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Population variation in the A chromosome distribution of satellite DNA and ribosomal DNA in the grasshopper *Eyprepocnemis plorans*

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Abstract

The double FISH analysis of two repetitive DNAs (a satellite DNA and ribosomal DNA) in 12 natural populations of the grasshopper *Eyprepocnemis plorans* collected at the south (Granada and Málaga provinces) and south-east (Albacete and Murcia provinces) of the Iberian Peninsula has shown their widespread presence throughout the whole genome as well as extensive variation among populations. Both DNAs are found in most A chromosomes. Regularly, both DNAs occurred in the S_{11} and X chromosomes, rDNA in the S_{10} and satDNA in the L_2 and M_3. No correlation was found between the number of satDNA and rDNA clusters in the A genomes of the 12 populations analysed, and both figures were independent of the presence of B chromosomes. The genomic distribution of both DNAs showed no association with the geographical localization of the populations analysed. Finally, we provide evidence that the supernumerary chromosome segment proximally located on the S_{11} chromosome is, in most cases, the result of satDNA amplification but, in some cases, it might also derive from amplification of both satDNA and rDNA.

Introduction

Repetitive or satellite DNA (satDNA) constitutes a considerable genome fraction in higher eukaryotes and represents the major component of heterochromatin (Miklos 1985, Charlesworth *et al.* 1994). These repetitive DNA sequences and specific associated proteins have been suggested to be involved in important processes such as stability of genome structure, position effect variegation, recombination, chromosome pairing and segregation, suprachromosomal organization, as well as genetic differentiation and karyotypic evolution (Miklos 1985, Weiler & Wakimoto 1995, Ren *et al.* 1997). The existence of multiple copies of repetitive sequences, such as satDNA or ribosomal DNA (rDNA), constitutes the basis for a wide variation found between species, populations and individuals (De Lucchini *et al.* 1993, Castro *et al.* 2001, Lanfredi *et al.* 2001).

In the grasshopper *Eyprepocnemis plorans* ($2n♂ = 22 + X0$ and $2n♀ = 22 + XX$), C banding

reveals the presence of heterochromatin in proximal regions of all standard chromosomes and interstitial regions of the M_7, M_8, S_9, S_{10}, S_{11} and X chromosomes. In some individuals, heterochromatic B chromosomes and/or a supernumerary chromosome segment proximally located on the S_{11} chromosome can also be observed (Camacho *et al.* 1991).

Physical mapping of repetitive DNA (e.g. satDNA or rDNA), by *in-situ* hybridization, is essential for ascertaining the origin, differentiation and evolution of chromosomes. DNA composition of different B chromosome variants in *E. plorans* has previously been analysed by fluorescence *in-situ* hybridization (FISH), reaching the conclusion that they are mainly composed of two repetitive DNA sequences, a 180-bp satellite DNA and rDNA (López-León *et al.* 1994, Cabrero *et al.* 1999) both of which are also present in the A chromosomes. For the population at Salobreña (Granada, Spain), *in-situ* hybridization performed on mitotic embryo cells showed that the 180-bp satDNA was present at proximal regions of most A and B chromosomes. In addition, a ribosomal DNA probe hybridized strongly with the chromosomes bearing active NORs (nucleolus organizer regions) (X, S_9, S_{10} and S_{11}) and weakly with the centromeric regions of most remaining A chromosomes (López-León *et al.* 1994).

In this paper, we report on a FISH analysis in spermatocytes to elucidate the distribution and relative positions of two repetitive DNA families (rDNA and 180-pb satDNA) on the A chromosomes of the grasshopper *Eyprepocnemis plorans* in 12 Spanish populations. The observed chromosome variation shows genome differentiation between populations and provides new insights about B chromosome origin.

Materials and methods

Adult males of the grasshopper *Eyprepocnemis plorans* were collected from 12 populations in four Spanish provinces, i.e. Granada (Salobreña), Málaga (Fuengirola and Torrox), Murcia (Calasparra, Benamor-1 and Salmerón) and Albacete (Ayna, Claras, Casa Mina, Chopillo, Liétor and Socovos). The Granada and Málaga populations were collected at the coast; others in the inland

Figure 1. Geographical localization of the 12 populations sampled. Three were from the Granada and Málaga coast, and nine from the inland part of the Segura river basin.

part of the Segura river basin (Figure 1). Testes were fixed in freshly prepared 3 : 1 ethanol : acetic acid and stored at 4°C. Two to ten males were analysed per population.

Chromosome preparations were made following the methods described in Cabrero *et al.* (1999). Two different DNA probes were used for fluorescence *in-situ* hybridization (FISH): pTa71, which contains a 9-kb EcoRI repeat unit of rDNA isolated from *Triticum aestivum* (Gerlach & Bedbrook 1979), and pEpD15 with a 180-bp DraI fragment of tandem repetitive DNA from *E. plorans* (López-León *et al.* 1994, 1995a). DNA was labelled by nick translation with fluorogreen 11-dUTP or fluorored 11-dUTP, using standard techniques. Double FISH was performed following the technique described in López-León *et al.* (1994). Photographs were taken on Fujichrome 400 Provia colour film. Slides were digitized with a Hewlett Packard Photo Smart scanner and the figures were composed with Adobe Photoshop.

To compare chromosome localization of the two repetitive DNAs among the 12 populations analysed and to explore possible geographical patterns, we scored the presence (1) or absence (0) for each of both DNAs on each of the 12 chromosomes of the A complement. This provided us with 24 binary variables for each population. A matrix of disagreement between populations, based on percentages of differences for these 24 variables, was built and compared, by means of Mantel tests, with two matrices of geographical distances, one built with distances measured along river courses for the Segura river basin populations and distances through the coast line for the three coastal populations, and another matrix with distances measured in a straight line ('as the bird flies') between all populations. The significance of the Mantel test was obtained by permutation.

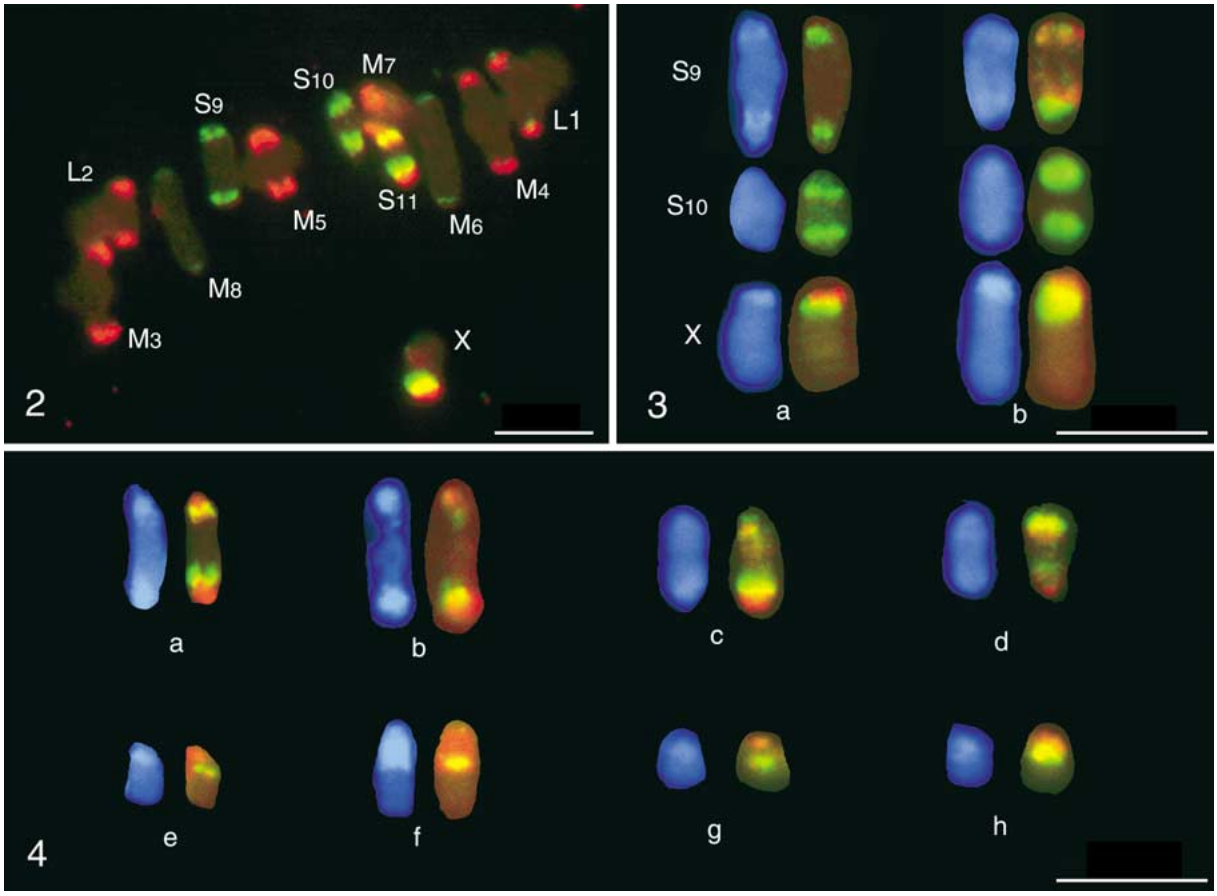
Results and discussion

The standard chromosome complement of *E. plorans* specimens in all populations studied consisted of $2n♂ = 22 + X0$ and $2n♀ = 22 + XX$ telocentric chromosomes, with three long (L_1, L_2 and X), six medium (M_3–M_8) and three short (S_9–S_{11}) chromosome pairs. The use of double FISH allowed us to localize the relative chromosomal positions of rDNA clusters and the 180-bp satDNA in the *E. plorans* karyotype (Figure 2). Although FISH is not usually a fully quantitative technique, differences in the strength of the hybridization signal at different loci suggest differences in the copy number of tandem repeats they contain (Suzuki *et al.* 1990, Leitch & Heslop-Harrison 1992).

Extensive variation in the number, size and position of rDNA and satDNA loci was found among the 12 populations. Table 1 summarizes, for each population, the number and type of chromosomes that hybridized with one or both probes.

180-bp satDNA

This repetitive DNA hybridized, in all populations analysed, to regions close to the centromeric regions of the four longest chromosomes of the complement (L_1, L_2, X and M_3) and the smallest one S_{11} (Table 1). Its presence in the five remaining chromosomes (M_4–M_8 and S_9), however, showed interpopulational variation. The total number of satDNA hybridization signals, in the haploid complement, ranged from 7 in Salobreña to 10 in Fuengirola and, as Table 1 shows, the specific chromosomes harbouring the satDNA blocks may differ between populations bearing the same total number of loci. Almost all chromosomes of the complement contained satDNA in one population or another. The only exception is the S_{10} chromosome that was never found to contain detectable amounts of this satDNA (Figure 3). Remarkably, this chromosome also lacks satDNA in Moroccan and Caucasian *E. plorans* specimens (Cabrero *et al.* 2003). Considering that the satDNA is specific for *E. plorans*, since it has not been detected in *E. unicolor* and three other species of Eyprepocnemidinae (Cabrero *et al.* 2003), its absence from the S_{10} chromosome suggests that this is an ancestral condition or that there exists a functional constraint against satDNA colonization for S_{10}. Interestingly, the presence of the satDNA on the S_9 chromosome was constant at the three Mediterranean coastal populations (Salobreña, Torrox, Fuengirola) but it was not present in the nine populations from the Segura river basin (see Table 1 and Figures 2 & 3). The satDNA is also present on the S_9 in Moroccan



Figures 2–4. Double FISH for satDNA (red) and rDNA (green) in testis cells of the grasshopper *Eyprepocnemis plorans*. *Figure 2*. Metaphase I from a male of the Benamor-1 population. *Figure 3*. Selected S_9 and S_{10} bivalents and X univalents from metaphase I spermatocytes, showing their most usual pattern (**a**) and the different patterns (**b**) observed for Torrox (S_9), Casa Mina (S_{10}) and Salmerón (X) populations. *Figure 4*. Selected S_{11} metaphase I bivalents (**a**–**d**) and spermatogonial mitotic chromosomes (**e**–**h**) showing the two repetitive DNAs in the same order as on the B chromosome (**a**: Benamor-1, **b**: Fuengirola) or the reverse (**c** & **d:** heteromorphic bivalents in Torrox). The size of the two homologues was frequently different in some males heterozygous for the proximally located supernumerary segment (**c**–**f**). The supernumerary segment was most frequently composed mainly of satDNA (compare **e** and **f**) but sometimes of similar amounts of satDNA and rDNA (compare **g** and **h**). All bars = 5 μm.

populations but not in a Caucasian population (Cabrero *et al*. 2003). This suggests that the S_9 chromosome, like S_{10}, lacked the satDNA in the ancestral condition, but gained satDNA in western and south Mediterranean populations. Hybridization signal size seemed to remain more or less constant in nearly all chromosomes, with the exception of the paracentromeric region of the S_{11} pair, where we observed variation for satDNA amount (Figure 4) which corresponded with the polymorphism for a supernumerary segment previously described for this chromosome (López-León *et al*. 1991, 1995b, Perfectti *et al*. 2000). This suggests that the supernumerary segment is composed mainly of satDNA.

Qualitative and quantitative differences in the satDNA content per chromosome may lead to genetic differentation and karyotypic evolution (Baimai 1998, Elisaphenko *et al*. 1998). The chromosomal locations that are conserved in all populations (L_1–M_3, S_{11}, X) could reflect the ancestral condition that has been maintained during the interpopulation divergence process. In fact, Caucasian specimens carry the satDNA in eight chromosome pairs (Cabrero *et al*. 2003), as do most Spanish specimens. A subsequent expansion

Table 1. Presence of satDNA (s) and rDNA (r) in the A chromosomes of the grasshopper *Eyprepocnemis plorans*.

	Chromosome												Total	
Population	L_1	L_2	M_3	M_4	M_5	M_6	M_7	M_8	S_9	S_{10}	S_{11}	X	s	r
Inland populations														
Ayna	sr	s	s	s	s	r	sr	r	r	r	sr	sr	8	8
Benamor-1	sr	s	s	s	s	r	s	r	r	r	sr	sr	8	7
Calasparra	s	s	s	s	s	s	r	r	r	r	sr	sr	8	6
Casa Mina	s	s	s	s	s	s	r	r	r	r	sr	sr	8	6
Chopillo	sr	s	s	s	r	s	r	s	r	r	sr	sr	8	7
Claras	s	s	s	s	s	s	r	r	r	r	sr	sr	8	6
Liétor	s	s	s	s	s	s	s		r	r	sr	sr	9	4
Salmerón	s	s	s	r	r	s	s	s	r	r	sr	sr	8	6
Socovos	s	s	s	s	r	s	r	s	r	r	sr	sr	8	6
Coastal populations														
Fuengirola	s	s	s	s	s	sr	sr	r	sr	r	sr	sr	10	7
Torrox	s	s	s	s	s	r	sr	r	sr	r	sr	sr	9	7
Salobreña	s	s	s	s	r	r	r	r	sr	r	sr	sr	7	8

of this sequence (except for chromosome S_{10}) could be achieved by homologous unequal crossing over, unequal sister chromatid exchange, non-homologous recombination, replication slippage or transposition (Butler & Metzenberg 1989, Cross & Renkawitz 1990, Murreu *et al*. 1990, Charlesworth *et al*. 1994). Remarkably, all four populations lacking B chromosomes (Ayna, Benamor-1, Claras and Socovos) bore satDNA in eight chromosomes, although this figure was not significantly different from the 8.38 ± 0.32 observed in the eight populations harbouring B chromosomes (Student $t = 0.80$, $df = 10$, $p = 0.44$).

Ribosomal DNA

FISH analysis of spermatocytes showed that rDNA was invariably present in the four chromosomes bearing active NORs (X, S_9, S_{10} and S_{11}) at all populations analysed, and was also observed in small paracentromeric clusters of most remaining chromosomes, although its presence varied among populations (Table 1 and Figures 2–4). Since the pTA71 probe includes the intergenic spacers, the possibility remains that some of these minor FISH signals do not correspond to true NORs. But this is unlikely in *E. plorans* because: (1) the same paracentromeric regions showing minor FISH signals are occasionally able to produce small nucleoli (as seen by silver impregnation) (Bakkali *et al*. 2001), and (2) these signals are also apparent when FISH is performed with a probe obtained by PCR amplification of the *E. plorans* rDNA unit containing ITSs but not intergenic spacers (Cabrero *et al*. unpublished).

The total number of chromosomes that hybridized with the rDNA probe showed variation among populations. The highest number of rDNA clusters (8) was observed in Salobreña (M_5–M_8, S_9–S_{11}, X) and Ayna (L_1, M_6–M_8, S_9–S_{11}, X) and the lowest one (4) in Liétor (S_9–S_{11}, X). This variation was also independent of the presence (6.38 ± 0.42) or absence (6.75 ± 0.48) of B chromosomes in the populations (Student $t = 0.55$, $df = 10$, $p = 0.60$). The variable number of rDNA sites observed is in agreement with the general asumption that NORs behave as very dynamic chromosome regions showing differences in chromosome location between species or populations of the same species (Schubert & Wobus 1985, Souza & Moreira-Filho 1995, Jankun *et al*. 2001, Castro *et al*. 2001). This variability could be accomplished by means of mechanisms similar to those described for satellite DNA.

The adjacent chromosome location of rDNA and satDNA might favour their combined interchromosomal expansion. However, no significant correlation was found between the population number of rDNA and satDNA clusters ($r = -0.28$, $t = 0.91$, $p = 0.38$). Also, in a Caucasian

population, the rDNA is limited to only two chromosome pairs whereas the satDNA is present in most A chromosomes (Cabrero *et al*. 2003). This suggests that spreading of rDNA genes in Spanish populations is more recent than that of satDNA.

In four inland populations, one or two chromosome types exhibited remarkably higher signal strength for rDNA in a specific chromosome (S_{10} in Casa Mina, Chopillo and Liétor; X chromosome in Salmerón; see Figure 3).

Interpopulation differences for chromosome distribution of both repetitive DNAs

Both matrices of geographical distances showed no association with the matrix of between-populations disagreement percentages (Mantel test: $Z = 0.103$, $p = 0.253$ for the along-the-river distances, and $Z = 0.123$, $p = 0.221$ for straight-line distances). In addition, the tests with only the nine populations of the Segura basin (inland populations) showed similar results (Mantel test: $Z = 0.196$, $p = 0.182$ for the along-the-river distances, and $Z = 0.250$, $p = 0.065$ for straight-line distances). Thus, the variation in chromosome presence for these two repetitive DNAs over the genome does not show a geographical pattern. This could be due to: (1) the existence of effective barriers to gene flow, which might be also an explanation for the absence of B chromosomes in populations from the headwaters of the Segura river basin (see Cabrero *et al*. 1997), (2) the occurrence of random expansion of the repetitive DNAs yielding different patterns in each population, (3) adaptation to an unknown environmental factor not related to geographical distance between populations, or (4) a combination of several of these factors.

Relative order of the two repetitive sequences

Double FISH provided useful information on the simultaneous presence of both DNA types as well as their relative position to the centromere. Both DNAs were always present in the S_{11} and X chromosomes of all populations, in addition to a variable number of loci on other chromosomes (L_1, M_6, M_7 and S_9) depending on the population considered (Table 1). Different B chromosome types are mainly composed of proximally located satDNA and distally located rDNA (López-León *et al*. 1994, Cabrero *et al*. 1999). The X chromosome showed the same order (Figure 3). Other chromosomes, e.g. L_1, M_7 and S_{11}, are polymorphic for the relative position of the two DNA sequences (Figures 2 & 4).

We have previously proposed that the B_2 chromosome (predominant in the Salobreña population) originated from the X chromosome, according to the coincident location of both repetitive sequences (López-León *et al*. 1994). The present study, considering more individuals and populations, might also point to other A chromosomes (L_1, M_7 and S_{11}) as possible sources for B chromosome origin. However, the polymorphic order of the two repetitive DNAs in these chromosomes does not favour them as candidates for B origin.

Supernumerary chromosome segment

In four populations (Salobreña, Torrox, Fuengirola and Chopillo) we have found individuals with the supernumerary chromosome segment located proximally on the S_{11} chromosome. This segment became evident after hybridization with the satDNA probe. A large FISH signal was observed in one member of the heteromorphic pair (Figure 4e, f). In the Fuengirola population, the extra segment of S_{11} seemed to result from increased amounts of both satDNA and rDNA (Figure 4g, h). Thus the heterochromatic supernumerary segment may be heterogeneous and the result of amplification of more than a single type of repetitive DNA.

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