

Latitudinal clines in the grasshopper *Dichroplus elongatus*: Coevolution of the A genome and B chromosomes?

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Abstract

Argentine populations of *Dichroplus elongatus* (Orthoptera: Acrididae) are polymorphic for B chromosomes. Previous studies showed that B chromosomes affect body size and some fitness components in Northwestern populations. We studied phenotype and B's variation patterns along a latitudinal cline as well as the relationship between karyotype and body size related traits in 17 populations from East. Body size related traits showed a 'saw tooth' pattern of variation being small at low and high latitudes and large at intermediate latitudes in most of the analysed populations. Analyses of variance and principal components demonstrated that in most analysed populations B carrier males are associated with a decrease in body size related traits with respect to individuals with standard karyotype. Accordingly with the relationship between karyotype and body size, an opposite pattern of latitudinal variation in the frequencies of B's with respect to body size variation was observed in this area. *i.e.* smaller individuals tend to have a higher frequency of B chromosomes. The comparison of the differentiation of both karyotype and body size traits with molecular neutral markers demonstrated the relative importance of selection moulding chromosome and phenotype variation. The observed pattern of phenotypic variation is likely to be the result of local adaptation to season length along the latitudinal gradient. The observed contrary pattern of B's clinal variation may reflect the population ability to maintain this chromosome in relation to the local adaptation. The available evidence indicates that the distribution of B chromosome frequency was shaped by selective factors.

Introduction

Since their discovery (Wilson, 1907) the maintenance of B chromosomes in natural populations has been very much of a riddle for evolutionary biology (Jones & Rees, 1982; Jones, 1995; Camacho *et al.*, 2000; Puertas, 2002).

Nowadays, B –also named supernumerary– chromosomes are regarded as symbiont genome, because they are additional, dispensable chromosomes that are present in some individuals of some populations of a species. B chromosomes have probably arisen from A complement

members, but they follow their own evolutionary pathway (Camacho *et al.*, 2000). Some models that explain the maintenance of these chromosomes in nature as a trade-off between accumulation mechanisms and their possible effects on fitness were proposed. The parasitic or selfish model (Ostergren, 1945; Jones, 1995) states that the presence of B's in many eukaryote species is explained, in most cases, because of nonMendelian inheritance due to meiotic or mitotic instability; this fact would lead to their accumulation in the germ line (drive) despite deleterious effects on carriers' fitness. The heterotic model (White, 1973; Bougourd & Jones, 1997) suggests that it is the beneficial effects of B's, and not drive, which would lead to their accumulation. This would be balanced by detrimental effects caused by an excessive number of Bs. Both models assume the existence of equilibrium (but see Zurita *et al.*, 1998, for a nonequilibrium model of long-term evolution).

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Analysis of clinal variation along climatic or geographical gradients can indicate a possible contribution of directional selection to differences among populations. (Bubliy & Loeschcke, 2005). As thermal regimes are particularly sensitive to latitude and altitude, geographical location imposes profound selection on organism's metabolism, morphology and behaviour, leading to covariation between phenotypic traits and geographical gradients. One example of such variation is latitudinal differentiation of size related traits in several *Drosophila* species, with a genetically based correlation between body size and latitude (e.g. Acevedo *et al.*, 1998; Huey *et al.*, 2000; Hallas *et al.*, 2002).

Selection acts on phenotypes, therefore analyses of the effects of chromosome rearrangements on body size related traits allow gaining further insight about the adaptive significance of chromosome variation. The correlation of B chromosome frequency with ecological or geographical variables would be an indirect but compelling piece of evidence that natural selection is involved in the observed B chromosome distribution (Cabrero *et al.*, 1997).

Clinal variation in quantitative traits is often attributed to the effects of spatially varying selection across an environmental gradient (Endler, 1977). However, identical patterns can be produced by the interplay between purely stochastic processes (i.e. genetic drift in combination with spatially restricted gene flow). The comparison of the levels of population differentiation at putatively selected traits with respect to differentiation at neutral genetic markers allows disentangling adaptive from neutral phenotypic differentiation (for reviews see Leinonen *et al.*, 2008; Pujol *et al.*, 2006; Whitlock, 2008). If traits evolve neutrally, the proportion of their variation among populations should on average be similar to the proportion of among-population variation in allele frequencies at neutral loci (Antoniazza *et al.*, 2009).

A common method for evaluating whether phenotypic variation is due to selection is to test its correlation with probably neutral molecular variation. Although migration and genetic drift have an equal effect all over the genome, selection affects only regions harbouring the quantitative trait loci (QTL) underlying the phenotypic trait it acts on. Thus, if selection causes divergent evolution of phenotypes among populations, either because selection is exerted on morphology itself or on genetically correlated traits, phenotypic differentiation is expected to exceed neutral differentiation, especially if populations remain interconnected by gene flow such as in cline models (Roff & Mousseau, 2005).

Although B chromosomes are very frequent in nature; there are few examples of B chromosomes affecting exophenotype (Camacho *et al.*, 2000). Populations of a species with B chromosomes which affect body size related traits may be a useful model to study the relationship between such chromosome variant and pheno-

type as well as the magnitude of the effect in different geographical areas.

Dichroplus elongatus is a South American grasshopper widely distributed throughout Argentina, Uruguay, most of Chile and southern Brazil (Mariottini *et al.*, 2011) whose Argentine populations exhibit widespread polymorphisms for mitotically unstable B chromosomes (Loray *et al.*, 1991; Clemente *et al.*, 1994). Simultaneous morphometric and cytogenetic studies showed that B chromosome carriers are associated with a decrease in body size in both males and females from Northwestern Argentina natural populations. Furthermore, the uni- and multivariate analysis of mating success revealed the occurrence of phenotype and chromosome directional selection favouring larger non-carrier males (Rosetti *et al.*, 2007). The analysis of reproductive potential suggested that B chromosome carrying females have higher numbers of embryos per clutch and ovarioles per ovary (Rosetti *et al.*, 2007). These results support the hypothesis that the persistence of B chromosomes in natural populations is the result of trade-offs among opposite selective effects and interactions with their mitotic instability.

In this study we reported morphometric and B chromosome variation patterns in 17 Argentine populations of the grasshopper *Dichroplus elongatus* to provide evidence regarding the maintenance of the B chromosomes and body size differences in different environments.

Our objectives were: a) to provide an opening assessment of the patterns of chromosome and phenotype variation along a latitudinal cline and b) to analyse the relationship between morphometric and chromosome differences with respect to molecular variation to test the relative importance of selective processes on phenotypic/morphometric variation in *D. elongatus*.

Materials and methods

Population sampling

Adult males were collected in 17 natural populations: Venado Tuerto (VET) (from Santa Fe province), Carmen de Areco (CAR), Campana (CAM), Las Flores (FLO), Rauch (RAU), Cañuelas (CAÑ), Colón (COB) (from Buenos Aires province), Río Cuarto (RCU), Río los Sauces (RLS), Santa Catalina (SAC) (from Córdoba province), Gualaguaychú (GUA), Colon (COE), Concordia (CON) (from Entre Ríos province), Mocoretá (MOC), Monte Caseros (MCA), Yapeyú (YAP), Santo Tomé (STO) (from Corrientes province), in 2005, 2006, 2007 and 2008 (Table 1, Fig. 1).

Chromosome preparations

The standard karyotype of *Dichroplus elongatus* (Orthoptera: Acrididae) is composed of 22 acrocentric

Table 1 Body size in natural populations of *Dichroplus elongatus*.

Location	Total	Thorax	Femur	Tibia	Tegmen	<i>n</i>	Latitude	Longitude	Max Temp (°C)	Min Temp (°C)	Mean Temp (°C)
Venado Tuerto (VET)	8.991 (0.065)	4.380 (0.044)	11.809 (0.349)	9.013 (0.264)	17.807 (0.181)	38	33°45'S	61°57'W	23.50	10.38	16.83
Carmen de Areco (CAR)	8.270 (0.044)	4.192 (0.043)	11.149 (0.101)	8.390 (0.068)	16.831 (0.180)	33	34°49'S	59°50'W	22.20	10.31	16.40
Campana (CAM)	8.279 (0.043)	4.101 (0.026)	11.107 (0.068)	8.341 (0.039)	16.238 (0.152)	54	33°59'S	58°57'W	22.09	10.97	16.62
Las Flores (FLO)	8.149 (0.016)	4.007 (0.017)	10.906 (0.060)	8.295 (0.028)	16.502 (0.152)	43	35°55'S	59°07'W	21.69	8.94	15.33
Rauch (RAU)	8.041 (0.038)	3.998 (0.020)	10.615 (0.075)	8.224 (0.049)	15.283 (0.123)	42	36°47'S	59°06'W	20.96	7.74	13.92
Cañuelas (CAÑ)	8.113 (0.033)	4.013 (0.028)	10.735 (0.105)	8.217 (0.044)	16.483 (0.192)	34	35°03'S	58°46'W	21.92	10.50	16.06
Colon (Bs As) (COB)	9.052 (0.054)	4.505 (0.033)	11.865 (0.076)	8.937 (0.059)	17.963 (0.134)	40	33°52'S	61°05'W	22.84	10.34	16.40
Rio Cuarto (RCA)	8451 (0.037)	4130 (0.025)	11.241 (0.076)	8669 (0.135)	17.509 (0.172)	23	33°08'S	64°20'W	22.98	10.97	16.33
Rio los Sauces (RLS)	8361 (0.045)	4014 (0.048)	10.889 (0.190)	8500 (0.106)	17.139 (0.238)	15	31°40'S	63°55'W	23.17	9.86	16.56
Santa Catalina (SAC)	8.526 (0.037)	4.215 (0.035)	11.410 (0.125)	8.894 (0.104)	18.061 (0.114)	13	34°09'S	63°22'W	23.83	9.85	16.25
Guaquaychu (GUA)	8.131 (0.026)	3.964 (0.018)	10.760 (0.070)	8.407 (0.045)	17.053 (0.107)	35	33°06'S	58°32'W	23.06	11.46	17.63
Colon(Entre Rios) (COE)	8.130 (0.017)	3.989 (0.025)	10.792 (0.080)	8.391 (0.050)	17.063 (0.102)	40	32°13'S	58°09'W	23.77	12.18	17.88
Concordia (CON)	8.103 (0.024)	3.988 (0.027)	10.484 (0.049)	8.311 (0.051)	16.495 (0.111)	29	31°24'S	58°02'W	24.91	13.15	19.03
Mocoreta (MOC)	8.098 (0.025)	3.958 (0.029)	10.457 (0.090)	8.058 (0.087)	16.176 (0.156)	31	30°38'S	57°58'W	25.90	14.31	19.75
Monte Caseros (MCA)	7952 (0.231)	3885 (0.113)	11.313 (0.329)	8767 (0.256)	17.224 (0.500)	35	30°17'S	57°38'W	25.91	14.31	19.75
Yapeyu (YAP)	7974 (0.175)	3852 (0.085)	10.781 (0.247)	8406 (0.194)	16.707 (0.381)	46	29°28'S	56°49'S'W	26.72	14.60	26.60
Santo Tome (STO)	7500 (0.667)	3526 (0.309)	9577 (0.832)	7687 (0.673)	15.372 (1.336)	12	28°36'S	56°01'W	26.72	14.62	20.67

Mean length values (in mm.) and standard errors in parentheses for five morphometric traits and geographical and climatic data of natural populations of *Dichroplus elongatus* collected in Argentina. *n* = sample size.

autosomes in males and a XX/XO sex determination system (Remis & Vilardi 1986). Argentinean populations of these species are polymorphic for mitotically unstable B chromosomes. In the present article we examined cytologically 293 males from 13 populations. Males were dissected and their testes fixed in 3 : 1 ethanol: acetic acid. Preparations were made by squashing several follicles in acetic haematoxylin. A minimum of 10 cells at meiotic metaphase I per individual, were examined to determine karyotypes.

Morphometric measurements

All individuals were measured for five body size related traits: Total Length: length from the phastigium to the articulation between the third coxa and third femur, Femur Length: length of third femur, Tibia Length: length of third tibia, Thorax Length: length of protho-

rax, Tegmen Length: the length of tegmen. Traits were measured on the right side of the insect and by the same observer (NR) with a stereoscopic microscope and an ocular micrometre (1 mm = 48 ocular units).

Statistical analyses

Phenotype data analyses

Multiple comparisons of mean body size related traits among populations were carried out through a multivariate analysis of variance (MANOVA).

We tested for statistical significance among populations through conventional one-way ANOVAS, considering the population as the independent factor and body size related traits as the dependent variables. To analyse population variation on a reduced set of independent variants, avoiding the problem of pseudoreplication, principal component analysis (PCA) of body

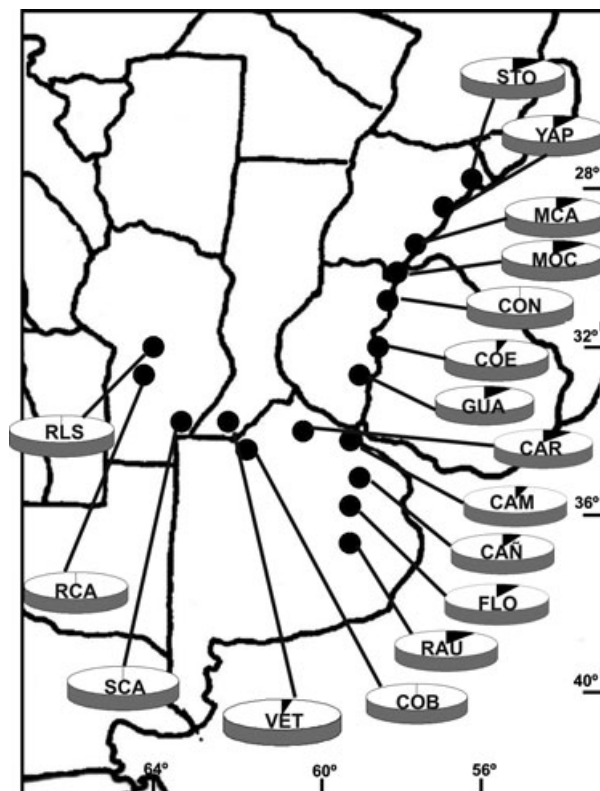


Fig. 1 B frequencies in Argentinean populations of *Dichroplus elongatus*. Geographical distribution of 17 natural populations showing the frequencies of B chromosomes. Abbreviations are according to Table 1.

size variables were also performed. We tested for variation in the first three principal components (PCs) by means of one-way ANOVAS considering the population as the independent factor and PCs as dependent ones.

The relationships between body size related traits with respect to geographical (latitude and longitude) and some climatic variables (mean annual temperature, maximum temperature and minimum temperature) were analysed by means of Spearman nonparametric correlation. Bonferroni's test for multiple comparisons was applied.

Chromosome data analyses

To assess spatial chromosome variation, we considered supernumerary chromosome frequencies from 17 populations phenotypically analysed in this study. Thirteen populations were analysed here and four populations were cytologically examined previously (VET; GUA; COL and MOC) (Rosetti & Remis, 2012). The relationship between B chromosome incidence and geographical and climatic variables were evaluated through Spearman nonparametric correlation.

Simultaneous chromosomal and body size variation

To estimate the phenotype differences between B and non B carriers the mean values of the five body size related traits were compared through a MANOVA. To analyse the effect of each body size related trait on karyotype, data were transformed to standardized deviations from the mean value for each trait within a population to avoid the interpopulation variation. Individuals ANOVAS were conducted considering karyotype as independent variable and the body size related trait as the dependent.

To avoid possible errors in the results attributable to bias caused by multicollinearity among traits, we also performed a principal component analysis (PCA) of body size variables. We tested for variation in the first three principal components (PCs) by means of two-way ANOVAS considering karyotype and populations as independent variable and the body size related trait as the dependent.

All the statistical analyses mentioned above were carried out with the program *STATISTICA* (Statistica Statsoft Inc., 1996), and *REAP* (McElroy *et al.*, 1991).

Analysis of morphometric and chromosome variation with respect to molecular neutral data

The assessment of statistical approximation of population differentiation based on different sets of data has been used to analyse the role of genetic drift and selection (Gillespie & Oxford, 1998; Roff & Mousseau, 2005). When there is significant correlation between one set of data assumed to be neutral and another set of data, interaction between genetic drift and migration may explain the pattern of variation in the second set of data. Alternatively, if there are significant differences between estimates of population differentiation from the two data sets genetic drift could not be responsible for differences in the second set of data. To analyse the relative importance of genetic drift and selection on phenotypic and karyotype variation, we compared statistical estimation of morphometric and chromosome differentiation with variation in neutral genetic markers.

We compared phenotypic and B-chromosome differences between populations with Nei's genetic distances previously estimated from the same populations analysed here using DAMD (Direct Amplification of Minisatellite DNA) markers (Rosetti & Remis, 2012).

The B-chromosome dissimilarities were estimated as Euclidean distances. The phenotypic differences between populations were estimated through both the differences in trait means and the mean percentage differences between the corresponding phenotypic variance-covariance matrices of each trait ($T\%$), according to Roff & Mousseau (2005).

Comparison between phenotypic and B chromosome differentiation with respect to Nei's genetic distances

were carried out through standard Mantel test and partial Mantel using the *PASSAGE* version 2 software (Rosenberg & Anderson, 2011). For all tests, the significance was estimated with 10 000 permutations. The goal of partial Mantel test is to evaluate the correlation between two matrices while controlling the effect of a third matrix, to remove spurious correlations. We correlated chromosome distances with phenotypic differences controlling the effect of genetic distances.

Relationships among populations were represented by multidimensional scaling (MDS) based on Nei genetic distances, B chromosome differences and T% values using the *STATISTICA* program (Statistica Statsoft Inc., 1996).

Results

Body size variation

To analyse phenotypic variation patterns, five traits related with body size were scored in individuals from 17 Argentinean populations of *Dichroplus elongatus* from 28°36' to 36°47'S latitude and from 56°01' to 64°20'W longitude (Table 1, Fig. 1).

There is considerable body size variation among populations (Wilks' $\lambda_{85;2692} = 0.12$, $P < 10^{-4}$). The individual ANOVAS revealed highly significant differences in all analysed traits (total: $F_{17;65} = 3.28$, thorax: $F_{17;33} = 0.93$, femur: $F_{17;12} = 6.40$, tibia: $F_{17;9} = 2.83$, tegmen: $F_{17;23} = 18.74$; $P < 10^{-4}$ in all cases). To analyse population variation on a reduced set of independent variables, a PCA was performed. The first principal component accounted for about 71% of the total variation, is positively associated with all body size related traits analysed and may be considered as a measure of overall size. The individual ANOVA of PCA1 showed again highly significant differences among populations ($F_{17;559} = 37.80$, $P < 10^{-4}$).

Wild adult males of the grasshopper *D. elongatus* tended to be larger from East to West. Comparisons of partial correlation of body size related traits on longitude support this assumption: total and thorax lengths are positively correlated with longitude ($r = 0.66$, $P = 0.004$; $r = 0.86$, $P < 10^{-4}$). The pattern of thorax length resembles the geographical gradient of temperature in the studied area. Significant negative correlations were demonstrated between the mean thorax length ($r = -0.73$, $P = 0.0007$) and mean temperature and minimum temperature ($r = -0.85$, $P < 10^{-5}$).

Sizes of wild adult males of *D. elongatus* tended to show contrasting patterns of body size variation with south latitude. From VET to RAU (from 33°45' to 36°47'S latitude) a decrease in body size related traits was evident when south latitude increases and mean temperature decreases (Table 1, Fig. 2). Significant negative correlations were detected between all mean body size related traits and south latitude (total: $r = -0.95$,

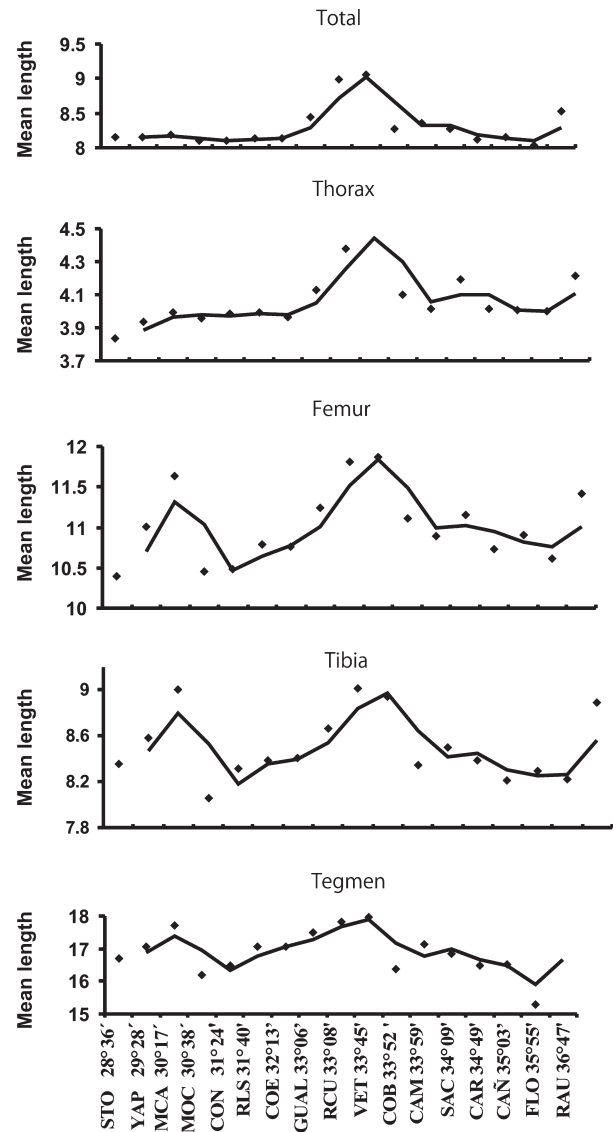


Fig. 2 'Saw tooth' latitudinal cline for five morphometric traits. Mean morphometric traits (in mm) as a function of latitude in Argentinean populations of *Dichroplus elongatus*. Abbreviations are according to Table 1.

$P = 0.0003$; thorax: $r = -0.95$, $P = 0.0003$; femur: $r = -0.96$, $P = 0.0002$; tibia: $r = -0.90$, $P = 0.0020$; tegmen: $r = -0.74$, $P = 0.037$). Significant positive correlations were also demonstrated between the mean body size related traits and maximum temperature (total: $r = 0.86$, $P = 0.006$; thorax: $r = 0.90$, $P = 0.0020$; femur: $r = 0.91$, $P = 0.0021$; tibia: $r = 0.86$, $P = 0.0065$; tegmen: $r = 0.88$, $P = 0.0038$).

From MOC to VET the (from 30°38' to 33°45'S latitude) body size related traits seem to increase with south latitude increase and temperature decrease (Table 1, Fig. 2). Significant positive correlations were detected between all

analysed traits and latitude (total: $r = 0.89$, $P = 0.007$; thorax: $r = 0.75$, $P = 0.051$; femur: $r = 0.86$, $P = 0.013$; tibia: $r = 0.89$, $P = 0.007$; tegmen: $r = 0.86$, $P = 0.013$). Significant negative correlations were demonstrated between the studied traits and mean temperature (total: $r = -0.89$, $P = 0.007$; thorax: $r = -0.79$, $P = 0.036$; femur: $r = -0.86$, $P = 0.013$; tibia: $r = -0.89$, $P = 0.007$; tegmen: $r = -0.86$, $P = 0.013$).

In populations above MOC, body size related traits tend to increase again, however, the number of analysed populations did not allow to study the relationship.

Chromosome variation

The incidence of B chromosomes in all populations was determined to analyse the pattern of chromosomal variation (Table 2, Fig. 1). There is a considerable variation in B incidence among analysed populations.

Populations at higher longitude exhibited higher incidence of B chromosomes. B incidence tended to increase with west longitude ($r = -0.61$, $P = 0.008$) when all populations were considered jointly. No significant association was detected between maximum temperature and B chromosome incidence ($r = -0.68$, $P = 0.089$).

In populations at higher latitude than VET, B chromosome incidence exhibited a positive association with south latitude ($r = 0.82$, $P = 0.013$) and a negative association with maximum temperature ($r = -0.77$, $P = 0.025$) (Table 2, Fig. 3a, b).

In populations at lower latitudes than VET B chromosome incidence seems to decrease when south latitude increase and maximum temperature decrease (Table 2). We detected a positive correlation between B chromosome incidence and maximum temperature ($r = 0.65$, $P = 0.04$) whereas a marginally significant negative correlation between B incidence and south latitude was detected when the results of GUA population are excluded ($r = -0.66$, $P = 0.05$) (Fig. 3c, d).

Relationships between B chromosomes and body size

To evaluate the relationships between chromosome constitution and phenotype, we scored body size related traits in B and non B carrier individuals in populations with B chromosomes (Table 2).

Given the significant associations between B incidence and body size variation with south latitude we considered three groups of populations for further analysis: populations at higher latitude than VET (Southern cline, eight populations), population located between VET and MOC (Northern cline, seven populations) and populations at lower latitudes than MOC (two populations).

The relationship between body size and B chromosome was analysed on those populations with at least two B carriers individuals, and considering the three

Table 2 Body size in different karyotypes from natural populations of *D. elongatus*.

Population	Traits	Mean	
		B	ST
VET	Total	9167	8929 (0.045)
	Thorax	4583	43 618 (0.048)
	Femur	11 458	12 187 (0.185)
	Tibia	9583	9263 (0.131)
	Tegmen	18 333	17 672 (0.213)
<i>n</i>		1	29
CAR	Total	8264 (0.139)	8314 (0.056)
	Thorax	4236 (0.184)	4244 (0.047)
	Femur	11 597 (0.250)	11 231 (0.111)
	Tibia	8542 (0.208)	8437 (0.067)
	Tegmen	17 292 (0.788)	16 922 (0.212)
<i>n</i>		3	22
CAM	Total	8021 (0.104)	8295 (0.064)
	Thorax	3958 (1.6E-10)	4200 (0.044)
	Femur	10 521 (0.104)	11 122 (0.120)
	Tibia	8333 (1.6E-10)	8343 (0.071)
	Tegmen	15 313 (0.521)	16 259 (0.359)
<i>n</i>		2	44
FLO	Total	8125 (0)	8125 (0)
	Thorax	3888 (0.069)	3958 (0)
	Femur	10 347 (0.069)	11 041 (0)
	Tibia	8194 (0.069)	8438 (0)
	Tegmen	15 416 (0.636)	16 666 (0.104)
<i>n</i>		3	33
RAU	Total	8055 (0.138)	8058 (0.048)
	Thorax	4027 (0.069)	4008 (0.028)
	Femur	10 555 (0.184)	10 725 (0.098)
	Tibia	8194 (0.069)	8308 (0.062)
	Tegmen	15 208 (0.318)	15 392 (0.172)
<i>n</i>		3	25
CAN	Total	8125 (0.208)	8117 (0.038)
	Thorax	4167 (0)	4004 (0.032)
	Femur	10 937 (0.521)	10 663 (0.120)
	Tibia	8437 (0.104)	8194 (0.053)
	Tegmen	16 875 (1.042)	16 312 (0.212)
<i>n</i>		2	27
COB	Total	–	9020 (0.073)
	Thorax	–	4483 (0.041)
	Tibia	–	11 805 (0.086)
	Femur	–	8881 (0.071)
	Tegmen	–	17 901 (0.1181)
<i>n</i>		–	27
RCA	Total	–	8464 (0.043)
	Thorax	–	4123 (0.026)
	Femur	–	11 228 (0.081)
	Tibia	–	8706 (0.161)
	Tegmen	–	17 511 (0.202)
<i>n</i>		–	19
RLS	Total	–	8542
	Thorax	–	4375
	Femur	–	11 250
	Tibia	–	8958
	Tegmen	–	18 125
<i>n</i>		–	11
SCA	Total	–	8 50 379

Table 2 (Continued)

Population	Traits	Mean		
		B	ST	
<i>n</i>	Thorax	–	4 18 561	
	Femur	–	11 28 788	
	Tibia	–	878 788	
	Tegmen	–	17 95 455	
				11
GUA	Total	8056 (0.069)	8148 (0.032)	
	Thorax	3888 (0.069)	3981 (0.023)	
	Femur	10 625 (0.241)	10 826 (0.084)	
	Tibia	8263 (0.184)	8441 (0.054)	
	Tegmen	16 875 (0.240)	17 114 (0.135)	
<i>n</i>		3	27	
	COE	Total	8125	8125 (0.023)
		Thorax	3958	3994 (0.031)
		Femur	11 250	10 818 (0.102)
		Tibia	8750	8405 (0.063)
Tegmen		17 708	17 047 (0.129)	
<i>n</i>		1	29	
	CON	Total	–	8102 (0.029)
		Thorax	–	3969 (0.036)
		Femur	–	10 497 (0.070)
		Tibia	–	8333 (0.065)
Tegmen		–	16 493 (0.161)	
<i>n</i>		–	22	
	MOC	Total	8125 (0)	8095 (0.036)
		Thorax	3958 (0.120)	3948 (0.039)
		Femur	10 347 (0.347)	10 426 (0.114)
		Tibia	7639 (0.455)	8115 (0.106)
Tegmen		16 666 (0.524)	16 111 (0.197)	
<i>n</i>		3	21	
	MCA	Total	8333 (0)	8135 (0.018)
		Thorax	4062 (0.104)	3979 (0.021)
		Femur	11 875 (0.208)	11 468 (0.128)
		Tibia	9166 (0.208)	8875 (0.102)
Tegmen		18 125 (0.625)	17 510 (0.135)	
<i>n</i>		2	20	
	YAP	Total	8229 (0.147)	8161 (0.102)
		Thorax	4063 (0.147)	3958 (0.154)
		Femur	11 146 (1.031)	11 132 (0.787)
		Tibia	9167 (0.884)	8705 (0.679)
Tegmen		17 708 (1.178)	17 391 (1.104)	
<i>n</i>		2	23	
	STO	Total	7917	8171 (0.046)
		Thorax	3750	3842 (0.050)
		Femur	10 000	10 439 (0.195)
		Tibia	7916	8403 (0.200)
Tegmen		15 833	16 782 (0.301)	
<i>n</i>		1	9	

Means and standard error (in parentheses) of morphometric traits (mm) measured in males with standard (ST) and B karyotype in populations of *Dichroplus elongatus*. *n* = sample size.

body size related traits (femur, tibia and tegmen lengths) which exhibited significant association with karyotype according with previous results (Rosetti *et al.*, 2007).

Within the first group we can assess the relationship between body size and B chromosome in five popula-

tions because they exhibited two or more B carrier individuals (CAR, CAM, CAN, FLO, RAU). We noticed that B carriers are associated with a decrease in body size (Fig. 4). The statistical analyses based on transformed data to avoid interpopulation variation revealed significant differences between karyotypes in multivariate (MANOVA) (Wilks' $\lambda_{5;186} = 0.93$, $P = 0.03$) and individual (ANOVA) approaches. Femur length, as indicative of body size, showed significant differences between B and non B carriers individuals ($F_{1;190} = 7.26$, $P = 0.007$).

In the second group we were able to analyse the association between phenotype and B chromosome only in two populations (GUA and MOC), where B carrier individuals were smaller than non B carriers (Fig. 4). MANOVA on transformed data revealed significant differences between karyotypes (Wilks' $\lambda_{5;48} = 0.70$, $P = 0.035$) and individuals ANOVAS showed that tibia length, as indicative of body size, exhibited marginally significant differences between B and non B carriers individuals ($F_{1;52} = 3.30$, $P = 0.07$).

In the remaining populations (MCA and YAP), further north than the MOC population, B carriers individuals exhibited higher mean body size related traits with respect to non B carrier individuals (Table 2). However, MANOVA based in transformed data showed nonsignificant differences between karyotypes (Wilks' $\lambda_{5;41} = 0.84$, $P = 0.18$).

To analyse relationship between karyotype and phenotype on a reduced set of independent variables, a PCA was performed. The PCA analysis was conducted using the seven populations where a significant association was detected between B chromosomes and body size related traits. The first principal components, which accounted for nearly 78% of the total variation, is positively associated with all body size related traits analysed and may be considered as a measure of overall size. As a general factor, B carrier individuals showed lower PCA values with respect to standard individuals (Fig. 4).

The individual ANOVAS showed significant differences between karyotypes for PC1 ($F_{6; 202} = 4.10$, $P = 0.04$) whereas neither significant differences among populations ($F_{6;202} = 1.69$, $P = 0.13$) nor significant interaction 'population \times karyotype' ($F_{6;202} = 0.66$, $P = 0.68$) were detected.

Relationship between molecular data, body size and chromosome variation

Phenotypic differences between populations were estimated through both the differences in trait means and the mean percentage differences between the corresponding phenotypic variance-covariance matrixes (P) of each trait (T%).

Variation in the P matrix, evaluated through T% did not correlate with genetic distances matrix, neither in

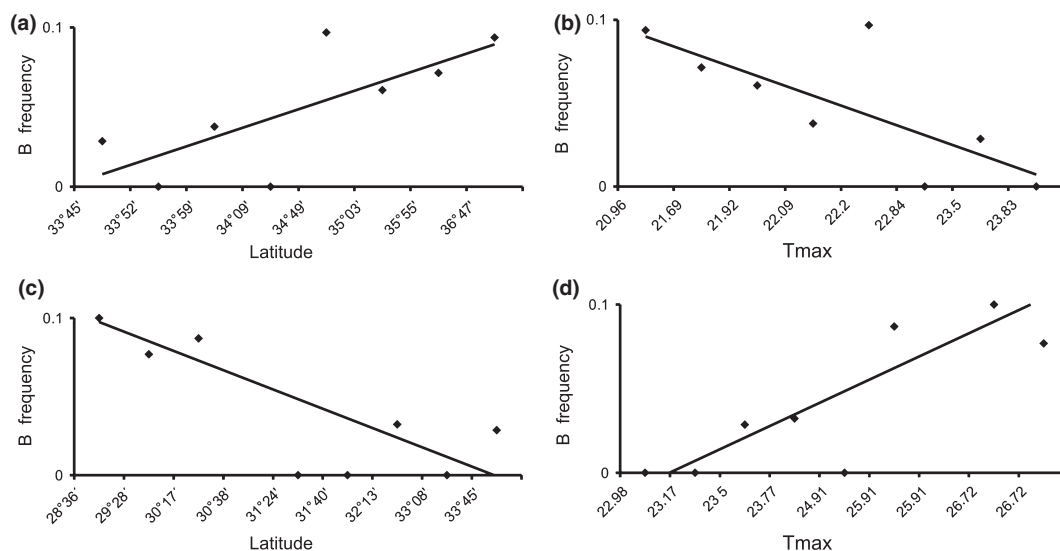


Fig. 3 Relationships between karyotype with geographical and climatic variables. B chromosome frequency vs. latitude (a and c) and B chromosome frequency vs. maximum temperature (Tmax, in °C) (b and d) in Argentinean populations of *Dichroplus elongatus*.

the Northern cline populations ($r = 0.14$, $P = 0.30$) nor in the Southern ones ($r = 0.23$, $P = 0.25$), indicating that genetic drift has not played an important role in shaping the P matrix.

For the Southern cline populations, there was no significant correlation between the difference in trait means and the difference in Nei's genetic distances (total: $r = -0.10$, $P = 0.73$; thorax: $r = -0.07$, $P = 0.60$; femur: $r = -0.19$, $P = 0.80$; tibia: $r = -0.08$, $P = 0.62$; tegmen: $r = -0.12$, $P = 0.66$), suggesting the importance of natural selection in shaping differences in mean values of analysed traits in this group of populations.

For the Northern cline populations, similar results were detected for tegmen and tibia length (tegmen: $r = 0.16$, $P = 0.27$; tibia: $r = -0.47$, $P = 0.07$) pointing out again the significance of natural selection on the mean value of these traits. Chromosome B frequencies evaluated through Euclidean distances did not correlate with genetic distances in both latitudinal clines (northern: $r = -0.03$, $P = 0.48$; southern: $r = -0.12$, $P = 0.76$) suggesting that variation in B chromosome frequencies cannot be moulded only by genetic drift.

Moreover, a significant relationship between differences in trait morphometric means and B chromosome distances was evident in southern cline populations (total: $r = 0.39$, $P = 0.01$; thorax: $r = 0.28$, $P = 0.06$; femur: $r = 0.29$, $P = 0.04$; tibia: $r = 0.43$, $P = 0.01$; tegmen: $r = 0.52$, $P = 0.002$). Partial Mantel tests demonstrated that the relationship was detected even after statistically controlling for the effect of neutral variation (total: $r = 0.38$, $P = 0.02$; thorax: $r = 0.28$, $P = 0.06$; femur: $r = 0.28$, $P = 0.045$; tibia: $r = 0.42$, $P = 0.01$; tegmen: $r = 0.52$, $P = 0.002$). Results of partial Mantel

test confirmed that migration- genetic drift interaction is not a sufficient explanation of the latitudinal pattern of clinal size and chromosome variation in *D. elongatus*.

MDS (Multidimensional Scaling) based on Nei's genetic distances, using DAMD (Direct Amplification of Minisatellite DNA) data, between populations in the Northern and Southern clines demonstrated the relationship between populations within groups (Fig. 5). MDS between populations of the Northern cline tend to cluster eastern and western populations separated by the Paraná River suggesting that restriction in gene flow may shape genetic interpopulation major differences. MDS between populations of the Southern cline represented two clusters grouping populations according to their geographical proximity.

MDS using T% and B chromosome differences did not agree with the MDS obtained through genetic distances suggesting that the same processes may not explain the detected patterns of variation. Some populations at similar latitude but geographically and/or genetically distant are clustered together (RLS-GUA; FLO and RAU in MDS for T%; SCA-COB and RLS-SCA in MDS for chromosome differences).

Discussion

Body size related traits represent valuable characters to analyse phenotypic intraspecific variation. Several examples demonstrated that arthropod populations of the same species differ phenotypically and that such variation is frequently correlated with some geographical and/or environmental variables (for review see Blanckenhorn & Demont, 2004). These traits are not likely to be the agents of variation by themselves, and

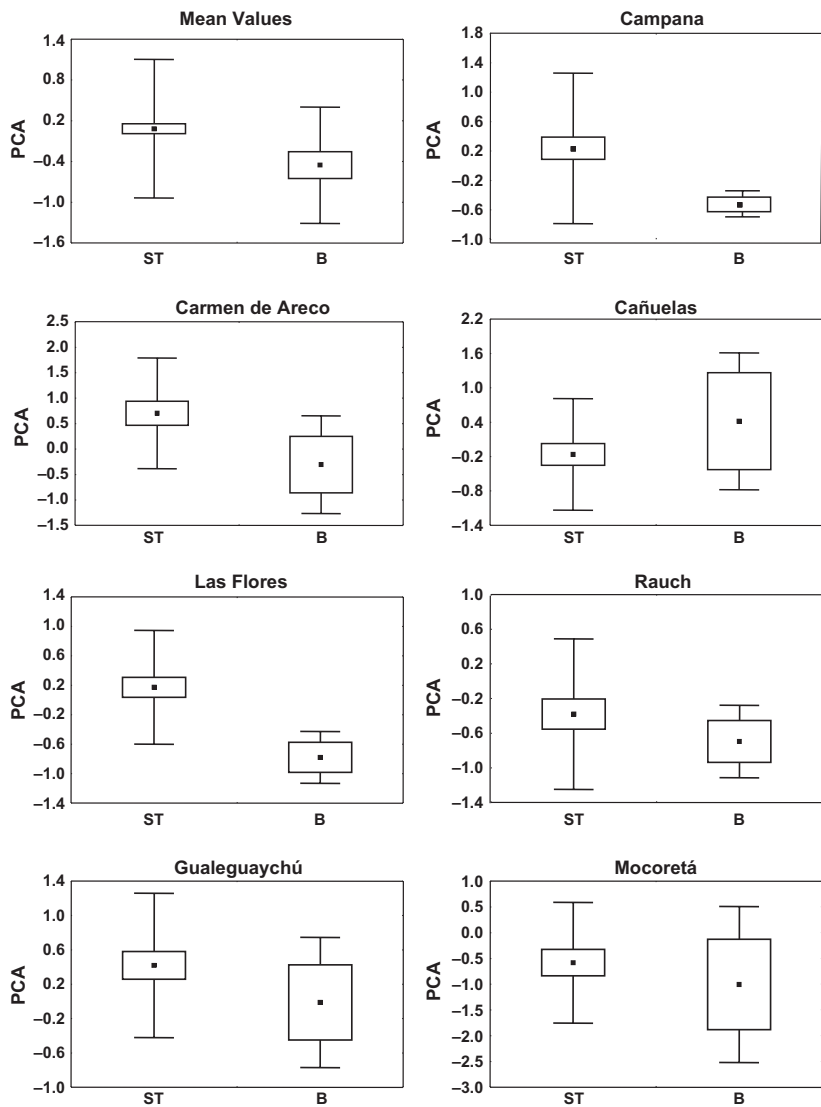


Fig. 4 Population box-plots for principal components in different karyotypes. Mean values (primary symbols), standard errors (boxes) and standard deviations (bars) for the first principal component (PC) in standard (ST) and B-carriers (B) males of *Dichroplus elongatus* from Argentinean populations

may be considered as indicator variables of some other factor that directly exerts a selective pressure on the population (Roff & Mousseau, 2005). It was accepted that latitudinal clines are adaptive and temperature may be the mean selection agent for phenotypic variation. Temperature may be related to both body size related traits themselves and the time in which growth and reproduction can take place (season length) (Roff, 1980).

Many species of arthropods showed a decrease in body size with increasing latitude or altitude. This pattern is known as 'converse Bergmann's rule' and has been widely described (Mousseau & Roff, 1989; Blanckenhorn & Fairbairn, 1995; Telfer & Hassall, 1999; Johansson, 2003; Bidau & Marti, 2007; Sesarini & Remis, 2008). In some examples, it was well demonstrated that body size differences among populations are very often genetically

based, as phenotypic variation is also expressed in the common garden laboratory (Masaki, 1967, 1972; Mousseau & Roff, 1989; Willot & Hassall, 1998; Telfer & Hassall, 1999; Schutze & Clarke, 2008).

In some species of insects a 'saw-tooth' pattern has been described. The 'saw-tooth' pattern occur where a species shift the life cycle from bivoltine (two generations per year) to univoltine (one generation per year) (Masaki, 1978; Mousseau & Roff, 1989; Brennan & Fairbairn, 1995; Groeters & Shaw, 1996; Mousseau, 2000; Johansson, 2003; Nygren *et al.*, 2007). According to this pattern, body size of insect tends to increase at lower latitudes in univoltine and bivoltine areas. However, body size tended to reduce at lower latitudes in the transition area from univoltine to bivoltine life cycle generating a marked decrease in body size (Roff, 1980).

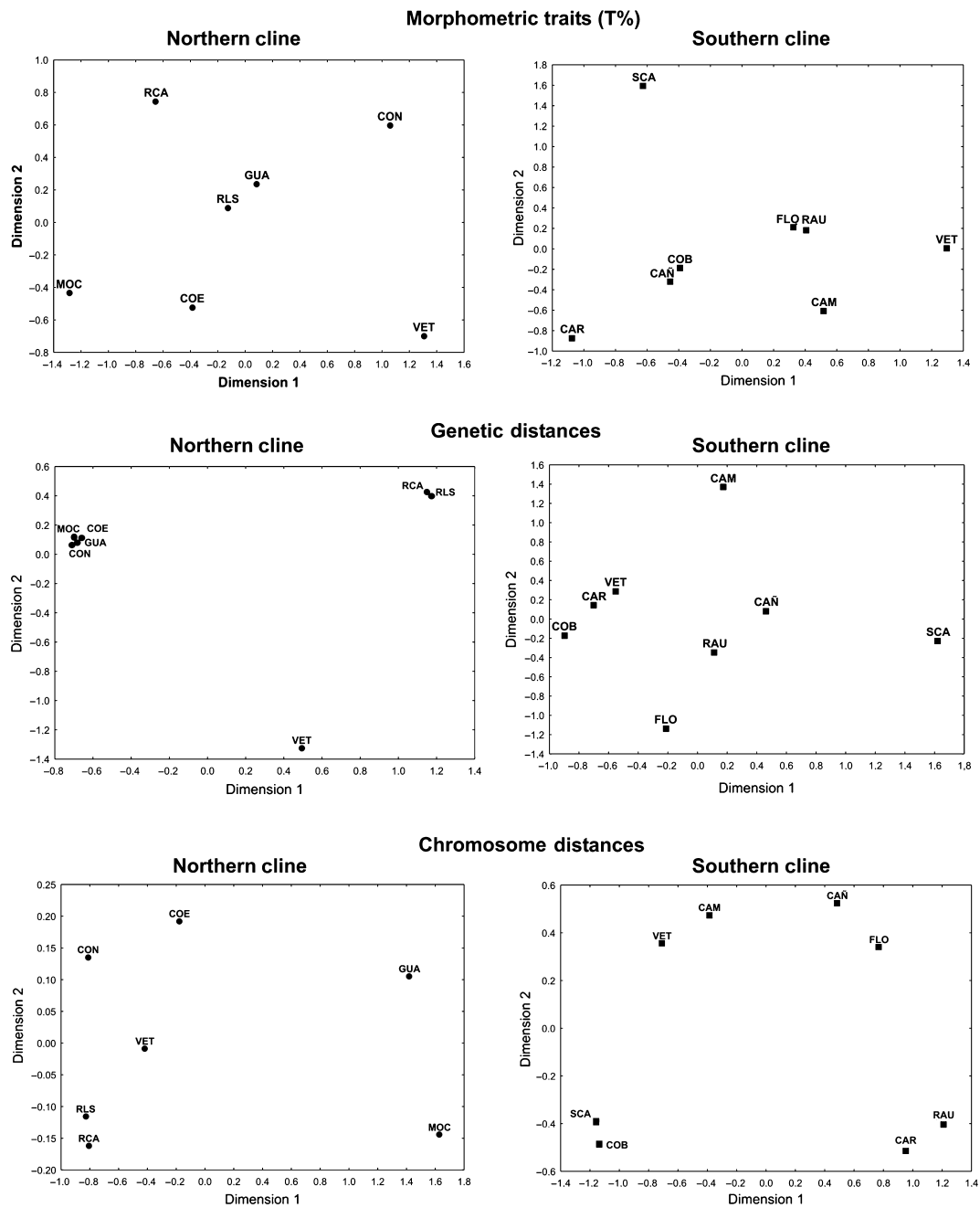


Fig. 5 Multidimensional Scaling Analysis (MDS) showing relationships among populations of *D. elongatus* in Northern and Southern Cline based on T% (stress values = 0.01 in both analysis); Nei's genetic distances (stress values = 0.00008; 0.09 respectively) and Euclidean chromosome distances (stress values = 0.0006; 0.000002 respectively).

The clinal pattern between VET (33°45'S) and RAU (36°47'S) populations should appear the variation pattern of univoltine populations. At higher latitudes the organisms have shorter seasonal length and development time and hence individuals are smaller. At lower latitudes in this area, individuals support larger favourable conditions to growth and development and

therefore are larger. As we continued moving northwards, from VET to MOC, body size related traits tended to decrease resembling the expected variation pattern in a transition area from univoltine to bivoltine life cycle.

Scarce information on how life cycle length varies with latitude is available in *D. elongatus*. Some studies

showed that the distribution of univoltine and bivoltine populations seem to support this scenario explaining body size variation in *D. elongatus* from Argentina (Barrera & Paganini, 1975; COPR (Centre for Overseas Pest Research), 1982; Lange & De Wysiecki, 2005; Luiselli *et al.*, 2002). However, additional studies are necessary to improve our understanding of the relationship between the number of generations per year and the detected body size variation in this grasshopper species.

In some examples chromosomal rearrangements are associated with effects on morphometric traits, establishing additional variation on which selection may be acting (White & Andrew, 1960; White *et al.*, 1963; Butlin *et al.*, 1982; Hasson *et al.*, 1992; Remis *et al.*, 2000; Colombo *et al.*, 2001, 2004; Werle & Klekowaki, 2004). Nevertheless, there are few examples of B chromosomes affecting body size. Morphometric studies in the mealy bug *Pseudococcus obscurus* showed that tibia length increased by increasing numbers of B's whereas in the grasshopper *Camnula pellucida*, B's are associated with a decrease in femur length (Camacho *et al.*, 2000). Moreover, B chromosomes of *Pseudococcus obscurus* delay male embryonic development supporting the increase in body size associated with B's (Nur, 1966). The B chromosome of the grasshopper *Myrmeleotettix maculatus* slows down the early prediapause development of males but no effect on morphometric measurements were detected between standard and B carrier individuals (Hewitt & East, 1978).

Previous studies in wild adult grasshoppers of *D. elongatus* from two Northwest Argentinean populations demonstrated that B chromosomes are associated with a decrease in body size related traits being more evident the effect on males than females (Rosetti *et al.*, 2007). Sex-specific effects of B chromosomes have also been detected in other organisms (Nur, 1969; Zima *et al.*, 2003; Bidau & Marti, 2008).

However, the effect of B chromosomes can vary spatially so the results in one population cannot be extrapolated to other population in the distribution range (Camacho *et al.*, 2000). For this reason, in the present article we carried out exhaustive cytogenetic and phenotypic simultaneous studies on 17 populations of *D. elongatus* from East Argentina to improve our understanding about B chromosomes in this species. Analysis of variance (uni and multivariate) and principal components demonstrated that in most analysed populations B carrier males are associated with smaller body sizes with respect to individuals with standard karyotype.

In *D. elongatus*, as in *Camnula pellucida*, B chromosomes are associated with a decrease in body size related traits. These results suggest that B's do not delay the male development but this conclusions require additional studies and should be considered carefully.

A clinal tendency towards a decrease in body size related traits with latitude was detected in *D. elongatus* populations between VET and RAU, and accordingly

with the relationship between B chromosomes and body size, an opposing pattern of latitudinal variation was observed in the frequencies of B's in this area. Similarly, contrary patterns of B chromosome with respect to body size variation may be apparent in populations with higher latitudes than the VET population. In populations between VET and MOC, it was observed that body size related traits increase and B chromosome decreases with latitude. These population approaches corroborate that B chromosomes are associated with a decrease in body size and suggest a consistent relationship among karyotype and exophenotype through a wide geographical area.

Several examples showed spatial chromosome differentiation associated with B chromosome clines (Hewitt & Brown, 1970; Shaw, 1983; Parker *et al.*, 1991; Zima & Macholan, 1995; Cabrero *et al.*, 1997). In some cases the relationships among karyotype, morphology and geographical/climatic variables were also analysed (Zima & Macholan, 1995; Zima *et al.*, 2003; Tsurusaki & Shimada, 2004).

In the harvestman *Psathyropus tenuipes* association between the number of Bs and external morphology was studied (Tsurusaki & Shimada, 2004). A northward increase in the number of Bs was detected throughout the Japanese Islands. Latitudinal gradients were also found in some external characters, whereas there were no correlations between those external morphologies and the number of Bs.

In the yellow-necked mice *Apodemus flavicollis* a relationship between the mean number of B chromosomes and body mass was found in males but not in females (Zima *et al.*, 2003). A clinal trend towards an increase in body size with latitude in *A. flavicollis* was detected in Europe following Bergmann's rule; consequently, with an adaptive scenario, a pattern of latitudinal variation was observed in the frequencies of B's in central and southeastern Europe (Zima & Macholan, 1995). It was proposed that this result can reflect a Bergmann's rule-like pattern, because, individuals with B's are better adapted to cold winter conditions owing increased growth rate and reaching larger body size.

The relative importance of adaptive and nonadaptive causes of clinal variation is an issue of central importance to our understanding of local adaptation and the determinants of geographical variation (Mayr, 1963; Felsenstein, 1976; Endler, 1977; García-Ramos & Kirkpatrick, 1997). In some cases, an inductive approach can be used to infer the causes of clinal variation in ecologically important traits. Because stochastic processes such as drift and gene flow are not expected to produce systematic patterns of variation in allelic frequencies, parallel clines in geographically isolated populations of the same species strongly implicate an adaptive basis for geographical differentiation. For example, parallel clines in body size of *Drosophila subobscura* on different continents implicate spatially varying selection as the driving

force of phenotypic divergence across latitudinal gradients (Huey *et al.*, 2000).

A more general approach to infer the role of selection in maintaining clinal variation is to compare relative levels of between-population divergence in quantitative traits and neutral DNA markers.

To gain insights into the evolutionary processes driving phenotypic divergences, we analysed the changes in trait means and in the phenotypic variance-covariance structure in relation to genetic differentiation detected at the molecular level. We used the genetic distance matrix from DAMD data as a predictor matrix for neutral variation patterns. We found no association between Nei's genetic distance and T% suggesting an important role of natural selection in the covariation pattern of morphometric traits in *D. elongatus*. Moreover, the lack of correlation found between genetic distances and mean value matrices in all analysed morphometric traits in the Southern cline and two morphometric traits in Northern cline support the importance of natural selection in shaping the clinal pattern of variation in mean values.

Similarly, no significant correlations were found between genetic distances and chromosome Euclidean distances in either Northern or Southern clines. The available evidence indicates that chromosome variation patterns would be shaped by selective factors. The patterns of chromosome variation in *D. elongatus* may be interpreted as changes of B-effects on carrier fitness across latitudinal variation. The frequency of B chromosomes may be a function of the mean body size, being more frequent in populations of lower body size. The partial Mantel test confirmed that phenotypic and chromosome clinal variation was not consistent with neutral divergence. Moreover, the patterns of variation in morphometric traits and chromosome frequencies appear to be related, suggesting the importance of selective forces shaping the evolutionary patterns of both variables. Previous results suggest that natural selection must have a relative important influence on B chromosome frequency in natural population of this species. In males it was demonstrated the existence of harmful effects of B chromosomes on mating success and fertility (Loray *et al.*, 1991; Remis & Vilardi, 2004; Rosetti *et al.*, 2007). Chromosome and phenotypic sexual selection favour larger males with standard karyotype. However, in females B chromosome is associated with a higher reproductive potential (Rosetti *et al.*, 2007). These results allow us to propose that B chromosome system in this species may be maintained by mitotic instability and selective effects involving some type of antagonistic selection. Changes in B-effects on carrier's fitness across latitudinal cline may explain the B chromosome pattern of variation. The selective disadvantage of B carriers on mating success in populations with relatively smaller body size may be weakened and thus B's are maintained at higher frequencies.

Another hypothesis may be related with the development time. If the small body size of B carriers is associated with a decrease in the development time it may be possible that B chromosomes are better tolerated in populations with shorter seasonal length. Further studies of male mating success and development time along latitudinal clinal variation will help us to understand this issue.

In most of the examples of clinal variation in the frequency of B's, a positive correlation with temperature was found (Jones & Rees, 1982). In some cases it was demonstrated that B chromosomes were widespread in regions where climate is more favourable for the species (Hewitt & Brown, 1970; Parker *et al.*, 1991). Our results demonstrated that the frequency of B's may be correlated negatively or positively with the latitude and hence with temperature depending the pattern of body size variation. Latitudinal contrasting chromosome patterns suggest that B frequencies might be moulded in relation to the pattern of phenotypic evolution.

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