

STRUCTURAL AND FUNCTIONAL REARRANGEMENTS IN POLYTENE CHROMOSOMES OF CHIRONOMIDS (DIPTERA) AS BIOMARKERS FOR HEAVY METAL POLLUTION IN AQUATIC ECOSYSTEMS

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Abstract

The polytene chromosomes of larvae of two geographically isolated populations of *C. riparius* (Bulgaria -Varna and Italy - Santena) from two trace metal polluted stations were studied from cytogenetical point of view. Many somatic rearrangements as well as functional alterations were detected in the salivary gland chromosomes of *C. riparius* of both populations. The chromosome rearrangements were not randomly distributed along the chromosomes. In 21 out of the 89 sites, breakpoints corresponding to inversion boundaries occurred repeatedly in both populations. These sites were considered as structural weak points. The deletions in a chromosome G led to the formation of pompon-like chromosome G. In 30 % of chromosomes G, changes in the functional activity of Balbiani rings was observed. This kind of alterations as well as occurrence of pompon-like chromosomes G may depend on the genotoxic action of polluting agents. All these cytogenetic damages can be considered as biomarkers which provide early warning signals of adverse long term genotoxic effects in organisms.

Introduction

Chironomids are a widely distributed and abundant group of species in freshwater ecosystems. Their larval stage is the most critical, responsive to environmental stress and metabolically active stage of their life. Chironomid larvae are prospective subjects for cytogenetic monitoring because their polytene chromosomes have a large size and a very good banding pattern (Michailova, 1989). The standard karyological characteristics of the polytene chromosomes of some species (Hägele, 1970, Michailova, 1989, Kiknadze et al., 1991) can be used as a basis for revealing chromosome aberrations and changes in aspect of heterochromatin and functional activity under the action of environmental mutagens.

Among chironomid species, *Chironomus riparius* (syn: *C. thummi*) has only four well banded polytene chromosomes (called AB, CD, EF, G) whose standard banding pattern has been mapped by Hägele (1970) and Kiknadze et al (1991). Larvae of this species can be reared in laboratory and used in dose response experiments.

In natural populations of *C. riparius* somatic rearrangements are almost absent: only one somatic deletion has been reported in a Novosibirsk population (Kiknadze et al., 1988) and a 0.4 % frequency of somatic inversions in an unpolluted Italian population (Michailova et al, in press). In a nine year inbred laboratory stock reared by Hägele (1984) the frequency of spontaneous aberrations was 0.84 %. Having in mind the advantages of this species as a model we used it for evaluating the genotoxic effects of environmental polluting agents, especially heavy metals, on the structural and functional organisation of the polytene chromosomes. We tried also to establish if some rearrangements of the polytene chromosomes can be used as biomarkers of chronic exposure to genotoxic agents.

Methodology

We analysed polytene chromosomes of larvae of two geographically isolated populations of *C. riparius* from two trace metal polluted stations: Santena, on the Banna river (Piedmont, Italy) and Varna in east Bulgaria (1565 cells from 56 larvae of the Santena population and 377 cells from 28 larvae of the Varna population). Chemical analysis showed that sediment and larvae from Santena had a concentration of Cr, Cu and Pb respectively six times and two times higher than the concentrations of the same metals in the sediment and larvae of a non-polluted station taken as a control (Sella et al., 1997; Michailova et al, 1998). In sediments of the Varna station the concentration of Pb was five times higher and those of Zn and Cu - two time higher than in an unpolluted sediment (Todorova, in press). Cytogenetic methods of analysis are reported by Michailova et al (1997, 1998) and Bovero (1999). We performed in situ hybridisation (FISH technique) with repetitive DNA clones (Alu and Hinf) following Schmidt, et al. (1988) method.

Results and discussion

Structural chromosome aberrations

In both populations a large number of somatic rearrangements of polytene chromosomes were discovered (Michailova et al., in press; Todorova, in press) (Table 1, table 2). No one specimen was found with the standard karyotype: both somatic and inherited heterozygous inversions, deficiencies and amplified sections as well as chromatid breaks were detected. In the Santena population we observed three types of deficiencies and three types of amplified sections and 40 different types of heterozygous inversions (Table 1). Twenty of these inversions were somatic (Michailova et al., 1998; Michailova et al., in press). In Varna 29 somatic heterozygous inversions, five deficiencies and one amplified section were detected (Table 2).

On the whole eight out of the 49 somatic inversions were pericentric (involving chromosomes AB and EF). The pericentric inversion in chromosome AB from the Santena larvae was connected with a positional effect: in the homologue, where the inversion was located, we found a pseudopuff, and in the other homologue the same region was in a standard condensed state. In the same population, three sections of chromosome EF (A4f-g A5d-g on arm E and B3h on arm F) showed variation in their size. Densitometric analysis of the DNA content of the three sections fell into several classes of density (Sella et al 1997). Such a large variation in DNA content of the three quoted sections has never been reported so far. Different types of structural chromosome alterations were observed in chromosome G in both studied populations (Michailova et al., 1998, Todorova, in press). The most frequent was a heterozygous inversion in section Da-Df (14%) and a change in the position of the nucleolus, which could be due either to a pericentric homozygous inversion in section Da-Ec (4,6 %) or a folding of the chromosome (Michailova et al. 1998). Also, in this chromosome different types of mosaic deletions were established (Michailova et al., 1998, Todorova, in press). Some of them can lead to the formation of pompon-like chromosomes G, for instance a deletion in section A1cd-A2bc together with a deletion in sections A1b-A2abc; deletions of BRc and of sections Cde-E1a-c, a large deletion including bands before and after the nucleolus organizer (Fig. 1a,b). In Varna region, a deletion of BRb and BRc, as well as a deletion in sections A1de and BRc converted chromosome G in a pompon chromosome (57,1%). In both populations chromosome G with a collapse of BRs also could be appeared as pompon chromosome. In both studied stations the pompon chromosomes appeared in two different aspects. In cells located in the main lobe of a salivary gland they were highly decondensed, while in cells of the lateral lobe they were in a condensed state.

Functional changes in chromosomes

Changes observed in a functional activity of chromosome G were very interesting. On this chromosome a system of three Balbiani rings (Bra, BRb, BRc) is located. Balbiani rings are permanently active puffs which code the major polypeptides of the salivary gland. Generally in an unpolluted sediment, the larvae of *C. riparius* (6-7 phase of the IV larval instar) the size of BRc is larger than that of BRb (Kiknadze & Panova, 1972). In contrast, in both studied populations a drastic regression of BRc, paralleled by an expansion of BRb was frequently observed (Michailova et al., 1998, Todorova, in press). While the level of regression of BRc is the same in all cells of the lateral and main lobe of the salivary gland, BRb activation shows some differences between main and lateral cells. In lateral cells very often BRb is highly condensed while in main cells it is highly active. Thus, in puffing of *C. riparius* Balbiani rings anthropogenic agents can induce changes which are similar to those induced by heat shock or sugar feeding (Diez et al., 1990). Apparently the BRs transcription mechanism reacts in the same way to different stressful situations.

In cells where BRs show different levels of activity, induction of a puff in the Dc section was detected. This puff can be activated either in a homozygous state (in 4.2 % of the Santena) or in a heterozygous state (in 15,1 % of the Santena population and in 1,1 % of the Varna population). This puff has been detected also by Kiknadze & Panova (1972) in larvae of a Novosibirsk population.

Furthermore, in some cells the nucleolar organizer showed a change in its activity: from very active on both homologues to a heterozygous active state (in 1,3 % of cells of Santena larvae and 1,9% of cells of Varna larvae) or slightly active (12,8 % of cells of Santena and 6,9 % of cells of Varna) or completely collapsed (1,8 % in cells of Santena larvae) (Michailova et al., 1998, Todorova, in press).

The telomeric region of chromosome G was decondensed (in 2.6 % of cells of the Santena larvae and 1,3 % of cells of the Varna). In many specimens of both populations chromosome arms had telomeres with a loose, granular structure, especially well observed in telomeres of arms A,B,C from Varna station (5,1 %, 4,5 % and 3,9 % respectively). In this population decondensed centromeres were observed in chromosomes AB, CD and EF with the frequency of 9,5 %, 5,3 % and 4,2 % respectively (Todorova, in press). In contrast, in the Santena population they

were observed only in some cells of three out of 56 examined larvae (Michailova et al, in press).

It is known that in *C. riparius* as many as 336 puffs appear during metamorphosis (Kiknadze & Panova, 1972). Novel puffs, not present in the standard map of puffs (Kiknadze and Panova, 1972) at phase 6-7 of the IV larval instar of Santena station were established in sections B1, B2, C2, C3, C4 of arm A; sections D3 of arm B; sections B1 of arm C; sections C4 and D1 of arm D and sections A5 of arm E.

Twenty one out of the 89 different chromosome breakpoints corresponding to boundaries of inversions were common to both populations (for instance the following breakpoints: Arm A, sections B4f, C2a; arm B, sections E1a, E2o, E3e, F1h, F2i, F3h; Arm C, sections B3c, B5a, C1d, arm E, sections B1c, B1r; arm F, sections B2q, B3h, B3b, B3o, B4d, C1a, C3d, C4d, C4f, arm G, A1d, BRc, BRb, Cc). Also common to both populations were breakpoints of three deficiencies (arms C,D,A) and deletions in arm G.

These coincidences suggest that in *C. riparius* not all chromosomal breaks occur randomly: the sites where they occurred repeatedly in different populations are probably structural weak points of the chromosomes. Following the method of Tonzetich et al (1988) chromosomes were subdivided in 18 arbitrary sections and location of breaks in each section in the chromosomes of Santena and Varna was compared. That breaks do not occur randomly along the chromosomes but concentrate in centromere proximate halves rather than in the distal regions is shown by the highly significant value of the Kruskal-Wallis test ($H = 44.21$; $df = 17$; $P < 0.001$).

We checked whether satellite DNA was located in the weak points by in situ hybridisation with probes of two satellite DNA elements, Alu and Hinf. Alu repeat unit is a sequence 170 pb long; Hinf repeat unit is a sequence 110 bp long. They are both composed by 70 % of AT (Hankeln, 1990).

Twenty Alu clusters in chromosomes AB, CD, EF and 33 Hinf clusters along the four chromosomes. (Figs. 2, 3) were found. They are structural components of *C. riparius* heterochromatin since they are present in the same heterochromatic sites in both populations. Centromeric regions of chromosomes AB, CD and EF as well as the chromosome domain B3b-B3o and B4d-C4a of arm F (i.e. sites of constitutive heterochromatin; Hägele, 1977; Michailova et al, 1997) are rich of Alu elements. Also Hinf clusters are observed in constitutive heterochromatic sites of chromosomes AB, CD, EF, G.

The break points of deletions in chromosome G are located in the same regions where Hinf elements are located. Two amplified sections of chromosome EF (A4f and B3h) are composed by Hinf and Alu elements respectively. Being composed by repetitive DNA, unequal crossing-over in these sections could have originated the different degrees of amplification we observed.

However, a comparison, chromosome by chromosome, the distribution of Alu and Hinf clusters with the distribution of the break points of inversions by means of the Kruskal-Wallis test (Sokal and Rohlf, 1981) shows that both in Santena and in Varna populations distribution of satellite DNA and inversion breakpoints did not differ in location nor for chromosome AB ($H = 4.5$ and 9.04 respectively; $P > 0.10$) nor for chromosome CD ($H = 1.2$ and 0.18 respectively; $P > 0.10$) nor for the chromosome EF ($H = 1.4$ and 0.4 respectively; $P > 0.1$). Breaks and satellite DNA clusters occur in similarly positioned regions i.e. on the proximal parts of the centromere region.

The aspect of the constitutive heterochromatin in the larvae of Santena was very interesting. We had evidence of a process of transformation of euchromatin into heterochromatin (Michailova et al., 1997). In this process if a gene is included in an inverted or translocated chromosomal segment its activity can be changed because its position in the genome is changed (Henikoff, 1990). Usually, as it moves near or into a heterochromatic region, an euchromatic section becomes inactivated and shows a mosaic expression. Position effects can be either enhanced or suppressed by different environmental conditions (Mottus, 1980). In *Drosophila melanogaster* position effects have been observed after ionising radiation (Henikoff, 1990) which produced chromosome rearrangements. In larvae from Santena in arms D and F another type of position effect was observed which is described by Zhimulev (1998): in small heterozygous inversions within a heterochromatic section part of heterochromatin becomes as euchromatin due to a position effect. It is possible that the observed position effects can be caused by the genome mobilisation induced by the polluting agents present in sediments of the Santena station. Such agents may exert some inhibiting effects on the synthesis of proteins which participate in chromatin condensation. That's why in some cells of all chromosomes of both populations the centromere heterochromatin as well as some C bands disappeared or looked decondensed.

Conclusions

We can now ask the question whether polytene chromosomes of Chironomids can be used as testers of the presence of genotoxic concentrations of polluting agents in aquatic ecosystems. The answer is positive because in the polytene chromosomes some biomarkers of environmental stressful conditions have been detected.

- reduction of activity or the complete inactivation of BRc in approximately 30 % of cells in a salivary gland;

- appearance of somatic rearrangements which are expected to occur more often in the weak points we detected ;
 - presence of pompon-like chromosomes G;
- Somatic cytogenetic damages observed in cells of salivary glands provide early warning signals of adverse long effects in organisms. These damages can occur also in germinal cells and therefore their occurrence in salivary gland cells is a signal of the extent of the risk of being transferred to the next generation.

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Table 1. Location and frequency of inversions and deficiencies in *Chironomus riparius* from the Santena station. Par. inv.= paracentric inversion; Per.inv.= pericentric inversion; * = hereditary inversion (from Michailova et al.,in press).

Arm	Aberration	Location	% Frequency of affected cells	% Frequency of affected larvae
A	Par. inv.	B1g-B2g	0.22	1.8
	Par. inv.	B4f-C2f	0.42	1.8
	Par. inv.	C2a-C2i	0.03	1.8
	Par. inv.	C2a-C3g	0.48	3.6
	Per. inv.	D1d-D3h	0.20	3.6
	Deficiency	A1a	0.08	1.8
	B	Par. inv.	E1a-E2o	0.20
Par. inv.*		E2e-E3e	-	5.4
Par. inv.		E2f-F3c	0.08	1.8
Par. inv.		E2m-F1h	0.31	3.6
Par. inv.*		F2i-F4d	-	1.8
Par. inv.*		F3c-F3h	-	1.8
Par. inv.*		F4d-G1d	-	10.7
Deficiency		G3p	0.06	1.8
C	Par. inv.	B2b-B4l	0.17	3.6
	Par. inv.	B3c-C1h	0.20	1.8
	Par. inv.*	B5a-C1d	-	5.4
	Par. inv.*	B5a-C2l	-	3.6
D	Par. inv.	C4e-C5f	0.03	1.8
	Par. inv.	C4f-C6d	0.06	1.8
	Par. inv.	C5a-C6a	0.03	1.8
	Par. inv.	C6a-D2g	0.08	1.8
	Deficiency	F2o	0.11	1.8
E	Par. inv.	A2i-B2a	0.08	1.8
	Par. inv.*	A3d-B1r	-	1.8
	Per. inv.	B1b-B4g	0.84	5.4
	Par. inv.*	B1c-B1o	-	3.6
	Per. inv.	B1o-B3a	0.08	1.8
	Per. inv.	B2b-B3o	0.11	1.8
	Per. inv.	B2f-B2o	0.06	1.8
F	Par. inv.	B2q-C1a	0.03	1.8
	Par. inv.	B3a-B3o	0.14	3.6
	Par. inv.	B3a-B4d	0.73	3.6
	Par. inv.*	B3c-B3h	-	8.9
	Par. inv.	B3d-B3i	0.42	3.6
	Par. inv.*	B3d-C1a	-	1.8
	Par. inv.*	B3j-C1e	-	1.8
	Par. inv.*	B4d-C3d	-	8.9
	Par. inv.	C2a-C3d	0.42	3.6
	Par. inv.*	C2a-C4f	-	5.4
	Par. inv.*	C3d-C4d	-	3.6

Table 2. Location and frequency of inversions, deletions and deficiencies in *Chironomus riparius* from the Varna station. Par. inv.= paracentric inversion; Per.inv.= pericentric inversion (from Todorova, in press).

Arm	Aberration	Location	% Frequency of affected cells	% Frequency of affected larvae
A				
	Par. inv.	B1a-B3g	0.5	3.6
	Par. inv.	B3i-B4f	1.6	14.3
	Par. inv.	C2a-C3a	1.1	3.6
	Par. inv.	C2a-C4a	0.5	3.6
	Per. inv.	D1e-D3q	0.5	7.1
	Per. inv.	D2b-D3d	3.2	7.8
	Deficiency	A1a	0.8	7.1
B				
	Par. inv.	E1a-E2o	1.6	
	Par. inv.	E2e-E3d	3.7	25.0
	Par. inv.	E3e-F1h	0.5	3.6
	Par. inv.	F2i-F3e	1.1	10.7
	Par. inv.	F3e-F3h	0.8	3.6
	Deficiency	G3p	0.5	3.6
C				
	Par. inv.	B1a-B4e	1.1	7.1
	Par. inv.	B3c-B5a	1.3	10.7
	Par. inv.	B3d-C1d	1.3	10.7
	Par. inv.	B5a-C1d	2.4	21.4
	Par. inv.	C2b-C4f	0.5	3.6
	Deficiency	A1a	0.8	3.6
D				
	Par. inv.	D3a-D3d	3.4	17.8
	Par. inv.	D3d-E1d	1.8	7.1
	Par. inv.	D3a-E1d	1.6	7.1
	Par. inv.	E2g-E4c	1.8	10.7
	Deficiency	F2o	1.3	10.7
E				
	Par. inv.	A2g-A4f	1.1	7.1
	Per. inv.	B1a-B3b	0.8	7.1
	Par. inv.	B1c-B1r	2.7	10.7
F				
	Par. inv.	B2q-B3h	0.5	3.6
	Par. inv.	B3b-B3o	0.5	3.6
	Par. inv.	B3o-B4d	0.8	7.1
	Par. inv.	C1a-C3b	0.8	7.1
	Par. inv.	C2-C4f	2.4	17.8
G				
	Deletion	1de + BRc	8.2	35.7
	Deletion	BRb+BRc	3.7	21.4
	Par. inv.	C-D	1.1	10.7
	Deficiency	A1a	0.5	7.1

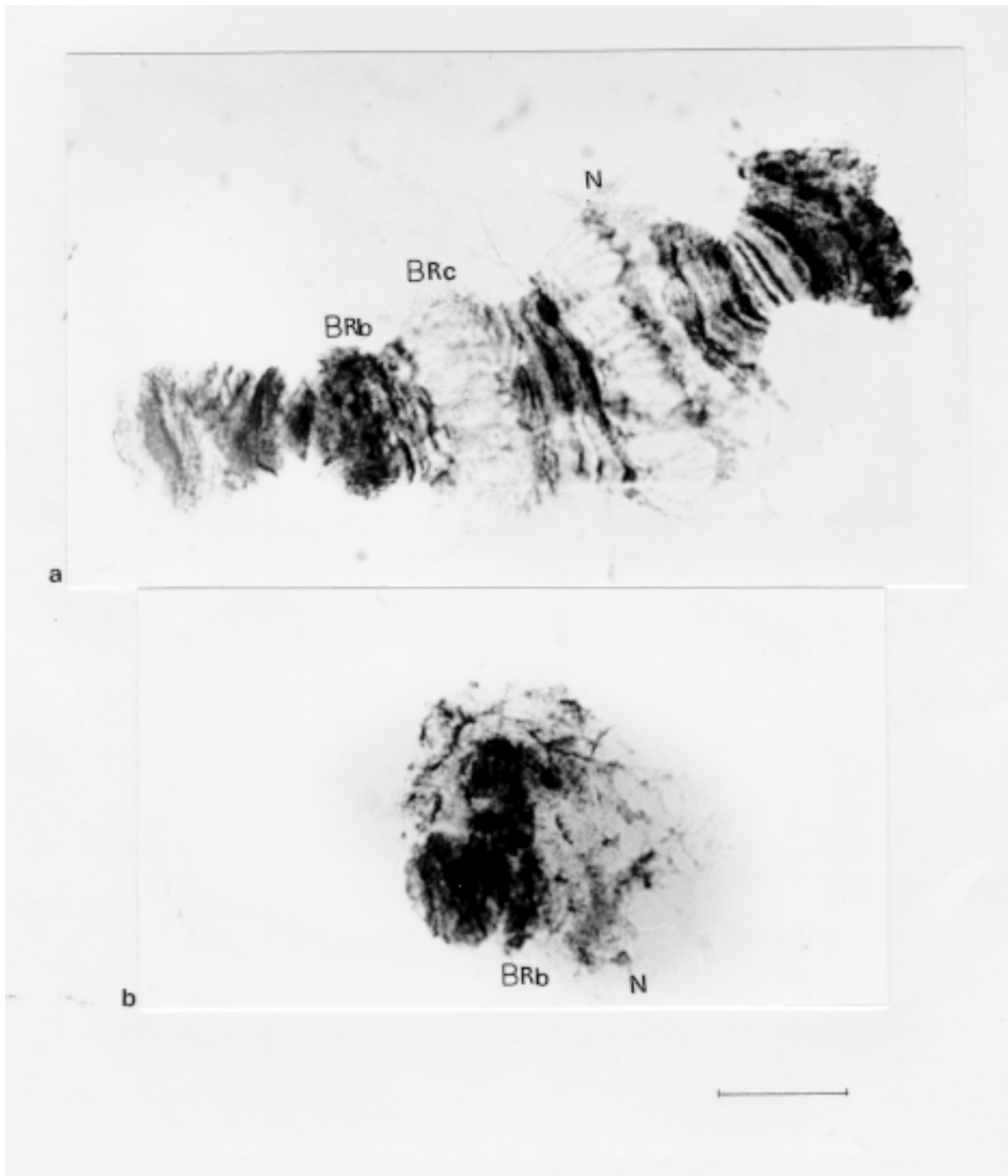


Fig. 1. Chromosome G
a. normal;
b. pompon (according to Michailova et al., 1998) ;
N- nucleolus; BR - Balbiani ring;

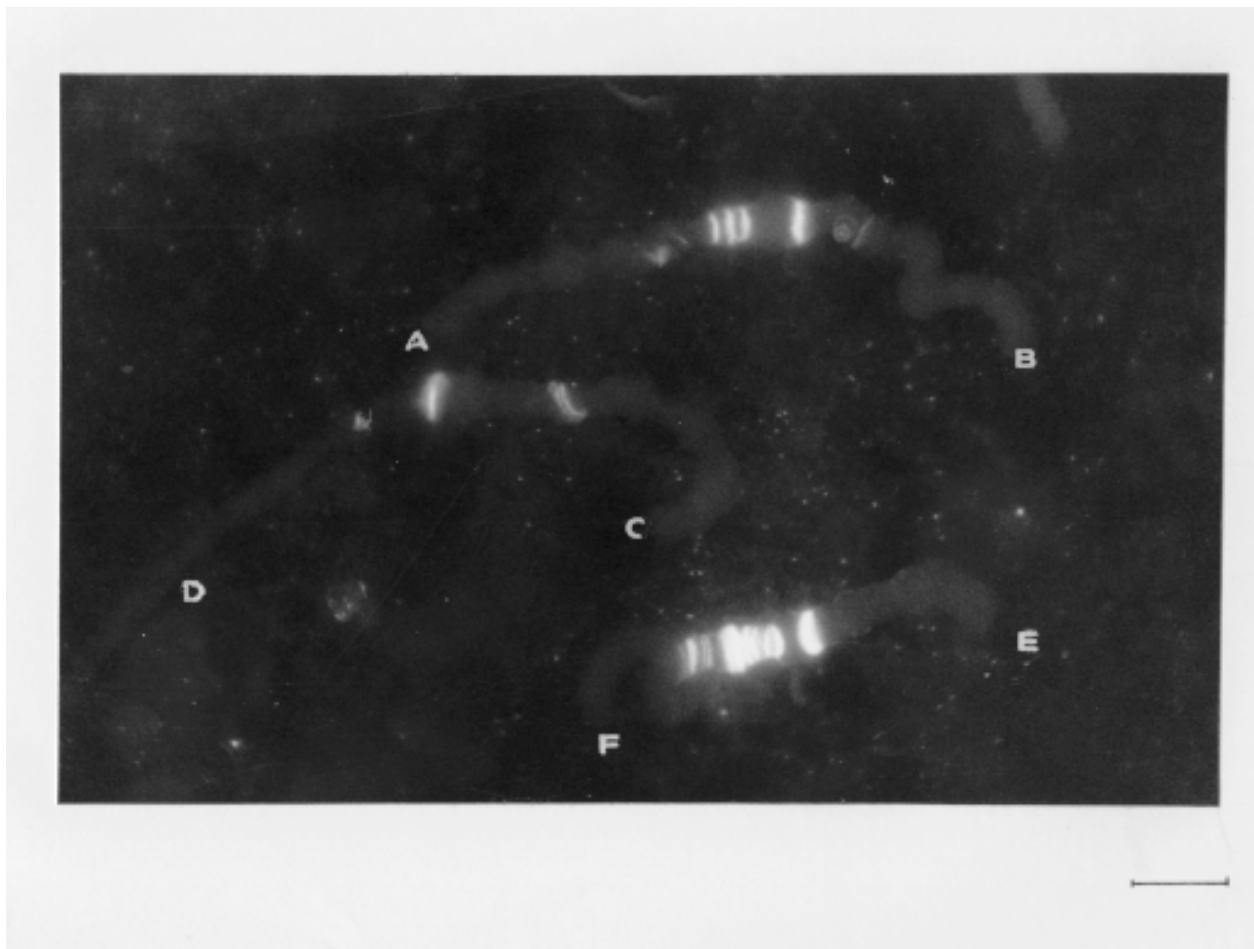


Fig. 2 . The distribution of Alu clustres along the polytene chromosomes: AB, CD, EF;



Fig. 3 . The distribution of Hinf clusters along some parts of chromosome arms: A, C, E, F;