

SPATIAL STRUCTURE AND HABITAT VARIATION IN A GRASSHOPPER HYBRID ZONE

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Abstract.—A hybrid zone between the grasshoppers *Chorthippus brunneus* and *C. jacobsi* (Orthoptera: Acrididae) in northern Spain has been analyzed for variation in morphology and ecology. These species are readily distinguished by the number of stridulatory pegs on the hind femur. Both sexes are fully winged and inhabit disturbed habitats throughout the study area. We develop a maximum-likelihood approach to fitting a two-dimensional cline to geographical variation in quantitative traits and for estimating associations of population mean with local habitat. This method reveals a cline in peg number approximately 30 km south of the Picos de Europa Mountains that shows substantial deviations in population mean compared with the expectations of simple tension zone models. The inclusion of variation in local vegetation in the model explains a significant proportion of the residual variation in peg number, indicating that habitat-genotype associations contribute to the observed spatial pattern. However, this association is weak, and a number of populations continue to show strong deviations in mean even after habitat is included in the final model. These outliers may be the result of long-distance colonization of sites distant from the cline center or may be due to a patchy pattern of initial contact during postglacial expansion. As well as contrasting with the smooth hybrid zones described for *Chorthippus parallelus*, this situation also contrasts with the mosaic hybrid zones observed in *Gryllus* crickets and in parts of the hybrid zone between *Bombina* toad species, where habitat-genotype associations account for substantial amounts of among-site variation.

Key words.—*Chorthippus*, habitat, hybrid zone, long-distance dispersal, mosaic.

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Hybrid zones are regions where differentiated genotypes are broken up by recombination and natural selection acts on the new combinations of alleles generated (Barton 1983; Harrison 1990). The simplest result of this interaction is the formation of a smooth sigmoid cline in allele frequency for each locus, the width of which is determined by a balance between dispersal into the zone and any selection that may be acting against hybrids. Many of the hybrid zones described to date show a close fit to this model in terms of allele frequencies at single loci, the distribution of multilocus genotypes through the zone, and the population means of phenotypic traits (Barton and Hewitt 1985).

The interaction between dispersal and selection determines the widths of clines for independent characters, and the rate of recombination relative to selection determines the degree to which clines for different characters influence each other's position and width. For example, in a hybrid zone between *Chorthippus parallelus parallelus* and *C. parallelus erythropus* in the Pyrenees, clines for different morphological and behavioral characters vary significantly in width and position (Butlin et al. 1991). In contrast, in hybrid zones between *Bombina* toad species, the contribution of many loci to hybrid fitness seems to restrict how much recombination between loci can occur before selection acts. Developmentally and adaptively distinct quantitative traits therefore show coincident clines of similar width (Nurnberger et al. 1995). Despite this, significant variation between populations in allele frequencies and levels of linkage disequilibria at five allozyme loci exist across some transects through the *Bombina*

zone, over and above the expectations of tension zone models (Szymura 1993; MacCallum 1994; MacCallum et al. 1998). A substantial proportion of these deviations result from habitat preference and/or habitat-specific selection in an ecologically variable region. The ecological variation maintains high levels of assortative mating even at the center of the hybrid zone, and results in significant associations between the mean and variance of a trait in a population and local habitat type (MacCallum et al. 1998).

Habitat-specific effects on the distribution of genotypes mean that simple models of hybrid zones may not be adequate predictors of variation in character score along a cline. Hybrid zones that show high levels of spatial structure as a result of genotype-habitat associations have been termed "mosaic hybrid zones" by Harrison and Rand (1989). Well-documented examples of such mosaic hybrid zones include those in *Gryllus* ground crickets (Harrison and Rand 1989) and *Allonemobius* crickets (Howard 1986). Local habitat has also been shown to be an important influence on the spatial distributions of different Louisiana *Iris* species and their respective hybrids (for review, see Arnold 2000). Such associations may be caused by differential survival in different habitats (provided that patches are sufficiently large relative to dispersal; Slatkin 1973), active habitat preference (MacCallum et al. 1998), or a combination of the two. Both processes reduce recombination within the hybrid zone and thus act to maintain local adaptation in the face of gene flow (Cain et al. 1999).

The study of mosaic hybrid zones allows investigation of the spatial scale at which local adaptation can persist in the face of gene flow and how this is affected by the distribution and nature of the loci that reduce hybrid fitness. However, factors other than ecological differentiation can also generate

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spatial deviations from a clinal pattern. These include population structure and history, long-distance dispersal, extinction and recolonization (Nichols 1989), and the possible movement of a cline under selection (Fisher 1937). For example, the patchy distribution of karyotypes in the *Mus domesticus* mouse hybrid zone in northern Italy can be explained by long-distance immigration in foodstuffs imported from neighboring areas, following the extinction of local populations by flooding (Hauffe and Searle 1993). Such processes may have important implications for the maintenance and spread of new adaptations by cline movement (Barton and Whitlock 1997; Pialek and Barton 1997) and may significantly influence the nature of quantitative trait variation available for selection in natural populations.

In this study, we fit a two-dimensional cline to morphological variation across a newly discovered hybrid zone between the grasshoppers *Chorthippus brunneus* (Thunberg) and *C. jacobsi* (Harz) (Orthoptera: Acrididae) in northern Spain. The potential of local habitat variation to explain the observed deviations from fitted clines is then tested, and possible explanations for the structure and position of the hybrid zone are discussed in more detail.

Chorthippus brunneus and *C. jacobsi* form part of the Biguttulus group of *Chorthippus* (Ragge and Reynolds 1998). Both species are fully winged, although flight is observed mainly as extended jumps when disturbed. These species are usually found below 1500 m and have parapatric distributions within Europe, which may reflect the position of refugia during the Pleistocene glaciation. *Chorthippus brunneus* is found throughout northern Europe, whereas *C. jacobsi* is restricted to the Iberian Peninsula. Previous sampling suggested that these species come into contact in a region close to the Cantabrian Mountains, which form a physical barrier between northern and central Spain (Ragge and Reynolds 1988). These mountains reach altitudes of more than 2500 m in the Picos de Europa and become lower and more spread out toward the east. Other regions of contact exist between these two species in the western Pyrenees (pers. obs.; D. R. Ragge, pers. comm.), although these have not yet been studied in detail.

Chorthippus brunneus and *C. jacobsi* are distinguished largely on the basis of the distinctive calling song of males, but they also differ in a number of morphological characters, in particular the number of stridulatory pegs on the hind femur (Ragge and Reynolds 1988). In Spain, their geographical distributions overlap with that of *C. yersini*, although this species is typically restricted to higher-altitude habitats (Ragge and Reynolds 1988; pers. obs.). The differences in stridulatory peg number were maintained in laboratory-reared individuals and were intermediate in first generation crosses, suggesting a genetic basis to this character (Bridle 1998). Previous crossing studies have also shown that this character is polygenic in *C. brunneus* and other *Chorthippus* species (Butlin and Hewitt 1986, 1988) and that peg number varies independently of song characters in backcrosses involving *C. brunneus* (von Helversen and von Helversen 1975, 1994; Elsner 1984).

Although *C. brunneus* and *C. jacobsi* are closely related to *C. parallelus*, they differ substantially in their ecology and behavior. In particular, both sexes of *C. brunneus* and *C.*

jacobsi are able to fly distances of at least 25 m when disturbed (pers. obs.) and are rarely found in the lush pastureland typical of *C. parallelus*. Instead, they are common in drier, ruderal habitats such as roadside verges and dry pasture or hay meadows. Species of the Biguttulus group also show greater divergence in male calling song than those described for *C. parallelus* subspecies (Ragge and Reynolds 1998). These song differences are known to be important in male mating success and in the fitness of hybrids (Perdeck 1958; von Helversen and von Helversen 1975).

MATERIALS AND METHODS

Collection of Males and Morphological Analysis

A total of 1302 *C. brunneus* or *C. jacobsi* males were collected from 90 sites across northern Spain during July and August of 1994, 1995, and 1996. According to distribution maps from Ragge et al. (1990), this region was chosen as an area of likely contact between *C. brunneus*, *C. jacobsi*, and *C. yersini*. The study area is bounded by the towns of Oviedo and San Vicente to the north and Benavente and Palencia to the south and includes the Picos de Europa mountain range, which extends almost to the coast of northern Spain. Twenty-two of the sites were visited more than once in subsequent years. In addition, many sites were visited where no *C. brunneus* or *C. jacobsi* individuals were heard, suggesting that the sites sampled represent the full range of habitats inhabited by these species in this region. All sites were less than 50 m \times 50 m in area and were typically between 5 km and 15 km apart, although for an intensively studied area at the center of the hybrid zone, most sites were less than 2 km apart. The exact locations of these collection sites are available from the authors. They vary in altitude from 0 m to 1450 m above sea level.

Individuals were located within a site by male calling song, and where possible a recording was made of the calling song of each individual before capture (J. R. Bridle and R. K. Butlin, unpubl. ms.). Initially, *C. yersini* individuals were also recorded and collected if present. However, analyses of several morphological and calling song characters for these individuals provided clear evidence that *C. yersini* hybridizes rarely, if at all, with the other two species. In contrast, phenotypic intermediates were common between *C. brunneus* and *C. jacobsi* (Bridle 1998). Subsequent analysis therefore excluded all those individuals classified by song as *C. yersini*.

Numbers of stridulatory pegs were counted for each *C. brunneus* and *C. jacobsi* individual using a Zeiss Stemi SR microscope (Zeiss, Oberkochen, Germany). All measurements were made by J. R. Bridle without knowledge of the site at which each individual was collected. Numbers of pegs are 99.6% correlated with a discriminant axis generated for the two species based on seven morphological characters (Bridle 1998). Pegs were counted under 50 \times magnification, and a natural log transformation was used to improve the normality of the peg data (hereafter referred to as peg score). All pegs that were visible were included in the count, without allowance for irregular spacing at the end of the stridulatory file.

Fitting a Two-Dimensional Cline to Variation in Mean

A two-dimensional cline was fitted to variation in peg number using a maximum-likelihood approach. This procedure estimates the cline parameters that best explain spatial variation in site mean across a two-dimensional area by fitting a linear chain of segments to the data, the perpendicular cross-section of each of which is a tanh cline. These segments can vary in orientation 360° from a fixed starting point for the first segment or from the junction for subsequent segments. This means that a geographically complex cline center, which meanders in orientation across the sampled area, can be fitted. The position of each site can then be specified as a perpendicular distance from the fitted center of the cline, allowing the cline to be collapsed into one dimension. The technique is similar to that used by Sites et al. (1995) to fit a cline to a hybrid zone between *Sceloporus* lizards.

The fits were carried out using ANALYSE version 1.30 (Barton and Baird 1996, available at <http://helios.bto.ed.ac.uk/evolgen/Mac/Analyse/index.html>). Although previously used for the analysis of marker locus data, the program was extended for use with quantitative trait data. To our knowledge, this is the first full exploration of such an analysis based on a quantitative trait, although a similar approach has been applied to the *Bombina* system (Atkinson 2000).

The existing methods for fitting clines in allele frequencies were extended in a simple way for quantitative traits. A wide variety of models of selection on single loci or quantitative traits predict tanh curve clines (Barton and Gale 1993). A tanh curve of given width and center is used to provide an expectation for a trait (allele frequency or quantitative trait mean) at a point some distance from the center, allowing us to calculate the likelihood of an observed trait value at that point (Edwards 1972). The full two-dimensional model is a series of these simpler fits around the linear chain of segments that approximates the position of the cline center. Where the trait is quantitative, we assume sampling from a normal distribution around the expected mean to calculate the likelihood. The expected variance was estimated from polynomial regression of site variance on site mean for all sites included in the model. This regression uses data from the whole zone to provide a prediction for the variance at any one site, based on the observed elevation in variance at the cline center due to segregation between loci affecting peg number (Barton and Gale 1993). We might also expect that selection against hybrids, or assortative mating, could be acting to produce bimodal distributions of phenotypes near the center of the hybrid zone. In this case the assumption of normality will assign extreme phenotypes near the center of the zone lower likelihoods than a more complex (bimodal) model of variation, meaning that we might find an excess of likelihood outliers in this region. There is no reason, however, to expect that this simpler model of variation would introduce a bias in the estimation of the cline parameters, which are based entirely on population mean.

Fitting such a model involves the simultaneous estimation of a number of interacting parameters: cline width, mean values for the quantitative trait outside the zone (P_{\min} and P_{\max}), and the orientation of each segment from its starting point (0–360°). Interactions between these parameters gen-

erate a complex high-dimensional likelihood surface particular to the dataset, which may be highly rugged in topography. ANALYSE uses the Metropolis algorithm (Kirkpatrick et al. 1983; Szymura and Barton 1986) to search this likelihood surface for the model with the maximum likelihood. This simulated annealing, hill-climbing algorithm allows downhill movement at a frequency that is reduced as the algorithm proceeds, preventing the model from becoming trapped at suboptimal local peaks during a search for the global optimum of the likelihood surface. Such algorithms are recognized as the most general solution to complex optimization problems (Press et al. 1992).

The validity of the fitted model can be checked by repeat runs of the algorithm, starting at different parameter settings (equivalent to different initial points on the likelihood surface), which should always eventually converge to the same global optimum (Barton and Baird 1996). The topography of the surface around the optimal model can then be explored by measuring the reduction in likelihood caused by random changes to the parameters. A projection of the volume of the explored parameter space that lies within two log-likelihood units of the optimum, onto the plane of a given parameter, gives the support interval for that parameter (approximately equivalent to a 95% confidence interval). In all cases, these estimates were made by the examination of models generated by making 10,000 random parameter changes (referred to as “tries”), with the best-fitted model as a starting point for all parameters.

Fits were restricted to the geographical region that was sampled in detail and to sites where more than one individual was collected. This meant that a total of 1213 males from 80 sites were considered in this analysis. Because a two-way ANOVA of individuals collected in 1995 and 1996 gave no evidence for systematic variation in peg number between years (Bridle 1998), data from all three years of sampling were combined. Individual measurements of peg score were transformed into a scale of zero (47 stridulatory pegs) to one (156 stridulatory pegs), resulting in population means that varied across the study area from 0.24 to 0.82. Sample sizes for each site varied from two to 66 individuals, with a mean sample size of 15.2 and with 10 sites represented by less than five individuals. The spatial position of each site was denoted by two coordinates, measured to the nearest kilometer. The population means and positions of these sites are shown in Figure 1.

Testing Alternative Cline Positions

The position of the cline center can be approximated by a range of increasingly complex patterns, from a single straight line to a complicated line with many segments. Fitting more segments to the data should always increase the overall fit of the model, because a greater number of smaller segments allows the cline center to vary at a finer spatial resolution. However, care must be taken regarding the nesting of hypotheses: a multisegment hypothesis is only nested within a simpler hypothesis if it can potentially reproduce the best fit of the simpler hypothesis. Therefore, stepwise increments of segment number (e.g., {1, 2, 3, 4 . . . }) involve comparing different, nonnested, families of hypotheses and may give

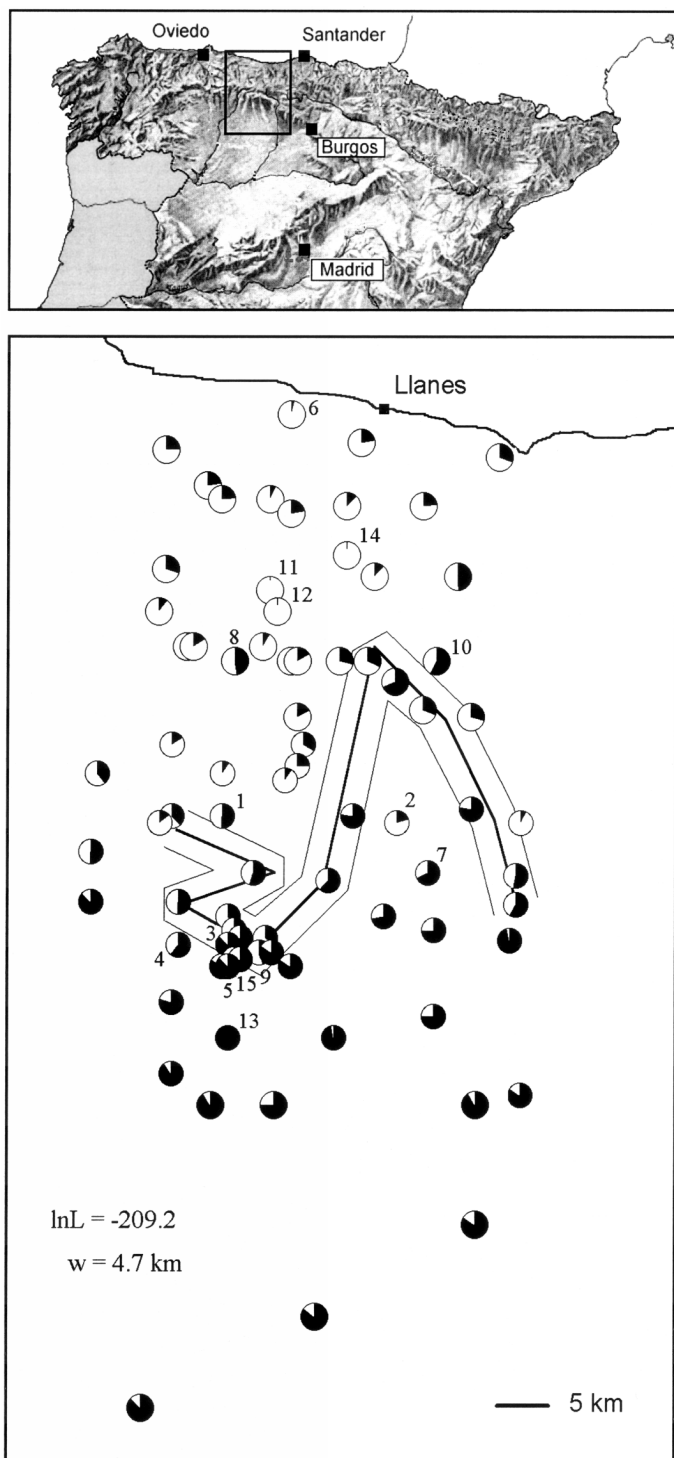


FIG. 1. The width and cline center position of the best fitting nine segment two-dimensional cline for mean peg number across a region of northern Spain (boxed). Observed means for 80 of the sites sampled during 1994–1996 are represented by pie charts. These means have been transformed so that minimum values are equivalent to empty pies for clarity. Heavy lines mark the estimated position of the cline center; lighter lines show fitted cline width. Sites with leverages of at least five likelihood units in the model are ranked from 1 to 5, depending on their contribution to the final likelihood. Sites ranked 1–5 contribute 78.9 likelihood units to the final model; sites ranked 6–15 contribute 67.1 units.

the appearance of nonconvergence as likelihoods oscillate, rather than smoothly increasing as parameters are added to the model. Comparison of a series of increasingly complex hypotheses therefore requires the subdivision of existing segments at each step. Examples of such nested series are {1, 2, 4, 8 . . .}, {1, 3, 6, 12 . . .}, {1, 5, 10, 20 . . .} segments of equal length, where the total length of the center line remains constant. Because the addition of new parameters decreases the number of degrees of freedom in the model, segment numbers should therefore be increased until there is no further significant improvement in likelihood using a likelihood-ratio test on $\Delta \ln L$ (the change in log-likelihood), with one degree of freedom for each new variable added (Edwards 1972; Hilborn and Mangel 1997). All models were run with a single fitted cline width across all segments.

Certain parameters must remain fixed in the model to make convergence to a likelihood peak computationally feasible for the range of models to be compared. These fixed parameters are the starting point of the two-dimensional cline at the eastern end of the study area, the total length of the segments used, and the requirement that each segment is of equal length. However, a number of criteria can be used to maximize the freedom of the model fitting process. These include fixing the starting point a long way from the best sampled region, maximizing freedom of cline center movement in this area, ensuring that the length of the central line can cover the span of the study area (even for a very tortuous path), and allowing the positioning of segment corners in regions where characters are changing in a complex way.

Ecological Analysis of Collection Sites

Ecological analysis was carried out at the 82 sites visited in 1995. These included seven sites that lay just outside the area included in the cline fits and seven sites that contained only *C. yersini* (which were excluded from clinal analysis). This meant that vegetation data were available at 68 of the 80 sites examined in the clinal analysis described above. At each site, different vegetation communities were identified based on the dominant plant species present (judged as those with the greatest percentage cover) and on the relative abundances of the two next most dominant species. The plant species present in each community were then classified to family or genus level using Hubbard (1954), Polunin and Smythies (1988), Blamey and Grey-Wilson (1989), and Rose (1989). The percentage cover of each taxon within each community was scored as absent, present, common, very common, or dominant. A full list of the 62 plant categories used for this analysis is given by Bridle (1998).

These rank abundances for each taxon were combined across all communities using the following ranking: 0 (not mentioned in site survey), 1 (present at site), 2 (common in one part of the site only), 3 (dominant or very common species in one community), 4 (dominant or common species in more than one community), 5 (common in all communities), and 6 (dominant in all communities). Variation between sites in these scores was analyzed using principal component (PC) analysis (Manly 1986), and a score for each site was calculated along each of the first 20 axes.

In addition to this vegetation analysis, the rank abundances

of two orthopteran species known to differ in their habitat requirements (Ragge and Reynolds 1998) were scored. These were *C. parallelus* (typically found in lush grass and meadowland) and *Oedipoda caerulescens* (typically found in dry, stony habitats). Abundances of the visually distinctive *O. caerulescens* were estimated by number of visual encounters; abundances of *C. parallelus* were estimated by frequencies of calling songs heard over a 10-min period at each site (Fischer et al. 1996). An estimate of the calling song frequency of *C. yersini* was also made.

Other environmental parameters measured included altitude, slope, aspect, ground condition (dry–wet), topographical variation within the site, and measurements of mean and mode vegetation height. Data for all of these measurements are available on request from the authors. All statistical analyses were carried out using the GENSTAT package (Lawes Agricultural Trust 1995).

Investigating Genotype-Habitat Associations

The models used to examine causes of geographical variation in population mean were also nested so simpler fits could always be recreated as a special case of more complex models. This allowed a direct comparison to be made between the importance of local genotype-habitat interactions and the simpler hypothesis of a broad-scale clinal change of characters within the study area. Maximum-likelihood parameters were therefore estimated for three types of models. The null model assumes that there is no spatial pattern to variation in a trait; observations are simply attributed to variation of the trait around its overall mean value. This is equivalent to drawing a flat cline through the study area with mean character value as a parameter of the model. The remaining models are modifications of this flat cline. The clinal model explains geographic variation by fitting a sigmoid curve with parameters specifying its center, width, and each of the extreme values of the character in the tails of the curve. A flat cline is equivalent to a sigmoid cline of infinite width, or alternatively a sigmoid cline of any width, whose start and end values are equal. The null model is therefore a special case of the clinal model and is nested within it. The habitat-only model explains local variation at each sampling site by modifying the null expectation for the character value relative to habitat attributes of the site. For example, if all sites with high values of PC1 deviated in the same direction from the null expectation, then the value of a weighting parameter for the influence of PC1 is free to vary such that the deviation is minimized (and the likelihood maximized). Where the weighting parameter is zero, the model is simply the underlying expectation, and the null model is a special case of the habitat-only model and is nested within it. The cline-and-habitat model is equivalent to the habitat model but modifies the clinal expectation rather than the null expectation. The clinal model is therefore nested within the cline-and-habitat model.

All models that tested the effect of habitat were based only on the 68 sites surveyed for vegetation that contained parental or hybrid *C. brunneus* or *C. jacobsi* individuals. Given the high levels of error associated with axes that explain small amounts of variation, PC axes that described less than 2% of

the total vegetation variation were excluded from further analyses. Because each vegetation PC axis describes a different orthogonal aspect of the vegetation matrix, different PCs can be fitted independently of each other, so that all models involve the same number of parameters and are unlikely to be affected by any problems of convergence that could arise from comparisons between models with different numbers of parameters.

The starting model for the cline fits (clinal model above) was the best model obtained for these 68 sites without the addition of any habitat effects. Subsequent fits (habitat-only and cline-and-habitat models above) then included a PC from PC1 to PC11 individually as a new parameter, and the algorithm was restarted from new starting values for all parameters. At least 20 separate runs were carried out for each new model, with between 200 and 10,000 tries.

Care must be taken when comparing the maximum likelihoods found for each model. First, the cline-only model and the habitat-only model are not nested, and so cannot be compared directly. Second, the habitat-only model includes both local and broad-scale patterns: The broad-scale character change may, by chance or due to deterministic processes, correlate with any broad-scale change in the habitat defined by each vegetation PC throughout the study area.

Figure 2 describes how environmental effects on character mean at local and broad scales are described by the different maximum-likelihood comparisons. Note that the relationship of likelihood gains $(1 + 2) = (3 + 4) = (5)$ should hold. Any improvement in likelihood to the clinal model obtained by the addition of a PC (nested comparison 2) should therefore be compared to the improvement in likelihood obtained by fitting the habitat variation alone (3). If the improvement to the clinal model is smaller than that obtained from habitat alone, this implies that the habitat variables fit (3) includes broad scale patterns that are explained well by the cline model, so that the addition of these variables to a cline fit (2) no longer results in a large increase in likelihood. In contrast, an improvement to the clinal model that is equal to or greater than that achieved by habitat alone suggests that the habitat variable only adds to the clinal model on a local scale. Such a result means that the increases in likelihood of adding cline (broad-scale) and habitat (local-scale) parameters are independent, implying that the cline is associated with an environmental transition and that significant habitat-genotype associations exist.

ANALYSIS AND RESULTS

Two-Dimensional Cline Fit for Peg Number

The effect of increasing the number of fitted segments in the two-dimensional cline model was examined first, relative to the maximum likelihood (least negative $\ln L$) reached for the previous number of segments (Table 1). Total length across all segments was kept constant at 138 km. All of these fits used the same starting position of the first segment, at the eastern end of the study area, allowing maximal movement of the cline center through the most intensively sampled area. This starting point was chosen after trials with several other starting points because it consistently generated the best likelihood estimates for the final model, as well as for models

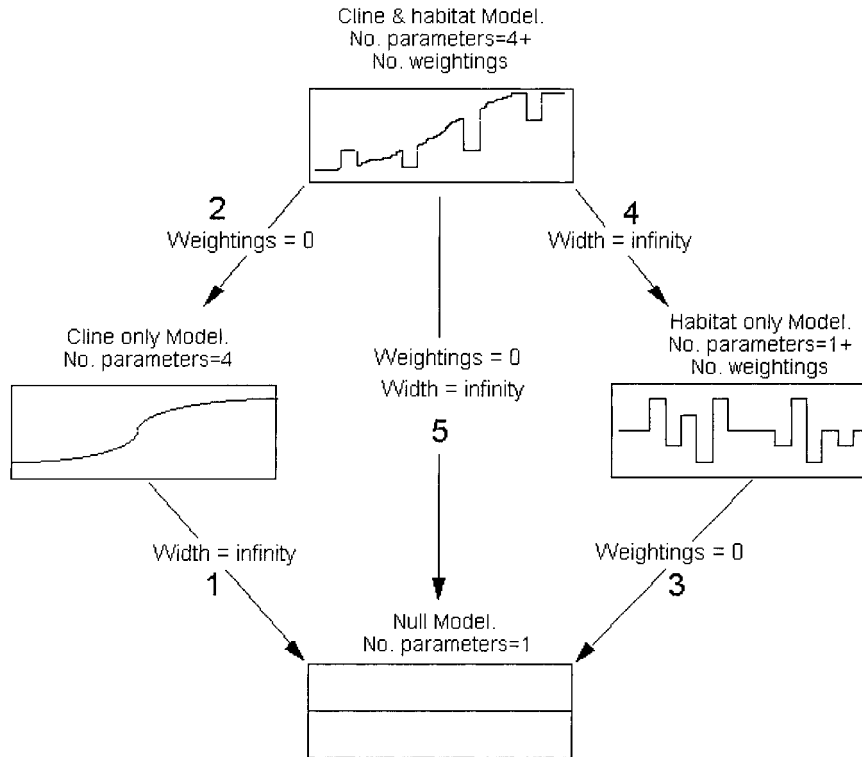


FIG. 2. Procedure for comparison of nested models for geographical variation in character states. Numbers refer to the change in maximum likelihood ($\Delta \ln L$) when comparing different nested combinations (see text for explanation).

with different numbers of segments, both for the 68-sites dataset (habitat sites only) and for the full (80-sites) dataset.

Increasing segment number in the nested series shown in Table 1 produced significant and consistent increases in like-

TABLE 1. Nested series of center line hypotheses for the 80-sites fit. Dots represent biologically unrealistic configurations such as loops and extreme folds. P_{min} and P_{max} represent the fitted values for peg mean (0–1 scale) at each end of the cline.

No. segments	P_{min}	P_{max}	Width (km)	$\ln L$
1	0.36	0.82	47.83	-445.31
2	0.32	0.91	71.47	-433.63
4	0.35	0.76	14.46	-287.83
8•	0.34	0.78	20.64	-236.05
1	0.36	0.82	47.84	-445.31
3	0.30	0.75	24.36	-312.51
6	0.36	0.71	2.95	-241.34
12	0.34	0.72	4.45	-214.00
1	0.36	0.82	47.72	-445.31
5	0.33	0.80	34.31	-343.45
10	0.35	0.72	3.10	-218.49
1	0.36	0.82	47.72	-445.31
7	0.35	0.72	4.61	-238.70
14	0.34	0.71	2.57	-202.85
14•	0.35	0.73	3.58	-193.09
1	0.36	0.82	47.72	-445.31
3	0.30	0.75	24.21	-312.34
9	0.34	0.72	4.74	-209.17
11	0.33	0.77	19.08	-223.56

lihood for both the 68- and 80-sites datasets. These increases in likelihood leveled off for all nested series at a similar likelihood level. This was mirrored by a steady reduction in fitted cline width with increasing segment number, which leveled off above nine segments at 4.74 km. Cline center hypotheses in the nested series {1, 3, 9 . . . } performed best, compared with other nested series.

Increasing the number of segments above nine typically produced biologically unrealistic models, such as loops and extreme folds in the fitted cline center. In this case, folding occurs because the intensively sampled area close to the cline center means that the best improvement in likelihood is obtained by pulling the final segment back into this region, rather than continuing to the western edge of the study area.

The best model available for this analysis therefore consisted of a chain of nine segments each 15.3 km in length. This was the lowest number of segments (i.e., degrees of freedom) that generated likelihoods close to the asymptote reached in Table 1.

The best model is shown in Figures 1 and 3 and estimates fitted width of the peg cline as 4.74 km (support limits: 3.65, 5.78). *Chorthippus brunneus* and *C. jacobsi* mean peg numbers outside the zone are estimated as 0.35 (equivalent to 85.2 pegs) and 0.72 (equivalent to 125.5 pegs) respectively (support limits: 0.34, 0.36 and 0.72, 0.73). A total of 15 sites showed strong deviations in peg mean from those expected from the final model, as shown by leverage contributions in the final model of five $\ln L$ units or more. These are marked in Figures 1 and 4 and are ranked according to their likelihood contribution.

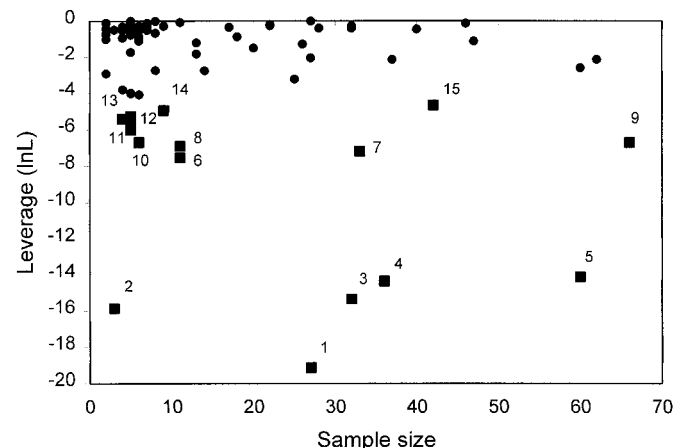
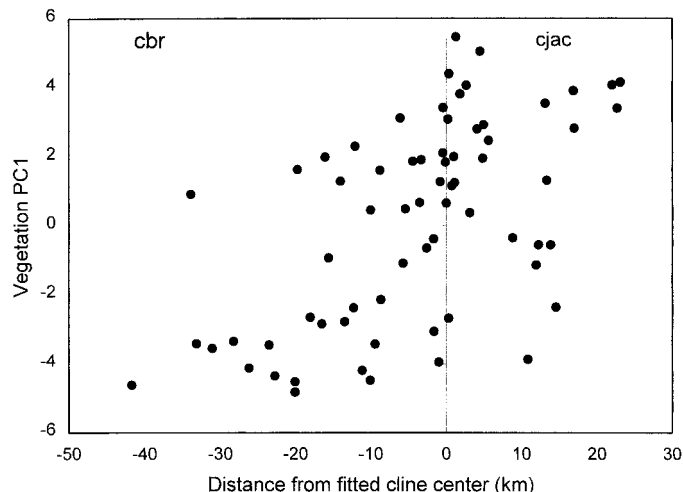
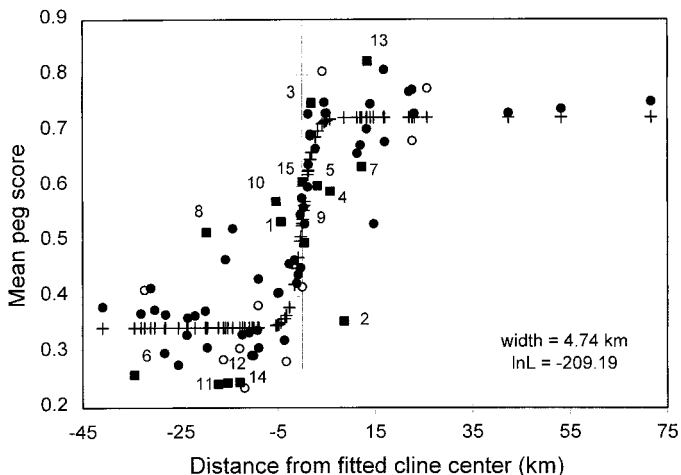


FIG. 3. Results from best-fitting nine segment two-dimensional cline model for mean peg score for all sites, collapsed into one dimension based on fitted distance to cline center. Leverage is also shown for each site, relative to sample size. Filled circles on the cline plot represent sites represented by at least five individuals. Filled squares represent sites that have leverages of more than five ln L units in the model. These sites are ranked according to their leverage, as in Figure 1.

Biological Significance of Habitat Principal Component Scores

The first 11 PC axes generated for the 62 vegetation categories each account for more than 2% of the variation in the vegetation matrix (Table 2). These 11 PCs together explain 80.4% of the total variation in vegetation present among sites.

Stepwise regression analysis of vegetation axes PC1–11 (dependent variables) with latitude, distance east along sampling area, altitude, ground condition, site topography, modal vegetation height, and aspect (degrees east or west) over 74 sites revealed that only three of these PCs were significantly correlated with these environmental parameters, after correction for multiple comparisons (Lindeman 1974). PC1 was positively correlated with distance south from the Atlantic coast ($P < 0.01$), explaining 36.3% of the variation in this axis; PC2 was positively correlated with site altitude ($P <$

FIG. 4. Broad-scale association of the hybrid zone with a region of environmental transition based on the perpendicular distance of each site from the fitted cline center ($r = 0.57$, $P < 0.01$, $df = 66$). Sixty-eight sites are included in total. Abbreviations cbr and cjac refer to the *Chorthippus brunneus* and *C. jacobsi* side of the cline, respectively.

0.05) and slope ($P < 0.01$), explaining 12.8% and 23.0% of variation, respectively. Changes in PC8 were negatively correlated with altitude ($P < 0.05$, explaining 12.2% of variation in this axis). The suggestion that variation in PC1 represents a broad latitudinal environmental change is further supported below by the highly significant increase in likelihood generated by adding PC1 to a model for the morphological data without the inclusion of clinal variation (Table 2).

Spearman's rank correlation of abundances of the grasshoppers *C. parallelus* and *O. caerulescens* with PC1 score at 79 sites showed that *C. parallelus* density is negatively cor-

TABLE 2. The effect of including habitat principal components (PCs) 1 to 11 to the best nine segment model for the 68 sites surveyed for habitat. Habitat PCs were added individually and retained in the final analysis if they significantly improved the clinal model. Numbers in parentheses refer to the different models for geographical variation described in the text and in Figure 2: [2] refers to the effect of including habitat PCs alone to peg and geographical data, [3] refers to the effect of including habitat PCs in a clinal model for variation in peg mean. $\Delta \ln L$ refers to the change in maximum likelihood generated by each comparison between models; values in parentheses refer to the maximum likelihood of the model within which these comparisons are nested.

Veg PC axis	% veg variation	$\Delta \ln L$ for PC [2]	PC weight [2]	$\Delta \ln L$ for cline + habitat [3]	PC weight [3]
None	—	(-999.9)	—	(-195.5)	—
+1	26.0	383.2	-0.44	19.1	-0.90
+2	13.0	5.8	0.05	0.9	0.08
+3	8.4	1.4	0.00	27.5	-0.68
+4	7.0	54.8	0.16	0.5	0.10
+5	5.6	1.6	0.01	0.6	-0.06
+6	5.3	43.4	-0.13	2.6	-0.55
+7	4.1	66.0	0.05	8.7	-0.50
+8	3.8	23.1	0.10	0.3	-0.08
+9	2.9	23.6	0.05	0.8	-0.04
+10	2.5	2.1	0.02	2.0	-0.26
+11	2.1	16.7	-0.08	0.7	0.12

TABLE 3. Support limits and best parameter estimates for the nine segment fits. Maximum-likelihood values for each model are given in parentheses. The All fit is for all 80 study sites; other fits (no-PC and +PC3) are for the 68 sites for which habitat data were available. Best fitting values for each parameter are given in italics, with the lower and upper support limits on either side.

Model	Width (km)	P_{min}	P_{max}	PC3 weight
All (-209.2)	3.65, 4.74, 5.78	0.34, 0.35, 0.36	0.72, 0.72, 0.73	—
No-PC (-193.8)	2.98, 3.89, 4.97	0.34, 0.35, 0.37	0.71, 0.71, 0.73	—
+PC3 (-168.5)	3.19, 3.69, 4.66	0.34, 0.36, 0.37	0.72, 0.73, 0.73	-0.85, -0.72, -0.55

related with PC1, whereas *O. caerulescens* density is positively correlated with PC1 ($r_s = -0.60$ for *C. parallelus*, 0.52 for *O. caerulescens*; $P < 0.001$ in both cases, $df = 77$). In addition, PC2 was significantly correlated with the estimated abundance ($r_s = 0.46$; $P < 0.001$, $df = 76$), and presence versus absence of the closely related species, *C. yersini* (one-way ANOVA, $F = 5.53$; $P < 0.05$; $df = 1, 80$).

Association of the Cline with an Environmental Transition

Of the first 11 PC axes, only PC1 showed a significant correlation with distance from the fitted cline center for the 68 sites included in the clinal analysis ($r = 0.57$; $P < 0.01$ for $df = 66$; Fig. 4). Again, this value was corrected for multiple comparisons (see above).

Testing for Habitat-Genotype Associations

The best model fitted for the subset of 68 sites with habitat data gave a maximum ln L estimate of -193.8. The fitted cline center and width were very similar to those obtained with the full dataset. The results from the addition of individual habitat PCs to the best starting model are shown in Table 2. These data indicate that PCs 1, 3, and 7 significantly improved the fit compared with the starting model. However, only PC3 had a significant effect over and above that ex-

plained by the fitting of the habitat data alone to the geographical and peg data, without the clinal model. This generated a final model with $\ln L = -168.5$ compared to -193.8 ($\Delta \ln L = 25.3$ for one extra degree of freedom), a highly significant increase ($P < 0.01$ after correction for multiple comparisons), and a best estimate of cline width of 3.69 km compared to 3.89 km for the no-PC model (Table 3). Both estimates for width lie well within the support limits of the width estimate for all 80 sites with no habitat variables fitted.

PC3 was weighted as -0.72 (support limits -0.85, -0.55) in this model, meaning that a high PC3 value for a site correlates with a reduced peg score relative to the expectation of a smooth cline at that position. Such a weighting would, for example, reduce the leverage of a site with a *C. brunneus*-like peg score close to the center of the hybrid zone. The value of PC3 at each site is negatively correlated with the relative abundances of lucerne (*Medicago sativa*) and broom/greenweed species (*Genista* or *Spartium/Cystisus*-like species; vector loadings in PC analysis: -0.68 or -0.11, respectively) and is positively correlated with the abundance of different grass species.

Figure 5 shows the effect of the inclusion of PC3 in the clinal model on the expected value for the mean at each site and on the distribution of residual variation in the dataset. Eleven sites show substantial deviations from the fitted model (contributing at least five ln L units to the maximum likelihood). These sites are marked as filled circles in Figure 5 and are listed in Table 4.

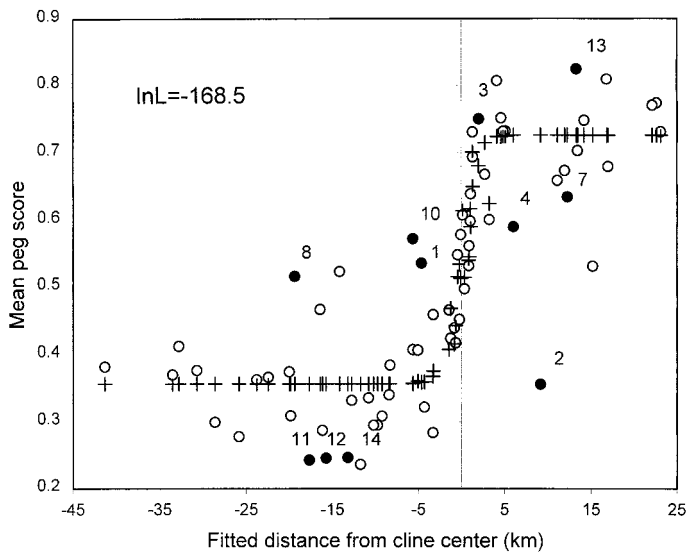


FIG. 5. The effect of adding vegetation PC3 to the shape of the fitted cline and to deviations from expected means. The remaining 11 sites with a leverage of more than five ln L units are shown as filled circles, and are numbered as in Figure 1. Crosses represent the expected mean at each site, given the fitted association with habitat PC3.

TABLE 4. The effect of including significant local habitat effects (PC3) on residual variation. Sites with leverages of more than five ln L units in the no-PC (no habitat) model are listed, along with their fitted distance from the cline center. Site numbers are as in Figure 1 and denote the rank of each site according to its leverage in the no-PC fit. Those sites whose leverage is reduced by more than two ln L units by the inclusion of habitat effects are shown in boldface and are all less than 4 km from the fitted cline center.

Site no.	Sample size	Fitted distance from center (km)	Leverage without PC3	Leverage after PC3 fit
1	27	-4.42	-18.56	-18.02
2	3	8.84	-15.23	-16.06
5	60	3.16	-15.03	-0.83
4	36	5.63	-13.14	-15.41
3	32	1.87	-12.36	-9.38
9	66	0.39	-7.92	-0.44
11	5	-18.05	-7.04	-7.58
12	5	-16.09	-6.41	-6.92
10	6	-5.70	-6.40	-6.22
13	4	13.37	-6.31	-5.23
8	11	-19.75	-6.29	-5.97
14	5	-13.49	-6.21	-6.71
7	33	12.25	-6.04	-7.53

Table 4 shows how fitting PC3 affects the leverage of individual sites that remained as strong outliers after fitting the no-PC model. Again, the distribution of these strong leverage sites is uncorrelated with sample size, suggesting that these deviations reflect real underlying variation. Including PC3 variation explains deviations from the fitted cline at a few sites, especially those close to the cline center (Table 4, Fig. 5). However, even after the inclusion of PC3, the model fails to predict the presence of some significant outliers grouped more or less symmetrically around the cline center, some of which actually increase in leverage after habitat fitting. These include sites with intermediate means up to 20 km north of the fitted cline center (site 8), and strongly *C. brunneus*-like or intermediate sites at the *C. jacobsi* end of the fitted cline (site 2).

Effect of Including Habitat on Cline Position

Superimposing the cline position for the no-PC fit onto the PC3 fit generated a $\ln L$ of -174.3 , compared to -168.5 ($\Delta \ln L = 5.8$ for nine fewer degrees of freedom; $P > 0.05$), indicating that the addition of habitat had no significant effect on estimated cline position.

Quantifying Residual Variation after Habitat Fitting

The amount of among-site variation in peg number remaining after fitting each clinal model was estimated by regressing observed population means on the predicted population means. Means were weighted by $(\text{standard error})^{-1.5}$ to allow for variation in sample size and within site variance.

These results showed that the best fitting two-dimensional cline for the no-PC fit ($\ln L = -193.8$) explained 80.6% of the among-site variance in peg number. The inclusion of vegetation PC3 ($\ln L = -168.5$) explained only an additional 1.8% of the among-site variance. The final model therefore accounted for 82.4% of the total among-site variance, with 17.6% of the among-site variance in the data remaining unexplained.

DISCUSSION

Broad-Scale Position of the Best-Fitting Cline

Many hybrid zones are associated with environmental transition, either due to the primary origin of clines along a selective gradient or because of the secondary trapping of neutral and selected clines at the density troughs that these ecotones represent (Hewitt 1975; Barton and Hewitt 1985; Nichols and Hewitt 1986; Harrison 1990). Alternatively, cline position may reflect the original location of postglacial contact or the current influence of local barriers to gene flow, which may only be coincidentally associated with an ecotone.

Any of these reasons could explain the current association of this hybrid zone with the environmental transition described by vegetation PCs 1 and 7. This ecotone is consistent with the transition in climate and vegetation across the Cantabrian Mountains, from lush pastureland in the mountainous north to mixed or arable agriculture in the elevated plains of the south (Polunin and Symthies 1988). The vegetation PC axes used in this study capture meaningful information about variation in local habitat, as shown by their significant cor-

relation with latitude, altitude, or mean slope at a given site, with the abundance of other grasshoppers known to differ in their ecological requirements and with *C. yersini* abundance, which is closely related to both *C. brunneus* and *C. jacobsi* (Ragge and Reynolds 1998). However, variation in PCs 1 or 7 fails to explain local variation in population mean, despite the fact that many sites close to the estimated cline center show vegetation scores typical of each end of the cline (Fig. 4). This makes it unlikely that variation in these PC axes has a direct influence on cline position, either by selection or by the trapping of clines in local regions of low density. This is also the case in the *C. parallelus* cline in the Pyrenees, which is broadly associated with a habitat transition, although detailed ecological analysis reveals no differences in habitat preference or use between the races (Virdee and Hewitt 1990).

The broad-scale ecological association of the *C. brunneus*–*C. jacobsi* cline therefore seems more likely to reflect the position of postglacial contact between these species, because the Picos de Europa Mountains probably limited postglacial expansion from the Iberian refuge while *C. brunneus* colonized northern Europe and the north coast of Spain (Ragge and Reynolds 1998). This region is highly complex topographically, with narrow valleys and gorges between massifs and some heavily forested areas to the east (Polunin and Symthies 1988).

Such complex topography close to the cline center could limit gene flow through the cline, so generating localized associations of the cline center with topography. In addition, small-scale topographical variation may contribute to the increased variation in habitat types suggested in Figure 4. However, a strong effect of local barriers to gene flow on cline position is unlikely because the fitted center often cuts across ridges, particularly as it moves north in the east of the study area. In addition, the dramatic changes in cline center position at the west of the study area are not associated with regions of high altitude (Fig. 1). However, local associations of the cline center with topography are difficult to test fully at this spatial scale, given the limited number of segments that can be fitted to a real dataset.

Width and Structure of the Cline

The best estimate of cline width (without habitat effects) is 4.74 km (support limits: 3.65, 5.78). This is reduced to 3.69 km (support limits: 3.19, 4.66) if significant habitat effects are included. If no selection is acting to slow introgression, cline width is proportional to the standard deviation of parent-offspring displacement per generation (σ), and the number of generations since contact (t ; $w = 2.51\sigma t^{0.5}$; Endler 1977; Barton and Gale 1993). Assuming contact between these species followed range expansion during the current interglacial (probably between 5000 and 10,000 generations ago; Hewitt 1993), this formula generates an estimate of σ of between 19 and 27 m $\text{gen}^{-0.5}$, or 13 and 33 m $\text{gen}^{-0.5}$ using the widest support limits for peg cline width (3.19 km to 5.78 km) and highest and lowest time estimates. In contrast, mark-recapture estimates of dispersal for flightless *C. parallelus* grasshoppers give values of σ that are typically higher than this, on the order of 30 m $\text{gen}^{-0.5}$ (Virdee and

Hewitt 1990). In addition, because both sexes of *C. brunneus* and *C. jacobsi* are fully winged, actual mean dispersal is likely to be higher than this direct estimate of $30 \text{ m gen}^{-0.5}$. If so, a cline wider than 5 km would be expected under neutral introgression. This suggests that selection may be acting to limit introgression of alleles affecting peg number across the hybrid zone, probably through selection on loci associated with those contributing to peg variation, rather than on peg variation itself. Certainly, the estimated width of this cline is narrower than that for stridulatory pegs in *C. parallelus* (4.2 km or 9.0 km at Col de Quillane and Col de Portalet, respectively), where F_1 males are completely sterile (Hewitt 1993) and postglacial contact is likely to have occurred at a similar time. Further work is underway to measure the fitness of F_1 and later generation crosses between *C. brunneus* and *C. jacobsi* in the laboratory (H. Tatsuta, pers. comm.).

Habitat-Genotype Effects on Population Mean

Previous studies of mosaic hybrid zones have shown that spatial deviations in population mean from the expectations of smooth clines can often be explained by the effect of environmental heterogeneity on genotypes that vary in fitness in different habitats (e.g., Harrison and Rand 1989; MacCallum et al. 1998). These associations can be generated either by direct environmental selection against certain combinations of alleles in habitat patches above a certain size (e.g., *Gryllus* crickets; Harrison and Rand 1989; *Sceloporus* lizards; Sites et al. 1995) or by the nonrandom dispersal of genotypes among different habitats (e.g., in *Bombina* toads; MacCallum et al. 1998).

In this hybrid zone, the inclusion of PC3 explains a significant amount of the residual variation in population mean. PC3 is negatively correlated with the abundance of lucerne (often grown as fodder in improved pastures) and broom/greenweed species (which commonly grow in dry, stony habitats at the edge of fields) and is positively correlated with the abundance of different grass varieties normally associated with naturally regenerating pasture (Blamey and Grey-Wilson 1989). This suggests some discrimination between improved pasture and drier edge habitats, which are more typical of sites dominated by *C. jacobsi* alleles, and pasture/meadow habitats, which are more typical of sites dominated by *C. brunneus* alleles. This effect of habitat appears confined to the cline center, because no broad-scale effect of PC3 was shown without the clinal model and no significant correlation was detected with distance from the fitted cline center.

Despite the statistically significant effects of PC3, habitat-genotype associations explain only a small and biologically insignificant fraction of the departures from the best fitting cline. Regressions of observed means on expected means indicate that the addition of PC3 to the model explains only an additional 1.8% of the among-site variance, in addition to the 80.6% of the among-site variance already explained by the best-fitting two-dimensional cline. Furthermore, the estimated cline width is not significantly reduced following the inclusion of these habitat parameters, as would be expected in a hybrid zone structured by strong ecological differentiation (Harrison and Rand 1989). Again, this result is consistent with a relatively trivial effect of the local envi-

ronment on spatial variation in peg score throughout this hybrid zone.

A total of 17.6% of the among-site variation in peg number remains unexplained after habitat effects are included. This contrasts with a similar regression carried out for the Pyrenean *C. parallelus* clines at Col de la Quillane and Col du Portalet, where one-dimensional clinal models for mean peg number explained 91% and 94% of the among-site variance respectively (Butlin et al. 1991). The residual variation in the *C. brunneus*-*C. jacobsi* hybrid zone is largely the result of 11 sites that are at least one cline width from the cline center and show high deviations from expected means (Figs. 1, 5; Table 4). The leverage of these outliers is not related to their sample size, indicating that the deviations are unlikely to be caused by inadequate sampling of these populations.

Limits of Fitting Two-Dimensional Clines

The intensity of sampling over a geographical area limits the spatial complexity of the two-dimensional cline that can be fitted reliably using maximum likelihood. Fitting a relatively simple model to a complicated cline center may result in some samples showing large deviations from the expected mean when, in fact, the local position of the cline center is wrong. In the field, a real cline center can theoretically move in position at a spatial scale related to the neighborhood size of the organism (Nagylaki 1975) or on a smaller scale if local barriers to gene flow affect dispersal. In such situations, fitting a clinal model with more segments could potentially explain more of variation and reduce the estimated cline width as the path of the cline center becomes more tortuous. However, in this case, models fitted with up to 14 segments (Table 1) failed to improve the final likelihood or to significantly reduce fitted cline width further, although this may be a reflection of the spatial scale of sampling.

The Effect of Population History and Long-Distance Dispersal

Large residual deviations in population mean seem unlikely to be due to genetic drift in a grasshopper species with large local populations. However, if suitable habitat is continually created and destroyed within the zone, long-distance colonization of vacant habitat from either parental population could cause significant spatial structure if the rate of extinction is rapid relative to recombination and the spread of alleles by diffusion. Alternatively, the high levels of spatial structure may be a legacy of the establishment of populations by long-distance dispersal when the zone initially formed (Nichols and Hewitt 1994; Ibrahim et al. 1996).

Such metapopulation-type dynamics are consistent with the ecology of *C. brunneus* and *C. jacobsi*. Males and females of both species are fully winged and tend to occupy disturbed, ruderal sites instead of the permanent meadow-grassland habitats typical of *C. parallelus* (Ragge and Reynolds 1998). Long-distance dispersal could also explain the occasional observation of single individuals with peg numbers typical of *C. jacobsi* some 30 km north of the fitted cline center, surrounded by areas of vacant habitat (Bridle 1998).

This study demonstrates high levels of spatial structure in the *C. brunneus*-*C. jacobsi* hybrid zone, of which only a small

proportion is explained by some aspect of local habitat. Instead, high levels of residual among-site variation probably result from a complex historical or contemporary pattern of contact between parental populations, which may be strongly influenced by long-distance dispersal and extinction-recolonization. This contrasts with previously studied mosaic hybrid zones, where spatial ecological variation is clearly implicated as the main cause of observed deviations from smooth clines (Harrison and Rand 1989; MacCallum et al. 1998).

This situation also contrasts with the smooth clines observed in hybrid zones between *C. parallelus* subspecies (Hewitt 1993; Butlin 1998). Although these closely related species are likely to share a similar genetic basis for peg number, they differ markedly in ecology. In particular, *C. brunneus* and *C. jacobsi* probably inhabit more ephemeral habitats, which would provide more opportunities for colonization by long-distance dispersal, bringing parental genotypes further into the zone without being broken up by recombination. This continual expansion into vacant habitat may have important implications for the movement of clines, and therefore for the spread and fixation of new adaptations (Hewitt 1996; Barton and Whitlock 1997; Pialek and Barton 1997). The resulting population structure may also provide more possibilities for speciation by reinforcement (Cain et al. 1999), because contacts between parental combinations of alleles are much more frequent than in smooth tension zones.

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