Supernumerary Chromosome Variants in Dichroplus elongatus (Acrididae): Fluorescent Banding and Cline Variation Pattern

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Supernumerary chromosome variants in Dichroplus elongatus (Acrididae): fluorescent banding and cline variation pattern

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Abstract

Dichroplus elongatus is a South American grasshopper that shows simultaneous polymorphisms for B chromosomes and several supernumerary segments located in chromosome pairs S10, S9, and M6 (SS10, SS9 and SS6 respectively), in natural populations from Argentina. Heterochromatin characterization with DAPI-CMA_3 banding reveals that B chromosomes show an interstitial GC-rich band. The distal SS10 segment seems to also be GC-rich, whereas the proximal supernumerary segments (SS6 and SS9) show two different heterochromatin regions: one GC-rich region (CMA_3+/DAPI-) and the other that has no specificity to AT or CG base pairs (CMA_3/DAPI dull). The results obtained permit us to distinguish two types of constitutive heterochromatin in the supernumerary segments of *D. elongatus*. The variation in the frequency of these chromosome polymorphisms was analysed with respect to some climatic and geographic variables. There is considerable B chromosome variation between populations, and this variation correlates negatively with latitude and positively with altitude and mean annual temperature. The incidence of the proximal SS9 segment is positively correlated with altitude. The pattern of chromosome variation is discussed in relation to the population's ability to maintain supernumerary DNA in more climatically favorable geographic regions.

Key words

B chromosomes, supernumerary segments, CMA_3/DAPI banding, clinal pattern

Introduction

The evolution of many Orthoptera groups is associated with chromosome rearrangements and in several cases, variation in the amount of heterochromatin has played an important role in these chromosome changes.

Constitutive heterochromatin can be observed in the genome during the cell cycle of all types of cells as C or fluorescent bands, which occur in different parts of individual chromosomes (Shibata *et al*. 1999). The pattern of heterochromatic bands is detected as differences in the number of bands and/or the amount of heterochromatin (Vershinin *et al*. 1996). Moreover, in Orthoptera, as in other animal and plant species, the genome also contains heterochromatic supernumerary elements. These chromosome variants are dispensable elements for the normal growth and development of the organism and may occur as B chromosomes or supernumerary segments.

B or supernumerary chromosomes are generally heterochromatic, fail to pair with the normal member of the complement (A chromosomes) during meiosis (unlike most chromosome mutations) and often show accumulation (*i.e.*, drive) and follow their own evolutionary pathway (Jones & Rees 1982, Beukeboom 1994, Camacho *et al.* 2000). Supernumerary segments are usually heterochromatic elements integrated into the normal complement and according to their position within the involved chromosome, are usually nominated as distal, interstitial, proximal or extrinsic (Hewit 1979, John 1981). Simultaneous polymorphisms for B chromosomes and supernumerary segments were found in many grasshopper species (Hewitt 1979, Colombo 1989, Cabrero *et al.* 1997, Perfectti *et al.* 2000).

The DNA composition of heterochromatic regions has been intensively investigated in order to explain their genetic properties. Common procedure involves the use of different fluorochromes able to differentiate DNA regions based on their nucleotide composition. Notorious examples include 4'-6-diamidino-2-phenylindole (DAPI), which highlights AT-rich regions, and chromomycin A3 (CMA_3), which shows preference for GC-rich regions (Sumner 2003). These techniques have been used to cytogenetically analyze both intra- and interspecific differences in several Orthoptera species (Rufas *et al.* 1988; Bella *et al.* 1990, 1991, 1993; Lopez Fernandez *et al.* 1989, 1992; Loreto & de Souza 2000).

The simultaneous analysis of B chromosomes and supernumerary segments variation in different populations offers the opportunity to analyze patterns of chromosome variation in nature. *Dichroplus elongatus* (Orthoptera: Acrididae) is a South American grasshopper that shows simultaneous polymorphisms for B chromosomes and several supernumerary segments located in the chromosome pairs S10, S9, and M6 in natural populations from Argentina (Remis & Vilardi 1986, Loray *et al*. 1991, Clemente *et al*. 1994). Both forms of supernumerary heterochromatin were found to influence chiasma frequency and distribution (Loray *et al*. 1991, Clemente *et al*. 1994). Moreover, B chromosomes also affect male fertility by increasing the production of macro- and microspermatids (Loray *et al*. 1991). They also affect other fitness components, such as mating success, viability and female fecundity (Rosetti *et al*. 2007, 2008).

Previous population cytogenetic studies on the grasshopper *D. elongatus* demonstrated different degrees of differentiation among populations for each heterochromatic variant (Remis *et al.* 1998, 2004). Furthermore, the hierarchical analyses of molecular differentiation at the mitochondrial DNA level, an essentially neutral marker, revealed a discrepancy with respect to chromosome differentiation and suggested that the pattern of B chromosomes might not be explained by historical factors (Clemente *et al*. 2000). These results suggest that the pattern of chromosome variation cannot be explained by the interaction between genetic drift and migration.

In the present work, we characterize different chromosome variants using fluorescent banding and analyze the pattern of simultaneous variation in relation to climatic and geographical factors in natural populations of *D. elongatus* from eastern Argentina.

Material and Methods

Biological material.—The standard karyotype of *D. elongatus* is composed of 22 telocentric autosomes and a XO/XX sex-determination system (Remis & Vilardi 1986). Natural Argentinean populations of this species are polymorphic for mitotically unstable B chromosomes and supernumerary segments in M6, S9 and S10 pairs (SS6, SS9 and SS10 respectively). Adult males of this grasshopper were collected from the Argentinean natural populations in Raco and Horco Molle from Tucumán province, Venado Tuerto located in Santa Fé province, Gualeguaychu and Colón from Entre Ríos province, and Mocoretá located in Corrientes. Specimens were collected during the years 2002, 2003, 2004, 2005 and 2007.

Chromosome preparations.—Males were dissected, and their testes fixed in 3:1 ethanol: acetic acid and stored at -20°C. Conventional chromosome preparations were performed by squashing several follicles in propionic hematoxylin (Remis & Vilardi 1986). A minimum of 10 meiotic cells per individual were examined to determine chromosome constitution.

Fluorescent staining with CMA_3 (chromomycin A3) and DAPI (4′-6-diamidino-2-phenylindole) was carried out on male individuals from Raco, Horco Molle and Venado Tuerto populations. For this, a few testes follicles were squashed in 45% acetic acid; the coverslip was then removed using the dry-ice method, and the slide air dried. Sequential DAPI-CMA_3 banding was performed, using the technique described by Rebagliati *et al.* (2003).

Photographs.—The best preparations were photographed using an analog Leica Wild MPS52 camera on a Leica DMLB fluorescence microscope, with filters for DAPI and CMA_3. Images were also taken using Kodak color ASA400 film and processed with Adobe Photoshop version 7.0.

Geographic chromosome pattern analysis.—B chromosome frequencies were transformed according to Christiansen *et al.*'s (1976) formula:

$$X_i = (p_1 - p_0)\sqrt{\left(\frac{N_1}{p_0(1-p_0)}\right)}$$

where p_0 is the mean B-chromosome frequency, p_1 represents the B-chromosome frequency in the i^{th} population and N_1 is the number of individuals sampled per population. This transformation normalizes the distribution and weights sampling sizes.

The relationships between chromosome frequencies and geographical (latitude, altitude and longitude) and climatic (mean annual temperature) variables were analyzed by means of nonparametric correlation using the STATISTICA program (STATISTICA STATSOFT Inc. 1996). Bonferroni's correction for multiple testing was applied.

Temperature data were obtained using the Local Climate Estimator from www.fao.org/sd/dim_en3/en3_051002_en.

Results

Chromosome polymorphisms.—Frequencies of simultaneous polymorphisms for B chromosomes and supernumerary segments in the chromosome pairs M6 (SS6), S9 (SS9) and S10 (SS10) were analyzed in four populations of the grasshopper *D. elongatus* from Eastern Argentina, together with their incidences. They are summarized in Table 1.

Fluorescent banding patterns.—Sequential banding with CMA_3/DAPI revealed positive CMA_3 (CMA_3+) and negative DAPI (DAPI-) bands in most of the autosomes. The comparison of the two different fluorochrome banding patterns showed that CMA_3+ and DAPI- bands were colocalized in terminal regions (Fig.1A, B). In some bivalents, terminal CMA_3+/DAPI- bands are displayed in both centromeric and telomeric positions.

The sex chromosome (X) showed proximal and distal CMA_3+/DAPI- bands (Fig. 1C, D and Fig. 2A, B), whereas B chromosomes are characterized by showing an interstitial CMA_3+/DAPI- band (Fig. 1C, D).

Heterozygote individuals for the distal SS10 segment showed a heteromorphic bivalent with telomeric CMA_3+/DAPI- bands of different sizes (Fig. 2A, B). Most of the SS10 segment is CMA_3+/DAPI-, revealing a bigger bright band in the heteromorphic bivalent.

Heterozygote individuals for the proximal SS9 and SS6 segments showed a bigger CMA_3+/DAPI- band than standard bivalents (Fig. 2C-F). Both proximal segments are composed of a CMA_3+/DAPI- region more proximal to the centromere and a CMA_3+/DAPI- region.

Geographical distribution pattern.—The geographical distribution of chromosome polymorphisms was analyzed from 2002 to 2007 in six *D. elongatus* populations, four of which are analyzed in the present paper, two being analyzed previously (Rosetti *et al.* 2007) (Table 1).

B chromosomes and proximal SS6 segments are geographically widespread in the studied area, whereas distal SS10 and proximal SS9 were absent in some populations. Supernumerary segments are always present in heterozygous condition, probably due to their low frequency in the population. SS6 and SS10 segments may occur simultaneously in the same individual. Three out of 246 analyzed males were double heterozygote for these variants, exceeding the expected frequency of this karyotype.

The geographic distribution of chromosome polymorphisms was analysed with respect to some climatic and geographic variables. There is considerable B-chromosome variation between populations and this variation correlates negatively with latitude (r = -0.943,

Table 1. Frequencies of B chromosomes (B) and supernumerary segments in M6 (SS6) S9 (SS9) and S10 (SS10), along with geographic and climatic data from natural populations of *D. elongatus* collected in Argentina. N= sample size. * Data from Rosetti *et al.* 2007.

Location	B	SS10	SS6	SS9	N	Latitude	Longitude	Altitude	Mean °C
Venado Tuerto	0.029	0.014	0.043	0.014	35	33°45′	61°57′	40	16.83
Gualeguaychú	0.091	0.015	0.030	0.00	33	33°06′	58°32′	40	17.18
Colón	0.032	0.00	0.016	0.00	31	32°13′	58°09′	40	17.88
Mocoreta	0.111	0.019	0.019	0.019	27	30°38′	57°58′	60	19.01
Raco- Nogues*	0.132	0.026	0.046	0.026	76	26°40′	65°10′	650	19.19
Horco Molle-Yerba Buena*	0.136	0.045	0.034	0.023	44	26°49′	65°19`	550	18.83

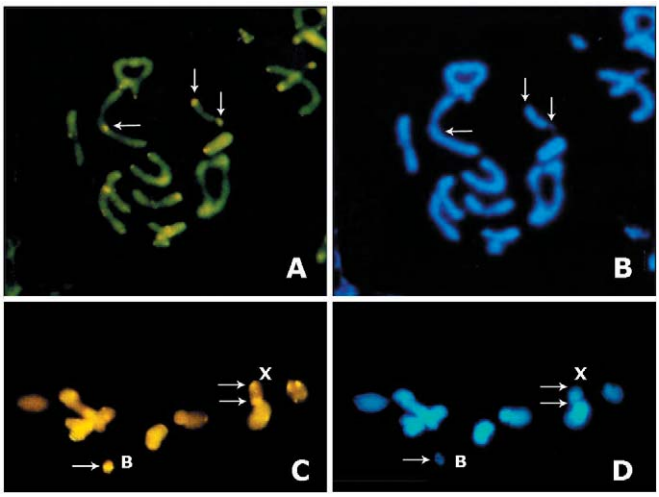

Fig. 1. *D. elongatus*. CMA_3 staining (A, C); DAPI staining (B, D). A, B) Standard complement at late diplotene. C, D) Metaphase I showing a B chromosome (B). White arrows show CMA_3 bright - DAPI negative. Bar= 10µm. For color version, see Plate IV.

P= 0.0048) and positively with altitude (r = 0.941, P = 0.005). The frequency of the proximal SS9 segment is positively correlated with the altitude, whereas the SS10 distal segment seems to be positively associated with longitude, though no significant correlation was detected (r = 0.77, P = 0.07). No significant associations were detected between geographic variables and the frequency of the SS6 segment. With respect to climatic variables, B chromosomes showed a significant positive correlation with the mean annual temperature (r = 0.88, P = 0.018).

The analysis of geographic chromosome patterns revealed that B chromosomes tend to decrease from the North towards the South, while both B chromosomes and SS9 have a tendency to increase with altitude.

Discussion

Natural populations of *D. elongatus* from Argentina are polymorphic for the presence of several additional dispensable elements.

Our results, based on fluorescence banding, demonstrate heterogeneity in the constitution of the supernumerary heterochromatin. In insects, especially grasshoppers, chromosomal regions with differential fluorescence may be rich in GC base pairs. All fluorescent bands are rich in GC base pairs, a condition commonly found in grasshoppers (Bella *et al.* 1991, 1993; Loreto & de Souza 2000). The distal SS10 segment is mainly composed of GC-rich heterochromatin, whereas proximal supernumerary segments (SS6 and SS9) showed two different heterochromatic regions: one GC-rich (CMA_3+/DAPI-) and another that has no specificity to AT or CG base pairs (CMA_3/DAPI dull). The centromeric region of the S6 and S9 autosomes, as well as the telomeric region of the S10 pair, showed a bright CMA_3+/DAPI- band. These results are in agreement with the tentative hypothesis arguing that supernumerary chromosomal segments in the genome of *D. elongatus* may have originated by duplication of pre-existing heterochromatic regions.

Different heterochromatin constitutions were also detected in the supernumerary segments of another grasshopper, *Dociostaurus genei*, which appear differentially stained with DAPI banding and thus seem to be AT-rich (Rodriguez Iñigo *et al.* 1993, 1996, 1998).

Previous studies in *D. elongatus* showed that the nucleolus is always associated with the proximal end of the S10 pair (Remis 1989). An association between CMA_3+ bands and NORs is rather common in insects, especially grasshoppers (Camacho *et al.* 1991; Bella *et al.* 1990, 1993; Loreto & de Souza 2000; Bakkali *et al.* 2001). However, in the present paper there is no evidence of any differential positive CMA_3 band in the proximal region of the S10 pair.

B chromosomes showed an interstitial GC-rich band. However no standard chromosome of the complement exhibited CMA_3 positive regions in interstitial position. It is widely accepted that B chromosomes are derived from polysomic A chromosomes (Jones & Rees 1982, Camacho *et al.* 2000). Contemplating this hypothesis of the origin of such supernumerary chromosomes, our results would seem to indicate that B chromosomes should have suffered posterior rearrangements to explain their banding pattern, compared to that of the chromosomes of the normal complement.

The prevalence of supernumerary variants within a species depends on the degree to which the species tolerates these extra genetic elements and the probable existence of an accumulation mechanism of the supernumerary variants (Remis *et al.* 2004). In the present paper some supernumerary variants of *D. elongatus* exhibited chromosome interpopulation variation in their frequencies. Here, as in previous population cytogenetic studies (Remis *et al.* 1998, 2004), we detected different degrees of differentiation among populations for each heterochromatic variant. Under a neutral scenario (interaction between genetic drift and migration) the differentiation degree is expected to be similar between distinct markers. Therefore, different factors may be moulding chromosome frequencies in natural populations of *D. elongatus*. There is no reason to reject the most likely hypothesis to explain SS9 chromosome variation patterns through historical factors in the studied area.

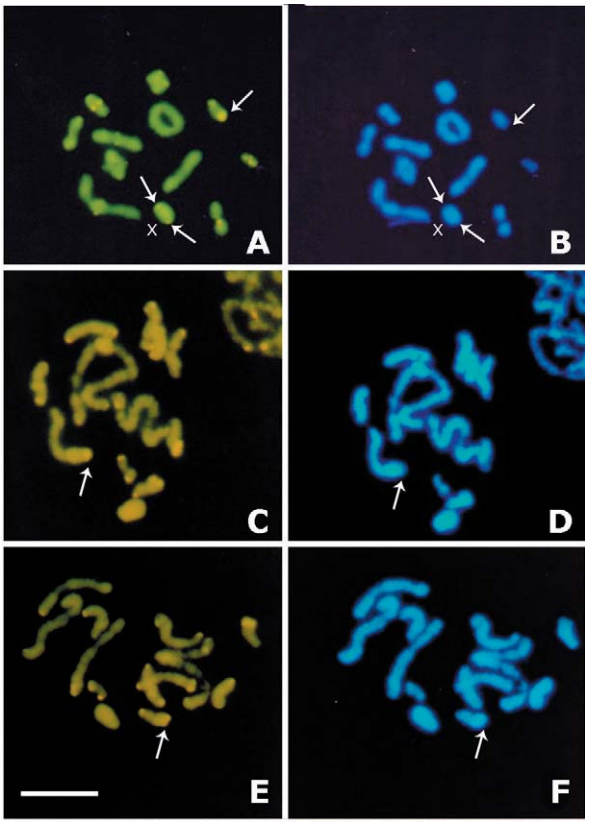

Fig. 2. *D. elongatus*. CMA_3 staining (A, C, E); DAPI staining (B, D, F). A, B) Late diakinesis showing distal SS10 segment. C, D) Diplotene showing proximal SS6 segment. E, F) Diplotene showing proximal SS9 segment. White arrows show CMA_3 bright - DAPI negative. Bar= 10µm. For color version, see Plate IV.

Both the size and frequency of these supernumerary segments may be shaped by their age, so that the older the segment is, the more likely it is to have experienced duplications and thus increase in size, and the closer its frequency will be to equilibrium.

The frequency of the B chromosomes in *D. elongatus* seem to decrease with latitude and increase with altitude and temperature. Supernumerary chromosome frequency often shows geographical variation which may be attributed to the geographic origin and evolutionary history of the B chromosome and/or the ability of the populations to maintain B chromosomes in climatically more favorable regions (Camacho *et al.* 2000). Indeed, several reports show spatial chromosome differentiation with B-chromosome clines (Hewitt & Brown 1970; Parker *et al.* 1991; Zima & Macholan 1995; Cabrero *et al.* 1997; Tsurusaki & Shimada 2004; Bakkali *et al.* 1999, 2002).

Some studies analyzed the relative significance of these factors for the patterns of geographic variation (Hewitt & Brown 1970; Bougourd & Parker 1979; Cabrero *et al.* 1997; Colombo 1989; Tsurusaki & Shimada 2004; Colombo & Confalonieri 2004; Perfectti *et al.* 2004a, 2004b; Bakkali *et al.* 1999, 2002; Bakkali & Camacho 2004). In the grasshopper *Myrmeleotettix maculatus*, B chromosomes were common in climatically more favorable regions (Hewitt & Brown 1970). The distribution of the B chromosomes in British populations may be attributable to the overall level of stringency or favorability of the environment. Cytogenetic population analysis of the grasshopper *Eyprepocnemis plorans* along four rivers belonging to the Segura basin (Spain), demonstrated that the geographical distribution of B chromosomes was molded mainly by historical nonselective events (Cabrero *et al.* 1997), a conclusion supported by similar studies on the B chromosomes of the same species in Moroccan populations (Bakkali *et al.* 1999, 2002; Bakkali & Camacho 2004). Clinal variation of B-chromosome frequency was also reported in the harvestman *Psathyropus tenuipes* from Japanese Islands (Tsurusaki & Shimada 2004). Previous studies demonstrated an association between the number of B chromosomes and resistance to gregarine infection in natural populations of this species (Gorlov & Tsurusaki 2000). The authors suggested that the clinal variation in B-chromosome frequency might be attributed to variability in the geographic abundance of gregarines.

Given that we have a negative correlation between latitude and altitude in the studied area and that the mean temperature is higher at higher altitudes, we suspect that the effects of these three variables on B-chromosome frequency in *D. elongatus* is due to the effect of just one of them. *A priori*, the mean temperature would be a good candidate, since at higher, closer to optimal, temperatures any negative effect of the B chromosome on the fitness of the carrier would be less pronounced. In other words, the harmful effects of the B chromosome on the fitness of the carrier are probably worse at nonoptimal temperatures.

The pattern of B-chromosome distribution in *D. elongatus* may be more complex and further studies, to relate B chromosome distribution to their geographic distribution in the light of different geographic/climatic variables, may help us understand the maintenance of such chromosome variants in nature.

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