Equilocality and heterogeneity of constitutive heterochromatin: *in situ* localization of two families of highly repetitive DNA in *Dociostaurus genei* (Orthoptera)

E. RODRÍGUEZ IÑIGO, B. FERNÁNDEZ-CALVÍN†, J. CAPEL‡ & C. GARCÍA DE LA VEGA*

Unidad de Genética, Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, †Unidad de Genética, Departamento de Biotecnología, E.T.S.I. Agrónomos, Universidad Politécnica de Madrid, 28040 Madrid and ‡Laboratorio de Biología Molecular y Virología Vegetal, C.I.T.-INIA, 28040 Madrid, Spain

The chromosome complement of the grasshopper *Dociostaurus genei* is characterized by the presence of constitutive heterochromatin (C-bands) located in the centromeric regions of all the chromosomes and in the distal regions of some autosomes in the form of supernumerary segments. A sequence analysis was carried out to obtain information about the molecular characteristics of both heterochromatic regions. Two families of tandemly repetitive DNA (DgT2 and DgA3) from *D. genei* were cloned and characterized. Data obtained from *in situ* hybridization indicate that these families are located solely in the regions of constitutive heterochromatin. The DgT2 clone is representative of a family of sequences which mainly forms the centromeric C-bands in each chromosome of the complement. The DgA3 family is the major component of the distal C-bands (supernumerary segments) present in most of the autosomal pairs. These results show the existence in *D. genei* of two different families of repetitive DNA restricted to different chromosomal domains. We discuss these results in the light of the possible role of chromosomal disposition in the maintenance of the differences between heterochromatic DNA from different chromosomal regions and the homogenization of DNA sequences from equilocal chromosomal domains.

Keywords: constitutive heterochromatin, insect cytogenetics, satellite DNA.

Introduction

Eukaryotic genomes are characterized by the variable proportions of highly repetitive DNA families that they contain. These usually correspond to regions of constitutive heterochromatin that are revealed by C-banding (John, 1988). Other banding methods prove that these sequences are homogenized when they occupy identical chromosome distribution within a given complement (equilocality) and are different in distinct chromosome domains (heterogeneity) (King & John, 1980; Greilhuber & Loidl, 1983; Schweizer *et al.*, 1983; Bella *et al.*, 1986). Acridoid grasshoppers are a well-known example where constitutive heterochromatin is present in the

form of prominent centromeric or distal blocks (Hewitt, 1979). However, much less is known about the molecular organization of these regions compared with other plant and animal species. There are a few examples where *in situ* hybridization has enabled the localization of particular families of highly repetitive DNA sequences in grasshoppers (Brown & Wilmore, 1974; Arnold *et al.*, 1986; John *et al.*, 1986; López-León *et al.*, 1994).

Dociostaurus genei is a grasshopper which displays a complex polymorphism of distal supernumerary segments (Rodríguez Iñigo *et al.*, 1993) besides the characteristic pericentromeric C-bands present in every chromosome. We have shown that both kinds of heterochromatin differ not only in location but also in composition. Although both are AT rich, as revealed by DAPI staining, the centromeric hetero-

*Correspondence.

chromatin is extensively *in situ*-digested by the endo-nucleases *Sau*3AI and *Taq*I, whereas the distal segments are mostly sensitive to *Alu*I (Rodríguez Iñigo *et al.*, 1993). These data prompted us to analyse the structure and the genome organization of the sequences included in both kinds of constitutive heterochromatin. The results also allow us to consider the role of chromosome disposition in the promotion of the homogeneity of sequences located in equilocal regions and the heterogeneity of sequences located in opposite chromosome domains.

Material and methods

Adult males and females of the grasshopper *D. genei* were collected from natural populations in the Central region of Spain. The chromosome complement is composed of 11 acrotelocentric autosome pairs and a single acrotelocentric sexual (X) chromosome in males. The sex chromosome system is of the X0 (males), XX (females) type.

Cytological procedures

Mitotic chromosomes were obtained from ovariole tips of females. These were injected intra-abdominally with 0.05 per cent colchicine in saline solution and left for 5 h. Fixation was carried out in 3:1 ethanol:acetic acid. To carry out cytological analysis, squash preparations were made in a drop of acetic acid 45 per cent. The coverslips were removed after freezing in liquid nitrogen and the slides were then air-dried.

C-banding was performed according to López-Fernández & Gosálvez (1981).

Isolation of genomic DNA

To isolate genomic DNA, adult individuals were homogenized in WCLB (10 mM Tris-HCl, 10 mM NaCl, 20 mM EDTA), SDS 2 per cent and protein-ase-K, incubated at 37°C for 16 h and then placed on ice for 1 h after adding NaCl to a final concentration of 2 M. The homogenate was then centrifuged at 2240 *g* for 15 min. The resultant supernatant was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and 1 volume of chloroform-isoamyl alcohol (24:1) and precipitated with 2 volumes of ethanol. The precipitated nucleic acid was finally washed with 70 per cent ethanol, air-dried, dissolved in TE (10 mM Tris/1 mM EDTA) pH 8 at a concentration of 1 μg μL^{-1} and digested with RNAse (0.1 μg μL^{-1}).

DNA blot hybridization

For Southern blotting, 10μg of total DNA was digested with the appropriate enzyme at 2 U μg^{-1} DNA for 2–8 h. The resulting fragments were fractionated on a 1.5 per cent agarose gel in 1 × TAE buffer (Tris-acetate/EDTA) and transferred to Hybond™$^{+}$ nylon filters as recommended by the manufacturer. The DNA was fixed at 80°C for 2 h.

Probes were amplified by PCR under standard conditions (Sambrook *et al.*, 1989) and radiolabelled with ^{32}P-dCTP by random priming (Feinberg & Vogelstein, 1983). Filters were prehybridized for 2 h with 5 × SSPE, 5 × Denhardt's solution, 0.5 per cent SDS and 100 μg mL^{-1} denatured salmon sperm DNA at hybridization temperature (65°C). The radiolabelled denatured probe was added and hybridization performed at 65°C for 16 h. The filters were washed twice in 2 × SSPE, 0.1 per cent SDS; then once in 1 × SSPE, 0.1 per cent SDS; and then once in 0.1 × SSPE, 0.1 per cent SDS. All washes were performed for 15 min at 65 °C. Finally they were exposed to X-ray film at -80°C for 1–25 h.

Cloning of repetitive DNA

For molecular cloning digested total DNA from *D. genei* was fractionated on 1.5 per cent agarose gel in 1 × TAE buffer under standard conditions (Sambrook *et al.*, 1989). The bands of interest were excised and DNA was purified and ligated in the pBluescript SK^{-} vector. The resulting ligations were used to transform competent cells of *E. coli* JM 103 recA^{-}. Recombinant clones were selected using standard protocols (Sambrook *et al.*, 1989). Mini-preparations of DNA from eight of the recombinant colonies from each ligation were made following Sambrook *et al.* (1989).

DNA sequencing

Sequencing was performed by the chain termination method (Sanger *et al.*, 1977) using the Automated Laser Fluorescent A.L.F. DNA sequencer (Pharmacia LKB Biotechnology).

Fluorescence *in situ hybridization (FISH)*

For *in situ* hybridization the probes were amplified by PCR, under standard conditions (Sambrook *et al.*, 1989) and labelled with digoxigenin 11-dUTP by nick translation (Rigby *et al.*, 1977) according to the manufacturer's instructions (Boehringer-Mann-

heim). Slides were pretreated prior to FISH with RNAse and pepsin according to Wiegant *et al.* (1991). *In situ* procedures were carried out following Pendás *et al.* (1993) with minor modifications. The preparations were denatured in 70 per cent formamide in 2 × SSC at 90°C for 5 min and dehydrated in ethanol series. Next 15 μL of hybridization mixture consisting of 50 per cent (v/v) deionised formamide-2 × SSC, 10 per cent (w/v) dextran sulphate, 50 mM phosphate buffer, 100 μg mL^{-1} of salmon sperm DNA, yeast t-RNA and 10 ng μL^{-1} of denatured labelled probe were placed on each slide, sealed with rubber solution and chromosomes and probe were denatured again together at 90°C for 10 min. Slides were incubated at 37°C for 16 h. After hybridization, slides were washed and the digoxigenin-labelled probes detected with fluorescein isothiocyanate conjugate (FITC). Depending on the degree of hybridization we used one (antidigo-FITC), two (antidigo mouse: antimouse-FITC) or three (antidigo mouse: antimouse digo: antidigo-FITC) antibodies. Chromosomes were counterstained with propidium iodide.

Results

Sequence analysis

After digesting the genomic DNA of *D. genei* with *Alu*I, or *Taq*I endonucleases, monomeric bands of about 190 bp were purified from 1.5 per cent agarose gels stained with ethidium bromide. These DNA bands were ligated, respectively, to the *Sma*I and *Cla*I sites of the pBluescript SK⁻ vector. Recombinant clones were screened again to test the presence of repetitive DNA by hybridization with undigested DNA of *D. genei*. Clones with stronger signals (data not shown) were selected for subsequent sequence analysis (Fig. 1). The DgA3 sequence (Fig. 1a), cloned from the *Alu*I band, is 160 nucleotides long and consists of 58.3 per cent AT. The DgT2 clone (from the *Taq*I band) is 217 bp long and contains 59.2 per cent AT (Fig. 1b). No significant internal repeats have been observed in either sequence. A computerized comparison revealed no similarities between the two sequences. Moreover, none of the sequences showed significant homology with those in the EMBL Data Library.

The genome organization of these two sequences was analysed by DNA blot hybridization with digestions of *D. genei* DNA. These results, which are summarized in Fig. 2, show clear differences between the DgA3 and DgT2 genomic organization. The former shows the typical ladder pattern in the

digestions of some enzymes which is indicative of a tandemly arranged repetitive DNA family (Fig. 2a). The results are in accordance with the restriction map obtained for the DgA3 sequence (Fig. 1a) such that *Alu*I, *Pvu*II (its target contains *Alu*I's target) and *Sau*3AI, which have one recognition site in the cloned element, cleave within almost all the repeats. This results in a monomeric unit of approximately 204 bp and the respective dimers and trimers. Digestions of DNA with enzymes that lacked cleavage sites in DgA3 yield instead two distinct patterns of hybridization: in treatments with *Eco*RI and *Dra*I, the majority of homologous DNA remains undigested. On the other hand, *Taq*I digestion displays a series of bands ranging from about 670 bp to undigested DNA.

DNA blot hybridization using DgT2 as a probe (Fig. 2b) shows a pattern of fragments that are in agreement with the restriction map obtained from the DgT2 sequence (Fig. 1b). Despite the fact that DgT2 lacks the *Dra*I target, other clones obtained from DNA digestions with *Taq*I do contain it (data not shown). This would explain the band obtained when the DgT2 clone is hybridized with *Dra*I digestions. Furthermore, DgT2 shows doublets in the *Taq*I lane separated by about 50 bp and is thus in accordance with its restriction map (Fig. 1b). Treatments with REs that lacked cleavage sites in pDg T2 (*Eco*RI, *Alu*I, *Pvu*II) yielded hybridization signals in regions of high molecular weight. However, *Sau*3AI digests show an intense hybridization band (289 bp) despite not possessing the *Sau*3AI target.

The differences found in nucleotide sequence and genomic organization between the two clones and their possible relation with different heterochromatic regions was tested by *in situ* hybridization.

In situ *hybridization (FISH)*

Probes from the DgA3 and DgT2 clones were labelled and hybridized to mitotic chromosomes of *D. genei*. The DgT2 sequence is localized in the centromere region of each chromosome where constitutive heterochromatin is sited (Fig. 3b). The size of the hybridization fits well with the size of the corresponding C-bands and is similar in size among the distinct members of the complement (compare Figs 3a and b). These results suggest that this sequence is a representative of the family which mainly forms those regions of constitutive heterochromatin.

The DgA3, in turn, hybridizes with the distal regions of some autosomes (Fig. 3c). In this case there are visible differences in the size of the hybrid-

(a)

*Alu*I   *Sau*3A
—————   ————
AGCTGCCTGTATTCAGAACAGCAAGTGGATCACTGTGCATACCTCTGGAA

ACGCAATTTGACGTGAAATATCAATTATAATTGTAGTTCCTCGTCCATAC

AACGCAGTTTATGGTGCTTTTTACACGGCTGGGCCAAATATGCAGAAAAT

      *Alu*I
      ————
TCAAGCAGCT

(b)

*Taq*I
————
TCGAAATGCAAATAACGGCTTAACCGTTCATACGAGACGAAAATAGATTA

CCCAGACTTGTAGAGGAGAAAGTTCTCTACAACTTTGCTCTCTTATGTTT

TTTCGGAATCTCGCACCGTTTCAGAGATATTTGTAAAAATATCGGAGATT

       *Taq*I
       ————
TGACGACGCGTGTCTGTCGATAGACAATCCGGTTTCGGAAACACTACGTT

     *Taq*I
     ————
CCAATGTGCAGTTTCGA

Fig. 1 Nucleotide sequences of two clones obtained from (a) *Alu*I and (b) *Taq*I digestions of the genomic DNA of *Dociostaurus genei*. Relevant targets of restriction endonucleases are marked. EMBL accession numbers: DgA3: X83723; DgT2: X83724.
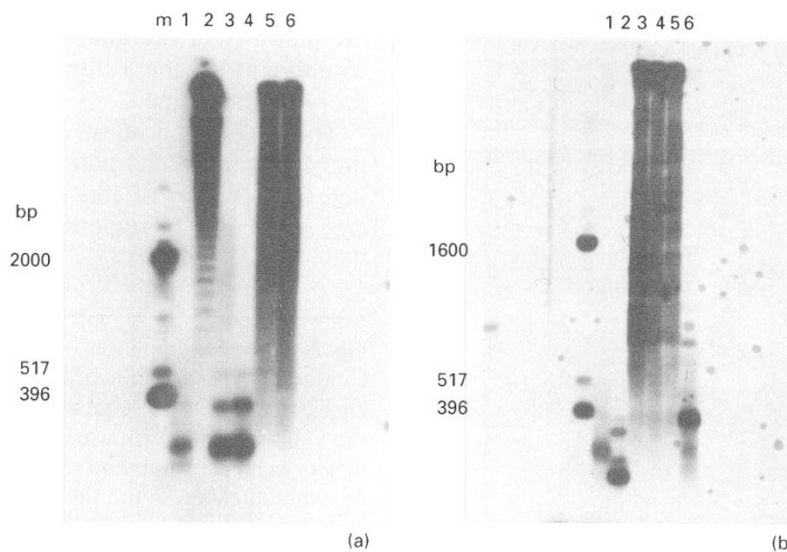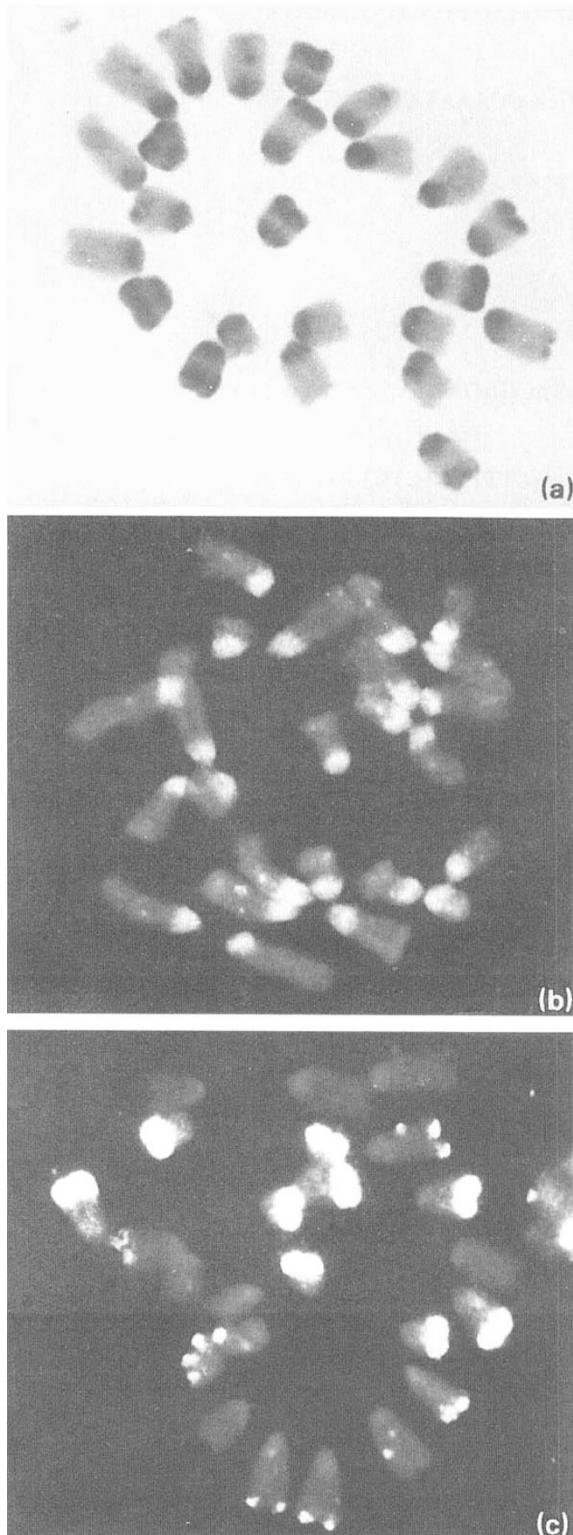

Fig. 2 Southern hybridization of probes from (a) DgA3 and (b) DgT2 clones. Marker (m); *Sau*3AI (1); *Taq*I (2); *Alu*I (3); *Pvu*II (4); *Eco*RI (5); *Dra*I (6). Note in (a) the typical hybridization pattern of monomers, dimers and trimers with *Alu*I (3) and *Pvu*II (4) digestions and in (b) the doublet hybridization bands obtained with *Taq*I (2) digestion.

Fig. 3 Mitotic metaphases of *Dociostaurus genei* after (a) C-banding, and *in situ* hybridization of probes from (b) DgT2 and (c) DgA3 sequences. The comparison between the C-banding pattern, which includes centromeric bands in every chromosome and distal supernumerary segments in some autosomes, and the localization of each hybridization reveals the correspondence of a particular sequence with a distinct heterochromatic chromosome domain. The DgT2 probe hybridizes with the centromere regions whereas the DgA3 probe is located exclusively in the distal regions of some autosomes.
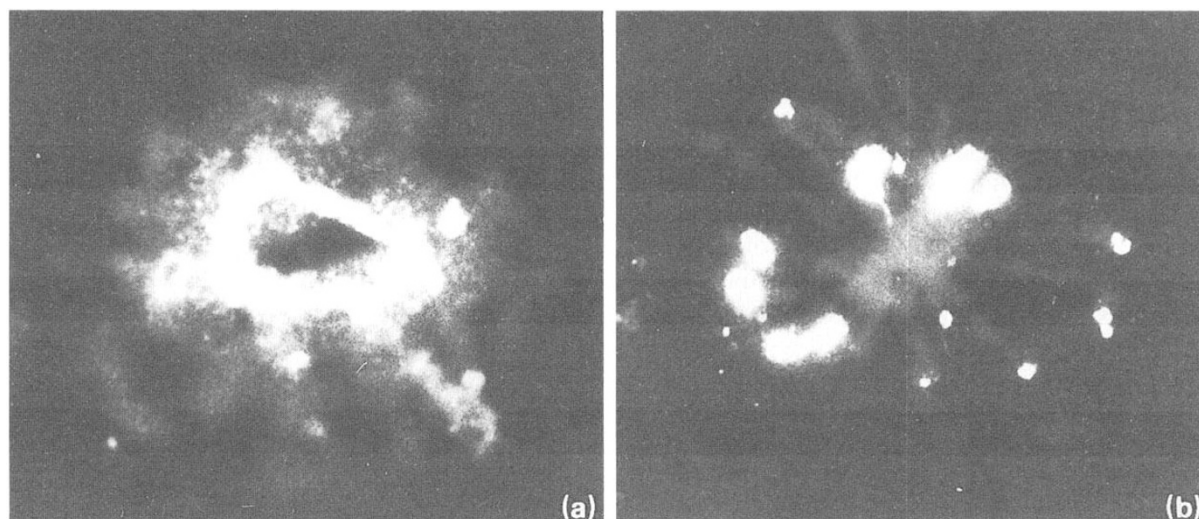
ization signals depending on the chromosome. These observations agree with the differences in size which characterize the distal supernumerary heterochromatic segments present in the majority of the autosomes of this species. The small chromosomes which commonly have large segments show prominent hybridization signals, while medium-sized chromosomes which have smaller segments display smaller signals (compare Figs 3a and c). Moreover, this correspondence also affects those cases where the segment is present in only one of the homologues. Interestingly, hybridization of this probe in the distal tips of some of the longer autosomes is also visible although these chromosomes do not show prominent C-bands in that region. These results clearly indicate that the DgA3 probe is a representative of the family of the highly repetitive DNA family which is the major component of the supernumerary segments.

Moreover, the analysis of the hybridization in telophase nuclei also shows the different location of both sequences and the Rabl polarization of the chromosomes (Fig. 4).

Discussion

The sequence analysis of representatives of repeated DNA families in *D. genei* has allowed the characterization of two of them. The *in situ* localization of probes from these sequences has confirmed that they must represent distinct families. Particularly, they occupy two distinct chromosome domains of constitutive heterochromatin: DgA3 is the major constituent of the distal supernumerary segments of the majority of the autosomes, and DgT2 is a major component of the centromeric heterochromatin of every chromosome.

These results confirm previous analyses based on the cytogenetic characterization of the constitutive heterochromatin of this species (Rodríguez Iñigo *et al.*, 1993). Both regions show bright fluorescence

Fig. 4 Mitotic telophase nuclei of *Dociostaurus genei* after *in situ* hybridization of (a) DgT2 and (b) pDg A3 probes. Note the Rabl polarization in both cases.

with DAPI staining which indicates AT richness. This correlates with the percentages of AT of both sequences (58.3 per cent in DgA3 and 59.2 per cent in DgT2). Moreover, the endonucleases which intensively digest a particular kind of constitutive heterochromatin region in fixed chromosomes yield restriction patterns of repetitive sequences in gels. Thus, the chromosome regions occupied by the centromeric heterochromatin and the supernumerary segments are digested by *Taq*I and *Alu*I endonucleases, respectively (Rodríguez Iñigo *et al.*, 1993), and the sequences (DgT2 and DgA3) come from prominent bands of DNA digestions with these same enzymes (Fig. 1).

These sequences are sequestered in two distinct and compartmentalized chromosome domains of constitutive heterochromatin. This chromosome location concords with two properties defined for the heterochromatin in other organisms: heterogeneity and equilocality (John, 1988).

Thus, a particular chromosome domain (centromere region and distal supernumerary segments in the case of *D. genei*) shows homogeneous composition among the members of the chromosome complement. On the other hand, the composition of both kinds of heterochromatic regions is clearly different. Moreover, as they represent two distinct families of repetitive DNA, the origin and evolution of these sequences agree with models based on the spatial organization of chromosomes in the nuclei (Schweizer & Loidl, 1987; Schweizer *et al.*, 1987). The Rabl polarization which predicts the colocalization of the centromeres throughout the mitotic cycle

is a powerful model by which the heterochromatin transfer between homologous and nonhomologous chromosomes may occur (see Fig. 4). In the same way, the proximity of heterochromatic DNA in the early meiosis ('bouquet polarization') may provide a cytological basis for the molecular processes which are responsible for the maintenance of satellite DNA sequence homogeneity between equilocal groups of C-bands on both homologous and nonhomologous chromosomes.

The distal regions of acrotelocentric chromosomes would occupy the opposite domain to that of the centromeres within nuclei (Fig. 4). The differences in size of the distal blocks present in most of the autosomes of *D. genei* may also reflect the origin and evolution of this component of the constitutive heterochromatin. Schweizer & Loidl (1987) suggested that the heterochromatin transfer between equilocal regions of a complement should occur between nonhomologues of similar size. In *D. genei* it is reasonable to suppose that this process would have originated in the smaller members of the complement, and then in a sequential fashion the supernumerary material would invade increasingly longer chromosomes. Thus one finds at present that the supernumerary segments are bigger in the shorter chromosomes and decrease in size in longer chromosomes. In fact, *in situ* hybridization shows that the sequence representative of these segments is also present in the longest chromosome pairs where no supernumerary segments or prominent C-bands are detected (compare Figs 3a and c).

Supernumerary heterochromatic segments have

been defined in terms of a portion of heterochromatin which is amplified and that is clearly dispensable as the organism can develop normally in its absence (John, 1983). Thus the supernumerary segments of *D. genei* are a tenable example of sequences that originate and accumulate by an efficient mechanism of amplification. The 'infective process' has reached most of the autosomes and, as supposed for parasitic DNA sequences, seems to elude the selective forces against its incorporation and maintenance.

Acknowledgements

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