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B chromosome prevalence and physical mapping of 18S rDNA and H4 histone sites in the grasshopper *Xyleus discoideus angulatus* (Romaleidae)

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ABSTRACT. We sampled 11 natural populations of the grasshopper *Xyleus discoideus angulatus* in Northeastern Brazil to analyze B chromosome frequency and meiotic behavior. We observed a single large B chromosome, resembling the X chromosome, in 29 of the 402 specimens. Eight of the 11 populations had B chromosomes, with a rather broad geographical distribution, suggesting that this is an ancient polymorphism; significant differences were observed in B chromosome prevalence among the populations. Presence of the B chromosome was associated with increased frequency of macrospermatids. Fluorescent *in situ* hybridization revealed 18S rDNA sites in the pericentromeric regions of the X and L_3 chromosomes, although some populations had an additional locus on the M_4 chromosome. No variation was found for chromosome location of H4 histone genes, which were always observed in paracentromeric regions of the L_2, M_4 and X chromosomes, a rather

unusual location compared to locations known from the families Acrididae and Proscopiidae. These B chromosomes lacked these two kinds of repetitive DNA, at least in amounts that can be visualized by fluorescent *in situ* hybridization, suggesting that these B chromosomes did not originate from any of the four chromosomes carrying rDNA or H4 histone genes.

Key words: Cytogenetics; FISH; Chromosome polymorphism; rDNA; Histone gene

INTRODUCTION

Some eukaryotic species have additional genetic elements known as B or supernumerary chromosomes, which are characterized by a non-Mendelian pattern of inheritance and absence of homology with other members of karyotypic complement. Although generally consisting of heterochromatin, they are not genetically inert elements, and their occurrence in the karyotype of some organisms can modify the frequency and distribution of chiasmas, normal spermatid production, fertility, and fecundity. In some cases, exophenotypical characteristics can also be affected (Jones and Ree, 1982; Camacho et al., 2000).

Regarding the origin of these extra elements, 2 hypotheses have been proposed. According to the first one, cytological and molecular studies support the idea that many B chromosomes originated from chromosomes of the diploid complement itself (intraspecific origin), arising through polysomies or centric fragments generated by chromosomal rearrangements (Teruel et al., 2009a,b). The second one suggests that B chromosomes can be derived from A chromosomes of closely related species via interspecific hybridization (Perfectti and Werren, 2001).

In the animal kingdom, approximately 80% of the species with supernumerary chromosomes are found in the class Insecta, especially in the orders Coleoptera, Diptera, and Orthoptera. In the latter, within the superfamily Acridoidea, the family Acrididae, which have been most intensively investigated, has a higher number of species with polymorphism of this genetic element, while, in Romaleidae family, this type of chromosome has been described only in *Zoniopoda tarsata* and *Xyleus angulatus* (= *Xyleus discoideus angulatus*) (Jones and Ree, 1982; Vilardi, 1986; Souza and Kido, 1995; Camacho, 2005).

X. d. angulatus is an endemic species from Northeast Brazil, which presents polymorphisms of B chromosomes. Because of its wide distribution through different ecosystems such as Caatinga, Cerrado, and Atlantic Forest (Carbonell, 2004), this grasshopper is regarded as an important organism for population studies concerning the extra genetic element. The karyotype of *X. d. angulatus* was first described by Mesa et al. (1982), with diploid number of 2n = 23, X0/2n = 24, XX. Subsequently, conventional and molecular cytogenetic techniques, such as C-banding, base-specific fluorochromes [chromomycin A_3/distamycin A/4,6-diamidino-2-phenylindole (CMA_3/DA/DAPI)], silver nitrate ($AgNO_3$) impregnation, and fluorescent *in situ* hybridization (FISH) with 45S rDNA probe were used in karyotypic analyses of heterochromatin and nucleolar organizer regions (Souza and Silva-Filha, 1993; Souza and Kido, 1995; Souza et al., 1998; Loreto et al., 2008).

This study aimed to better understand the evolutionary dynamics of B chromosomes in *X. d. angulatus*; meiotic chromosomes of different population were analyzed in order to evaluate the prevalence, distribution, and influence of B chromosome in the meiotic process in this species. Additionally, the location of 18S rDNA and H4 histone sites were determined.

MATERIAL AND METHODS

In this study, we analyzed 402 male individuals of the grasshopper *X. d. angulatus* (Stal, 1873) collected from 11 different locations in the State of Pernambuco, Brazil (Table 1).

Table 1. Different locations of the grasshopper *Xyleus discoideus angulatus* in the State of Pernambuco (Brazil).

Microregion	Locality	Number of individuals	Geographic coordinates	Altitude
Litoral	Cabo de Santo Agostinho (Gurjaú)	23	8°17'13"S 35°02'06"W	29 m
Zona da Mata	Goiânia (IPA)	33	7°33'38"S 35°00'09"W	13 m
	Nazaré da Mata	25	7°44'31"S 35°13'40"W	89 m
	Lagoa do Carro	60	7°50'41"S 35°19'11"W	128 m
	São Lourenço da Mata (EET)	15	8°00'08"S 35°01'06"W	58 m
Agreste	Bezerros	44	8°14'00"S 35°47'49"W	470 m
	Caruaru (PEPJVS)	58	8°17'00"S 35°58'34"W	800 m
	Gravatá	26	8°12'04"S 35°33'53"W	447 m
	João Alfredo	33	7°51'21"S 35°15'18"W	328 m
	Saloá (FB)	28	8°58'33"S 36°41'15"W	725 m
	Surubim	57	7°49'59"S 35°45'17"W	394 m

IPA = Instituto de Pesquisa Agrícola; EET = Estação Ecológica de Tapacurá; PEPJVS = Parque Ecológico Professor João Vasconcelos Sobrinho; FB = Fazenda Brejo.

The testes of the individuals were removed and fixed in Carnoy (ethanol and acetic acid, 3:1, v/v). For chromosomal analyses, slides were prepared using the classic testicular follicle squashing technique. The slides for conventional analysis were stained with 2% lacto-acetic orcein, while those used for $AgNO_3$ impregnation and FISH were prepared by adding one drop of 45% acetic acid. Slides without dye were covered with coverslips and immersed in liquid nitrogen.

Analysis of prevalence of B chromosomes

Chromosomal analysis and identification of individuals with or without B chromosomes was performed using conventional staining. For each individual, approximately 12 follicles were analyzed. Whether the prevalence of B chromosome differed among populations was determined using the χ^2 test.

The $AgNO_3$ impregnation was performed according to Rufas et al. (1983), with some modifications. Cytological preparations were pre-treated with 2X SSC at 60°C for 10 min and impregnated with aqueous silver nitrate solution. Slides were covered with coverslips and incubated in a moisture chamber at 70°C for 2 min.

Bright field images were obtained using a Canon Powershot A430 digital camera coupled to the ocular of an Olympus microscope. The images of the captured cells were adjusted for brightness and contrast in Corel Photo-Paint X4 (Corel Corporation).

FISH

Genomic DNA was extracted from the muscles of the saltatory legs of *X. d. angulatus* according to Aljanabi and Martinez (1997). Subsequently, the DNA was used to produce probes of 18S rDNA as described by Cabral-de-Mello et al. (2010) and the H4 gene as described by Pineau et al. (2005) by using universal primers. Probes were labeled with digoxigenin (dig-11-dUTP).

The FISH technique was carried out according to Moscone et al. (1996) in 10 indi-

viduals with B chromosomes. Slides containing cytological preparations were subjected to a series of alcoholic dehydration (70 and 96%). Subsequently, they were treated with RNase for 1 h at 37°C and pepsin for 20 min at 37°C. After each treatment, slides were washed with 2X SSC. Next, 1 µL probe was added to 9 µL hybridization mix (5 µL 100% formamide, 2 µL 50% dextran sulfate, 1 µL 20X SSC, 1 µL DNA blocking) and denatured at 75°C for 10 min. The hybridization solution was applied, and the slides were coated with 24 x 24-mm coverslips. The hybridization occurred in a moisture chamber at 37°C for 48 h. In the post-hybridization baths, slides were subjected to successive washes in 2X SSC at room temperature (approximately 25°C) for 5 min, 2X SSC at 42°C for 5 min, 0.1X SSC at 42°C for 5 min, and 2X SSC at room temperature for 10 min.

Sheep anti-digoxigenin (Roche 1,207,741) was used as the primary antibody and rabbit anti-sheep conjugated with FITC (Dako F0135) as the secondary antibody to detect hybridizations signals. After each antibody was applied, the slides were washed 3 times (5 min) with 1x PBS/0.1% Tween 20. Chromosomes were counterstained with DAPI and mounted with Vectashield (Vector Burlingame, CA, USA). Photomicrographs were obtained using a Leica DMLB epifluorescence microscope with a camera Leica DFC 340FX and the Leica CW4000 software. Subsequently, captured images of the cells were adjusted for brightness and contrast with the Adobe Photoshop CS5 software (Adobe Systems Incorporated).

RESULTS

The standard karyotype of *X. d. angulatus* consists of 22 acrocentric autosomes and an X0/XX sex determination system. In this species, a B macro-chromosome, being very similar to the X chromosome in terms of size (B is only slightly smaller than X), morphology (both are acrocentric), meiotic behavior (both are heteropycnotic), was observed in 29 of 402 karyotyped individuals (Figure 1a and b). Side-by-side or end-to-end associations were visualized between B and X chromosomes (Figure 1a), which were the most frequent at the beginning of meiosis (leptotene) but decreased in frequency from zygotene to metaphase I (Table 2).

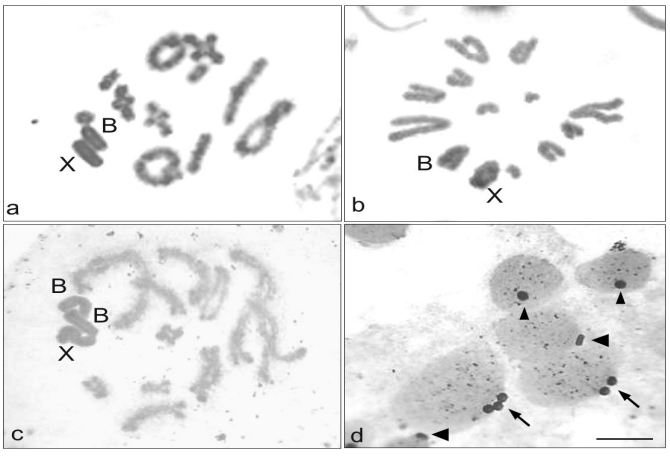

Figure 1. Conventional staining (**a-c**) and silver nitrate impregnation (**d**) in individuals with B chromosome. **a.** Diplotene presenting associated side-by-side between chromosomes X and B. **b.** Metaphase II. **c.** Pachytene with two B chromosomes of the individual from Lagoa do Carro. **d.** Normal spermatids with one centriole adjunct (arrowheads) and abnormal (arrows) with two or more centriole adjuncts. Bar = 5 µm.

Table 2. Frequency of association between X and B chromosomes of *Xyleus discoideus angulatus* in different stages of male meiosis.

	Leptotene		Zygotene		Pachytene		Diplotene		Diakinesis		Metaphase I	
	X+B	X/B	X+B	X/B	X+B	X/B	X+B	X/B	X+B	X/B	X+B	X/B
Number of cells	735	284	354	195	373	248	333	323	149	259	68	118
%	72.1	27.8	64.5	35.5	60.1	39.9	50.8	49.2	36.5	63.5	36.6	63.4

X+B = association; X/B = no association. Data obtained from six 1B individuals.

The distribution and prevalence of B chromosomes were analyzed in 11 different populations from 3 distinct regions, on the basis of climate and vegetation: Litoral, Zona da Mata, and Agreste. The B macro-chromosome was found in 8 populations (Table 3), and 28 of the 29 B-carrying individuals had a single B chromosome. One individual from the Lagoa do Carro population showed intra-individual variation for the number of B chromosomes, suggesting that the B chromosome was mitotically unstable in this individual. Separate analysis of 14 testis follicles showed the intra-follicular stability but inter-follicular variation of B number, since 3 follicles lacked B chromosomes, whereas 5 carried 1B and 6 carried 2B (Figure 1c). Prevalence of B chromosomes ranged from 0 to 26.67% (São Lourenço da Mata - EET) (Table 3), with significant differences among populations ($\chi^2 = 25.68$, d.f. = 10, P = 0.0042).

Table 3. Prevalence of B chromosome in eleven populations of *Xyleus discoideus angulatus* from different localities of the State of Pernambuco.

Microregion	Locality	0B individuals	1B individuals	Total	Prevalence (%)
Litoral	Cabo de St. Agostinho (Gurjaú)	23	0	23	0.0
Zona da Mata	Goiânia (IPA)	32	1	33	3.03
	Nazaré da Mata	25	0	25	0.0
	Lagoa do Carro	53	7[a]	60	11.67
	São Lourenço da Mata (EET)	11	4	15	26.67
	Bezerros	38	6	44	13.64
	Caruaru (PEPJVS)	55	3	58	5.17
Agreste	Gravatá	22	4	26	15.38
	João Alfredo	30	3	33	9.09
	Saloá (FB)	27	1	28	3.57
	Surubim	57	0	57	0.0
Total		373	29	402	7.21

IPA = Instituto de Pesquisa Agrícola; EET = Estação Ecológica de Tapacurá; PEPJVS = Parque Ecológico Professor João Vasconcelos Sobrinho; FB = Fazenda Brejo. [a]One individual showed inter-follicular variation of 0-2 B chromosomes.

B-carrying males showed the presence of abnormal spermatids (macrospermatids), and silver nitrate impregnation revealed that macrospermatids had 2 or more centriolar adjuncts, unlike normal spermatids that show a single centriolar adjunct (Figure 1d). To investigate whether B chromosome presence is associated with a higher frequency of macrospermatids, we analyzed five individuals from the Lagoa do Carro population, each having a different class of karyotype (0B and 1B). Additionally, the frequency of macrospermatids was analyzed in follicles 2B of the male showing inter-follicular variation for B chromosome number (0B-2B). Results showed that individuals without B chromosomes had lower rate of abnormal spermatids (2.39%) compared with the frequency found in carriers with an extra element (1B = 7.44%; 0B-2B = 11.49%).

Physical mapping of 18S rDNA and H4 histone genes performed using FISH in 10 individuals carrying B chromosomes revealed the presence of 2 different patterns for 18S rDNA location: 4 individuals presented 2 loci in the pericentromeric regions of the X chromosome

and the L_3 bivalent (Figure 2a), whereas 6 individuals additionally carried a third locus in the pericentromeric region of the M_4 bivalent (Figure 2b). One individual was heterozygous for the rDNA locus on the L_3 bivalent (Figure 2c). Physical mapping of H4 histone genes revealed their presence in the pericentromeric region of L_2 and M_4 bivalents, as well as in the X chromosome, in all individuals analyzed (Figure 2d). B chromosomes, however, apparently lacked rRNA and histone genes.

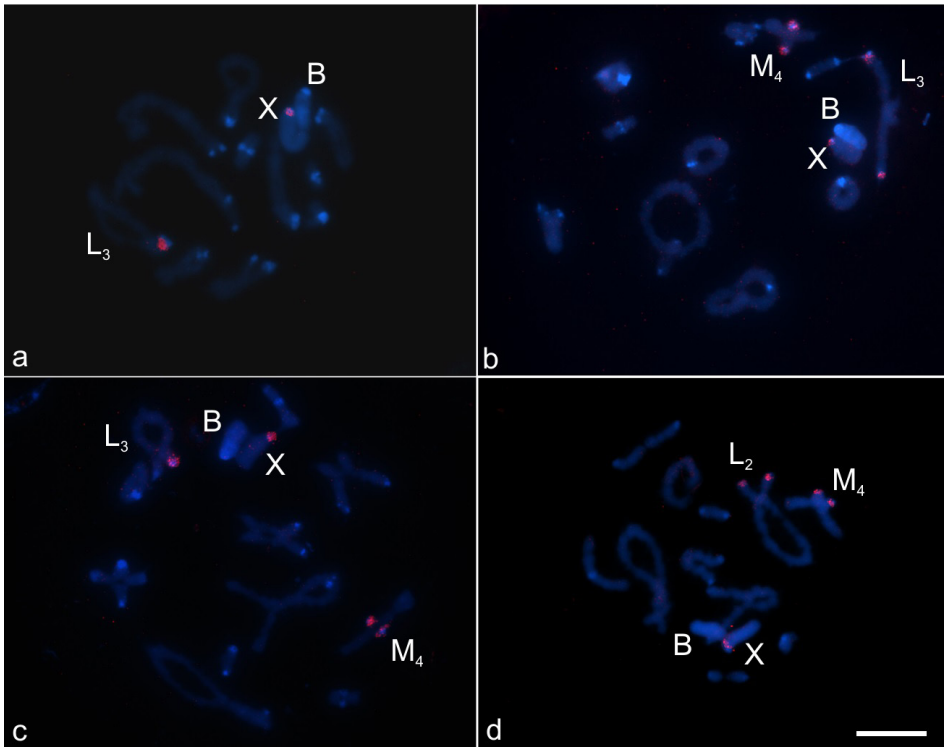

Figure 2. FISH using the 18S rDNA (**a-c**) and H4 histone (**d**) probe. **a.** Cell with two markers, one in the L_3 bivalent and the other on the X chromosome; **b.** cell with three sites located in the L_3, M_4 bivalents and X chromosome; **c.** heteromorphism to the site present in the L_3 bivalent; **d.** H4 histone sites present in the L_2, M_4 bivalent and X chromosome. Bar = 5 µm.

DISCUSSION

The occurrence of B chromosomes in *X. d. angulatus* was first described by Souza and Kido (1995) in a female population from Igarassu (Pernambuco, Brazil), where a partially heterochromatic nature of the extra element was identified by C-banding. Additionally, numerical instability of B chromosomes was found in females, probably due to a meiotic mechanism of accumulation. On the other hand, males analyzed in our study showed no numerical variation of B chromosomes, indicating meiotic stability characterized by the absence of such accumulation mechanisms.

B chromosomes were present in 8 of 11 populations of *X. d. angulatus*; this indicated wide distribution of these extra elements in the populations from Pernambuco State. Due to

the physical distance among collection areas and the fact that *Xyleus* species have low flight ability (Carbonell, 2004), a recent occurrence of gene flow among populations is very unlikely. Thus, this wide distribution of B chromosomes might be the result of an ancient polymorphism, occurring in an initial population that dispersed with the migration of individuals to other habitats.

The occurrence of significant differences in the prevalence of B chromosomes could be related to the different characteristics of the habitats sampled, since the carrier populations are divided into 3 climate zones, having characteristic features and differences in climate and vegetation. However, no correlation between prevalence of B chromosome and environment of the populations was observed. According to Camacho et al. (2000), the prevalence of B chromosomes does not depend exclusively on ecological factors; other factors such as historical events (number of generations since the appearance of this element), transmission (intensity of accumulation among populations), and random events (genetic fluctuations experienced by finite-sized populations) that occur in natural populations need to be considered as well. Therefore, the variation in prevalence observed in this study might be affected by these 3 factors either independently or simultaneously.

Our data suggest that the increase of macrospermatid frequency might be attributed to different events, such as inhibition of cytokinesis and cell or nuclear fusion that can be induced by the presence of B chromosomes. The frequency values of abnormal spermatids are similar to those observed in other grasshopper species, such as *Sphingonotus coerulans* (3-14%) (Gosálvez et al., 1985), *Metaleptea brevicornis adspersa* (8.59%) (Bidau, 1986), and *Dichroplus pratensis* (3-10%) (Bidau, 1987). The ability of B chromosomes to induce the production of abnormal spermatids affects the fitness of carriers, since its actual effect on the process of spermiogenesis can be significant. On the other hand, a diploid spermatid with 2 centriolar adjuncts implies in the elimination of 2 possible normal spermatids, thereby affecting the fertility of the carrier (Bidau, 1987). Moreover, the absence of microspermatids in individuals with B chromosomes indicates the maintenance of the extra element in the species, since B chromosomes will not be eliminated during the meiotic division in the form of micronuclei (microspermatids).

Similarities between B and X chromosomes on the meiotic behavior, size, morphology, and heteropicnosis were also observed in *X. d. angulatus*. According to Camacho et al. (2000), this type of similarity and association between B and X chromosomes are frequently noted in different species. In most cases, this situation occurs due to the origin of B chromosomes, which can have the sex chromosome as an ancestor. An example of this derivation is the B_2 chromosome of *Eyprepocnemis plorans* (López-León et al., 1994), where repetitive DNA and rDNA sequences are located respectively at the proximal and distal regions as in X chromosome.

However, FISH using probes of 18S rDNA and H4 histone in *X. d. angulatus* reinforces the idea that the occurrence of similarities between B and X chromosomes, besides a strong meiotic association observed during prophase I, may not be the result of the origin of B chromosomes from X. This is because both 18S rDNA and H4 histone are present in the pericentromeric region of X and absent in B chromosomes. Additionally, C-banding and fluorochrome staining with CMA_3/DA/DAPI showed different patterns of heterochromatic block distribution and composition among these chromosomes (Souza et al., 1998; Loreto et al., 2008).

Rebollo et al. (1998) suggested another hypothesis to explain the similarities between X and B chromosomes. According to the authors, univalent chromosomes need strategies to

remain in the genome, preventing the occurrence of errors during chromosome orientation in the equatorial plate (metaphase I) and increasing their efficiency of transmission to daughter cells. Therefore, the achiasmatic association observed between B and X chromosomes in *X. d. angulatus* might have contributed to the transmission of the extra element with its consequent permanence in the genome.

Interindividual variation in the number of 18S rDNA sites was also observed by FISH, showing 2 patterns of distribution: individuals with 2 and 3 sites. Mechanisms, such as translocation and transposition, have been responsible for the dispersion of ribosomal sequences in the genomes and have been described for other grasshopper species (Cabrero and Camacho, 2008; Loreto et al., 2008). However, heteromorphism of the 18S rDNA is not uncommon in grasshoppers and variability in size, number, and location of sites has been described in several species (Souza and Moura, 2000; Cabral-de-Mello et al., 2010). Unequal crossing-over and deletions involving segments of chromosomes are usually considered as mechanisms that are responsible for the structural modifications of NORs (Carvalho and Dias, 2007). One of these events is probably responsible for the loss of the rDNA site in one of the homologues of the L_3 bivalent in the heterozygous individual of *X. d. angulatus.*

In *X. d. angulatus*, FISH revealed that H4 histone sites were located on the L_2 and M_4 bivalents and X, an unusual situation for most of the grasshoppers that have such genes located on a single site, as revealed by Cabrero et al. (2009) in Acrididae species. They found that 21 of 35 species studied of Acrididae had a karyotype 2n = 23, X0, and only a H3 site, usually in the interstitial region of chromosome 8, was identified in an autosome. Additionally, a double FISH with probes for H3 and H4 genes was performed in 11 randomly chosen species, and the results revealed that, in all cases, both genes were co-located. The same result was found for the grasshopper *Locusta migratoria* by Teruel et al. (2010), in which the H3/H4 site was located on chromosome 8. Cabral-de-Mello et al. (2011) analyzed H3 sites in 4 species of the family Proscopiidae and revealed the existence of a single site, located on the pair 4, unlike that observed in representatives of the family Acrididae. These differences found among representatives of the families Acrididae and Proscopiidae and the species *X. d. angulatus* should be associated with chromosomal rearrangements. However, further studies are needed to determine the nature of these variations.

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