Nonclinality of Molecular Variation Implicates Selection in Maintaining a Morphological Cline of *Drosophila melanogaster*

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**ABSTRACT**

One general approach for assessing whether phenotypic variation is due to selection is to test its correlation with presumably neutral molecular variation. Neutral variation is determined by population history, the most likely alternative explanation of spatial genetic structure, whereas phenotypic variation may be influenced by the spatial pattern of selection pressure. Several methods for comparing the spatial apportionment of molecular and morphological variation have been used. Here, we present an analysis of variance framework that compares the magnitudes of latitudinal effects for molecular and morphological variation along a body size cline in Australian *Drosophila* populations. Explicit incorporation of the relevant environmental gradient can result in a simple and powerful test of selection. For the Australian cline, our analysis provides strong internal evidence that the cline is due to selection.

*Drosophila melanogaster* populations from several continents show a positive relationship between body size and latitude (COYNE and BEECHAM 1987; CARRY et al. 1993; IMASEVA et al. 1994; VAN’T LAND et al. 1999). The repetition of body size clines provides evidence that they are due to selection as opposed to population history. Body size clines in *Drosophila*, therefore, present an attractive context for evaluating methods for detecting selection on the basis of internal evidence from single clines. Here, we describe a method for determining if latitudinal variation in a trait (in this instance body size) is a likely consequence of natural selection, or, instead, of demographic history, the main competing hypothesis. We compare latitudinal effects on microsatellite allele frequency and on wing area (a trait highly correlated with body size) in populations of *D. melanogaster* previously reported to show a latitudinal cline in body size in Eastern Australia (JAMES et al. 1995, 1997).

Previous studies (PROUT and BARKER 1993; SPITZER 1993; LONG and SINGH 1995; PODOLSKY and HOLTFSORD 1995; LYNCH et al. 1999) have employed a test for natural selection that was developed by PROUT and BARKER (1993). This test relied on a comparison of *F* sub t values, based on (neutral) molecular and (possibly adaptive) morphological variation, denoted, respectively, *F* sub s and *Q* sub s (LANDE 1992). In this approach, selection was implicated if the confidence intervals for *Q* sub s and *F* sub s differed. LONG and SINGH (1995) generalized this approach by testing whether *Q* sub s and *F* sub s maintained proportionality in a comparison of population pairs. This formulation allowed for population structure, reflected by the degree of molecular and morphological difference between different population pairs. For some situations, however, apportioning diversity with *F*-statistics is not ideal. For example, in the case of clinal variation, we should like to include in our test the hypothesis of a specific environmental gradient (e.g., altitude or latitude) responsible for the spatial variation in selection pressure. Such a measure of “clinality” was used by BERRY and KREITMAN (1993) to describe latitudinal variation of haplotype frequencies at the Adh locus. Similar to this approach, we provide a test for natural selection by comparing the magnitudes of latitudinal effects for molecular and morphological variation along a body size cline in Australian *D. melanogaster* populations.

**ANALYSIS AND RESULTS**

In this study, we analyzed populations of flies collected at 11 different latitudes from a 2000-km north–south transect along the east coast of Australia in January 2000 (Table 1). One to four locations (0.005–35 km apart) were sampled from each latitude. Latitudinal sites varied in spacing along the transect and were densely clustered in a section (32.6–37.5° S) where the steepest slope of morphological change had been observed previously (JAMES et al. 1995). The southernmost samples were derived from the island of Tasmania (43° S) and were therefore necessarily more distant from their nearest continental neighbor (37.5° S). Altitudinal differences between populations were minimized by sampling at coastal sites as close to sea level as possible. Isofemale

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lines were set up from wild-caught flies and, after two to three generations in the laboratory, lines were cultured at a standard larval density (50 larvae per vial) at 25° using methods described in James et al. (1995). A single vial was cultured for each isofemale line, and 10–20 lines were cultured from each latitude. From each vial, the right wings of five randomly selected individuals of each sex were removed and the area was determined using methods described in Gilchrist and Partridge (1999).

Molecular variation in these populations was assessed using 19 polymorphic microsatellite loci, distributed across the second and third chromosomes (Table 2; further information can be obtained from the microsatellite database at http://www.ucl.ac.uk/biology/goldstein/mlist1.htm). Due to the fact that these loci were specifically selected for a high number of repeat units, some markers show variences in repeat number that are unusually high for D. melanogaster. Genotypes at these loci were scored from DNA extracted from individual wild-

### TABLE 1

<table>
<thead>
<tr>
<th>Site</th>
<th>Code</th>
<th>Latitude (°S)</th>
<th>Longitude (°E)</th>
<th>Wing area Populations</th>
<th>Microsatellites Populations Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cape Tribulation</td>
<td>MEG</td>
<td>16.85</td>
<td>145.46</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Townsville</td>
<td>TOW</td>
<td>19.37</td>
<td>146.00</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>Rockhampton</td>
<td>ROC</td>
<td>23.40</td>
<td>150.49</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Coolangata</td>
<td>COO</td>
<td>28.29</td>
<td>153.53</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Belmont</td>
<td>BEL</td>
<td>32.60</td>
<td>151.16</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>Picton</td>
<td>PIA</td>
<td>34.11</td>
<td>150.34</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Milton</td>
<td>MIL</td>
<td>35.18</td>
<td>150.27</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Termiel</td>
<td>UPB</td>
<td>35.30</td>
<td>150.13</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Melbourne</td>
<td>KOC/YY</td>
<td>37.48</td>
<td>145.27</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Hounville</td>
<td>HUO</td>
<td>43.09</td>
<td>147.04</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Tasmania</td>
<td>SCR</td>
<td>43.04</td>
<td>147.04</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

Cytological and genetic locations are according to GenBank. Expected heterozygosities were calculated for all flies listed in Table 1. Significant proportions of variation explained by latitude are given after Bonferroni correction (NS, not significant; *, significant at the 0.05 level).
the PCR reaction, amplifications were prepared in the prealiquoted plates (Advanced Biotechnologies, Surrey, UK), each containing 0.2 mm dNTP, 1.5 mm MgCl₂, 75 mm Tris-HCl, 20 mm (NH₄)₂SO₄, 0.01% Tween 20, and 0.31 units of Taq polymerase. Primer combinations are listed in Table 2. One microliter each of DNA extract and primer mix were added for a final reaction volume of 15 μl. Concentrations for each primer pair in the mix were adjusted and final concentrations in the PCR reactions ranged from 0.04 to 0.54 μg/μl. All amplifications were carried out in a GeneAmp PCR 9700 system (Applied Biosystems, Foster City, CA) using the following cycle profile: 94°C, 4 min; (94°C, 30 sec; 53°C, 30 sec; 72°C, 30 sec) × 25; 72°C, 8 min. For specificity and sensitivity of the PCR reaction, amplifications were prepared in the following order: DNA was pipetted into the lids of 96-well plates (0.2 ml); primer mix was added to the prealiquoted PCR reaction mix; lids were attached, and the plate was centrifuged 1 min at 1000 rpm and placed in the preheated thermocycler (“hot start” PCR). Allele sizes were scored on an automated sequencer (ABI 377) by using GeneScan 2.1 and Genotyper 2.5 software (Perkin-Elmer). The results of this survey revealed a high degree of variability within loci. Expected heterozygosities ranged between 0.40 and 0.99 and populations shared 44% of their alleles on average.

The relationship between population mean body size and latitude was assessed by employing a simple linear regression model (\( Y = b_0 + b_{\text{Lat}} + \varepsilon \)). To estimate the proportion of the total microsatellite variation explained by latitude, frequencies of the most common allele (MCA) were calculated for each locus and used as dependent variables in linear regression. Although only the MCA was scored for each locus it is still possible to detect clinality, as the majority of alleles at each locus are at such low frequency that they do not show a clinal pattern. In an analysis of an arbitrary set of three loci the inclusion of the full set of alleles did not increase the apparent clinality. Distributions of the residuals resulting from these regression models (body size measurements and MCA frequencies) did not show deviations from normality (Kolmogorov-Smirnov test, all \( P > 0.05 \)). All regression models were analyzed using the software package STATISTICA (5.5 A; StatSoft, Tulsa, OK). Nested analysis of molecular variance (AMOVA) was carried out using the software package ARLEQUIN (version 2.0; SCHNEIDER et al. 2000).

Apportioning variances due to latitude revealed highly contrasting results between the molecular and morphological data. The amount of variation explained by latitude for wing area was 81 and 82% (\( P < 0.001 \)) for males and females, respectively. Flies from extreme northern populations were typically 15% (3–4 standard deviations) smaller than those from the southernmost population (Figure 1). These results can be compared with the findings of JAMES et al. (1995), who measured flies from similar latitudes that were collected in 1993 and maintained in the laboratory as cage populations and found that latitude explained 63.0% (males) and 64.2% (females) of the variation among population means along the cline. The lower proportion of variation explained by latitude in the earlier study may reflect the lower intensity of sampling over the latitudes where morphology showed the greatest clinal variation. The proportion of variation explained by latitude for the 19 microsatellite loci ranged from 1% (AC005115) to 76% (DMU25686), with a mean of 21% (Table 2). After correction for multiple comparisons, latitude explained a significant proportion of the total variation for loci DMU14395, AC008193, AC004759, DMTRXIII, and DMU25686.

To test the null hypothesis of morphological variation according to neutral expectations, the overall explanatory power of latitude for morphological and molecular variation was compared. Confidence limits for the morphological coefficients of determination (\( R^2 \)) were calculated for an empirical distribution obtained by bootstrapping. In each of 1000 iterations, observed residuals from the linear model were drawn at random and used to calculate new regression coefficients. Upper and lower 95% confidence limits (CLs) were determined for these 1000 \( R^2 \) values over all loci were calculated and 95% CLs were determined for these 1000 \( R^2 \) means. The upper CL for the microsatellite \( R^2 \) was 0.28. In contrast, the lower CLs for wing area were considerably higher in both sexes (males, 0.75; females, 0.76). AMOVA revealed 1.95% of variation between populations that were grouped by latitude. In an analogous analysis, 56% (females) and 56.5% (males) of the total wing area variation were explained by differences among populations. The envi-
Environmental gradient (in this case latitude) had a much greater impact on the distribution of morphological variation. The data also confirm the presence of the body size cline in two independent samples collected 7 years apart.

CONCLUSIONS

The test for natural selection along an environmental cline, suggested by Prout and Barker (1993) and later modified by Long and Singh (1995), was based on a comparison of population structures between quantitative characters, on which selection may act, and molecular markers that are assumed to evolve at a neutral rate. However, this method does not explicitly incorporate the relevant environmental variable. The difference between the methods can be illustrated by an example in which we imagine a sampling regime with spatially clustered populations along a gradient, which might reflect the situation of most field collections. The differences and $F$ values from all populations would then contribute equally to the dataset. However, for selection pressures that change systematically along a cline, neighboring populations are predicted to be more similar to each other than to distant ones. We expect the greatest mismatches between the molecular and morphological diversity for the greatest separations along the environmental gradients, exactly as observed in this study. For the example of latitudinal variation in body size in eastern Australia, populations were not sampled at equal distance, but more densely in the south, and with the natural gap between the southernmost continental and Tasmanian sites. To account for this gradually varying dependency of samples within the dataset, one should test for the influence of the environmental variable (in this case latitude) on the morphological and molecular variation between populations. By investigating size changes along a predefined latitudinal transect we add a post hoc component to our statistical analysis. However, the main focus of this study was to carry out two specific tests: First, is there a significant relationship between the morphological trait and the environmental gradient and, second, is there a relationship with neutral molecular markers along the same gradient? Hence, we do not address the question of whether this trait is under direct selection pressure. Future work is needed to clarify whether body size is a direct target of selection or genetically correlated with another trait under direct selection. As the main focus of this study is a comparison of the proportion of variation explained by an environmental gradient between two different sets of data, we deliberately chose a simple linear regression model. However, to describe the shape of the cline in more detail, different slope-fitting models could be employed.

Microsatellites provide high resolution of population structure (e.g., Goldstein et al. 1999), and are therefore particularly useful for this kind of study. If a trait is influenced by the environmental gradient, while the microsatellites are primarily influenced by demographic factors such as migration and colonization history, then we would expect the explanatory value of the environmental gradient to be statistically distinguishable for the two types of data. However, five loci (DMU14395, AC008193, AC004759, DMTRXIII, and DMU25686) showed significant variation due to latitude. It will be interesting to see if one or more of these loci are linked to a quantitative trait locus associated with changes in body size. Clinality of markers such as observed for AC004759 could be due to linkage disequilibrium with a linked quantitative trait locus (QTL) that it is under clinal selection. In this case one would expect a window of clinality involving multiple markers surrounding a QTL. The expected size of such a window of clinal markers would depend on many factors, including the pattern of linkage disequilibrium in the founding population and the strength and timing of clinal selection, and is impossible to predict a priori. Nonetheless, the observation of clinality for multiple closely linked markers would strengthen considerably the case for a linked QTL under clinal selection. This could be the case for loci DMU25686 and AC008193, which are located in close proximity on the third chromosome (cytological locations 93F and 94D, respectively).

Variation along the cline can be explained by two alternative hypotheses: First, both morphology and molecular variation may have been shaped by demographic processes, including the colonization process, independent of any selective effects on the phenotypic variation. Second, the morphological variation may have been shaped by selection, in which case it would not be expected to be correlated with molecular variation that is influenced only by demography. On the basis of the clear difference in variance explained by the environmental gradient between molecules and morphology, we reject the first hypothesis. It should be noted, however, that rejecting a common demographic explanation for both the molecules and the morphology does not rule out nonequilibrium configuration of the molecular data. In particular, analyses of genetic structure along the cline show some support for at least two differentiated populations, which could reflect multiple colonization events (data not shown).

We were able to show that the magnitude of the morphological cline and its shape has been conserved over a time period of at least 7 years. Even local variations such as the slight increase in body size at the sample site in Bowen (20.01° S) in the 1993 sample are rediscovered in the recent collection at Townsville (19.37° S). Although population fluctuations or colonization patterns along the cline are unknown, we conclude that the overall evolutionary response is not only predictable (Huey et al. 2000) but also well conserved over long periods of time.
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