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Possible origin of a B chromosome deduced from its DNA composition using double FISH technique

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Double fluorescent *in situ* hybridization (FISH) with two DNA probes (a 180 bp tandemly repeated DNA and ribosomal DNA) was performed in embryo cells of the grasshopper *Eyprepocnemis plorans*. Repetitive DNA was present in most standard chromosomes (excepting 7, 8 and 10) and in the proximal two-thirds of the B chromosome, which was its major location in the complement. Ribosomal DNA was present distally on the B, and in the active nucleolar organizer regions (NORs) of the X, 9, 10 and 11 chromosomes. A small number of rRNA gene clusters was also observed in the pericentromeric regions of chromosomes 1–8. The double FISH technique showed that the B chromosome (B_2 type) is mainly composed of a 180 bp tandem repeat and ribosomal DNA, the minute short arm being the only region that does not hybridize with them. The location and order of the centromere and both the DNA sequences on the B chromosome coincide only with those in the X chromosome, indicating that the B most probably derives from the X.

Key words: B-chromosome, *Eyprepocnemis plorans*, FISH, grasshoppers, *in situ* hybridization, repetitive DNA, ribosomal DNA

Introduction

Many plant and animal species are polymorphic for the presence of additional dispensable chromosomes in some individuals from some populations. They are called supernumerary, accessory, or B chromosomes to distinguish them from the standard (A) chromosomes. The fact that they usually do not recombine with A chromosomes means that they may evolve more independently. Thus, Bs usually show properties very divergent from the As, and so it is difficult to determine their exact origin, though most of them are presumably derived from the A chromosomes.

The DNA composition of B chromosomes has been intensively investigated in recent years in order to explain both their origin and genetic properties. The data hitherto available present a puzzling picture, with each B chromosome showing special features. Most satellite DNA sequences analysed in several *Glossina* species are shared by both A and B chromosomes (Amos & Dover 1981). Maluszynska & Schweizer (1989) have shown by *in situ* hybridization that B chromosomes of *Crepis capillaris* contain ribosomal DNA (rDNA) sequences. Other examples of B chromosomes with rDNA are found in Green's paper (1990). In rye (Sandery *et al.* 1990) and *Brachycome dichromosomatica* (John *et al.* 1991), B-specific DNA sequences have been isolated. In the B chromosome of *Nasonia vitripennis*, both shared and specific DNA sequences have been found (Nur *et al.* 1988, Eickbush *et al.* 1992).

A simple explanation for the origin of shared sequences is that Bs are derived from As. However, B chromosomes harbouring their own specific DNA sequences may have resulted from interspecific hybridization (John *et al.* 1991, Eickbush *et al.* 1992). Of course, other mutational processes may be at work, including transposition and amplification.

The grasshopper *Eyprepocnemis plorans* has a very widespread B chromosome polymorphism along the Mediterranean and Atlantic coasts of Iberia, and one or more B chromosome variants are present in almost all the populations analysed (Camacho *et al.* 1980, Henriques-Gil *et al.* 1984, Henriques-Gil & Arana 1990,

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López-León *et al.* 1992). In this paper we present evidence that B chromosomes in *E. plorans* are composed primarly of two DNA sequences that are also present at a lower level in the A chromosomes.

Material and methods

Embryos and chromosome preparation

Ten-day-old embryos were obtained from several egg pods layed by gravid females of *Eyprepocnemis plorans* collected at Jete and Salobreña (Granada, Spain). Fixation and chromosome preparation were as described in Camacho *et al.* (1991). Preparations showing well-spread metaphase cells were selected by phase-contrast light microscopy. They were then dehydrated in an ethanol series, air-dried and placed in an oven at 37°C overnight.

Isolating and cloning of the repetitive DNA

A set of 15 different restriction enzymes was tried for the production of a repetitive DNA band pattern. Genomic DNA was digested with 10 units of enzyme per μg of DNA overnight. The DNA fragments were resolved electrophoretically in 1.5% agarose in TBE buffer (0.009 M Tris-borate, 0.002 M EDTA) and stained with ethidium bromide. Seven of the 15 enzymes showed repetitive DNA bands, namely *Eco*RI, *Eco*RV, *Taq*I, *Alu*I, *Mbo*I, *Msp*I, and *Dra*I. The restriction endonuclease *Dra*I yielded a prominent 180 bp band that was eluted from the agarose gel slice and purified according to the method described by Koenen (1989). A total of 50 ng of DNA were used in the ligation reaction to the *Sma*I sites of the plasmid PGEM 3Z f (+) (Promega, USA). Competent *Escherichia coli* cells were transformed, and the recombinant clones containing the repetitive DNA fragments of interest were selected by colony colour and digested fragment size.

DNA probes

Two probes were used for the double fluorescent *in situ* hybridization (FISH) technique. 1) pTa71 from wheat (*Triticum aestivum*) is a complete 9 kb rDNA gene unit containing the 5.8S, 18S and 26S genes and the intergenic spacer (Gerlach & Bedbrook 1979) and was kindly provided by R. B. Flavell & M. O'Dell. 2) pEpD15 is a 180 bp *Dra*I fragment of repetitive DNA from *E. plorans* (see above). Both pTa71 and pEpD15 were labelled by nick translation with digoxigenin 11-dUTP (Boehringer, Mannheim, Germany) and Fluorored 11-dUTP (Amersham, UK), respectively.

Fluorescent *in situ* hybridization

Preparations were incubated in 100 μg/ml DNase-free RNase in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) for 1 h at 37°C in a humid chamber, washed three times in 2 × SSC at room temperature for 5 min, dehydrated, and air-dried as described above.

The hybridization mixture contained 50% (v/v) high grade formamide, 5% (w/v) dextran sulphate, 0.1% (w/v) sodium dodecyl sulphate in 2 × SSC, and 5 ng/μl of autoclaved salmon sperm DNA (hybridization stringency of 78%). Then 100 ng of digoxigenin 11-dUTP pTa71 and 150 ng of Fluorored 11-dUTP pEpD15 were added to 30 μl of hybridization mixture for each slide and denaturated at 70°C for 10 min. The hybridization mixture was loaded onto the slide preparation and covered with a plastic coverslip. Combined denaturation of chromosomes and probes was carried out at 65°C for 10 min using a programmable temperature controller (Heslop-Harrison *et al.* 1991). The slides were then incubated at 37°C overnight for hybridization. After hybridization, coverslips were carefully floated off by placing the slides 2 × SSC at 42°C for 3 min. They were then given two stringency washes in 20% formamide in 0.1 × SSC at 42°C for 5 min each, washes in 0.1 × SSC (3 min) and in 2 × SSC (2 × 5 min) at 42°C, and finally allowed to cool to room temperature.

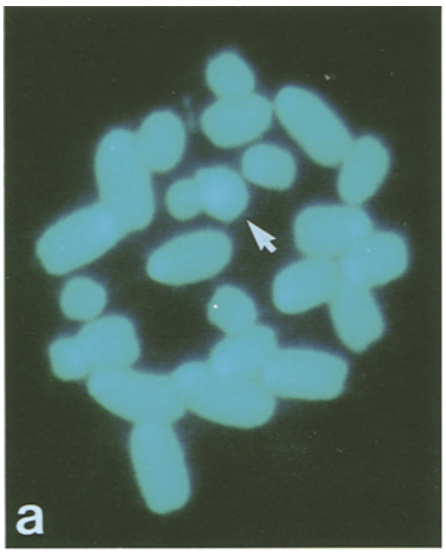
Sites of digoxigenin-rDNA probe hybridization were detected with sheep antidigoxigenin-FITC (fluorescein isothyocyanate) (Boehringer Mannheinm, Germany); the labelling system of the repetitive DNA using a directly labelled nucleotide (Fluorored 11-dUTP, Amersham, UK) does not need any detection steps. Slides were transferred to detection buffer (4 × SSC, 0.2% (v/v) Tween-20) for 5 min and blocked with 5% (w/v) BSA (bovine serum albumin) in detection buffer for 5 min. Slides were treated with 20 μg/ml FITC conjugated sheep antidigoxigenin in detection buffer containing 5% (w/v) BSA for 1 h at 37°C and washed in detection buffer three times, 5 min each at room temperature. The signal was amplified by incubating the preparations in 10 μg/ml of FITC-conjugated rabbit-antisheep (Dakopatts, UK) in detection buffer containing 5% (w/v) normal rabbit serum as a blocking agent, for 1 h at 37°C and by subsequently washing three times in detection buffer for 5 min.

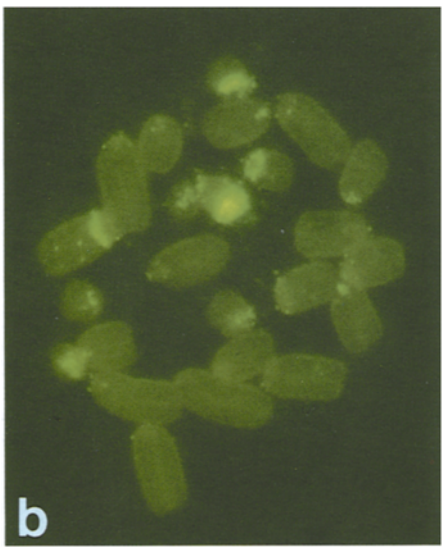
Slides were counterstained with 4'6-diamidino-2-phenylindole (DAPI) (2 μg/ml in McIlvaines citrate buffer, pH = 7) (100 μl/slide, 10 min at room temperature) and then they were mounted in antifade solution (AF1, Citifluor) to reduce the fading of the fluorescence. Preparations were examined with a Leitz Aristoplan epifluorescence microscope, and photographs were taken on Fujicolor Super HG 400 colour print film.

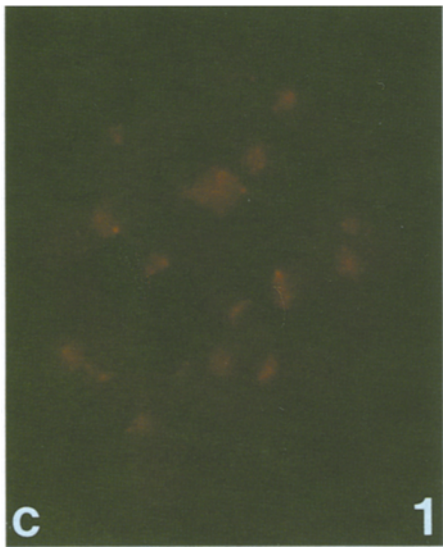
Results

Eyprepocnemis plorans specimens possess $2n = 2+X♂/XX♀$ standard A chromosomes and a varying number of additional B chromosomes.

DAPI-staining preferently binds AT-rich DNA sequences (Schweizer 1981) and shows almost uniform







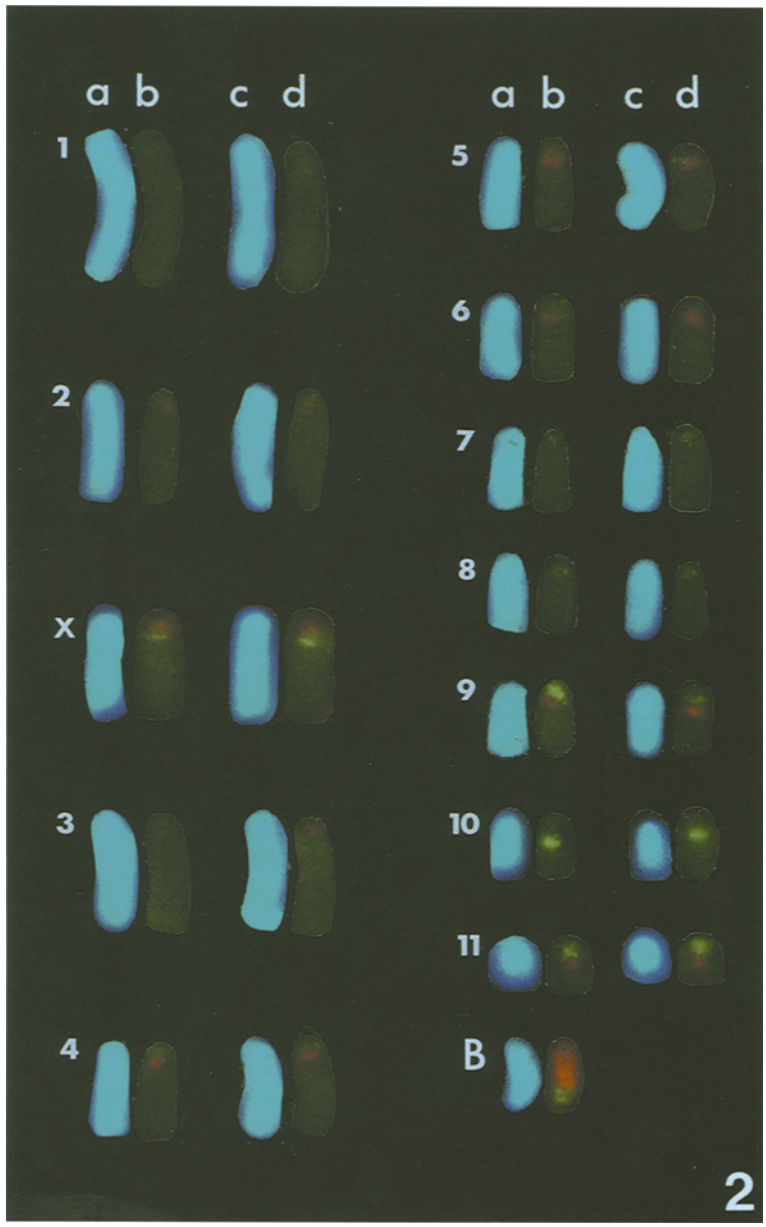


Figure 1. Double FISH of an embryo metaphase cell in the grasshopper *Eyprepocnemis plorans*. **a** DAPI staining showing positive bands at most centromeres and strong staining of the B chromosome. **b** Ribosomal DNA (pTa71 probe) localization as detected by FITC. **c** Repetitive DNA (pEpD15 probe) localization detected by Fluorored. Arrow points to the B chromosome.

Figure 2. Diploid karyotype showing DAPI staining (a,c) and a double exposed photograph for FITC showing rDNA hybridization sites (green) and Fluorored showing repetitive DNA sites (red) (b,d).

fluorescence in all the chromosomes after *in situ* hybridization, with the exception of most centromeres and the first two-thirds of the B chromosome which fluoresce more intensely (Figure 1a). FITC-excitation showed rDNA hybridization sites as yellow-green fluorescence and was present on the NOR (nucleolar organizer region)-carrying chromosomes (X, 9, 10 and 11) and the distal third of the B chromosome (Figure 1b). When the same cell was excited for Fluorored, red fluorescence was observed in the centromeric regions of most chromosomes and a larger segment in the proximal two-thirds of the B chromosome (Figure 1c). Double exposure photographs of both excitations in the same cell show the relative positions of both DNAs on the chromosomes. As Figure 2 shows, the 180 bp repeat is present close to the centromeric regions of all chromosomes. Chromosomes X, 4, 5, 6, 9, 11 and B had larger amounts than others (1–3), the B being the chromosome with the greatest amount of repetitive DNA. The ribosomal DNA showed weak hybridization signals in the centromeric regions of chromosomes 1–8 but strong signals in the X, 9, 10, 11 and B chromosomes.

Discussion

The most remarkable feature of the double FISH results is the almost complete painting of the B chromosome (the proximal two-thirds are red and the distal third is green) the only non-bright region being the minute short arm (Figure 2). Thus the B seems to be mainly composed of DNA sequences (the repetitive DNA and rDNA) that are also present in the standard chromosomes. Molecular cytogenetic investigations using *in situ* hybridization provide the optimal method of showing the distribution of particular DNA sequences within the genome. It is not yet known which sequences are present in the short arm of the B chromosome, and further investigations are required to show whether other sequences—repetitive or single copy—are interspersed with the rDNA and 180 bp tandem repeat sequences on the long arm.

The present results support, clarify, and extend previous observations from G-banding, which suggested that proximal C-bands on most chromosomes in *Eyprepocnemis plorans* are composed of a G^- pericentromeric region containing G-C rich DNA sequences, and a G^+ paracentromeric region containing A-T rich DNA sequences (Camacho *et al.* 1991). The G^- regions showed bright fluorescence with A_3 chromomycin, as was expected for heterochromatin containing G-C rich DNA sequences (Schweizer 1981). They also hybridized with the ribosomal DNA probe, as did the active NORs in chromosomes X, 9, 10, and 11. Interestingly, an exceptional *E. plorans* male showing nucleoli associated with most chromosomes has recently been found in the Salobreña population (J. S. Rufas, personal communication). Thus, small and potentially active rDNA clusters can be present in the centromeric regions of autosomes 1–8. The G^+ regions were expected to show bright fluorescence with DAPI if they contained A-T rich DNA sequences, but they did not (Camacho *et al.* 1991). The present paper demonstrates that these regions hybridize with the repetitive DNA probe, and this repeat is somewhat biased in AT content (59%) (López-León *et al.*, in preparation). Perhaps differential DNA extraction during *in situ* hybridization (Maluszynska & Heslop-Harrison 1993), sequence structure, the AT bias in the repeat, or else the DAPI concentration used, prevented differentiation of these chromosome regions by DAPI staining. These results uphold our previous conclusion that proximal C-bands on most standard chromosomes are heterogeneous. The B chromosome, however, showed the only $DAPI^+$ region in the chromosome complement (Camacho *et al.* 1991) and, in parallel, showed the strongest hybridization with the AT-based repetitive DNA probe. Perhaps the higher concentration of these repeats in the B chromosome facilitated differential staining with DAPI.

The B chromosome (type B_2) has been shown to be mainly composed of two fractions: 1) repetitive DNA in the proximal dark C and G bands, and 2) ribosomal DNA contained in the distal region, which stains lightly with C and G banding. The order of these two types of DNA sequences in the B is the same as that in the X chromosome only. No other chromosome is similar. Hence, this B most probably originates from an X chromosome, with the whole euchromatic region being deleted and the two types of DNA sequences subsequently being amplified. The theory that the B chromosome originates from the X chromosome in several grasshopper species has been proposed mainly on the basis of morphology, dosage, and meiotic behaviour (see Hewitt 1973, 1979 for reviews). In *E. plorans*, B chromosomes associate with the X chromosome in more than 20% of meiotic prophase I cells, but the association only persists in 7% of meiotic metaphase I cells, suggesting that it is not a chiasmatic association (Camacho *et al.* 1980). However, the present results demonstrate that although chiasmata are not formed, this association might be by homology, because the X and B univalents share these two types of DNA sequences. On the other hand, in *Glossina* species, prophase I association between Bs and the Y chromosome occurs in the absence of satellite DNA similarity between them (Amos & Dover 1981). The most likely explanation for the B and X or Y association is their heteropycnosity, where the chromosomes form a heterochromatic group. Obviously, the origin of the B chromosome may not be ascertained only by studying its morphology or meiotic behaviour. The best way to do this is to compare the DNA composition of the A and B chromosomes. However, the origin of the B could not be easily deduced in several recent DNA studies (see Introduction). In *E. plorans* the theory that the B originates from the X chromosome is plausible because the B chromosome organization for the two DNA sequences investigated

seems very simple and coincides uniquely with that in the X chromosome. However, the fact that B chromosomes in *E. plorans* frequently undergo a variety of chromosomal rearrangements, i.e. centromere misdivision, inversions, deletions, etc (López-León *et al.* 1993) prevents complete confidence in this hypothesis because the real pathway of the origin of the B may have been obscured by such evolutionary changes in the B itself.

The presence of rDNA coding sequences in the distal third of the B chromosome supports our previous hypothesis that the B possessed a distal NOR that is usually inactive (Cabrero *et al.* 1987, López-León *et al.* 1991). Silver-staining revealed NOR activity only in the X, 9, 10 and 11 chromosomes, and on an exceptional B chromosome that was fused to the largest autosome in a single male (Cabrero *et al.* 1987). Thus, the rRNA genes contained in the B retain the capability to express even though they are usually inactive. This suggests that either rRNA genes in the B are submitted to concerted evolution operating on this gene family, or that the B is young, so that there has not been enough time to completely silence them. However, we have data indicating that the B is not young, namely the wide distribution of the B chromosome polymorphism, including Mediterranean regions over Europe and North Africa (Henriques-Gil 1984), and the absence of a drive mechanism (López-León *et al.* 1992) that would have been necessary for its initial spread, but which has presumably been lost through selection of modifier genes (Shaw 1984). Hence, Muller's Ratchet (Green 1990) does not seem to have operated on the rRNA genes of the *E. plorans* B chromosome, perhaps because these genes are affected by the same concerted evolutionary process as the rRNA genes on the standard chromosomes.

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References

Amos A, Dover G (1981) The distribution of repetitive DNAs between regular and supernumerary chromosomes in species of *Glossina* (TseTse): a two step process in the origin of supernumeraries. *Chromosoma* **81**: 673–690.

Cabrero J, Alche JD, Camacho JPM (1987) Effects of B chromosomes on the activity of nucleolar organizer regions in the grasshopper *Eyprepocnemis plorans*: activation of a latent nucleolar organizer region on a B chromosome fused to an autosome. *Genome* **29**: 116–121.

Camacho JPM, Carballo AR, Cabrero J (1980) The B chromosome system of the grasshopper *Eyprepocnemis plorans* subsp. *plorans* (Charpentier). *Chromosoma* **80**: 163–176.

Camacho JPM, Cabrero J, Viseras E, López-León MD, Navas-Castillo J, Alché JD (1991) G banding in two species of grasshoppers and its relationships to C, N and fluorescence banding techniques. *Genome* **34**: 638–643.

Eickbush DG, Eickbush TH, Werren JH (1992) Molecular characterization of repetitive DNA sequences from a B chromosome. *Chromosoma* **101**: 575–583.

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Res* **7**: 1869–1885.

Green DM (1990) Muller's Ratchet and the evolution of supernumerary chromosomes. *Genome* **33**: 818–824.

Henriques-Gil N (1984) El sistema de cromosomas accesorios de *Eyprepocnemis plorans* (Acrididae: Orthoptera). PhD Thesis. Universidad Complutense de Madrid.

Henriques-Gil N, Santos JL, Arana P (1984). Evolution of a complex B chromosome polymorphism in the grasshopper *Eyprepocnemis plorans*. *Chromosoma* **89**: 290–293.

Henriques-Gil N, Arana P (1990) Origin and substitution of the B chromosomes in the grasshopper *Eyprepocnemis plorans*. *Evolution* **44**: 529–539.

Hewitt GM (1973) The integration of supernumerary chromosomes into the orthopteran genome. *Cold Spring Harbor Symp Quant Biol* **38**: 183–194.

Hewitt GM (1979) Grasshopper and crickets. *Animal Cytogenetics Vol 3: Insecta 1, Orthoptera*. Gebruder Borntraeger, Berlin-Stuttgart, Germany.

Heslop-Harrison JS, Schwarzacher T, Anamthawat-Jónsson K, Leitz AR, Shi M, Leitz IJ (1991) *In situ* hybridization with automated chromosome denaturation. *Technique (Phil)* **3**: 109–115.

John UP, Leach CR, Timmis JN (1991) A sequence specific to B chromosome of *Brachycome dichrosomatica*. *Genome* **34**: 739–744.

Koenen M (1989) Recovery of DNA fragments from agarose gels using liquid nitrogen. *Trends Genet* **5**: 137.

López-León MD, Cabrero J, Camacho JPM (1991) A nucleolus organizer region in a B chromosome inactivated by DNA methylation. *Chromosoma* **100**: 134–138.

López-León MD, Cabrero J, Camacho JPM, Cano MI, Santos JL (1992) A widespread B chromosome polymorphism maintained without apparent drive. *Evolution* **46**: 529–539.

López-León MD, Cabrero J, Pardo MC, Viseras E, Camacho JPM, Santos JL (1993) Generating high variability of B chromosomes in *Eyprepocnemis plorans* (grasshopper). *Heredity* **71**: 352–362.

Maluszynska J, Schweizer D (1989) Ribosomal RNA genes in B chromosomes of *Crepis capillaris* detected by non-radioactive *in situ* hybridization. *Heredity* **62**: 59–65.

Maluszynska J, Heslop-Harrison JS (1993) Physical mapping of rDNA loci in *Brassica* species. *Genome* **36**: 774–781.

Nur U, Werren JH, Eickbush DG, Burke WD, Eichbush TH (1988) A "selfish" B chromosome that enhances its trans-

mission by eliminating the paternal genome. *Science* **240**: 512–514.

Sandery MJ, Foster JW, Blunden R, Jones N (1990) Identification of a family of repeated sequences on the rye B chromosome. *Genome* **33**: 908–913.

Schweizer D (1981) Counterstain-enhanced chromosome banding. *Hum Genet* **57**: 1–14.

Shaw MW (1984) The population genetics of the B-chromosome polymorphism of *Myrmeleotettix maculatus* (Orthoptera: Acrididae) *Biol J Linnean Soc* **23**: 77–100.