Morphological, Genetic, and Chromosomal Variation at a Small Spatial Scale within a Mosaic Hybrid Zone of the Grasshopper *Dichroplus pratensis* Bruner (Acrididae)

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Abstract

Hybrid zones are regions where genetically different populations meet and mate, resulting in offspring of mixed characteristics. In organisms with limited dispersal, such as melanopline grasshoppers, hybrid zones can occur at small spatial scales (i.e., <500 m). We assessed levels of morphological, chromosomal, and molecular variability in adult males of the grasshopper *Dichroplus pratensis* Bruner (*N* = 137 males, 188 females) collected at 12 sites within a mosaic hybrid zone in a heterogeneous environment in Sierra de la Ventana, Argentina. In this hybrid zone, 2 Robertsonian chromosomal races, polymorphic for different centric fusions, meet (the “Northern race” at low altitudes and the “Southern race” at higher altitudes), forming hybrids that show monobrachial homologies during meiosis. High morphometric variation in 6 traits was revealed among grasshoppers of both sexes, with male body size positively and significantly correlated with increasing altitude. Frequency of Robertsonian fusions characteristic of the Southern race increased significantly with altitude. Moreover, fusion frequencies covaried between samples. Considerable genetic variation was revealed by random amplification of polymorphic DNA markers, with heterozygosity ranging from 0.3477 to 0.3745. Insects from low-altitude and high-altitude populations showed significant genetic differentiation, as indicated by *F*<sub>ST</sub> values. The proposed model for *D. pratensis*, involving the generation and maintenance by chromosomal fusions, of gene complexes adaptive in different environments, could explain the observed clinal patterns within the contact zone.

Key words: chromosomal fusions, grasshopper, morphometric traits, RAPDs, variability
the characteristics of individuals and populations through the hybridization zone, mainly at intermediate habitats (Ross and Harrison 2002). Mosaic hybrid zones may be maintained by selection together with habitat preferences, resulting in changing patterns of variation within specific spatial scales. In organisms with limited dispersal or vagility, it is at small scales that parental types meet, mate, and form hybrid offspring. Thus, sampling at different spatial scales is important when studying the constitution of hybrid zones (Ross et al. 2008).

*Dichroplus pratensis* Bruner, 1900 is a Neotropical grass-hopper with a very large latitudinal, longitudinal, and altitudinal distribution in Argentina (Bidau and Marti 2002; 2007; Cigliano and Orte 2003). *Dichroplus pratensis* has a standard telocentric karyotype of 2n = 19 (X0) male/20 (XX) female telocentric chromosomes, upon which 8 Robertsonian (Rb) fusions are superimposed (Bidau 1990, 1991; Bidau and Marti 2002). Argentine populations of this species, except the ones in the extreme margins of its distribution within the country, are polymorphic for 1–4 Rb fusions of different quality and frequency, involving the large telocentric autosomes (L1–L6) (Bidau and Marti 2002). Populations of *D. pratensis* occupying the center of the geographical distribution in Argentina display higher frequencies of Rb polymorphisms, and this is referred to as “central-marginal” distribution (Marti and Bidau 2001; Bidau and Marti 2002, 2005). Secondary contact between populations of *D. pratensis* in which the same telocentrics are involved in different arm combinations (monobrachial homologies) produces hybrid descendants that exhibit higher order multivalents (quadrivalents and quinquivalents) during meiosis (Bidau 1991; Tosto and Bidau 1991; Bidau and Marti 1995). Fusion multivalents show high frequencies of nondisjunctional orientation and segregation (Bidau 1991).

In males and females of *D. pratensis*, all heterozygous and homozygous Rb configurations show reduced and shifted chiasma patterns with respect to standard telocentrics (Bidau 1990; Bidau and Marti 1995; Chiappero et al. 2004). This chiasma repatterning causes intrachromosomal recombination to be less frequent and restricted to distal regions of fusion products and creates large recombination-free regions, which could house adaptive superfamilies (Bidau 1990; Bidau and Marti 1995; Marti and Bidau 1995). Through its effects on recombination, each fusion system would protect a given set of coadapted gene complexes adaptive to specific environments (Bidau 1990, 1991; Bidau and Marti 2002). Those coadapted gene complexes were proposed to be associated to the wide ecological tolerance and Marti (2002). Those coadapted gene complexes were proposed to be associated to the wide ecological tolerance of *D. pratensis*, as the chromosomal polymorphisms in nature (Bidau and Mirol 1988; Mirol and Bidau 1994; Bidau and Marti 1995), as has already been proposed for *Drosophila* (Brussard 1984). In *D. pratensis*, Rb fusions have a central-marginal distribution: populations inhabiting ecologically marginal areas where the environment is changing and unpredictable (such as the ones in the extreme North or extreme South of Argentina), usually do not exhibit fusions. In fusion-free populations, all chromosomes may be allowed to freely recombine and, in turn, more genetic variability can be released to act as the material for adaptation by natural selection (Bidau and Marti 2002, 2005).

Environmental gradients related to latitude, altitude, or other ecological constraints correlated with the organism’s reaction norm and life-history traits may promote morphological, chromosomal, and genetic clines at large and small scales (Brussard 1984; Endler 1986; Brown 1995; Gaston and Blackburn 2000; Bidau and Marti 2002, 2005). In this work, we studied a *D. pratensis* chromosomal hybrid zone within a heterogeneous environment spread over the southern portion of the transitional zone between wet and dry pampas of Argentina—Sierra de la Ventana, Buenos Aires province. The Sierra de la Ventana region presents a great diversity of microclimates (Kristensen and Frangi 1995; Luzzi et al. 2007), soils, and environments (Cappannini et al. 1971; Vargas Gil and Scoppa 1973) related to topography (Luzzi et al. 2007) and it shows a highly variable annual rainfall regime, transitional between continental and subtropical Atlantic type (Krepper et al. 1989). Sixteen different vegetation units have been described in Sierra de la Ventana and interpreted as a result of the interaction among those climatic and geological factors (Luzzi et al. 2007). Ecological gradients such as the one in Sierra de la Ventana can exhibit a wide variety of selection pressures that may generate, depending on the sources of natural selection in the milieu, different kinds of genetic responses (McAllister et al. 2009).

In Sierra de la Ventana a geographically restricted “Southern” race of *D. pratensis*, polymorphic for fusions between telocentric chromosomes L1 and L2 (L1/L2; the largest chromosomes of the karyotype, L3 and L4 (L3/L4), and L5 and L6 (L5/L6), contacts a “Northern” race, vastly distributed in central Argentina, polymorphic for fusions between telocentrics L1 and L6 (L1/L6) and L3/L4. As a result, complex Rb heterozygotes with reduced fertility occur at the contact zone (Bidau 1991). Within the Sierra de la Ventana mosaic hybrid zone, chromosome frequencies change steeply over relatively short distances and altitudes (ca. < 1000 m and < 500 m, respectively) with fusions L1/L2 and L5/L6 associated to higher altitudes (Tosto and Bidau 1991).

Heterogeneous environments can also contribute to the origin of an additional dimension of variation at the intraspecific level: morphological variation between sexes or Sexual Size Dimorphism (SSD) (Fairbairn 1990). SSD can arise either by differences in the direction and/or intensity of sexual selection between males and females (Darwin 1871) or by natural selection, if males and females show different ecological preferences (Butler et al. 2000; Bidau and Marti 2008). In most insects, as in *D. pratensis*, SSD is manifested as females being larger than males. Furthermore, if environmental factors produced differences on general size among races, different amounts of SSD would be
expected as shown for *D. pratensis* at a large geographic scale (Bidau and Martí 2007, 2008).

If chromosomal rearrangements play an active role in ecological adaptation of *D. pratensis* through the protection of coadapted gene complexes (Bidau and Mirol 1988; Mirol and Bidau 1994; Bidau and Martí 1995), we hypothesize that Sierra de la Ventana grasshoppers with different Rb fusion systems would also differ in their patterns of morphological and genetic variation at the molecular level. To investigate this, we assessed levels of morphometric, chromosomal, and molecular genetic variability in grasshoppers collected within the mosaic hybrid zone of Sierra de la Ventana. The study focused on samples from low and high altitudes within the area, in which significant changes in chromosomal composition within short distances are expected according to previous work (Tosto and Bidau 1991).

**Materials and Methods**

**Sampling**

We collected 188 female and 137 male adult grasshoppers at 12 sites within the Sierra de la Ventana hybrid zone (geographical midpoint at lat 38°09′S, long 61°47′W) in a transition area between low- and high-altitude populations. Two samples (DB 1 and DB 2) were taken at a site with mean elevation of 251.5 m above sea level; 8 samples (CC1–CC 8) were taken at Cerro Ceferino, a hill reaching 456 m. Samples were 30 m apart from one another, being CC1–2 taken at the foot of the hill and CC3–8 taken at the hill top, with the remaining samples taken at the hill slope (Figure 1). Two further samples (CCB1 and CCB2) were taken at a neighboring hill separated from Cerro Ceferino by approximately 200 m and a semipermanent stream (Figure 1). Complex chromosomal hybrids are expected to occur at DB1–2 and CC1–2 (Bidau 1991). Sample sizes were as follows: CC1: 142, 135; CC2: 172, 145; CC3: 182, 155; CC4: 182, 115; CC5: 165, 155; CC6: 182, 85; CC7: 152, 125; CC8: 125, 155; CCB1: 142, 145; CCB2: 192, 135; DB1: 125, 45; DB2: 152, 35.

**Morphometric Analyses**

We measured and log-transformed mean values of 6 morphometric traits: total body length (BL), pronotum (PL), tegmina (TeL), and monobrachial homologies (CV; Sokal and Rohlf 1995) and estimated SSD. We assessed morphometric variability through the coefficient of variation (CV = s × 100/μ; Sokal and Rohlf 1995) and applied a general linear model (GLM) and simple correlation/ regression analysis in SPSS v. 13.0. We calculated the ratio between the arithmetic mean of each character in females and that corresponding to males to visualize deviations from isometry (Smith 1999) and estimate SSD. We regressed log10 (male size) on log10 (female size) (Fairbairn 1997) to describe the scaling of SSD with body size by using reduced major axis (RMA) regression to estimate slopes (βRMA) with the software of Bohonak and van der Linde (2004), Java version. We tested the hypothesis that βRMA = 1.0 with

![Figure 1](https://academic.oup.com/jhered/article-abstract/102/2/184/785337) **Figure 1.** Representation of the study area. The location of Sierra de la Ventana within Buenos Aires Province (black) is indicated by a white dot. The circle in the lower-left part of the figure shows a general view of the sampling site. A detailed representation of the sampling scheme is provided, in which the 2 sampled hills (Cerro Ceferino Hill and CCB Hill) are represented by black triangles. Sampling sites for CC1-8 and CCB1-2 are indicated along the triangles; the distance between each sample (ca. 30 m) is also indicated. The crossroads between route 72 and route 76, where DB1-2 samples were taken, is shown. “Sauce Grande” and “El Negro” are a permanent and a semipermanent stream, respectively.

Clarke’s *T* statistic with adjusted degrees of freedom (Clarke 1980). For some analyses, we extracted factors from the 6 morphometric traits using the principal components analysis (PCA).

**Chromosomal Analyses**

Karyotypes were obtained as described in Bidau (1990). We characterized each sample by its *F* value (mean number of different homozygous or heterozygous fusions per individual per sample) (Bidau 1990). *F* ranges between 0 and 3 in nonhybrid populations, as 3 is the highest possible number of different fusions. In hybrid populations, due to monobrachial homologies, *F* can be >3. We explored the possibility that fusion frequencies were correlated with sampling location (altitude: ALT) by performing Mantel tests (Mantel 1967) in XLSTAT 2008. Using this test, the calculated statistic would represent the intensity of the relationship between the variables as in a Pearson *r* (Reynolds and Houle 2002).
DNA Isolation and RAPD-Polymerase Chain Reaction Amplification

We extracted DNA from 37 adult grasshoppers from Cerro Ceferino (CC1 N: 12; CC6 N: 13, and CC8 N: 12) and 9 from Don Bosco following Levitan and Grosberg (1993). We transferred femur muscles to a 1.5 ml sterile micro-centrifuge tube with 300 µl CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2 mercaptoethanol, 20 mM ethylenediaminetetraacetic acid [EDTA], and 10 mM Tris pH 8.0) and grinded them with a sterile glass pestle. We added 0.12 mg of Proteinase K (Promega, MD) and incubated tubes at 65 ºC for 90 min; following addition of 3 µl of RNAase (Sigma-Aldrich Co., Argentina) and incubation of tubes at 37 ºC for 1 h. We extracted samples twice with 300 µl of phenol:chlorophorm:isoamyl alcohol (25:24:1) and once with 300 µl of chloroform:isoamyl (24:1). To precipitate DNA, we added 30 µl of 10 M sodium acetate pH 6 and 300 µl of 100% ethanol and stored tubes at -20 ºC for an hour. We centrifuged samples for 20 min at 14 000 rpm; following rinsing of DNA pellets with 75% ethanol, drying, and resuspending in 200 µl of sterile distilled water.

Levels of molecular genetic variation were examined using randomly amplified polymorphic DNA (RAPD) markers. We initially screened 22 commercial 10-mer primers of arbitrary sequence to evaluate nuclear genetic variability in D. pratensis 10 from the A series (A1–A10) and 10 from the B series (B1–B10) (Biodynamics S.R.L., Buenos Aires, Argentina) and 2 from the OPB series (OP-B01 and OP-B03) (Operon Technologies, Alameda, CA). We performed RAPD-polymerase chain reactions (PCRs) in 25 µl final volume with: 10 mM Tris–HCl pH 9.50 mM KCl, 2.5 mM MgCl₂, 4% dimethyl sulfoxide, 0.2 µM each DNTP, 15 ng primer, 1 U Tag polymerase (Invitrogen, Carlsbad, CA) and 10 ng DNA. We included a negative control containing all reagents except DNA in each group of amplifications. We performed reactions in a UNO-II thermalcycler (Biometra, Goettingen, Germany) with a cycling program of: denaturing at 92 ºC for 1 min, 49 cycles each of 1 min at 92 ºC, 35 ºC for 1 min, and 2 min at 72 ºC; and a final extension step of 10 min at 72 ºC. We amplified each sample at least twice on different days to test for band repeatability. We loaded PCR products on 1.2% agarose gels stained with ethidium bromide and electrophoresed them in 0.5 Tris–borate–EDTA buffer, at 2 V/cm for 6 h. We photographed gels under UV light using a KODAK-DC290 (Eastman Kodak, Rochester, NY) digital camera. We sized PCR products by comparison with a standard DNA marker (Promega, Madison, WI). For subsequent analyses, we choose RAPD primers with bright, consistent, intense, polymorphic, and reproducible amplified bands, following criteria by Williams et al. (1990) and Ballinger-Crabtree et al. (1992).

Molecular Data Analysis

We constructed a binary matrix scoring amplified fragments as present (1) or absent (0) for each individual. We estimated allele frequencies using BIOSYS-1 version 1.7 (Swofford and Selander 1981), assuming Hardy–Weinberg equilibrium of genotypes and following Lynch and Milligan (1994). We estimated mean heterozygosities (̂H) by the TFGPA program (Miller 1998) and compared ̂H values for different samples using the Friedman test (Friedman 1937) contained in INFOSTAT (Infostat Group 2002). We calculated Wright’s (1951) fixation index (̂FST) values for samples from CC (CC1, CC6, and CC8) and Don Bosco, using the RAPDFST program (Black 1995), correcting for small and different sample sizes. We calculated Wright’s (1978) modification of Roger’s (1972) genetic distance using the same program. We verified the homogeneity of the theta (0) estimator of Wright’s ̂FST statistic by the “jackknife” methodology of nonparametric resampling and homogeneity of the ̂FST statistic by calculating the contingency chi square. To analyze if genetic divergence within the hybrid zone was correlated with chromosomal variation, we performed a Mantel (1967) test between the genetic (̂FST, matrix A) and chromosomal (P and fusion frequencies, matrix B) dissimilarity matrixes using DB, CC1, CC5, and CC8 as sources of data. To test for isolation by distance (IBD), the relationship between genetic distance matrix (measured by ̂FST) and geographic distance matrix was analyzed using the ISOLDE program implemented in the GENEPOP package (Raymond and Rousset 1995).

Results

Morphometric Variation

Grasshoppers showed extensive morphometric variation at a micro-spatial scale (see Supplementary Table S1). The GLM model, with all 6 morphometric characters separately measured in males and females from each sample as dependent variables, revealed highly significant differences between samples and sexes (Table 1). All characters, excepting tegmina length, showed highly significant differences between males and females (Table 2). Only body length was significantly different between samples (Table 2) (however, see below). “Sex” was considered as a fixed factor and “sample” as a covariate considering that microgeographic location was correlated to morphometric (and chromosomal) variation. Sample was not used as a fixed factor because of the high number of levels that would produce unwieldy results. Thus, sex × sample interaction

<table>
<thead>
<tr>
<th>Table 1</th>
<th>A GLM applied to the morphometric data of Table S1. Multivariate tests using Pillai’s trace statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect</td>
<td>Statistic Value</td>
</tr>
<tr>
<td>Intercept</td>
<td>Pillai’s Trace</td>
</tr>
<tr>
<td>Sex</td>
<td>Pillai’s Trace</td>
</tr>
<tr>
<td>Sample</td>
<td>Pillai’s Trace</td>
</tr>
</tbody>
</table>

Design: Intercept + Sex + Sample; df, degrees of freedom.
could not be statistically quantified. Nevertheless, a GLM (results not shown) reducing the sample variable levels to 6 by pooling pairs of localities by geographic proximity, allowed us to use sample as a fixed factor. In this case, the sex sample interaction was not significant.

Body length was significantly positively correlated with altitude in males ($r = 0.762$, $P = 0.004$, Figure 2a). To further analyze the relationship between body size and altitude, we performed PCAs including the 6 measured morphometric traits. Using the first PC, which showed the highest loadings for the majority of traits (Table 3) as size estimator, a positive marginally significant correlation was evidenced ($r = 0.555$, $P = 0.061$) for males. PC2, which included the remaining traits, showed a positive non-significant trend. We found no significant correlations between altitude and body size for females that showed a slightly increasing trend ($r = 0.272$, $P = 0.392$; BL, $r = 0.205$, $P = 0.524$). Strictly within the Cerro Ceferino hill (samples CC1–CC8), the trend for male body size was significant and positive (BL, $r = 0.725$, $P = 0.018$; PC1, $r = 0.664$, $P = 0.036$; PC2, ns). Again, female size was not significantly correlated with altitude although body length showed a slightly positive trend ($r = 0.484$, $P = 0.177$).

The GLM (Table 1) showed that SSD was part of the morphometric variation in our samples. Supplementary Table S1 showed that SSD was female biased for all traits in all samples with a few exceptions. RMA regression analyses performed to analyze the scaling of SSD with body size showed RMA slopes smaller than 1.0 for body length and third femur length, although the differences were not statistically significant (Table 4). Pronotum length showed a nonsignificant >1.0 slope (Table 4). Third tibia length, tegmina length, and pronotum height showed negative slopes, indicating that these characters decreased in size in males as general body size increased.

RMA regressions performed to study allometric relationships indicated that trait proportions did not vary concomitantly with respect to body size in males and females and only 2 trends in males (pronotum length and pronotum height) showed significantly higher than 1.0 RMA slopes (Table 5). In general, SSD did not show any relationship with altitude (ALT) although third tibia length was significantly correlated with ALT in an inverse function ($r = 0.589$, $P = 0.047$, $\log_{10} T3L = 0.991 + [14.69/ALT]$).

**Table 2** A GLM applied to the morphometric data of Table S1. Tests of between-subjects effects for each independent variable

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>BL</th>
<th>F3L</th>
<th>T3L</th>
<th>TeL</th>
<th>PL</th>
<th>PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>2</td>
<td>8.42, 0.002</td>
<td>12.65, &lt;0.001</td>
<td>37.18, &lt;0.001</td>
<td>1.29, 0.295</td>
<td>10.50, 0.001</td>
<td>9.10, 0.001</td>
</tr>
<tr>
<td>Intercepts</td>
<td>1</td>
<td>24 758.9, &lt;0.001</td>
<td>13 019.4, &lt;0.001</td>
<td>12 736.2, &lt;0.001</td>
<td>14 455.8, &lt;0.001</td>
<td>26 61.3, &lt;0.001</td>
<td>14 31.6, &lt;0.001</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>10.70, 0.004</td>
<td>23.52, &lt;0.001</td>
<td>72.70, &lt;0.001</td>
<td>0.14, 0.714</td>
<td>19.37, &lt;0.001</td>
<td>18.01, &lt;0.001</td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>6.14, 0.022</td>
<td>1.78, 0.196</td>
<td>1.66, 0.212</td>
<td>2.45, 0.132</td>
<td>1.62, 0.217</td>
<td>0.196, 0.663</td>
</tr>
<tr>
<td>Error</td>
<td>21</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>0.445</td>
<td>0.546</td>
<td>0.780</td>
<td>0.110</td>
<td>0.500</td>
<td>0.464</td>
</tr>
</tbody>
</table>

BL: body length; F3L: left femur 3 length; T3L: left tibia 3 length; TeL: tegmina length; PL: pronotum length; PH: pronotum height. In each cell, the Pillai’s trace value and its respective significance is given. $r^2$ coefficients are indicated in the bottom row; df, degrees of freedom.

**Figure 2.** Nonlinear regressions between altitude and $\log_{10}$ body length in 12 samples of the *Dichroplus pratensis* hybrid zone of Sierra de la Ventana. In both cases, the regression equation, correlation coefficient and statistical significance, are included in the figure. (a) $\log_{10}$ male body length versus altitude; (b) $\log_{10}$ female body length versus altitude.** Highly significant; ns, nonsignificant.
**Table 3** PCA of morphological data of males and females of *Dichroplus pratensis* from Sierra de la Ventana

<table>
<thead>
<tr>
<th>Principal components</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trait</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LGBLM</td>
<td>0.787*</td>
<td>0.280</td>
</tr>
<tr>
<td>LGTELM</td>
<td>0.027</td>
<td>0.828*</td>
</tr>
<tr>
<td>LGF3LM</td>
<td>0.677*</td>
<td>−0.511*</td>
</tr>
<tr>
<td>LGT3LM</td>
<td>0.918*</td>
<td>0.255</td>
</tr>
<tr>
<td>LGPLM</td>
<td>0.329</td>
<td>0.684*</td>
</tr>
<tr>
<td>LGPHM</td>
<td>0.857*</td>
<td>0.057</td>
</tr>
<tr>
<td>%VE</td>
<td>48.10</td>
<td>23.95</td>
</tr>
</tbody>
</table>

Factors were extracted and rotated with the VARIMAX procedure with Kaiser Normalization for 6 morphometric variables (BL: body length; F3L: left femur 3 length; T3L: left tibia 3 length; TeL: tegmina length; PL: pronotum length; PH: pronotum height). Values correspond to correlation coefficients between variables and factors. Relatively high loadings (|r| > 0.5) are marked with an asterisk.

**Chromosomal Variation**

The 4 fusions characteristic of the hybrid zone varied widely in the 12 samples, with mean frequency values (F) ranging from 1.9 (DB1) to 3.0 (CC6 and CCB2) (Supplementary Table S2). Fusion L1/L6 was only recorded in DB and in CC2 (Supplementary Table S2). The frequencies of L1/L2 and L5/L6 increased toward the top of both hills reaching fixation in most samples; fusion L3/L4 showed high frequencies in all samples (Supplementary Table S2). F and frequencies of fusions L1/L2, L3/L4, and L5/L6 were positively correlated with altitude (Figure 3a–d), whereas the restricted fusion L1/L6 did not show a significant correlation (Figure 3e).

The Mantel test indicated that the frequency of fusion L1/L2 was correlated with fusion L5/L6: r(AB) = 0.778, P < 0.0001. When the comparison included the chromosomal distance matrix (C) for frequency of fusion L3/L4, the result was, again, highly significant: r(AB.C) = 0.724, P < 0.0001.

In males from Cerro Cefénero, body length increased significantly and linearly with mean fusion frequency (r = 0.784, P = 0.017), with frequency of fusion L5/L6 (r = 0.949, P = 0.002) and with frequency of fusion L3/L4 (r = 0.788, P = 0.015). No trend was statistically significant in females.

**Molecular Variation**

After screening, 4 RAPD oligomers were chosen for molecular genetic diversity assessment: A04 (forward: 5’-CTTCACCCGA-3’), B03 (forward: 5’-ACTTCGACAA-3’), B04 (forward: 5’-TGCCCATCAG-3’), and OPB03 (forward: 5’-CATCCCGCCTG-3’). Amplification with these primers produced a total of 27 polymorphic bands varying in size between 700 and 2803 bp.

Mean H values were 0.3477 and 0.3745 for samples CC1 and CC8, respectively, and of 0.4311 for CC5 slope; Don Bosco’s samples showed a mean H value of 0.3111. Comparison of mean heterozygosities revealed significant differences between samples (Friedman test, P < 0.005). Modified Roger’s genetic distances ranged from 0.1325 to 0.1695. Wright’s (1951) FST revealed significant differences between samples CC1 and CC8 and between Don Bosco’s samples and each of the other samples (Table 6).

No significant trend was evidenced between genetic and chromosomal variation, as indicated by Mantel tests (Table 7). Similarly, no significant correlation between genetic and geographical distances was evidenced among the studied samples (ISOLDE program, P = 0.1140).

**Discussion**

Chromosomal polymorphisms in natural populations of animals and plants can occur as a means of controlling genetic recombination (Carson 1975). Robertsonian translocations can create, by restricting recombination (White 1973, 1978; King 1993), large genomic regions carrying alleles in linkage disequilibrium, including those adaptive to specific conditions (Noor et al. 2001; Rieseberg 2001; Noor and Bennett 2009). In this study, we analyzed *D. pratensis* grasshoppers sampled within the Sierra de la Ventana chromosomal hybrid zone, considered an ecologically favorable area for this species, that is, with more food availability and less strenuous environmental conditions.
than less favorable (marginal) areas (Bidau and Martí 2002).

The high frequencies of centric fusions found in the present work are consistent with the hypothesized model for *D. pratensis*, which predicts that populations located in central areas would show higher frequencies of chromosomal rearrangements (Chiappero et al. 2004). Despite its habitat heterogeneity, the Sierra de la Ventana area belongs to the central ecologically favorable range of the species, thus, selective pressures against chromosomal hybrids are relatively low with respect to marginal environments where resources are scarce, seasonality is high and reproductive season is much shorter (Bidau and Martí 2002, 2007). For these reasons, moderate levels of variability would be enough for *D. pratensis* populations to adapt to the central environment on Sierra de la Ventana, while in marginal habitats, high genetic variability would be essential for adaptation to harsh and unpredictable conditions (Bidau and Martí 2005). Thus, in spite of imposing low levels of recombination, the polymorphic Rb rearrangement system would be most likely maintained in these populations. As expected from the geographical distribution of the analyzed samples, mean number of fusions per sample, as well as the frequency of fusions L1/L2, L3/L4, and L5/L6 increased significantly with altitude, whereas fusion L1/L6 did not. Furthermore, frequencies of the 3 fusions covaried between samples, as evidenced by Mantel tests: Significant correlation was found among the frequencies of fusions L1/L2 and L5/L6, characteristic of the “Southern” or high elevation race, with L3/L4. In this context, it is worth noting that “pure” Southern race is a coastal form of restricted distribution that eventually reached the Sierra de la Ventana transition zone where it now occupies more low-resource areas (hill tops) than the “pure” Northern race, a form typical of grasslands. It is probable that the fusion system of the Southern form allowed its adaptation to a relatively harsher environment than that of the Northern race, and following competition within the hybrid zone, the former could only adapt to a restricted environment somewhat similar to the original one due to its already selected Rb system. Interaction between both races in small-scale transition zones would indeed produce covariation of centric fusions toward higher altitudes, to which, progressively, the Southern system became more adaptive.

Only male body length showed a positive and significant correlation with increasing altitude, indicating that they tend to be larger with higher altitude within a very short horizontal span (Figure 2a). Morphometric PC1, which might be a better representation of general body size, shows positive, but nonsignificant correlation with altitude. No trend was significant in females. Additionally, we found a correlation between *F* and body length in males from the study area (*r* = 0.784, *P* = 0.017). The nonsignificant trend observed for this correlation in females may probably be due to the fact that in females of this species, body size variability is usually lower than in males (Bidau and Martí 2007). The body size/karyotype correlations detected for males supports the hypothesis for *D. pratensis* because populations presenting more fusions, such as those in the hill top, would suffer restricted release of genetic variability, which in turn may also reduce the amount of morphological (quantitative) variation (Bidau 1991; Bidau and Martí 1995, 2005). This is in contrast with the trend in body size in *D. pratensis* at a large geographic scale where size shows an inverse correlation with altitude as well as latitude due to the shortening of the time available for development (Bidau and Martí 2007). Thus, the observed trend in Sierra de la Ventana is more probably associated to habitat segregation of 2 well-adapted forms to contrasting microhabitats within the hybrid zone which, although environmentally heterogeneous, only represents a very small fraction of total geographic range environmental variability of the species.

## Results of RMA regression of log10 (trait length) on log10 (male or female BL) for population means of 5 morphometric traits of *Dichroplus pratensis* populations from Sierra de la Ventana (F3L: left femur 3 length; T3L: left tibia 3 length; TeL: tegmina length; PL: pronotum length; PH: pronotum height)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Trait</th>
<th><em>r</em></th>
<th><em>t</em></th>
<th>df</th>
<th><em>P</em></th>
<th><em>r</em></th>
<th>df</th>
<th><em>P</em></th>
<th>95% CI</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>F3L</td>
<td>0.246</td>
<td>0.87</td>
<td>10</td>
<td>0.405</td>
<td>0.931</td>
<td>0.26</td>
<td>14.11</td>
<td>0.799</td>
<td>1.617</td>
</tr>
<tr>
<td></td>
<td>T3L</td>
<td>0.841</td>
<td>4.92</td>
<td>10</td>
<td>0.0006</td>
<td>1.185</td>
<td>0.020</td>
<td>1.13</td>
<td>16.89</td>
<td>0.275</td>
</tr>
<tr>
<td></td>
<td>TeL</td>
<td>0.268</td>
<td>0.88</td>
<td>10</td>
<td>0.400</td>
<td>0.609</td>
<td>0.35</td>
<td>13.40</td>
<td>0.200</td>
<td>0.196</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>0.194</td>
<td>0.63</td>
<td>10</td>
<td>0.543</td>
<td>1.763</td>
<td>0.34</td>
<td>13.21</td>
<td>0.023*</td>
<td>2.982</td>
</tr>
<tr>
<td></td>
<td>PH</td>
<td>0.572</td>
<td>2.20</td>
<td>10</td>
<td>0.050</td>
<td>1.881</td>
<td>0.488</td>
<td>1.35</td>
<td>14.80</td>
<td>0.003*</td>
</tr>
<tr>
<td>Female</td>
<td>F3L</td>
<td>0.529</td>
<td>1.96</td>
<td>10</td>
<td>0.078</td>
<td>0.885</td>
<td>0.291</td>
<td>0.33</td>
<td>14.30</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>T3L</td>
<td>0.486</td>
<td>1.76</td>
<td>10</td>
<td>0.109</td>
<td>0.634</td>
<td>0.175</td>
<td>1.39</td>
<td>13.40</td>
<td>0.198</td>
</tr>
<tr>
<td></td>
<td>TeL</td>
<td>0.112</td>
<td>0.36</td>
<td>10</td>
<td>0.726</td>
<td>1.368</td>
<td>0.430</td>
<td>1.30</td>
<td>13.07</td>
<td>0.216</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>0.009</td>
<td>0.03</td>
<td>10</td>
<td>0.977</td>
<td>1.104</td>
<td>0.349</td>
<td>0.35</td>
<td>13.00</td>
<td>0.732</td>
</tr>
<tr>
<td></td>
<td>PH</td>
<td>0.531</td>
<td>1.97</td>
<td>10</td>
<td>0.077</td>
<td>1.427</td>
<td>0.382</td>
<td>1.68</td>
<td>14.55</td>
<td>0.136</td>
</tr>
</tbody>
</table>

*P*, Pearson’s correlation coefficient; *t*, Student’s *t* statistic; *β*, slope of the RMA regression line; *T*, Clarke’s *T* statistic; df, degrees of freedom; df, Clarke’s adjusted degrees of freedom for *T*; *a*, intercept of the RMA regression line; 95% CI, 95% confidence intervals; SE, standard error; *P*, probability.
altitudes (Bidau and Martí 2008). However, the altitudinal trends of SSD were not significant for most characters, except third tibia length where SSD decreased with larger body size (and altitude) as expected. The causes for this pattern remain unknown but may reflect modifications of allometric growth patterns of each race due to hybridization within the zone.

At the molecular level, RAPD loci provided a useful tool to explore the molecular genetic diversity within and among

**Figure 3.** Nonlinear regressions between altitude and frequency of centric fusions in 12 samples of the *Dichroplus pratensis* hybrid zone of Sierra de la Ventana. In all cases, the regression equation, correlation coefficient, and statistical significance are included in the figure. (a) Mean frequency of different fusions per population (F) versus altitude; (b) Frequency of the 1/2 fusion versus altitude; (c) Frequency of the 3/4 fusion versus altitude; (d) Frequency of the 5/6 fusion versus altitude; (e) Frequency of the 1/6 fusion versus altitude. **Highly significant; *Significant at the 0.05 level; ns, nonsignificant.

*D. pratensis* samples differing in Rb rearrangements, as in other acridoids (Confalonieri et al. 2002; Zhang and Kang 2005; Sesarini and Remis 2008). Grasshoppers from Sierra de la Ventana hybrid zone showed moderate genetic variation at RAPD markers. In spite of the relatively few RAPD loci (27) examined in this study, the levels of heterozygosity found were higher, on average, than those reported for chromosomally distinct populations of other grasshoppers, using a higher number of loci (Confalonieri
than in other regions, and moderate levels of genetic environmental pressures are expected to be less stringent range (Bidau and Martí 2002). In central areas as this one, Ventana inhabit an ecologically favorable area of the species (Bennett 2009). Third, the populations carrying the rearrangements (Noor and "hitch-hike" with them, reducing overall genetic diversity in linkage disequilibrium with those gene-complexes, might gene complexes, any large regions within the genome in ability changes) would result in allelic differences detectable by markers primarily due to differences in mutation rates (Brussard 1984). This finding is not surprising because both markers differ in the nature and amount of genetic variability that they detect (Apóstol et al. 1996). Allozymes may exhibit lower heterozygosity than DNA-based markers primarily due to differences in mutation rates because only non-synonymous substitutions (amino acid changes) would result in allelic differences detectable by differences in electrophoretic mobility of protein products. The hypothesized model for D. pratensis can also account for the overall low to moderate levels of molecular genetic variation observed in the studied samples, in a three-fold manner. First, the observed levels are not unexpected if we bear in mind that chromosomal rearrangements have an overall recombination-reducing effect, which reduces variability per se (Bidau and Martí 2002). Second, if the rearrangements are harboring favorably selected co-adapted gene complexes, any large regions within the genome in linkage disequilibrium with those gene-complexes, might “hitch-hike” with them, reducing overall genetic diversity in the populations carrying the rearrangements (Noor and Bennett 2009). Third, D. pratensis populations of Sierra de la Ventana inhabit an ecologically favorable area of the species range (Bidau and Martí 2002). In central areas as this one, environmental pressures are expected to be less stringent than in other regions, and moderate levels of genetic variation would be sufficient to cope with adaptation to those environments. This model for D. pratensis contrasts with that proposed for Drosophila, in which ecologically central populations are more variable, at least at the allozymic level (Brussard 1984).

Samples from the slope of Cerro Ceferino hill showed the highest mean heterozygosity, as expected for samples that can have gene flow with samples that differ in the nature and frequencies of Rb fusions (from the hill top and foot). If the distinctiveness of chromosomal constitution reflects local adaptation to different micro-geographical conditions, a small amount of gene flow between the slope populations and the nearest others might cause them to be slightly more variable. However, this would be in principle incompatible with the absence of IBD although the former could be a result of the low statistical power of our analysis (see below).

RAPD loci revealed significant genetic differentiation, as measured by Wright's (1951) $F_{ST}$ estimator (Table 6), between D. pratensis samples that also differ in the quality and quantity of their Rb fusions: Cerro Ceferino and Don Bosco (different in the frequency of fusions 1/2, 3/4 and 5/6). These results are in agreement with those obtained by Chiappero et al. (2004) whom used another type of molecular marker (allozymes) to compare samples from the Northern hybrid and Southern D. pratensis races from the same hybrid zone. In a neighbor-joining tree of genetic distances, D. pratensis samples clustered according to their origin (sampling location) and chromosomal composition (Chiappero et al. 2004). Thus, it appears that chromosomal differences may be promoting differentiation at the molecular level (Bidau and Martí 1995, 2002; Martí and Bidau 1995). A possible mechanism through which this might happen was proposed by Confalonieri et al. (2002) to explain why RAPD loci frequencies tended to follow similar patterns of variations as chromosome and enzymatic variability in the grasshopper Trimerotropis pallidipennis. This mechanism relies in the fact that RAPD primers’ annealing sites may be affected by rearrangements (inversions in the case of T. pallidipennis, Rb fusions in the case of D. pratensis) as they involve large parts of the grasshoppers’ genomes (Confalonieri et al. 2002). Pattern changes of RAPD bands due to chromosomal rearrangements should occur when priming sites span rearranged chromosomal segments (e.g., band(+) in 1/2 fused metacentric, band(−) in unfused chromosomes 1 and 2). Thus, cytogenetic distinctions between Cerro Ceferino and Don Bosco samples may be reflected also by differences in RAPD patterns between them. Furthermore, considering that chromosomal rearrangements promote generation and maintenance of large chromosomal regions with reduced recombination (Bidau 1990; Bidau and Martí 1995, 2002; Noor et al. 2001; Rieseberg 2001), and that this, in turn, probably reduces the genetic diversity within populations, relative divergence measures, such as $F_{ST}$, are expected to increase among subpopulations bearing different rearrangements (Noor and Bennett 2009). Although the Mantel test performed to test the hypothesis that the observed genetic variation was

---

**Table 6** Wright's (1951) $F_{ST}$ estimator of genetic differentiation (standard deviation) based on RAPD analysis among Dichroplus pratensis populations from Sierra de la Ventana mosaic hybrid zone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CC1</th>
<th>CC6</th>
<th>CC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>0.00000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC6</td>
<td>0.0155</td>
<td>0.00000</td>
<td></td>
</tr>
<tr>
<td>CC8</td>
<td>0.0380**</td>
<td>0.0014</td>
<td>0.00000</td>
</tr>
<tr>
<td>DB</td>
<td>0.1872*</td>
<td>0.1172*</td>
<td>0.1479*</td>
</tr>
</tbody>
</table>

CC1: Cerro Ceferino foot; CC6: Cerro Ceferino slope; CC8: Cerro Ceferino top; DB: Don Bosco.

* $P < 0.05$; ** $P < 0.01$.

---

**Table 7** Results of Mantel tests for the comparison of genetic and chromosomal dissimilarities within the Sierra de la Ventana hybrid zone of Dichroplus pratensis.

<table>
<thead>
<tr>
<th>Chromosomal trait</th>
<th>t(AB)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>0.713</td>
<td>0.140</td>
</tr>
<tr>
<td>1/2</td>
<td>0.895</td>
<td>0.072</td>
</tr>
<tr>
<td>5/6</td>
<td>0.686</td>
<td>0.154</td>
</tr>
<tr>
<td>3/4</td>
<td>0.679</td>
<td>0.172</td>
</tr>
<tr>
<td>1/6</td>
<td>0.945</td>
<td>0.050*</td>
</tr>
</tbody>
</table>

* Significant.

---

et al. 2002, 47 RAPD loci; Sesarini and Remis 2008, 84 loci). Heterozygosity values found in this study were also higher than those reported for D. pratensis of Sierra de la Ventana using allozymes as genetic markers (see Table 3 in Chiappero et al. 2004). This finding is not surprising because both markers differ in the nature and amount of genetic variability that they detect (Apóstol et al. 1996). Allozymes may exhibit lower heterozygosity than DNA-based markers primarily due to differences in mutation rates because only non-synonymous substitutions (amino acid changes) would result in allelic differences detectable by differences in electrophoretic mobility of protein products. The hypothesized model for D. pratensis can also account for the overall low to moderate levels of molecular genetic variation observed in the studied samples, in a three-fold manner. First, the observed levels are not unexpected if we bear in mind that chromosomal rearrangements have an overall recombination-reducing effect, which reduces variability per se (Bidau and Martí 2002). Second, if the rearrangements are harboring favorably selected co-adapted gene complexes, any large regions within the genome in linkage disequilibrium with those gene-complexes, might “hitch-hike” with them, reducing overall genetic diversity in the populations carrying the rearrangements (Noor and Bennett 2009). Third, D. pratensis populations of Sierra de la Ventana inhabit an ecologically favorable area of the species range (Bidau and Martí 2002). In central areas as this one, environmental pressures are expected to be less stringent than in other regions, and moderate levels of genetic variation would be sufficient to cope with adaptation to those environments. This model for D. pratensis contrasts with that proposed for Drosophila, in which ecologically central populations are more variable, at least at the allozymic level (Brussard 1984).

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related to chromosomal distinctiveness was nonsignificant, it is important to bear in mind that our analysis had low statistical power. Alternatively, and probably more likely, it can be that significant $F_{ST}$ between DB and CC sites are due to spatial isolation, such as in a nonselective IBD model (Wright 1943). However, the test performed to inspect if the observed pattern in \textit{D. pratensis} could be explained by IBD was nonsignificant. We have to bear in mind, though, that our data set had limited statistical power and that IBD applies to populations under equilibrium between gene flow and genetic drift, which might not be the case in the studied \textit{D. pratensis} samples.

We cannot rule out the possibility that the significant genetic differentiation observed between subpopulations is product of genetic drift acting on populations with restricted gene flow. For drift to be responsible for differentiation, severe limitation to gene flow, and thus genetic isolation, must be ongoing in these populations. If restricted gene flow is present, it most likely be caused by the different chromosomal composition of each population but not enough evidences exist at present to ensure this. Moreover, the samples studied in this work are representatives from a continuum within a greater hybrid population, and not small geographically isolated populations necessary for genetic drift being a strong force.

In conclusion, the present study analyzed a chromosomal hybrid zone with the simultaneous use of 3 kinds of traits that can evolve at different rates. Our data revealed that chromosomal, morphological, and molecular variation is that can evolve at different rates. Our data revealed that hybrid zone with the simultaneous use of 3 kinds of traits force. If restricted gene flow is present, it most likely be caused by the different chromosomal composition of each population but not enough evidences exist at present to ensure this. Moreover, the samples studied in this work are representatives from a continuum within a greater hybrid population, and not small geographically isolated populations necessary for genetic drift being a strong force.

In conclusion, the present study analyzed a chromosomal hybrid zone with the simultaneous use of 3 kinds of traits that can evolve at different rates. Our data revealed that chromosomal, morphological, and molecular variation is widespread in \textit{D. pratensis} populations from Sierra de la Ventana hybrid zone and supported differentiation patterns within very short distances. We must stress, however, that additional studies examining more RAPD loci and/or different kinds of genetic markers are needed before more robust conclusions can be made on the nature of the selective forces that act to maintain differentiation. Although the underlying causes of these differences remain to be elucidated, we propose that local adaptation can persist in the face of partially restricted gene flow due to negative heterosis in chromosomal hybrids and of the effects of environmental heterogeneity on specific combinations of genotypes that vary in fitness. The Sierra de la Ventana hybrid zone remains an excellent model to test these predictions.

**Supplementary Material**

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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**References**


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