved to be a good measure of the amount of rDNA (Schmid et al. 1982).

Genetic factors related to the large chromosome number of this species, the presence in the karyotype of some chromosome pairs varying between uniarmed and biarmed (pairs 8, 9, and 10), and finally, the lack of an adequate technical methodology to obtain enough good chromosome spreads in some cases, as outlined by Thorgaard (1976, 1983), could account for the controversial data obtained in karyotypic studies on *S. trutta*.

Variation in Ag-NOR pattern

Although salmonids seem to have evolved from a tetraploid ancestor (Allendorf and Thorgaard 1984), most species within this group exhibit a well established single Ag-NOR chromosome pair (Hartley 1987). Chromosome rearrangements or inactivation have been invoked to explain the reduction in the expected number of Ag-NORs (Phillips and Ihssen 1985). In general, there is good concordance within salmonids between Ag-NOR positions and CMA3-positive staining (Phillips and Ihssen 1985; Mayr et al. 1988), which is claimed to stain NOR chromatin regardless of its activation status (Amemiya and Gold 1986). A single Ag-NOR pair is particulary evident in those species where, starting from the tetraploid ancestor, there is a more evolved karyotype as in the case of *Oncorhynchus* and *Salmo salar*, which both show low chromosome numbers (Phillips et al. 1986). The situation is quite different in the genus *Salvelinus*, whose karyotype is above 80, and which shows a multichromosomal location of Ag-NORs (Phillips and Ihssen 1985).

S. trutta represents an intermediate position possessing two main Ag-NORs, and quite frequently a third one, but with several additional CMA3-positive bands in a number of subtelocentric pairs (Mayr et al. 1986, 1988; Martínez et al. 1991). In-situ hybridization, a more precise technique to detect less conspicuous rDNA sites, has confirmed this finding, and new rDNA locations have been detected in the brown trout (Pendás et al. 1993).

In the present study we have analyzed a substantial number of individuals and populations of *S. trutta* from Northwestern Spain to ascertain the possible existence of new Ag-NORs in this species, and to obtain a more accurate picture of the NOR expression pattern at the population level. Most populations, excluding those from the Miño drainage, have shown the typical Ag-NOR pattern of this species, with differences among populations in the presence of the third Ag-NOR. Veral stock, a population which shows great genetic divergence with regard to the native ones (Martínez et al. 1993 a), has also shown a higher expression percentage of the third Ag-NOR.

Populations from the Miño basin, and especially the Chamoso sample, clearly showed a multichromosomal Ag-NOR pattern, with a higher number and new locations, always telomeric, of Ag-NORs. The reduced incidence of this atypical pattern within the Incio reservoir, relative to the Miño basin, can be explained, at least partially, by the high introgression levels observed in this population as a consequence of stocking with Veral individuals (Martínez et al. 1993 a).

Different explanations have been put forward to account for the detection of new Ag-NORs (Gold and Amemiya 1986; Phillips et al. 1988; 1989). The activation of previously silent rDNA sites could be an interesting hypothesis in brown trout, because of the additional inactive rDNA locations revealed by CMA3 and in-situ hybridization. These inactive positions have been demonstrated to comprise the short arm of several acrocentric-subtelocentric chromosomes. However, although in some case the new Ag-NORs from the Miño drainage correspond to these positions in our study (Fig. 3 h, j), many others involve the telomeres of the long arm of several acrocentric and metacentric chromosomes. Therefore, their appearance is not easily explained by the activation of silent rDNA sites. Furthermore, some individuals from the Chamoso river show a chaotic Ag-NOR distribution with up to ten randomly-activated Ag-NORs in different metaphases. This atypical distribution probably fits better to a model of transposition, as proposed by some authors in other species (Schubert and Wobus 1985; Phillips et al. 1988; 1989).

Finally, it is important to emphasize the activation of specific clusters within particularly-long NOR regions of the main Ag-NOR chromosome 11, which also occurs in some individuals of the Chamoso population. These clusters are always located in the same position, while the short arm remains mostly inactive, and appear activated in all metaphases analyzed within particular specimens. The whole short arm of this chromosome pair has proved to consist of tandemly-repeated rDNA units as revealed by in-situ hybridization (Pendás et al. 1993). Furthermore, individuals with a standard Ag-NOR pattern show activation of rDNA genes all along the short arm of pair 11 (Martínez et al. 1993). These facts probably indicate that regulation

of rDNA genes in these particular chromosomes from the Chamoso river can be controlled at specific clusters within a long NOR region. Also this regulation pattern is inherited through mitosis, since the same cluster appears activated in all metaphases within these special individuals.

The Ag-NOR pattern in the Chamoso population, and possibly also in the Miño basin, represents a very different one within *S. trutta*, a salmonid species with an intermediate NOR distribution pattern between the single pair location of most salmonids and the multichromosomal one found in the genus *Salvelinus*. Although a transposition mechanism could be involved in this atypical pattern, new investigations will be required to assess this possibility. Inheritance studies and a detailed analysis with different banding techniques are being performed in our laboratory to resolve these open questions.

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