

The U2 snDNA Is a Useful Marker for B Chromosome Detection and Frequency Estimation in the Grasshopper *Abracris flavolineata*

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Abstract

In this study, we describe a strategy to determine the presence of B chromosomes in the living grasshopper *Abracris flavolineata* by FISH using U2 snDNA as a probe in interphase hemolymph nuclei. In individuals without B chromosomes, (0B) 2 dot signals were noticed, corresponding to A complement U2 snDNA clusters. In +1B and +2B individuals, 4 or 8 additional signals were noticed, respectively. In all cases, the absence or presence of 1 or 2 B chromosomes correlated in hemolymph and in somatic or germline tissues, validating the efficiency of the marker. Our data suggest that the B chromosome of *A. flavolineata* is present in all somatic tissues. B-carrying individuals showed the same number of B chromosomes in germ and somatic cells, suggesting that the B is mitotically stable. The marker was used to compare B chromosome frequency in the analyzed population with a sample collected previously, in order to test for B frequency changes and differences of B chromosome prevalence among sexes, but no statistically significant differences were noticed. The identification of living animals harboring B

chromosomes will be very useful in future studies of B chromosome transmission, as well as in functional studies involving RNA analysis, thus contributing to the understanding of evolutionary history and the possible role of the B chromosome in *A. flavolineata*.

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Supernumerary or B chromosomes occur in addition to the standard chromosome complement (A chromosomes) in approximately 15% of eukaryotic species. In most species, they are characterized by enrichment in repetitive DNAs, lack of meiotic pairing with chromosomes of the standard complement, and non-mendelian inheritance patterns [Camacho, 2005; Houben et al., 2013, 2014]. One of the most important parameters that influence B chromosome evolution is its transmission rate along generations, but it has been determined in only a few species [Jones and Rees, 1982; Camacho, 2005]. This analysis is difficult due to the need for the maintenance of living animals after determining the presence of B chromosomes. To facilitate this analysis and thus the possibility of performing controlled crosses, it is necessary to develop markers to detect B chromosomes in living animals without reducing the animals' reproductive success [Cabrero et al., 2006].

Some individuals of the grasshopper *Abracris flavolineata* (Ommatolampidinae, Acrididae) harbor 1 or 2 submetacentric B chromosomes in individuals sampled at the Rio Claro/SP population [Bueno et al., 2013]. The B chromosome of this species has been studied to estimate its frequency, and several repetitive DNA sequences have been mapped trying to unveil its origin and evolution [Bueno et al., 2013; Milani and Cabral-de-Mello, 2014; Palacios-Gimenez et al., 2014; Menezes-de-Carvalho et al., 2015]. In the 65 individuals studied by Bueno et al. [2013], sampled between August, 2011, and August, 2012, the B chromosome frequency was 30.7%, with only a slight difference between males (31.5%) and females (29.7%). Concerning the B chromosome content for repetitive DNA, it has been shown that they contain some microsatellites and transposable elements, but the most remarkable marker found was the presence of 4 clusters of U2 snDNA in the B chromosome, contrasting with the single cluster present in A chromosomes, specifically in the longest autosome [Bueno et al., 2013].

Here, we describe a strategy to determine the presence of B chromosomes in live animals without compromising their life and reproductive capacity, as previously used in the grasshopper *Eyprepocnemis plorans* [Cabrero et al., 2006]. Our assay is based on obtaining hemolymph cells followed by FISH using U2 snDNA as a probe. To evaluate this strategy, we used it for B chromosome frequency estimation in individuals from the same population previously analyzed, to test for possible temporal changes in B chromosome frequency, besides differences of B chromosome prevalence between sexes.

Materials and Methods

A total of 86 adult individuals, 37 males and 49 females, of *A. flavolineata* were collected in Floresta Estadual Edmundo Navarro de Andrade and in the Campus of UNESP-Universidade Estadual Paulista, Rio Claro/SP-Brazil, and were analyzed to estimate the B chromosome frequency. These locations are the same as the previous sampling from Bueno et al. [2013]. Each animal was numbered and maintained in plastic boxes until the experiments were performed. A drop of hemolymph was obtained from the pleura of the thorax in the base of the third pair of legs using a needle [Cabrero et al., 2006]. The hemolymph was aspirated using a pipette and spread onto a clean slide. After drying, the slides were immersed in modified Carnoy's solution (3:1 absolute ethanol:acetic acid) for 15 min for proper material preservation.

Probes for U2 snDNA were obtained from genomic DNA of *A. flavolineata* using the primers described by Bueno et al. [2013] through polymerase chain reaction (PCR). The sequence was PCR-labeled using digoxigenin-11-dUTP (Roche, Mannheim, Germany). The FISH experiments were conducted with modifications proposed by Cabral-de-Mello et al. [2010]. Approximately

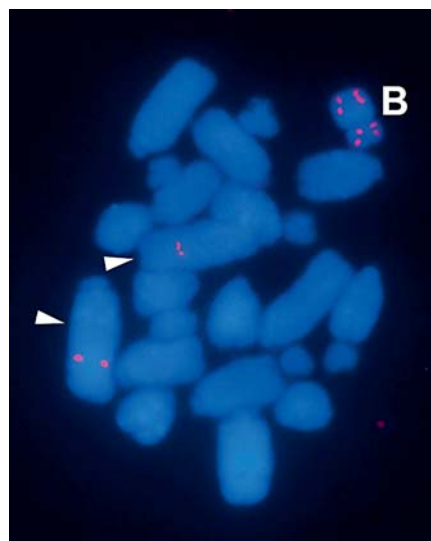


Fig. 1. Embryo mitotic metaphase chromosomes from a male showing the pattern of hybridization of U2 snDNA. Arrowheads show pair 1. Note the 4 clusters in the B chromosome.

100 ng of the probe was used, and the detection was undertaken using anti-digoxigenin rhodamine (Roche). The chromosomes were counterstained using DAPI and slides mounted in Vectashield (Vector, Burlingame, CA, USA). The chromosomes and signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. The images were captured using a DP70 cooled digital camera in gray scale and processed using Adobe Photoshop CS2.

To validate the efficiency of the marker, in addition to determining B chromosome presence/absence in hemolymph using FISH, we checked 2 tissues by conventional analysis using Giemsa (5%). This confirmation was done by the analysis of somatic and germ cells of females and males, respectively. For males, the meiotic cells were obtained from the testes, and for females, mitotic metaphase cells were obtained from gastric caeca following the protocol of Castillo et al. [2010].

After B chromosome estimation (prevalence and mean), the χ^2 test was performed to compare the frequency of the present sample and the sample obtained by Bueno et al. [2013], by means of a contingency table, using the R (R Core Team, <http://www.R-project.org/>). The prevalence of B chromosome was compared between the 2 samples, between males and females from the same sample, and among the same sex in the 2 samples.

Results and Discussion

Based on the presence of multiple clusters (4) of U2 snDNA in the B chromosome of *A. flavolineata* and its restriction to the largest chromosome pair in the A complement (Fig. 1), with 1 cluster per homolog, we expected to determine the B chromosome number on the basis of

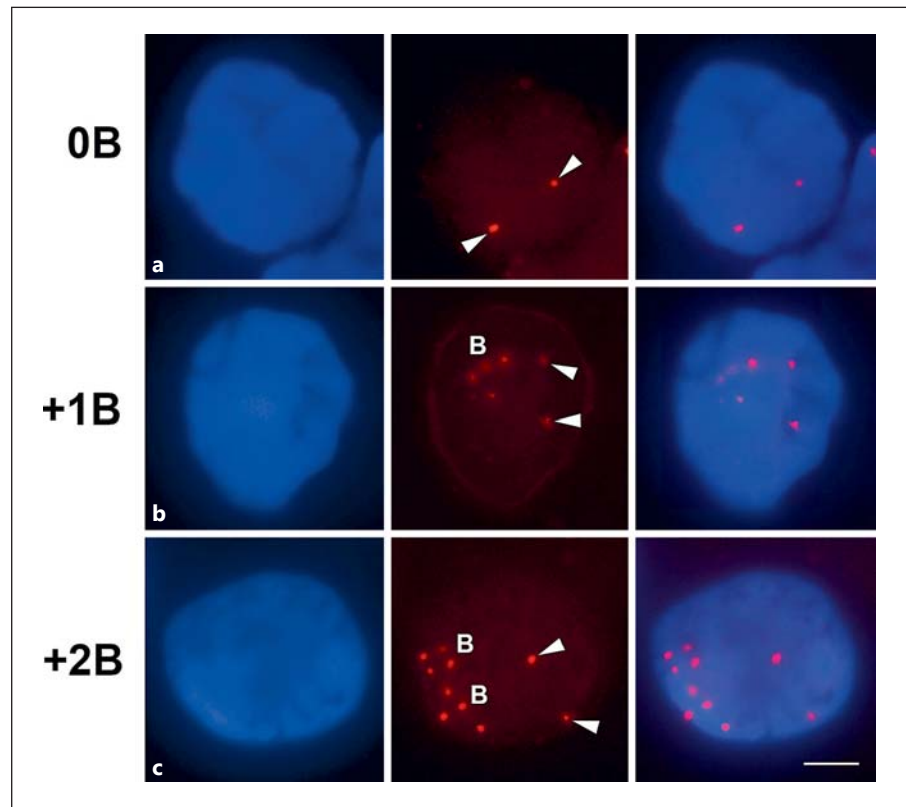


Fig. 2. Interphase hemolymph nuclei from individuals without (0B) and with B chromosomes (+1B and +2B) revealing the distinct patterns of hybridization with the U2 snDNA probe. Arrowheads show the signals from pair 1. Note the difference in signal amounts for each condition. Scale bar, 5 μ m.

the number of FISH signals for U2 clusters in hemolymph interphase nuclei. Consistently, in animals lacking B chromosomes (0B), only 2 dot signals were noticed (Fig. 2a) corresponding to the A complement U2 snDNA signals, whereas the individuals harboring 1 B chromosome (+1B) showed 6 signals (Fig. 2b). In animals with 2 B chromosomes (+2B), 8 additional signals were identified (Fig. 2c). In a previous study in the *E. plorans* grasshopper, Cabrero et al. [2006] suggested that the hemolymph is the best material to analyze living grasshoppers for the presence of B chromosomes using C-banding and fluorochrome staining. Here, we demonstrate that this type of cell is also useful for FISH experiments, allowing a reliable identification of the B chromosome and scoring the number of B chromosomes per nucleus.

Among the 86 individuals studied, we confirmed the accuracy of the marker to recognize animals harboring B chromosomes in 42 of them using the hemolymph cells by the analysis of testes and gastric caeca using standard staining. In fact, all males lacking B chromosomes in the testis showed 2 U2 dots in hemolymph nuclei submitted to FISH, whereas males carrying 1B showed 6 dots, and those carrying 2B showed 10 dots (Table 1). Likewise,

Table 1. Number of males and females with absence or presence of B chromosomes

	Individuals, <i>n</i>		
	0B (2 dots)	1B (6 dots)	2B (10 dots)
Males	32	4	1
Females	37	10	2
Total	69	14	3

0B, without B chromosomes; 1B, 1 B chromosome; 2B, 2 B chromosomes. Dots observed in FISH analysis in hemolymph nucleus.

hemolymph nuclei showed 2 U2 dots in females lacking B chromosomes in the gastric caeca, but 6 dots in those from 1B females (Table 1). The consistency of the B number between different tissues of a same individual (hemolymph and testis, and hemolymph and gastric caeca) suggests that the B chromosome is mitotically stable, thus being present at equal numbers in the germ and somatic lines. Bueno et al. [2013] documented an intraindividual

Table 2. Prevalence and mean of B chromosomes in *Abracris flavolineata*

	Individuals, <i>n</i>				Prevalence	Mean
	0B	1B	2B	Total		
Present sample						
Males	32	4	1	37	13.5%	0.16
Females	37	10	2	49	24.5%	0.29
Total	69	14	3	86	19.7%	0.23
Bueno et al., 2013						
Males	26	7	5	38	31.5%	0.45
Females	19	6	2	27	29.7%	0.37
Total	45	13	7	65	30.7%	0.41

0B, without B chromosomes; 1B, 1 B chromosome; 2B, 2 B chromosomes.

variation of the B chromosome number in meiotic cells of *A. flavolineata*, suggesting mitotic instability. Our present results indicate the contrary, so that mitotic instability of this B chromosome needs revision. As in *A. flavolineata*, the presence of B chromosomes in somatic tissues and germline cells was documented in the grasshopper *E. plorans* [Cabrero et al., 2006] and the plant *Puschkinia libanotica* [Vernon and Witkus, 1970], contrasting with B chromosomes that are not present in all somatic tissues, such as those in *Aegilops speltoides* [Mendelson and Zohary, 1972], and other B chromosomes which are restricted to the germline, as reported in the marsupial *Echymipera kalabu* [Hayman et al., 1969]. The occurrence of B chromosomes in the hemolymph, suggesting B chromosome presence in all somatic tissues, indicates that the B chromosomes are not eliminated from the somatic line, as also noticed in *E. plorans* [Cabrero et al. 2006], but other specific tissues should be investigated. Moreover, it would be interesting to analyze possible effects caused by the presence of B chromosomes in *A. flavolineata*.

Considering the frequency estimation among the 86 animals analyzed, 14 harbored 1, and 3 harbored 2 B chromosomes, corresponding to about 19.7% of the sample. The frequencies of B chromosomes were higher in females than in males, being about 24.5% (12 individuals) and 13.5% (5 individuals), respectively. In all cases, the frequency of B chromosomes is lower than described by Bueno et al. [2013], i.e., 30.7% considering both sexes, 31.5% in males and 29.7% in females (Table 2). Although we observed differences between B prevalence in the distinct samples and between sexes, they were not statistically significant as shown in the following: prevalence be-

tween the distinct samples ($\chi^2 = 1.86$, $p = 0.172$), prevalence between males and females in Bueno et al. [2013] ($\chi^2 = 0.028$, $p = 1$) and in the present sample ($\chi^2 = 0.984$, $p = 0.321$), prevalence among males in Bueno et al. [2013] and the present sample ($\chi^2 = 2.53$, $p = 0.111$), and prevalence among females in Bueno et al. [2013] and the present sample ($\chi^2 = 0.046$, $p = 0.829$). These data also indicate that there is no sex-specific mechanism for B accumulation in *A. flavolineata*. The fluctuation of B chromosome frequency in natural populations throughout the years was studied in a few species, i.e., in the grasshoppers *E. plorans* [Camacho et al., 2015], *Myrmeleotettix maculatus* [Shaw, 1983], and *Acrida lata* [Kayano et al., 1970], in the fish *Prochilodus lineatus* [Cavallaro et al., 2000], and the wasp *Trypoxylon albirtase* [Araújo et al., 2002]. In some species, the frequency remained constant throughout the years, while in other ones this frequency rapidly was changed, as in *P. lineatus* and *T. albirtase* caused by accumulation mechanisms or advantage for host. For *A. flavolineata* the fluctuation in frequency has no statistical significance and could not be clearly attributed to any specific factor, occurring randomly, as in genetic drift. The collection and B chromosome frequency estimation throughout other years will clarify this issue.

As demonstrated in other species, such as *E. plorans* [Cabrero et al., 2006], *Nasonia vitripennis* [Nur et al., 1988], *Partamona helleri* [Tosta et al., 2004], and in maize [Gourmet and Rayburn, 1996], the development of a strategy to detect the B chromosome would be useful to study the evolutionary dynamics of this element. Our strategy is based on a simple method well established in cytogenetic laboratories, i.e., FISH. Determining the presence of B chromosomes permits the accurate description of the number of B chromosomes per individual. Although some information has accumulated concerning the composition of the B chromosome in *A. flavolineata* [Bueno et al., 2013; Milani and Cabral-de-Mello, 2014; Palacios-Gimenez et al., 2014; Menezes-de-Carvalho et al., 2015], no studies of the transmission rate were performed in this species, and the absence of markers to detect B chromosomes in live animals poses a problem for analysis. In this way, the identification of living male and female grasshoppers harboring B chromosomes, without the need to kill them or severely injure their viability, will be very helpful in future studies involving controlled crosses focused on determining the B chromosome transmission rate and also in functional studies involving RNA analysis. Finally, the use of U2 snDNA in mitotic or meiotic chromosomes or in the interphase hemolymph nucleus will be useful to analyze spatial and temporal fre-

quency fluctuations of this B chromosome variant, thus, contributing to the understanding of evolutionary patterns and the possible biological role of this B chromosome in *A. flavolineata*.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare no conflicts of interest.

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