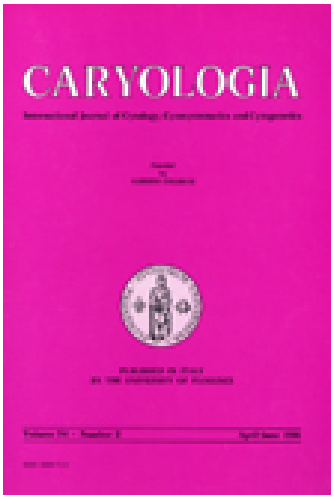

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M. Benazzi[a], D. Formenti[b], M.G. Manfredi Romanini[b], C. Pellicciari[b] & C.A. Redi[b]

[a] Istituto di Zoologia e Anatomia comparata, Università di Pisa, Pisa

[b] Istituto di Istologia, Embriologia e Antropologia, Centro di Studio per l'Istochimica del C.N.R., Università di Pavia, Pavia, Italy

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FEULGEN-DNA CONTENT AND C-BANDING OF ROBERTSONIAN TRANSFORMED KARYOTYPES IN *DUGESIA LUGUBRIS*

M. BENAZZI*, D. FORMENTI, M.G. MANFREDI ROMANINI, C. PELLICCIARI and C.A. REDI

*Istituto di Zoologia e Anatomia comparata, Università di Pisa, Pisa, and Istituto di Istologia, Embriologia e Antropologia, Centro di Studio per l'Istochimica del C.N.R., Università di Pavia, Pavia, Italy

SUMMARY — In the planarian species, *Dugesia lugubris*, two biotypes are found: E ($2n = 8$, $n = 4$) and F ($2n = 6$, $n = 3$); on the basis of karyometric studies it has been hypothesized that the second was derived from the first through a Robertsonian mechanism of centric fusion. The quantitative cytochemical data reported here confirm the hypothesis of karyotype evolution, since there are no significant differences between the DNA content of the nucleus in the two biotypes. The regenerative blastemas of both biotypes contain a number of cellular populations with a variable Feulgen-DNA content; these correspond to successive doublings of the 2C diploid content. In addition, metaphase plates with multistranded chromosomes have been found. A difference between the chromosome C-banding in the two biotypes has also been observed.

INTRODUCTION

As pointed out by BENAZZI, PUCCINELLI and DEL PAPA (1970), REINOLDSON and BELLAMY (1970) and BENAZZI *et al.* (1975), there are, within the *Dugesia lugubris-polychroa* group, four sibling species, which are chromosomically differentiated and reproductively isolated, and correspond to the A-B-C-D; E; F; G biotypes.

The first four biotypes (A-D) make up a homogeneous polyploid series, starting with the A biotype ($2n = 8$, $n = 4$); they can interbreed, and correspond to *D. polychroa* (O. Schimidt). The E biotype is diploid, with $2n = 8$, $n = 4$, while the F biotype is again diploid, but its chromosome number is $2n = 6$, $n = 3$; both biotypes have been tentatively assigned to *D. lugubris* (O. Schmidt). The G biotype is diploid, too, with a chromosome number of $2n = 8$, $n = 4$; it corresponds to *D. mediterranea* (BENAZZI *et al.* 1975). The morphology of the mitotic and bivalent chromosomes differentiate these three biotypes from each other and from the A-D biotypes (BENAZZI 1957;

BENAZZI and PUCCINELLI 1961; BENAZZI *et al.* 1970). (For further details, see BENAZZI and BENAZZI LENTATI 1976).

On the basis of karyometric analyses, BENAZZI and PUCCINELLI (1973) suggest that the F biotype may have originated from the E biotype through a Robertsonian fusion; in their view the formation of the karyotype may be

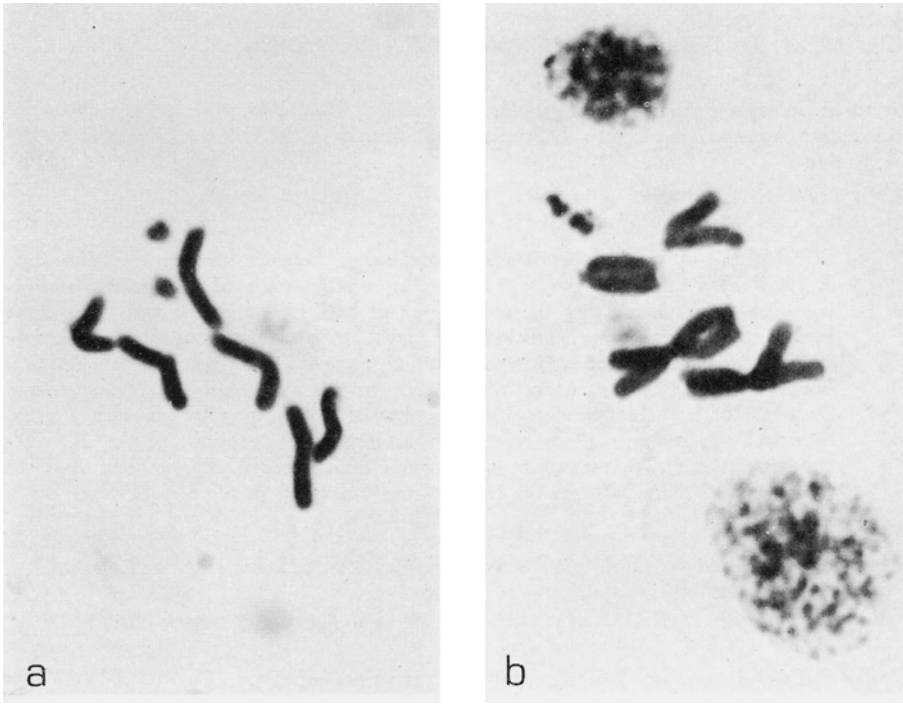

Fig. 1. — Mitotic metaphases of biotypes E and F of *Dugesia lugubris* after Feulgen reaction. × 100.

supposed to have been completed by a pericentric inversion of the tiny submetacentric in the E biotype, so giving rise to the tiny metacentric of the F biotype.

The haploid set of the E biotype (Fig. 1a), in fact, is made up of three acrocentric chromosomes of different lengths and a tiny submetacentric; the haploid karyotype of the F biotype (Fig. 1b) comprises a large metacentric, an acrocentric of medium length and a small metacentric.

The transformation of the karyotype has established an effective reproductive barrier (perhaps linked with morphological changes in the copu-

latory system), which now ensures a genetic separation between these two forms. So far, however, the specific individuality of the F biotype has not been codified by giving it a specific name.

It seemed to us that it was worth deepening the analysis of this model of karyotype evolution through an *in situ* cytochemical evaluation of the DNA content of the whole genome in each of the two biotypes, and of their individual chromosomes and chromosome arms.

Genome size, expressed in terms of the DNA content measurable through Feulgen reaction, has been a widely used parameter in studies on the evolution of various groups of animals (BACHMANN 1972; BACHMANN *et al.*, 1972; MANFREDI ROMANINI 1973; HINEGARDNER 1976). In particular, it has been repeatedly observed that in cases where chromosome restructuring is due to Robertsonian phenomena of fusion-fission (in Chiroptera, Lemurs and Rodents), no significant changes in Feulgen-DNA content have been found (MANFREDI ROMANINI and CAPANNA 1971; CAPANNA and MANFREDI ROMANINI 1971; MANFREDI ROMANINI *et al.* 1978; REDI and CAPANNA 1978).

A quantitative study on the DNA content of individual chromosomes — a parameter already recognized as being a reliable criterion for the identification of the components of the human karyotype (VAN DER PLOEG *et al.* 1974; DE STEFANO and MANFREDI ROMANINI 1975) and those of the single chromosome arms (MENDELSOHN *et al.* 1973) — seemed to us to be a useful cytochemical method for testing the validity of the model of karyotype evolution proposed for the E and F biotypes of *D. lugubris* by BENAZZI and PUCCINELLI (1973).

The results reported below bear out the concept that the transformation of the karyotype can be attributed to a Robertsonian mechanism, so endorsing the notion that the large metacentric in the F biotype derives from the fusion of two acrocentric components of the E biotype.

As in the other examples mentioned (Chiroptera, Lemurs and Rodents), there is no appreciable quantitative difference between the genome size of the two biotypes.

The data reported by us also show that the regenerative blastemas in both biotypes comprise cell populations whose Feulgen-DNA content is variable, with peaks corresponding to a series of doublings of the lowest (2C) values found. Some structural features of the chromatin within the metaphase have been observed and are analyzed in detail.

In the discussion we also consider the fact that the two biotypes contain different quantities of chromosome material that give a positive reaction to C-banding carried out with the method of SUMNER (1972).

MATERIALS AND METHODS

The biotype E specimens of *D. lugubris* were originally collected at Münster (Nordrhein Westfalen, West Germany), whereas those belonging to biotype F came from Pavullo (Modena, Italy); all the animals used in the present study had been kept for some time in the laboratory.

The study on DNA content (Feulgen positive material) was carried out on neoblasts from regenerative blastemas.

The planarians were cross-sectioned in their middle zone; after 3-4 days of regeneration the portions containing the blastema were removed and placed in 0.3% colchicine (Merck) for 3-4 hours; the blastemas were then hypotonized in distilled water for 10 minutes at 37°C, then fixed at 4°C with 3:1 methanol acetic acid for 15 minutes, and lastly disaggregated in 45% acetic acid.

To obtain preparations of cells and metaphase plates free from overlapping (a necessary precondition for a correct microdensitometric evaluation), instead of adopting the usual squash technique, we prepared slides by following the main principles of the " hot plate " technique: a suspension of neoblasts in 45% acetic acid was repeatedly allowed to evaporate on slides heated to 50°C, until this yielded a preparation that was sufficiently rich in cells. It may be noted that the population so obtained on each slide was representitive of all the stages of the regenerative process.

The Feulgen reaction was carried out simutaneously on a number of preparations of each biotype and on blood smears from the following animals: *Gallus domesticus, Xenopus laevis, Bufo boreas, Bufo regularis, Bufo cognatus*, of which the DNA amount in picograms has been reported (DAVIDSON *et al.* 1973; ROSEN *et al.* 1973; PELLICCIARI and BACHMANN 1979); these blood smears were included to make possible an estimate in absolute units of the genome size of *D. lugubris*.

The technical details for carrying out the Feulgen reaction were as follows: hydrolysis in 5N HCl at 23°C for 60 minutes; staining with Schiff reagent for 45 minutes; differentiation in sulphurous water for 30 minutes; dehydration and embedding in Eukitt (Kindler).

Assessment of the quantity of Feulgen positive material was carried out with a Vickers M85 scanning microdensitometer on a wavelength of 545 ± 5 nm. This instrument was used under the following conditions: 100× immersion objective, 10× eyepiece, dry condensor; area of spot projection 0.4 μm in diameter, 1 x 1 scanning frame. A square adjustable mask of variable size was used to measure the individual chromosomes and the chromosome arms of the large metacentric. All the measurements were corrected to avoid the photometric error due to glare (BEDI and GOLDSTEIN 1974, 1976).

The C-banding of the chromosomes was obtained by applying the technique suggested by SUMNER (1972). This technique, called BSG-banding, requires pretreatment for 15 minutes in 0.2 M HCl followed by treatment in a saturated (5%) solution of $Ba(OH)_2$ at 60°C for 60 to 90 seconds. After rapid rinsing in distilled

water, the slides were left overnight in a solution of 2 x SSC at 60°C in a moist-chamber. Staining was carried out with 10% Giemsa (Merck) in 0.1 M phosphate buffer at pH 7 for 15 to 20 minutes.

RESULTS

The histograms in Fig. 2 show the Feulgen-DNA content of the interphase nuclei and of the metaphases in neoblasts within the regenerative blastemas from the E and F biotypes of *D. lugubris*. The distribution of values

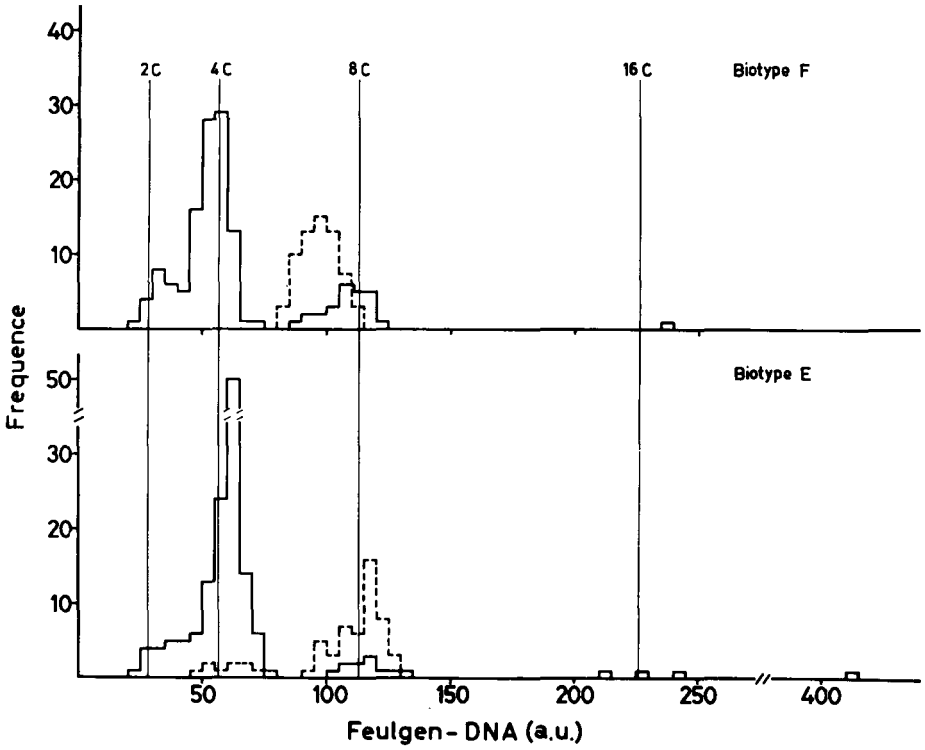

Fig. 2. — Distribution of Feulgen-DNA content of interphasic nuclei (———) and metaphases (— — —) of neoblasts from biotypes E and F of *Dugesia lugubris*.

for interphase nuclei (continuous line) turns out to coincide almost exactly in the two biotypes. More specifically, the values for DNA content show a series of doublings, as far as 16C in the F biotype and still further in the E biotype. The fact that the two distribution patterns almost coincide

suggests that the two biotypes have an almost equal DNA content. This is about 1.75 pg per diploid set, as calculated on the basis of the DNA content values in the control species expressed in arbitrary density units and in picograms. The calibration line (Fig. 3) has been calculated as the least square regression line through the origin (according to BACHMANN *et al.* 1972).

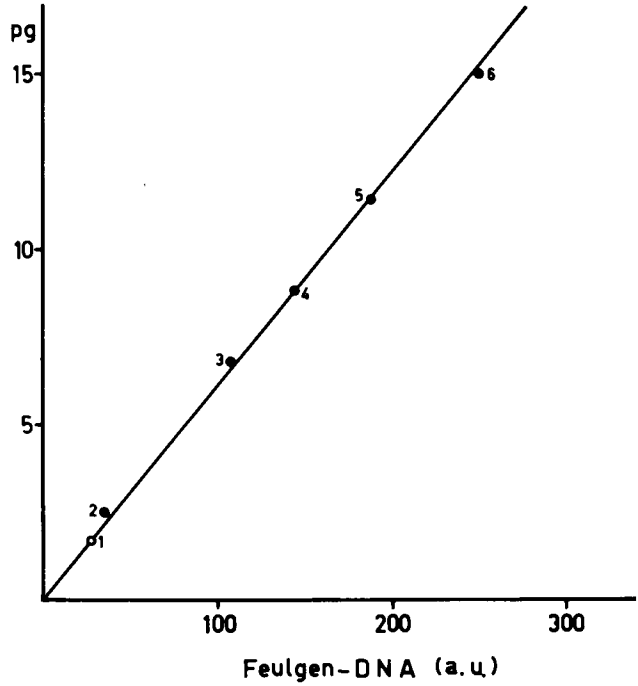

Fig. 3. — Calibration of genome size in arbitrary units against picrograms of DNA content. The line is a calculated regression line through the origin. 1 = *D. lugubris*; 2 = *Gallus domesticus*; 3 = *Xenopus laevis*; 4 = *Bufo regularis*; 5 = *Bufo cognatus*; 6 = *Bufo boreas*.

The two biotypes show a difference as regards the Feulgen-DNA content of their metaphases (Fig. 2, dashed line). In the F biotype only one type of metaphases was found, with a constant chromosome number, $2n = 6$, and a Feulgen-DNA content 11.7% below the 8C content of the corresponding interphase nuclei. On the contrary two types of metaphase plates were found in the E biotype, with a chromosome number of $2n = 8$ in both cases, but with a Feulgen DNA content exactly corresponding to the 4C and 8C content of the interphasic nuclei. In addition, in the E biotype the Feulgen-DNA content of the individual chromosomes in the 8C plates was

twice what it was in the single chromosomes of the 4C metaphases. The situation is thus identical with that reported by GAY *et al.* (1970) for the metaphase chromosomes of the ganglionic cells of the third larval stage of *Drosophila melanogaster* — a situation attributed by these authors to the presence of " multistranded or polynemic " chromosomes.

The almost exact coincidence noted above between the levels of the Feulgen-DNA content in the interphase nuclei in the two biotypes suggests that the shortfall in DNA content in the metaphase plates of the F biotype

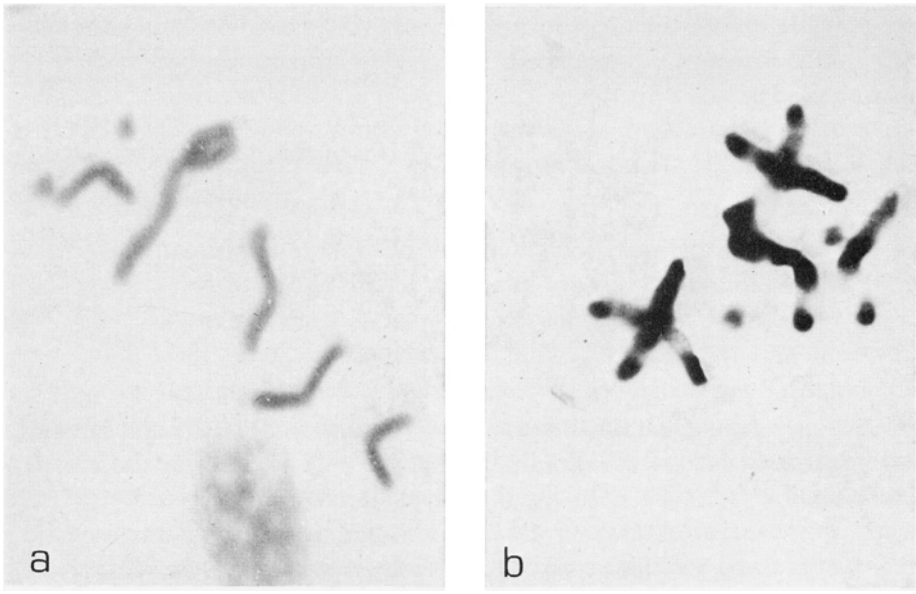

Fig. 4. — Mitotic metaphases of biotypes E and F of *Dugesia lugubris* after C-banding treatment and Giemsa staining. × 100.

(with respect to the expected level of 8C) is due less to any real difference in genome size than to the lower availability of the chromosome material to Feulgen reaction at the metaphase stage.

This presumably different availability to the hydrochloric hydrolysis of the Feulgen reaction could be ascribed to the greater " heterochromatinization " of the chromosome material in the F biotype with respect to that in the E biotype, as shown by their respective C-banding patterns (Fig. 4). In any case, this would be a kind of condensation not ascribable to constitutive heterochromatin (as defined by BROWN, 1966), but dependent on the peculiar behaviour of chromatin in one of the two biotypes during mitosis.

The Feulgen-DNA content values of the individual chromosomes (and in the chromosome arms of the large metacentric in the F biotype) as percentages of the total content in the karyotype (Tab. 1) are in full agreement

TABLE 1 - *Microdensitometric values and karyometric values for the relative lenghths of the chromosomes in biotypes E and F. Relative length: length of chromosome × 100/ total length of haploid genome. (Karyometric values are from* BENAZZI *and* PUCCINELLI *1973).*

Biotypes	Chromosome number	Microdensitometric values	Karyometric values
BIOTYPE E	1	34.82 ± 0.17	33.70 ± 0.29
	2	33.03 ± 0.07	31.45 ± 0.30
	3	26.72 ± 0.20	27.08 ± 0.28
	4	5.76 ± 0.07	7.76 ± 0.14
BIOTYPE F	1 long arm	32.52 ± 0.39	33.35 ± 0.35
	1 short arm	27.92 ± 0.40	26.73 ± 0.45
	2	31.76 ± 0.19	31.68 ± 0.27
	3	5.23 ± 0.07	8.23 ± 0.30

with the karyometric data of BENAZZI and PUCCINELLI (1973) and strengthen their hypothesis that a Robertsonian mechanism may account for the karyotype transformation from the E to the F biotype.

Lower values for Feulgen-DNA are found in the tiny metacentric of the F biotype and submetacentric of the E biotype than in the corresponding karyometric measurements. The present datum appears to show that there is a lower mean concentration of Feulgen-positive material along these chromosomes. It is likely, however, that the discrepancy between the karyometric and microdensitometric data, for such very small components, is the result of the different role played by the centromeric zone in the two types of analysis.

CONCLUSIONS

1) The data reported here confirm the model of Robertsonian transformation of the karyotype proposed for the E and F biotypes of *D. lugubris* by one of us (BENAZZI and PUCCINELLI 1973), and, at the same time, provide a further argument in favour of the hypothesis that this mechanism does not imply appreciable changes in the Feulgen-DNA content (MANFREDI ROMA-

NINI *et al.* 1978; CAPANNA and MANFREDI ROMANINI 1971; REDI and CAPANNA 1978). The karyometric measurements and the microdensitometric values are in full agreement as regards the large metacentric and the acrocentric of the F biotype, and the three acrocentrics of the E biotype; on the other hand, comparison between these two types of data reveals that the smallest component of each of the two karyotypes has a low Feulgen-DNA content per unit length (this behaviour is not influenced by the minor rearrangement through inversion that has occurred here). It may be stressed here that the technical resources of absorption cytophotometry can go no further than this, and that one will have to resort to different kinds of apparatus in analysing the role played by the centromere, and in determining whether is true that the chromatin material is distributed differently along these chromosomes. If the role of the centromere does not turn out to be influential, the model of *D. lugubris*, which is interesting in itself, would also supply an example of a different degree of DNA condensation in the various components of the karyotype.

2) The functional implication of the constant presence in the two biotypes (whose somatic and germinal cell lines are diploid in all cases) of cells whose DNA content corresponds to classes of successive doublings of the lowest value leads us to conclude that a high DNA content is a favourable condition for the onset and accomplishment of the regenerative process. One finding which supports this hypothesis is the fact that there are very few neoblasts with a 2C DNA content, and that no 4C metaphase plates have been found in the F biotype, while these are rare ($< 17\%$) in the E biotype, too.

On the other hand, the presence of a polynemic condition, which appears to explain the existence of metaphase plates with a diploid chromosome number and an 8C Feulgen-DNA content, shows evident physiological analogies with all the processes which require a considerable biosynthetic activity in the cell. The functional importance of polynemy (and, more generally, of polyploidy), in ensuring an intense biosynthetic activity through a more rapid availability of many active copies of the genome, has been stressed by BRODSKY and URYVAEVA (1977).

3) The Feulgen-DNA content measured in the metaphases of the F biotype is about 11% lower than that of the 8C plates in the E biotype, but it is also 11% lower than the content — which is statistically identical in the two biotypes — of the interphasic nuclei of the 8C neoblasts. Our preliminary data on C-banding allow us to interpret this shortfall as being due to the lower availability to the Feulgen reaction of the chromatin fraction that is most strongly condensed in the metaphases within the C-banding-positive zones, typical of the F biotype only. It is not, however, clear why

this condensation should emerge as a lower sensitivity to the Feulgen reaction at the metaphase but not at the interphase stage. The investigations reported here are therefore continuing in an attempt to identify the characteristics of the regenerative cycle of neoblasts, since the only explanation which can be hypothesized at present is that, during the metaphase, there is a highly specific condensation of zones which are " euchromatic " in interphase.

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