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Microdissection and Chromosome Painting of X and B Chromosomes in the Grasshopper *Eyprepocnemis plorans*

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Key Words
B chromosomes · Chromosome painting · *Eyprepocnemis plorans* · Grasshopper · Microdissection

Abstract
The relative location of 2 repetitive DNAs, i.e. ribosomal (rDNA) and a tandemly repeated satellite DNA (satDNA), with respect to the centromere, suggested that B chromosomes in the grasshopper *Eyprepocnemis plorans* derived intraspecifically from the X chromosome. To test this hypothesis, we microdissected X and B chromosomes and amplified the obtained DNA by 2 different procedures, the conventional DOP-PCR method and the single-cell whole-genome amplification GenomePlex® method. We then generated DNA probes to perform chromosome painting. Our results have confirmed that X and B chromosomes share many DNA sequences between them and with most of the autosomes, especially at locations where the satDNA and rDNA reside, in consistency with previous information. This supports the hypothesis of an intraspecific origin of B chromosomes in *E. plorans*. Nevertheless, the present results did not help to clarify whether Bs were derived from the X chromosome or else from 1 or more autosomes.

Nowadays, microdissection constitutes one of the most direct approaches to ascertain the molecular composition of certain chromosomes or chromosome regions. McQuade et al. [1994] were the first in using this technique for getting molecular information of B chromosomes in the marsupial *Petauroides volans*, but it has subsequently been employed for many other B chromosome systems [for recent reviews, see Camacho, 2005; Teruel et al., 2009]. By generating a mixture of DNA probes for fluorescent in situ hybridization (FISH), with the DNA microdissected from a certain chromosome, it is possible to get an idea of the DNA sequences shared among different chromosomes within the same genome [Guan et al., 1994]. This technique, chromosome painting, is usually the first step after microdissecting B chromosomes, since it rapidly provides valuable information on possible B ancestry.

The B chromosome system of the grasshopper *Eyprepocnemis plorans* is characterized by the existence of numerous B variants, some of which have become the most abundant variant in different natural populations [Henriques-Gil et al., 1984; Henriques-Gil and Arana, 1990; López-León et al., 1993; Bakkali et al., 1999; Bakkali and Camacho, 2004]. A second characteristic of the *E. plorans* B chromosomes is their almost ubiquitous presence in the subspecies *E. plorans plorans* (in the circummediterranean region and the Caucasus) and their absence in the

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E. plorans meridionalis subspecies (in South Africa) [see López-León et al., 2008]. The only known region lacking B chromosomes in *E. plorans* is the head of the Segura river basin in southeastern Spain [Cabrero et al., 1997].

The most widespread B variant in Spanish populations is B_1, so it is plausible that this was the ancestral B variant in the Iberian Peninsula [Henriques-Gil et al., 1984] and Balearic Islands [Riera et al., 2004]. But other variants seem to have replaced it in some small areas, such as B_2, which replaced B_1 in Granada and East-Málaga provinces, and B_5, which replaced B_1 in the Fuengirola (Málaga) area [Henriques-Gil and Arana, 1990]. One of these replacements was even witnessed in the Torrox (East-Málaga) population, since B_2 and B_{24} were both present in a sample collected in 1984 [Henriques-Gil and Arana, 1990] but only B_{24} was observed in samples from 1992 and 1994 [Zurita et al., 1998].

By employing fluorescence, G-, and N-banding, Camacho et al. [1991] showed that the proximal C-bands in all A chromosomes of *E. plorans* are actually composed of 2 different kinds of heterochromatin, one of them being $DAPI^+$ (A+T rich) and the other $CMA3^+$ (G+C rich). FISH later demonstrated that they correspond, respectively, with the location of a 180-bp tandemly repeated DNA (satDNA) and 45S rDNA, which are proximally located in most A chromosomes [López-León et al., 1994].

Most of the tens of B variants hitherto reported in *E. plorans* are made up of 2 main repetitive DNAs: 45S ribosomal DNA (rDNA) and a 180-bp satDNA, in addition to other unknown DNA sequences which putatively might be interspersed with the former 2, or in the small short arm. Their similar molecular architecture supports the hypothesis that all these variants derived from a single ancestral B variant [Cabrero et al., 1999].

All most widespread B variants in *E. plorans* carry the 2 repetitive DNAs, i.e. rDNA and satDNA, in the same order with respect to the centromere, i.e., the satDNA is closer to the centromere than the rDNA. The presence of these 2 repetitive DNAs in the pericentromeric regions of most A chromosomes suggests that, in principle, any A chromosome might be the B chromosome ancestor. However, since the centromere–satDNA–rDNA order observed in B chromosomes was only coincident with that in the X chromosome, López-León et al. [1994] suggested the possible origin of the *E. plorans* B chromosome from the proximal region of the X chromosome. Later on, however, this same order has been observed in some autosomes of some populations, suggesting that they might also conceivably be sources for B chromosome origin [Cabrero et al., 2003a].

To test the hypothesis of B origin from the X chromosome, we performed the microdissection of the B chromosome (B_{24} variant) and its putative ancestor, i.e. the X chromosome, and the subsequent chromosome painting with probes coming from both chromosomes.

Materials and Methods

Ten adult males of the grasshopper *E. plorans* were collected at Torrox (Málaga) in November 2006. The presence of B chromosomes in these males was assessed by analysing C-banded hemolymph interphase cells following the procedure described by Cabrero et al. [2006]. Microdissection of X and B_{24} chromosomes was performed on diplotene spermatocytes from 2 of these males, one of which carried 1 B_{24} and the other carried 2 B_{24} chromosomes. The males were anesthetized and dissected, and testes were fixed in 1:3 acetic-ethanol for 10 min and were then stored in 1.5 ml tubes containing 500 μl of 70% ethanol at –20°C until usage.

Just prior to microdissection, 2 testis tubules were immersed in a drop of 50% acetic acid to get a cell suspension which was spread onto a 24 × 60 mm coverslip (previously incubated overnight in 10× SSC) on a warm plate at 27°C. The X and B chromosomes were easily recognizable under phase contrast in unstained diplotene cells because of their positive heteropycnosis and different sizes (fig. 1).

Chromosome microdissection was performed in an Eppendorf TransferMan NK2 micromanipulator coupled to a Zeiss Axiovert 200 microscope, with glass needles made with a 2-step horizontal pipette puller (Bachofer), and sterilized by UV radiation. About 15 B chromosomes and 15 X chromosomes were separately placed in 9 μl DNase-free ultrapure water and were then amplified by means of the GenomePlex® Single Cell Whole Genome Amplification Kit (wga4-Sigma) [Langmore, 2002; Gribble et al., 2004]. A second round of microdissections was performed for each chromosome. The X chromosome was again amplified by the GenomePlex method, but the B chromosome was amplified this second time by the conventional DOP-PCR method [Telenius et al., 1992]. Our previous comparison of both techniques for microdissected X chromosomes in the migratory locust showed that the GenomePlex method provided DNA less enriched in repetitive sequences [Teruel et al., 2009]. Here we perform a similar test for B chromosome DNA sequences in the grasshopper *E. plorans*.

The microdissected chromosomes were collected into 2 different tubes (0.2 ml), 1 for Xs and the other for Bs, each containing 20 μl 1× buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3; Roche) when they were amplified by DOP-PCR, or else 9 μl DNase-free ultrapure water for the GenomePlex method. When the DNA was amplified by this last method, we immediately re-amplified it, following supplier instructions, and then marked it by nick translation with rhodamine-11-dUTP or fluorogreen-11-dUTP. When the DNA was amplified by DOP-PCR, we re-amplified and marked DNA with SpectrumOrange-dUTP (Vysis) [Marchal et al., 2004].

Chromosome painting was performed as described in Marchal et al. [2004] on mitotic metaphase cells from 1 embryo obtained

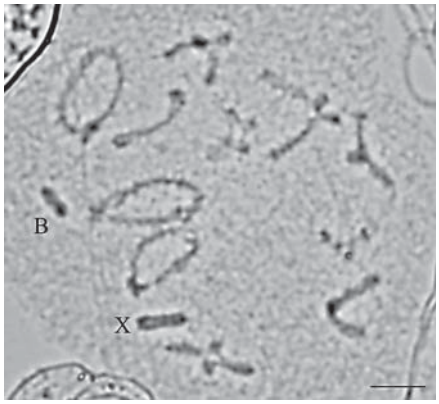

Fig. 1. Phase-contrast photography of an unstained diplotene cell of the grasshopper *Eyprepocnemis plorans* showing that X and B chromosomes are easily identifiable because of their positive heteropycnosis and different sizes.

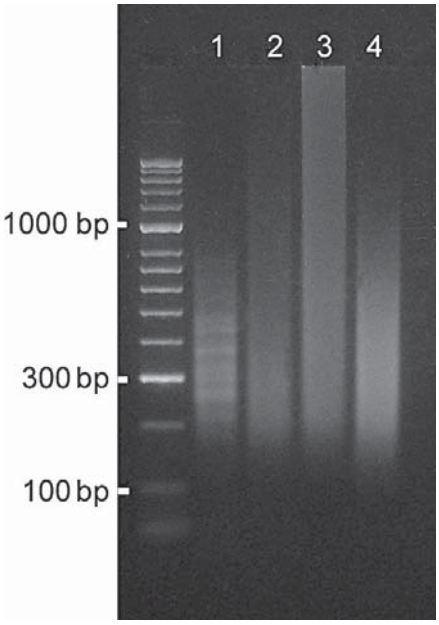

Fig. 2. Amplification of the microdissected DNA by the DOP-PCR (lane 1) and GenomePlex (lanes 2–4) methods. Lanes 1 and 2 correspond to the DNA microdissected from the B_{24} chromosome, and lanes 3 and 4 correspond to the DNA microdissected from the X chromosome.

in our laboratory carrying the B_1 (from the Mundo river population, Albacete, Spain) and 2 embryos carrying B_{24} (from Torrox) chromosomes. In addition, primary spermatocytes from a male collected at Goynuk (Turkey) were submitted to chromosome painting. At least 5 cells were captured from each individual on an Olympus microscope equipped with the DP70 cooled digital camera, and plates were mounted with The GIMP freeware.

Results and Discussion

The chromosome set of *E. plorans* consists of 2n = 22 + X0 in the male and 2n = 22 + XX in the female, all chromosomes being acrocentric [Camacho et al., 1980]. The X chromosome is the third element in order of decreasing size, and the 11 autosomes can be classified into 3 size groups: 2 long (L_1 and L_2), 6 medium (M_3–M_8) and 3 short (S_9–S_{11}). The X chromosome is shorter than L_2 but longer than M_3.

The DOP-PCR method for amplification of microdissected chromosomes preferentially amplifies highly repeated DNA sequences [Houben et al., 2001]. This was one of the reasons to try the GenomePlex single-cell whole-genome amplification kit for the B_{24} chromosome in *E. plorans*, and to compare it with the DOP-PCR results. Both amplification methods yielded B chromosome DNA fragments between 100 and 1,000 bp, with higher concentrations at about 300–500 bp (fig. 2). The main difference between both methods was a higher enrichment in repetitive DNA fragments for the DOP-PCR procedure (see bands in fig. 2, lane 1). The lower enrichment in repetitive DNA sequences from microdissected B DNA amplified by the GenomePlex method is consistent with the similar result obtained previously for microdissected X chromosomes in *Locusta migratoria* [Teruel et al., 2009].

Chromosome painting with the DOP-PCR-obtained B_{24} DNA probe (fig. 3a–c) painted almost the whole B_{24} chromosome, except the small short arm (see fig. 3c), and also the proximal regions of all A chromosomes, excepting the M_6 and M_8 autosomes (see fig. 3b), most likely because these chromosomes contain only small amounts of rDNA and no satDNA [Cabrero et al., 2003a], which are the 2 main DNA components of B chromosomes in *E. plorans* [Cabrero et al., 1999].

On the other hand, chromosome painting with the GenomePlex-generated B_{24} DNA probe (fig. 3d–f) painted the whole B_{24} chromosome, including the small short arm, as well as the proximal regions of all A chromosomes (see fig. 3e), suggesting that this method did amplify a higher variety of B DNA sequences. For this reason, we performed chromosome painting with other B chromosome variants using this DNA probe. The B_1 chromosome, a variant carrying less satDNA but more rDNA than B_{24}, was completely painted with this DNA probe (fig. 3g–i), and the same result was obtained for a B chromosome from a Turkish population (fig. 3j–l), which was almost completely composed of rDNA [see López-León et al., 2008]. These results indicate that the GenomePlex-

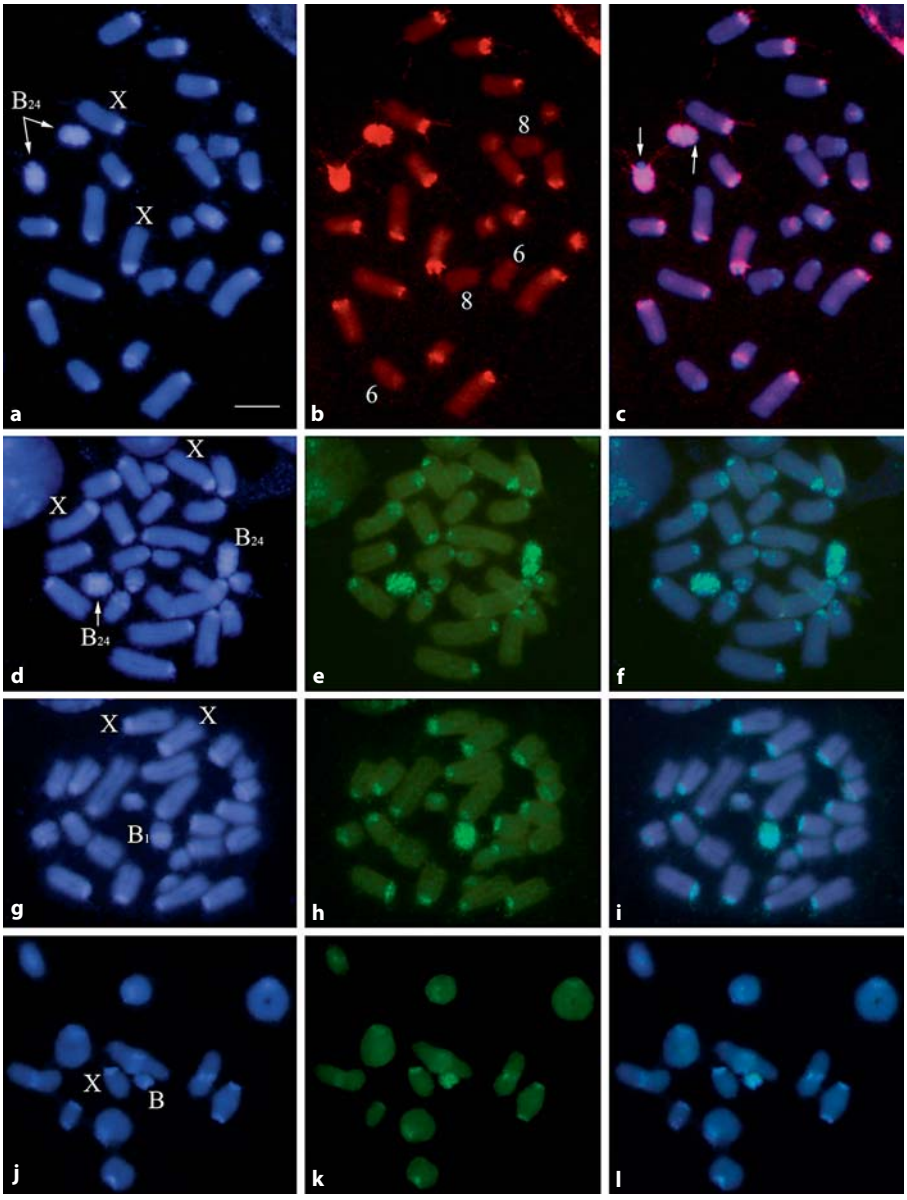

Fig. 3. Chromosome painting with the DNA probes obtained by microdissection of the B_{24} chromosome, performed on embryo mitotic metaphase cells (**a–i**) and spermatocytes at metaphase I (**j–l**). Left column shows DAPI pattern, central column shows FISH with the painting DNA probe, and right column shows the former 2 merged. **a–c** Mitotic metaphase cell from a female embryo carrying 2 B_{24} chromosomes, painted with the B-probe obtained by DOP-PCR. Note in **b** the absence of centromeric painting in chromosomes 6 and 8, and in **c** the absence of painting in the short arm of B_{24}. **d–f** Mitotic metaphase cell from a female embryo with 2 B_{24}, painted with the B probe obtained by the GenomePlex method. Note the presence of painting in the whole B chromosome length and in all pericentromeric regions of all A chromosomes. **g–i** Mitotic metaphase cell from a female embryo carrying 1 B_1 chromosome, painted with the B probe obtained by the GenomePlex method. **j–l** Metaphase I spermatocyte from a Turkish male carrying 1 B mainly made of 45S rDNA. Note that it is painted by the GenomePlex B_{24} probe. Bar in **a** represents 5 μm.

generated DNA contained a good representation of the main DNA sequences from the B_{24} chromosome.

Consistent with recent results in the migratory locust [Teruel et al., 2009], our present results in the grasshopper *E. plorans* confirm that the GenomePlex method is better than the conventional DOP-PCR for DNA amplification of microdissected chromosomes, since chromosome painting indicated that it provides DNA containing a higher variety of DNA sequences, marking all regions of the microdissected B_{24} chromosome, including the short arm, which was not painted by the DOP-PCR-generated probe.

The X chromosome was twice microdissected and amplified by the GenomePlex method. In both cases, the X-DNA probes painted more intensely the proximal region of the X chromosome (fig. 4). The X probe obtained from the first microdissection (fig. 4a–c) painted most of the B_{24} chromosome length, since this B variant is mostly composed of the 180-bp tandem DNA repeat also present in the pericentromeric region of the X chromosome [Cabrero et al., 1999], but it failed to paint the B_{24} distal region, mostly composed of rDNA (which is also present in the X chromosome proximal region), and the small short arm (see fig. 4c). In addition, it painted the $DAPI^+$

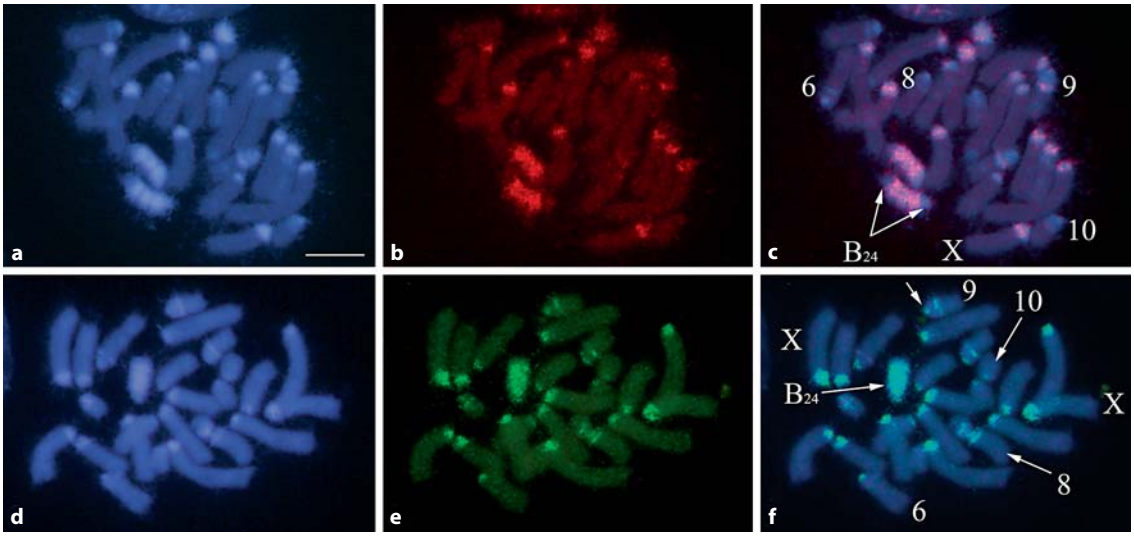

Fig. 4. Chromosome painting with the X chromosome DNA probes obtained by the first (**a–c**) and second (**d–f**) microdissections, performed on embryo mitotic metaphase cells. Left column shows DAPI pattern, central column shows FISH with the painting DNA probe, and right column shows the 2 former merged. **a–c** Mitotic metaphase cell from a male embryo carrying 2 B_{24} chromosomes (1 of the smallest autosomes, no. 11, is missing). Note the absence of painting at both B chromosome ends (arrows) and at proximal regions of chromosomes M_6, M_8, S_9 and S_{10}. **d–f** Mitotic metaphase cell from a female embryo carrying 1 B_{24} chromosome. Note the presence of painting over the whole B chromosome length and at proximal regions of all A chromosomes, including M_6, M_8, S_9 and S_{10}. Bar in **a** represents 5 μm.

proximal regions of most autosomes, except those in chromosomes M_6, M_8, S_9 and S_{10} (see fig. 4a–c). The probe obtained from the second X microdissection, however, painted the whole B_{24} chromosome and the $DAPI^+$ regions of all autosomes (fig. 4d–f).

Chromosome painting thus showed that the GenomePlex method provided samples of DNA sequences from the X chromosome which were enriched in repetitive DNA located in $DAPI^+$ proximal regions of the X chromosome and that were also present in most (first microdissection) or all (second microdissection) autosomes, and also in the B_{24} chromosome. The observed differences between the 2 X probes, however, suggest some caution with the GenomePlex method, so that at least 2 different amplifications are advisable to increase the likelihood of obtaining a good chromosome-DNA representation.

The X chromosome probes also contained a good representation of repetitive DNA elements scattered over the euchromatic length of A chromosomes (see fig. 4b, e). This pattern is coincident with that obtained by FISH for some mobile elements, such as *gypsy* and *LINEs* (Montiel et al., unpublished), resembling previous observations in *L. migratoria* [Teruel et al., 2009]. The fact that they were more apparent with the X probe (fig. 4) than with the B probe (fig. 3) is consistent with the higher amount of mobile elements in the large euchromatic region of the X chromosome, which makes it more likely to amplify them after microdissection.

The results of the microdissection of X and B_{24} chromosomes and the subsequent chromosome painting confirm previous observations that these chromosomes share some repetitive DNA sequences, specifically 45S rDNA and the 180-bp tandemly repeated DNA, which make up most of the B_{24} chromosome long arm [López-León et al., 1994; Cabrero et al., 1999]. The 180-bp satDNA is A+T rich [López-León et al., 1995], thus being $DAPI^+$, and is most likely one of the most represented DNAs in the probes obtained by microdissection. But our present results also suggest the existence of DNA sequences in the short arm of the B_{24} chromosome, which are painted with the X probe (see fig. 4e). Therefore, it is possible that other DNA sequences are also shared between X and B chromosomes. This might provide additional support for the hypothesis that Bs derived from the X chromosome. However, the fact that both X and B probes also painted the proximal regions of most autosomes indicates that they also contain the DNA sequences microdissected from the X and the B chromosomes.

In addition, the 180-bp tandem DNA repeat, which is the most abundant DNA in the B_{24} chromosome [Cabrero et al., 1999] and is also present in pericentromeric regions of the X chromosome and most autosomes [Cabrero et al., 2003a], seems to be restricted to *E. plorans* since it was not found by FISH in *E. unicolor* and 3 other Eyprepocnemidine species [Cabrero et al., 2003b], thus suggesting that an interspecific origin of these Bs is unlikely.

Therefore, all available data point to the intraspecific origin of B chromosomes in *E. plorans*, but additional information is needed to test the possibility for an autosomal origin of Bs, for which purpose the separate microdissection of each autosome, and the subsequent analysis of the DNA sequences shared with the B, might be a useful approach.

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