

Autosomal, sex and B chromosomes in *Eyprepocnemis plorans* (Orthoptera) viewed with restriction endonuclease *in situ* digestion

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Similarities and differences between different subsets of heterochromatin localized on the autosomes, sex chromosomes and a B-chromosome in the genome of the grasshopper *Eyprepocnemis plorans* were analysed by *in-situ* digestion with 23 different restriction endonucleases, three un-specific nucleases, C-banding and specific fluorescent DNA-ligands. Results show an equilocal distribution of the different heterochromatic subsets in this species and provide a possible evolutionary origin for the B chromosome.

Keywords: B-chromosomes, heterochromatin, insects cytogenetics, restriction endonucleases.

Introduction

B-chromosomes are a special class of chromatin in many species of animals and plants which show peculiar characteristic features. For example, (i) they are supernumerary to the main chromosome complement, (ii) they are often transmitted in a non-Mendelian fashion and (iii) they can affect the genetics of a species through their effects on meiotic recombination (see Jones & Rees, 1982 for review). To date, however, the molecular mechanism(s) through which the B-chromosomes act on the individuals carrying them, as well as their organization in terms of DNA-protein structure, are not known. In particular, the origin of B-chromosomes, and their relationship with other components of the genome, is a difficult problem. In this connection, Hewitt (1979) stated that 'the relationship of B-chromosomes to the regular complement will be determined through molecular based studies such as nucleic acid hybridization and specific chromosome banding procedures'. Unfortunately, more than 10 years later the nature, function and origin of such 'illegal members of inheritance' still remain largely unknown, despite many different investigations.

The discovery of banding techniques allowed the differentiation of metaphase chromosomes into visible areas that correspond to specific chromosome domains. Different types of chromatin could thus be

recognized depending on their reaction to a number of chemical, physical or enzymatic treatments (i.e. G-, R-, C-bands). Moreover, the improvement of such techniques permitted new detailed analyses and differentiation into discrete subclasses and blocks of chromatin. For instance, a positive homogeneous reaction to C-banding, usually identified as constitutive heterochromatin (Sumner, 1972), is now known not to represent the same type of material in a given organism (John *et al.*, 1985; Gosálvez *et al.*, 1987; López-Fernández *et al.*, 1989). In this study we used a number of techniques including *in-situ* digestion with restriction endonucleases, to analyse the different types of chromatin present in the genome of the grasshopper *Eyprepocnemis plorans*. This species was selected in order to study the origin of an extra B-chromosome as well as its relationship with the different classes of chromatin in its karyotype. The results obtained lead us to hypothesize that the origin of this particular B-chromosome might involve genomic material which was part of an autosome. This material seems to have undergone rearrangements and followed an evolutionary pathway different from that of the autosomes.

Materials and methods

Males of *Eyprepocnemis plorans* collected in Sardinia (Italy) were used in this study. Testes were fixed in 3:1

ethanol:acetic acid and some follicles were squashed in a drop of 45 per cent acetic acid. Coverslips were removed after immersing the preparations in liquid nitrogen. Fluorescence analysis was carried out using Chromomycin A3 (CMA3), Dystamycin (DA) and 4'-6 diamidine-phenyl indole (DAPI) according to the method developed by Schweizer (1976; 1980).

Twenty-three restriction endonucleases (REs) (see Table 1) were used to digest fresh air-dried preparations. The slides were incubated with 100 μ l of incubation buffer plus 30 units of each enzyme. All REs, except Taq I, were incubated overnight at 37°C. Incubation with Taq I was at 65°C overnight. The same times and temperatures were employed in control experiments using only the incubation buffer. Cytological preparations were stained with Giemsa 2 per cent in phosphate buffer (pH 6.8). C-banding was used for constitutive heterochromatin localization.

Table 1 The effect of different REs on the autosomes, X and B- chromosomes in *E. plorans*. Item 'type' groups similar bands obtained with different REs

Type	Enzyme	Restriction site	Autosome	X	B
I	Mbo I	GATC	+	+	+
	Msp I	C CGG	+	+	+
	Dde I	C TNAG	+	+	+
	Scr FI	CC NGG	+	+	+
	Sau 96A	G GNCC	+	+	+
	Taq I	T CGA	+	+	+
II	Sau 3A	GATC	+	-	-
	Hpa II	C CGG	+	-	-
	Bam HI	C GATCC	+	-	-
	Rsa I	GT AC	+	-	-
	Dpn I	GA TC	+	-	-
	Hind III	A AGCTT	+	-	-
	Hinf I	G ANTC	+	-	-
III	Hae III	GG CC	+	-	+
	Hin pI	GC GC	+	-	+
	Pvu II	CAG CTG	+	-	+
	Alu I	AG CT	+	-	+
	Pst NI	CTGCA G	+	-	+
IV	Hha I	GCG C	-	+	+
V	Bst NI	CC GG	-	-	+
VI	Ava II	G GWCC	-	-	-
	Dra I	TTT AAA	-	-	-
	Eco RI	G AATTC	-	-	-

(+) Presence of chromosome differentiation.

(-) Absence of chromosome differentiation.

Results

The chromosome complement

Eyprepocnemis plorans is an orthopteran species with 23 acrocentric chromosomes in males (XO) and 24 in females (XX). C-banding shows heterochromatic blocks in centromeric regions of all chromosomes (Fig. 1a). Populations of this species from the South-East of Spain show a complex polymorphism of B chromosomes (Henriques-Gil *et al.*, 1982). The population of *Eyprepocnemis plorans* that we analysed had only one type of acrocentric B-chromosome which was homogeneously and deeply stained after C-banding. In only a few cases was a little differentiation between centromeric heterochromatin and euchromatin observed but this was not as marked as that obtained between autosomes or the X chromosome (Fig. 1a). B is stable in the germ line but its number may vary from one individual to another.

CMA3, a fluorescent DNA ligand which preferentially binds to GC-rich DNA (Ward *et al.*, 1965; Berman *et al.*, 1985), shows intense fluorescence in the centromeres of all chromosomes. However, the B-chromosome fluoresces homogeneously and more brightly than the centromeric blocks (Fig. 1b). Not all the heterochromatin in the centromeric blocks of the autosomes show CMA3 fluorescence. Those regions close to the end of the chromosomes, in close relationship with the kinetochores, are brighter. DAPI, a fluorescent DNA ligand which preferentially binds to AT-rich DNA (Kapusinski & Skoczylas, 1978), produces relatively uniform positive fluorescence in all chromosomes, including the B (Fig. 1c). The level of fluorescence in this B is lower than that obtained in the autosomes.

Restriction endonucleases

Results obtained with REs are described first with reference to their ability to produce linear differentiation in the autosomes, sex chromosomes and B-chromosomes. Using this criterion, results can be grouped into six classes (Table 1).

1 REs producing linear longitudinal differentiation in all chromosomes (Fig. 2a).

2 REs producing longitudinal differentiation in only the autosomes (Fig. 2b).

3 REs producing longitudinal differentiation in autosomes and B-chromosome but not affecting the X-chromosome (Fig. 2c).

4 REs producing longitudinal differentiation in the B and the X chromosome (Fig. 2e).

5 REs only producing longitudinal differentiation in the B chromosome (Fig. 2d).

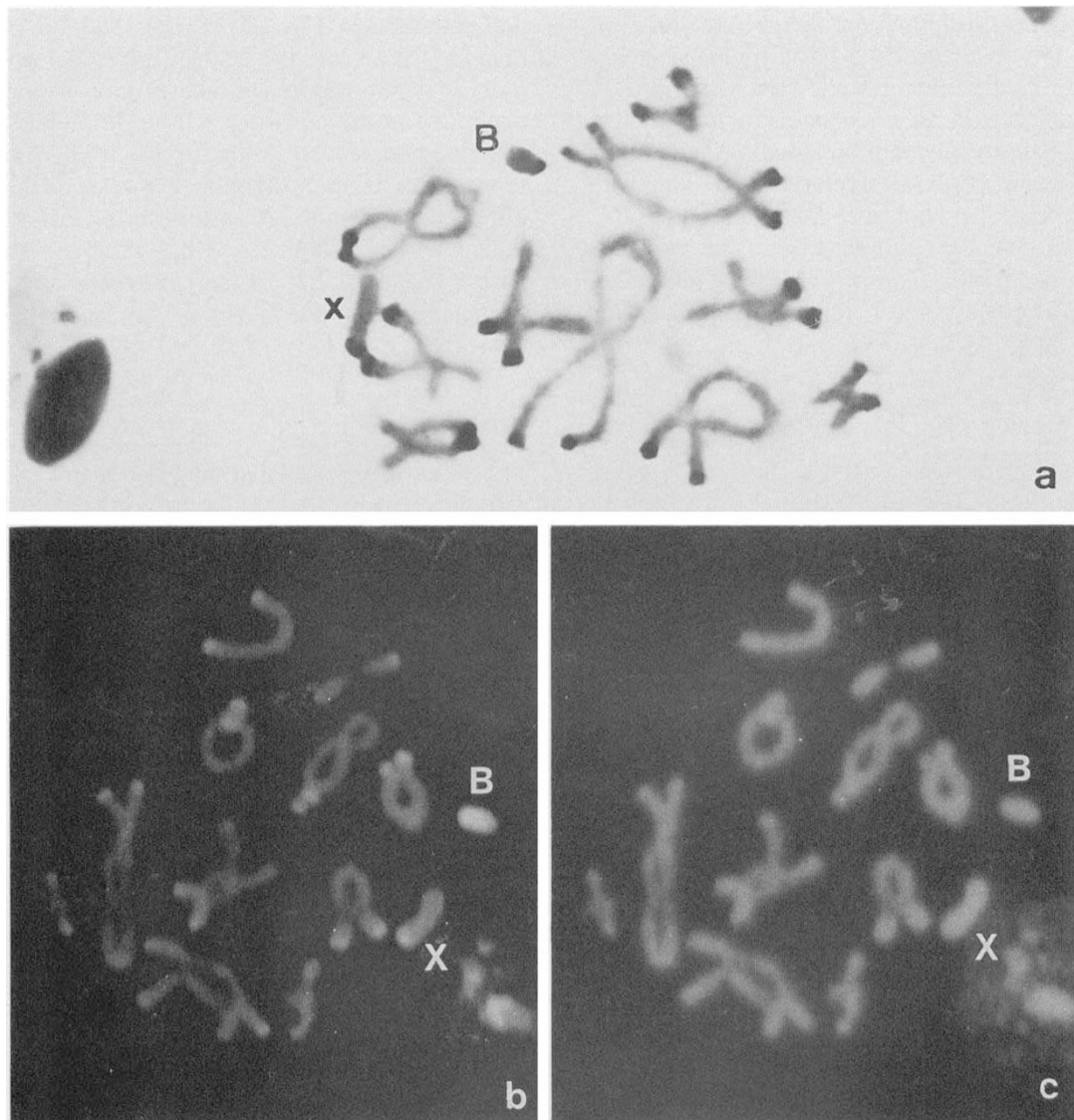


Fig. 1 Constitutive heterochromatin distribution in *E. plorans* with a B-chromosome. (a) C-banded diplotene. (b and c) Same diplotene stained with CMA3 (b) and DAPI (c).

6 REs not producing linear differentiation in any chromosome.

The longitudinal differentiation produced in each case is enzyme-dependent, i.e. the bands produced by a given RE are not necessarily identical in size, chromosomal localization or staining characteristics to those produced by a different RE. Thus, for example, REs such as Mbo I or ScrF1 give rise to longitudinal differentiation in all chromosomes but the banding pattern obtained in each chromosome is different (compare Fig. 2a with f). In order to give details on the above-listed points we describe the results obtained with different REs on autosomes, sex and B-chromosomes.

Banding patterns produced on the autosomes

REs capable of producing clear-cut differentiation in autosomes can be subdivided into two categories: (i) those inducing a C-like banding pattern (MboI, MspI, DdeI, BamHI, RsaI, HaeIII, AluI, HinfI) (Fig. 2a; 3a); and (ii) those removing part of the constitutive heterochromatin (diminished C-like bands), giving rise to small positively stained dots in each centromere region (HinfI, ScrF1, Sau 96A, TaqI) (Fig. 2f; 3b). The heterochromatic regions from which material was removed stained less than undigested heterochromatin but more than euchromatin (Fig. 3b). This pattern was produced

on all autosomes and also the X chromosome by certain REs. We thus refer to a basic model chromosome as being divided into three different regions: (i) heterochromatic region h1, not cleaved by REs such as *Hin* pI, *Scr*FI, *Sau* 96A or *Taq*I and localized at the end of the chromosome, probably in close relationship with the kinetochores, (ii) heterochromatic region h2, cleaved by the same REs and adjacent to the previous one. The chromatin block composed of both h1 and h2 accounts for all constitutive heterochromatin of each chromosome, and (iii) euchromatin, E (Fig. 3c).

Banding pattern produced on X chromosome

Fewer REs produced bands on the X chromosome (see Table 1). All these REs also produced a clear-cut differentiation on the autosomes, and the banding pattern obtained in this chromosome was similar to that produced on the autosomes. *Mbo*I, *Msp*I and *Dde*I produced C-like banding in sex chromosomes (Fig. 3d)

and autosomes (Fig. 2a). *Scr*FI, *Sau* 96A and *Taq*I digested part of the constitutive heterochromatic blocks (region h2) in a similar fashion to the digestion produced in the autosomes (Fig. 3e), while region h1 remained uncleaved. An exception to this banding pattern comes from *Hha*I, which produced longitudinal differentiation on the X and B but did not differentiate the autosomes (Fig. 2e). *Hha*I removed the heterochromatic block h2 on the centromere while leaving the euchromatic chromosome arm and the h1 region uncleaved (Fig. 3f).

Banding pattern produced on the B-chromosome

The heterogeneity of banding produced by digestion with different REs was more pronounced on the B than the X chromosome or autosomes. The results are classified as in Table 2 into (i) REs capable of revealing all centromeric heterochromatin (Fig. 4a) and (ii) REs capable of producing interstitial bands undetected by

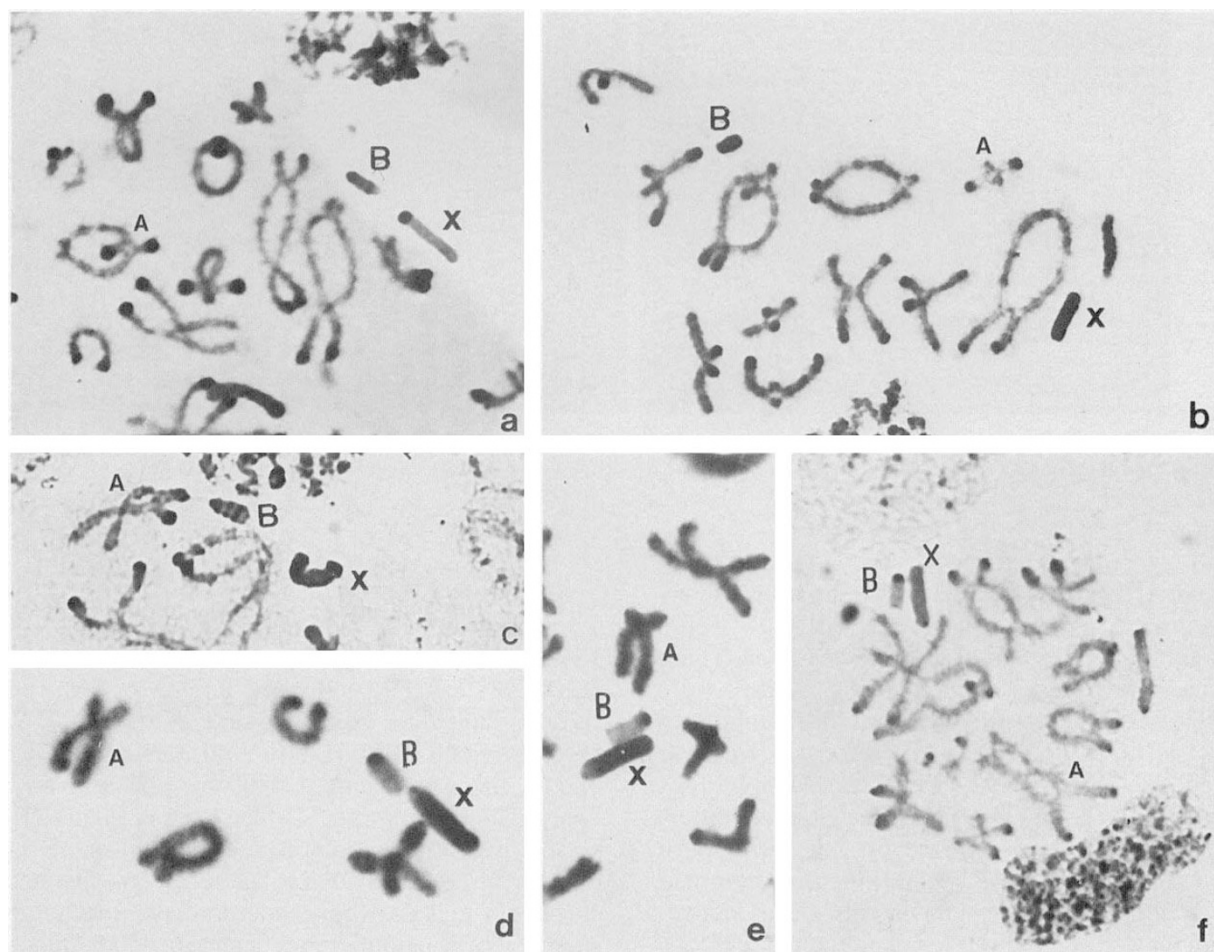


Fig. 2 Restriction nuclease banding in diplotene cells from individuals containing one B-chromosome. (a) *Mbo* I, (b) *Bamb* H1, (c) *Alu* I, (d) *Bst* NI, (e) *Hha* I, (f) *Scr* FI.

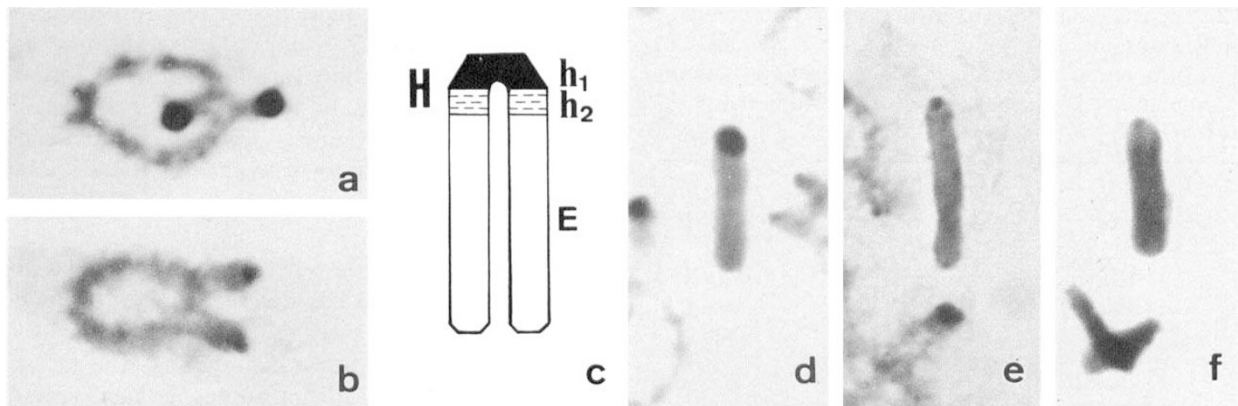


Fig. 3 Heterochromatin heterogeneity in *E. plorans*. Selected diplotene bivalents digested with Mbo I (a) and Scr FI (b). (c) A diagrammatic representation showing the distribution of different subsets of chromatin according to banding characteristics. (d-f) Selected X-chromosomes showing differential digestibility of chromatin: Mbo I (d), Scr FI (e) and Hha I (f).

other banding methods (Fig. 4b). In the latter case a centromeric plus two interstitial bands were obtained. The level of differentiation obtained in these interstitial bands varied from one RE to another. Thus, for example, while AluI left both bands uncleaved (Fig. 2c), MboI produced more intense digestion of the interstitial band near the centromere (Fig. 4b). When the B-chromosome was digested with MspI additional differences in these interstitial bands were detected depending on the incubation time. Short digestion (2 h) with this enzyme produced the two interstitial bands mentioned above (Fig. 4c). At increasing digestion times (7–8 h), the band closest to the centromere tended to lose stainability (Fig. 4d). Longer digestions (overnight) produced massive extraction of material from both bands and only the centromere remained stained. This effect confirms the existence of the structural differences between these bands observed with REs such as MboI.

Two points should be emphasized: (i) Hin pI, ScrFI, Sau 96A and TaqI, REs which remove part of the constitutive heterochromatin (h_2 region) in autosomes and in the X chromosome, do not digest the centromeric constitutive heterochromatin in the B-chromosome; (ii) additional interstitial bands are produced on the B-chromosome with REs such as MboI, MspI, DdeI, PvuII, AluI, Pst NI, HinfI and HaeIII; most of these REs give rise to paracentromeric bands on the autosomes. This fact could be of importance in understanding the origin of this extra chromosome as will be discussed later.

Digestion with non-specific nucleases

Incubation of chromosomes with S1 nuclease produced a pronounced C-like banding pattern in auto-

somes and in the X chromosome. In the B-chromosome, it was possible to observe a centromeric band as well as the two additional interstitial bands that were also revealed by some REs (Fig. 4e). In all cases, S1 digestion produced a dramatic loss of euchromatin in the chromosome arms and gave rise to high contrast between eu- and heterochromatin after staining (Fig. 4f).

Bal 31 and ExoIII do not induce any different staining in any chromosome; they do not remove DNA differentially from eu- and heterochromatin.

Discussion

In purified DNA REs act by cleaving specific base sequence targets and they also show the capability to act on the DNA of fixed chromosomes (Mezzanotte *et al.*, 1983; Miller *et al.*, 1983). The extraction of DNA from fixed chromosomes by REs can be affected by a series of factors such as fixation of the material (Burkholder, 1989), cleavage frequency (Miller *et al.*, 1983; 1984; Bianchi *et al.*, 1985; Mezzanotte, 1986) or structure of the RE and chromatin (Gosálvez *et al.*, 1989). Even so we believe that under homogeneous experimental conditions REs may be used to study processes that determine pathways of evolution of a given karyotype through the sensitivity of each chromosome region to a given RE. In this context, the results obtained in the present experiment, summarized in Table 2, indicate the similarities and differences that underlie the genome of this species. Thus, while the action of each RE on the autosomes produces a pattern that is both homologous and conservative, differences exist among autosomes, sex chromosome and Bs. This clearly points to a different evolution pattern for each type of chromosome.

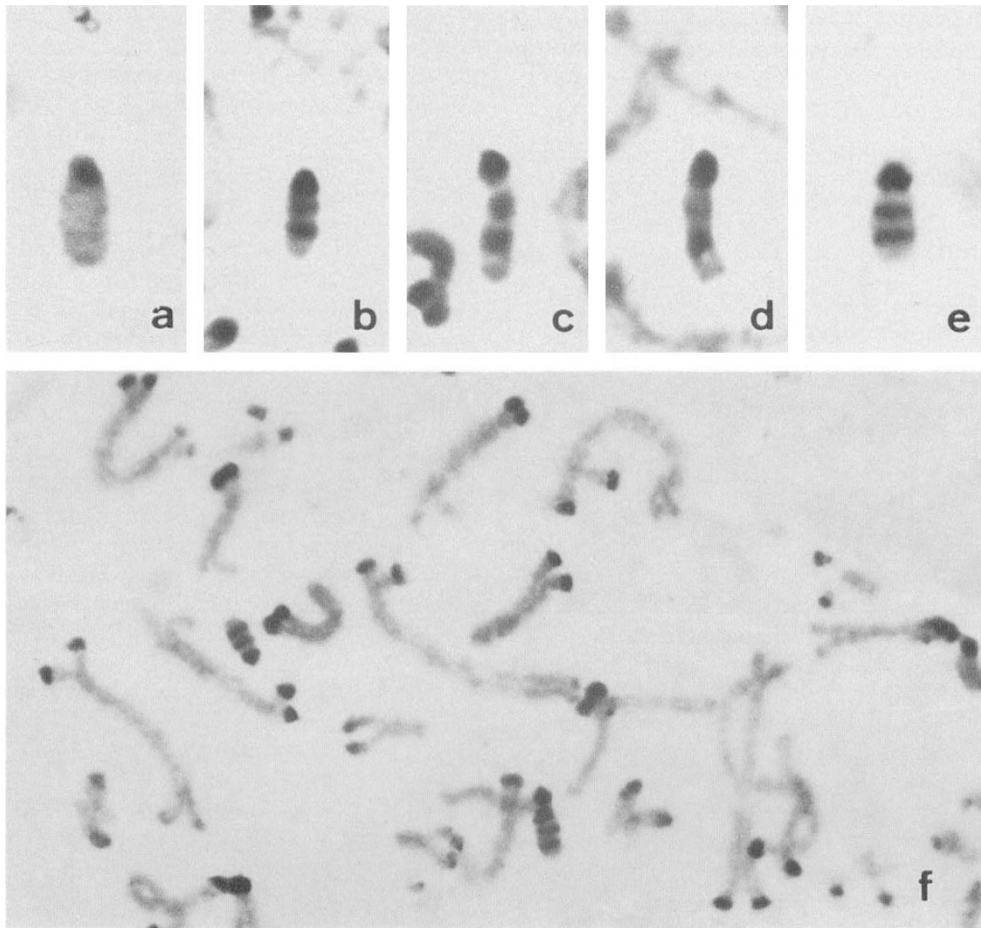


Fig. 4 Selected B-chromosomes in *E. plorans* after digestion with Bst NI (a), Mbo I (b), Msp I after 2 h digestion (c) or overnight (d) and S1 nuclease (e). (f) Early diplotene digested with S1.

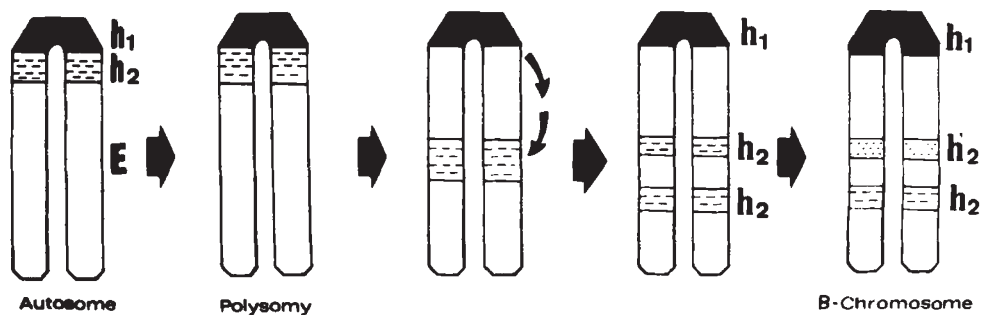


Fig. 5 Diagrammatic representation of the possible origin of the B-chromosome in *E. plorans* from a centric autosome fraction involving different chromosome rearrangements.

larity between specific regions, i.e. constitutive heterochromatin of autosomes and B-chromosome (see information summarized in Table 2). This leads us to hypothesize that the B-chromosome is derived through a series of events from an autosome. In particular, we postulate that the following steps have occurred: (i) production of polysomy; (ii) chromosome rearrange-

ment (inversion?) involving heterochromatic region h2 and part of eu-chromatic (E) and (iii) amplification of the proximal h1 region and reorganization of region h2 to give rise to an incipient differentiation between bands h2 and h'2 (Fig. 5).

In conclusion, our data indicate that evolutionary processes in the *E. plorans* karyotype have produced

some degree of heterogeneity within different fractions of chromatin, as in other cases studied. They also allow us to postulate that the material that forms this B-chromosome possibly arises from a number of evolutive steps involving amplification and reorganization of autosomal material.

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