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PATTERNS OF TRAIT DIVERGENCE BETWEEN POPULATIONS OF THE MEADOW GRASSHOPPER, *CHORTHIPPUS PARALLELUS*

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Abstract.—To understand the process of speciation, we need to identify the evolutionary phenomena associated with divergence between populations of the same species. A powerful approach is to compare patterns of trait differences between populations differing in their evolutionary histories. A recent study of genetic divergence between populations of the meadow grasshopper *Chorthippus parallelus,* from different locations around Europe has allowed us to use this species to investigate which aspects of evolutionary history are associated with divergence in morphology and mating signals. During the last glaciation *C. parallelus* was confined to a number of refugia in southern Europe and has subsequently recolonized the northern part of the continent. This process of isolation followed by range expansion has created populations differing markedly in their evolutionary pasts—some have been isolated from one another for thousands of years, others have undergone repeated founder events, and others now live in sympatry with a closely related species. Using laboratory-reared grasshoppers from 12 different populations with a range of evolutionary histories, we quantify differences in morphology, chemical signals, and male calling-song. The observed pattern of divergence between these populations is then compared with the pattern predicted by hypotheses about what drives divergence. This comparison reveals that long periods in allopatry and processes associated with repeated founder events are both strongly associated with divergence.

Key words.—Acoustic, cuticular hydrocarbons, founder effect, mating signal, morphology, orthoptera, refugia, reproductive isolation, speciation.

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Speciation occurs when populations of a single species diverge to form genetically independent groups. This process may involve the accumulation of genetic differences affecting many aspects of phenotype, but, centrally, it requires the evolution of barriers to gene exchange. These barriers promote genetic isolation and thus accelerate differentiation. A number of alternative, but not mutually exclusive, hypotheses have been proposed to explain why such divergence may occur between allopatric populations. These hypotheses can be divided into three broad areas. Divergence may be due to: (1) incidental effects of slow accumulation of incompatible mutations (e.g., Coyne 1992); (2) pleiotropic effects of adaptive divergence in response to environmental variation (e.g., Schluter 1996; Etges 1998) or to sexual selection (e.g., Lande 1982, Payne and Krakauer 1997); or (3) processes associated with founder events (Powell 1978, 1989; Bryant and Meffert 1990; Meffert 1995). This last category includes two distinct potential processes. First, population bottlenecks accelerate genetic drift and may cause major genetic reorganisation (Carson and Templeton 1984). Second, low population densities may increase the risk to females of failing to find a mate, thus making them less choosy and hence reducing selection on male sexual traits (Kaneshiro 1989).

The processes that have driven divergence between populations (and ultimately speciation) will be unique to each case. Nevertheless, to build up a picture of the general importance of different factors, we need to study individual systems. Past studies have tended to concentrate on a single existing hypothesis for divergence between populations. Few have set out to compare several available hypotheses, often because sufficient background information on evolutionary histories is lacking.

Chorthippus parallelus is a flightless, gomphocerine grasshopper inhabiting mesic grasslands throughout Europe, ranging from Turkey and southern Spain to within the Arctic Circle (Bellman 1988). The species has been studied extensively in a hybrid zone in the Pyrenees, where two divergent populations come into contact (Butlin 1998). Recent molecular phylogeographic studies comparing populations across much of Europe (Cooper et al. 1995; Lunt et al. 1998) indicate that during the last glaciation *C. parallelus* was confined to southern refugia on the Iberian Peninsula, Italy, the Balkans, Turkey, and at least one more independent refuge east of the Carpathian Mountains. At the end of this glaciation, around 9000 years ago, *C. parallelus* spread northward out of these refugia to recolonize Europe. However, rather than a general movement north, the molecular data indicate that northern Europe was colonized entirely by the descendants of grasshoppers from the Balkan refuge (Cooper et al. 1995; Lunt et al. 1998). This is likely to be because the grasshoppers from refugia in southern Italy and Spain were hampered in their expansion by the persistence of ice along the Alpine and Pyrenean Mountain chains. By the time the ice had melted on the high cols of the Pyrenees, southern France was already occupied by new populations descended from those grasshoppers that had survived in the Balkan refuge. The secondary contact between these two divergent populations has created the hybrid zone now found at the border between France and Spain.

Information about the evolutionary histories of grasshoppers in different areas enables us to use comparisons of present-day populations of *C. parallelus* to evaluate three hypotheses for divergence: (1) If divergence tended to occur solely as a by-product of long periods of geographic isolation, we would expect the largest differences to be found between populations derived from the different refugia, which have been isolated from one another for at least 100,000 years

(Based on sequence divergence and glacial history; Hewitt 1996). (2) If processes associated with founder events tended to drive divergence, we would expect populations in the north of Europe to be more divergent from other populations. Levels of genetic variation are lower within northern Europe than anywhere else in Europe (Cooper et al. 1995). To migrate more than 1500 km to Britain from the Balkans before the land bridge with France closed around 7500 years ago, the species' range must have expanded at about 1 km/year. *Chorthippus parallelus* is flightless, and field studies suggest typical movement rates of less than 20 m/generation (Virdee and Hewitt 1990). Therefore, it is highly probable that populations found at the northern edges of the current range are the products of numerous founder events when a single female or small group was carried ahead of the advancing front, presumably by natural events such as being carried by extreme winds or washed down rivers. (3) If adaptation to different environments drives divergence, we would expect differences between populations in environments with different selection pressures. For *C. parallelus,* a potentially significant environmental factor is the closely related species *Chorthippus montanus,* which is sympatric with *C. parallelus* across parts of its range in central Europe (Bellman 1988). *Chorthippus montanus* is morphologically very similar to *C. parallelus* (Reynolds 1980) and the two species can be hybridized in the laboratory (O. von Helversen, pers. comm.), although hybrids have not been found in the field. *Chorthippus montanus* and *C. parallelus* have very similar acoustic signals (Bauer and von Helversen 1989) and cuticular composition—a likely mating signal (see Results). The possibility of reproductive character displacement (Butlin 1989, Howard 1993) causing evolutionary change in mating signals in sympatry represents a potential environmental factor promoting divergence between populations of *C. parallelus.* If this is the case, we would expect to see differences between populations sympatric with *C. montanus* and populations outside its range.

A second environmental factor is altitude. *Chorthippus parallelus* occurs from sea level to 2000 m, even within the same latitudinal range (for instance, within northern Greece; pers. obs.), allowing us to control for latitudinal differences. If adaptation to altitude is important in driving divergence between populations, then we might expect to see differences between adjacent populations occurring at different altitudes.

Measuring Divergence

To characterize divergence between populations differing in their evolutionary histories, we measured aspects of morphology (see Materials and Methods) and two signaling modes: male calling-song and putative cuticular pheromones of both sexes. Mating signals are particularly relevant as measures of divergence because they are likely to contribute to reproductive isolation between populations. Partial reproductive isolation (e.g., assortative mating; T. Tregenza, V. Pritchard, and R. Butlin, unpubl. data) will accelerate divergence by reducing gene flow between populations.

Calling song is spontaneously produced by single males and is implicated in mate attraction and discrimination (Butlin and Hewitt 1985a, Ritchie 1990). However, even in the absence of song, females can discriminate between males from populations on either side of the Pyrenean hybrid zone (Ritchie 1990), indicating that a second signaling mode must exist. A likely candidate is a cuticular pheromone, which is known to exist in other orthopterans (Howard and Blomquist 1982; Bell and Carde 1984; Tregenza and Wedell 1997) and indicated in *C. parallelus* by the finding that males will court dead females, but cease to do so if their cuticular hydrocarbons are removed (Butlin 1998).

Both morphological and signaling traits are likely to be exposed to both natural and sexual selection, but to differing extents. However, certain morphological features, such as the relative sizes of the two parts of the pronotum, are probably not strongly sexually selected, whereas male calling-song is known to have a role in mate attraction (Butlin and Hewitt 1986). Cuticular lipids are likely to fall somewhere between the two, having functions both in waterproofing and as contact pheromones.

Materials and Methods

We collected adults of both sexes, with a minimum of 40 mated adult females from each of 12 sites (Fig. 1) and provided them with fresh grass (*Dactylis glomerata*) for food and pots containing 2 cm of damp sand as an egg-laying substrate. We collected newly laid egg pods daily and placed them in petri dishes containing damp sand, which were then stored in large sealed containers in a cool room. After at least 4 months at 4°C (corresponding to the winter diapause), we removed egg pods from the cool room in weekly batches, and after a week at 22°C, placed them on sand that was kept saturated with water. Eggs hatched approximately 10 days after being placed on the wet sand. Nymphs were reared under standard conditions, as described by Kelly-Stebbins and Hewitt (1972), with minor modifications. *Dactylis glomerata* was provided as a food plant. Offspring from different populations were reared simultaneously under the same conditions. Individuals from the same egg pod are not entirely independent, but are likely to be half-siblings because females mated multiply. As in previous studies, we did not rear individual pods separately because typically only two or three individuals per pod survived to adulthood and the number of cages required would have been prohibitive. All experiments and analyses were carried out on these laboratory-reared individuals so that environmental effects on phenotype could be standardized.

Morphology

All measurements were made under a binocular light-microscope with an eyepiece graticule, using dead specimens preserved by freezing. We measured four morphological characters of adults of both sexes: length and maximum width of the hind femur and length of the two elements of the pronotum—the prozona and the metazona (Reynolds 1980). Additionally, in males we counted the number of pegs on the inside of the hind femur and measured the lengths of these rows of pegs. The peg row makes up the stridulatory file that the male rubs against his fore wing to produce sound. A total of 694 males and 592 females were measured. In 614 individuals we measured hind femurs of both right and left legs

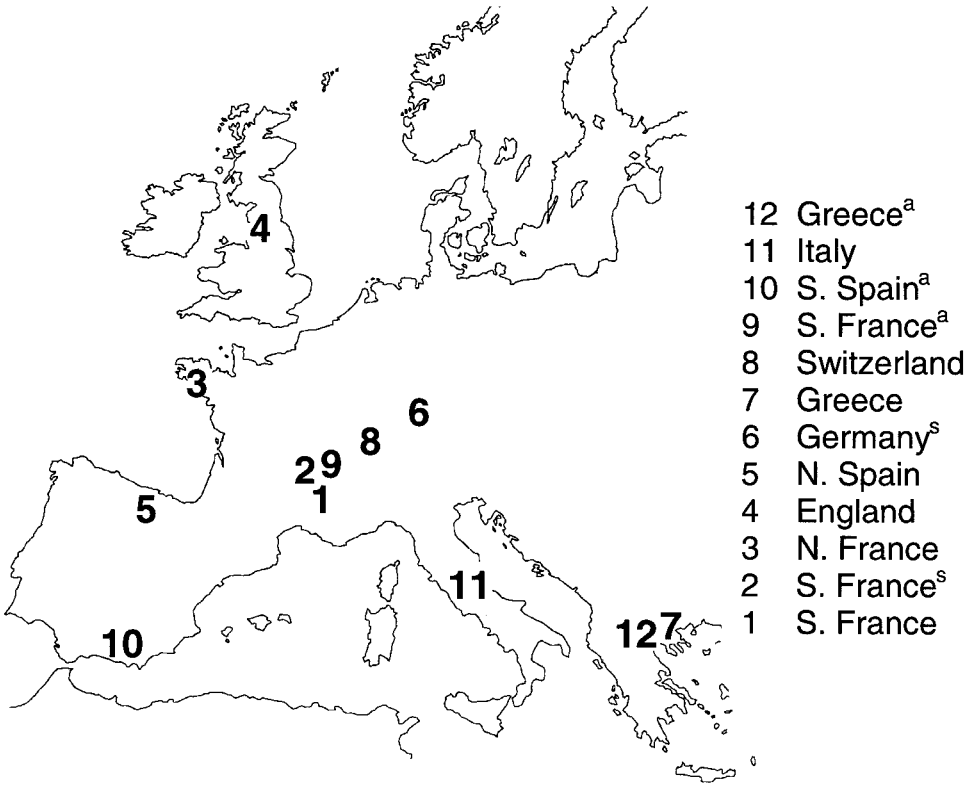

FIG. 1. Study populations. Numbers are allocated to retain consistency with Butlin and Tregenza (1998). a, populations at greater than 800 m altitude; s, populations sympatric with *Chorthippus montanus*. 1, Villefranche, Massif Central, France (2°3′E 44°21′N); 2, Tourniac, Massif Central, France (2°13′E, 45°11′N); 3, Roscoff, Brittany, France (48°43′N, 3°59′W); 4, Hard Knott Pass, Lake District, UK (54°25′N, 3°14′W); 5, Cervera de Pisuerga, León, Spain (4°29′W, 42°50′N); 6, Erlangen, Bavaria, Germany (10°48′E, 49°42′N); 7, Stavros, Thessaloniki, Greece (40°40′N, 23°42′E); 8, Bern, Switzerland, (46°54′N, 7°29′E); 9, Puy Mary, Massif Central, France (2°40′E, 45°6′N); 10, Las Sabinas, Granada, Spain (3°22′W, 36°58′N); 11, Ovindoli, Abruzzi, Italy (13°31′E, 42°8′N); 12, Karia, Thessaloniki, Greece (22°25′E, 40°0′N).

(for length and width and in males, peg number and file length), and used means of both legs in subsequent analyses. In other individuals the measured leg was chosen at random.

Male Calling-Song

We recorded calling-songs (Butlin and Hewitt 1985a) of 273 male *C. parallelus* from eight populations, and from 12 male *C. montanus* from sites 2 and 6, at a mean temperature of 29.5 ± 2.64°C (SD). Recordings were made in the laboratory on a Denon DRM-540 cassette tape deck (Nippon Columbia Co., Tokyo, Japan) with Beyerdynamic MCE80 microphones (Beyerdynamic, Farmingdale, NY). These analogue recordings were digitized using a CED 1401 A/D converter sampling at 20 KHz and analyzed on a PC using Spike 2 software (both Cambridge Electronic Design, Cambridge, UK). Temperature corrections (standardized to 30°C) were calculated from linear regression of the entire dataset which provided an equally good fit to nonlinear regressions. Four temporal song characters were measured, as in Dagley et al. (1994). These were: syllable length (SL); syllable number (SN); echeme length (EL); and echeme interval (EI). (A syllable is the sound produced by a complete up and down movement of the legs, and an echeme is a first-order assemblage of syllables.) For each male we measured song characters from five sequential echemes. To calculate syllable length, we measured the total duration of the five syllables prior to the last syllable in each echeme, and divided this time by 5, thereby avoiding the often indistinct first and last syllables in each echeme. Echeme intervals were measured as the time between the ends of the penultimate syllables in neighbouring echemes.

Cuticular Composition

To measure cuticular lipid composition, which will determine contact pheromone blend (Neems and Butlin 1995; Buckley 1998; Butlin 1998), cuticular components were analyzed from 446 females and 455 males. Hexane extracts from single hind femurs of individuals killed by rapid freezing were analyzed using a Varian 3400 gas chromatograph (Varian Associates Ltd., Walton-on-Thames, UK), following the procedures described by Neems and Butlin (1994). Individuals from different populations were analyzed in random order to avoid spurious differences between populations due to gradual changes in gas chromatograph performance (which might occur if each population were run in a block). All statistical analyses were carried out using log contrasts of peak areas (Aitchison 1986): Each peak area was divided by the area of the peak with the greatest mean area (peak K), and the $\log_{10}$ of this ratio was used in subsequent analyses.

TABLE 1. Population contrasts used in examining the proportion of between-population variance explained by different hypotheses for divergence (see text). Refugia (a) compares populations from Spain with all other populations; refugia (b) compares the Italian population with Greek populations and those derived from the Greek refuge. Founder (a) compares the refugial Greek populations with their descendants in the rest of Europe; founder (b) compares descendants of the Greek refuge that are relatively distant from the refuge with those nearer Greece. Altitude compares three pairs of neighboring populations, one at high and one at low altitude. Sympatry compares populations sympatric with *Chorthippus montanus* with altitudinally similar allopatric populations in the same area of southern Europe.

Hypothesis		Morphology	Calling-song	Cuticular composition
Refugia	(a)	5,10 vs. 1,2,3,4,6,7,9,11	5 vs. 1,2,3,4,6,7	5,10 vs. 1,2,3,4,6,7,8,9,11,12
	(b)	11 vs. 1,2,3,4,6,7,9	11 vs. 1,2,3,4,6,7	11 vs. 1,2,3,4,6,7,8,9,12
Founder	(a)	7 vs. 1,2,3,4,6,9	7 vs. 1,2,3,4,6	7,12 vs. 1,2,3,4,6,8,9
	(b)	3,4 vs. 1,2,6,8,9	3,4 vs. 1,2,6	3,4 vs. 1,2,6,8,9
Altitude		1,5 vs. 9,10	none	1,5,7 vs. 9,10,12
Sympatry		2,6 vs. 1	2,6 vs. 1	2,6 vs. 1,8

Comparing Divergence: Patterns and Hypotheses

To gain insights into the evolutionary processes driving divergence, we need to synthesize observed patterns of variation between populations. There are a number of possible ways to do this. One method is to organize data in matrices of distance between populations for particular traits (see Butlin and Tregenza 1998). These matrices can then be compared with prediction matrices based on the different hypotheses. For instance, the prediction matrix for the refugia hypothesis would have large distances between populations derived from different refugia, but no differences between descendants of the same refuge. The disadvantage of this approach is that it has very weak statistical power because it does not allow for variation within populations to be compared with variation between populations. We have chosen to use an alternative approach in which the hypotheses for divergence are treated as planned comparisons in an analysis of the variance between the different populations. This approach is also rather conservative, because each comparison reduces the degrees of freedom of the analysis, but it allows all the data collected from individuals from the different sites to be used and provides more readily comparable measures of the proportion of the total observed variance explained by the different hypotheses.

Treating each hypothesis as a planned comparison following a between-population analysis of variance (ANOVA) for each character set (Sokal and Rohlf 1995) allows us to ask the following question: How much of the variance between populations in a given character set can be explained if we divide populations into groups with different evolutionary histories? For instance, if we divide populations into those likely to have been through repeated founder events and those which have not, does this explain more of the morphological variance between populations than if we divide them according to whether they occur at high or low altitude?

This approach involves conducting an ANOVA for each character set separately. Each set is composed of several characters (e.g., morphology involves several different body-part measurements), these characters are analyzed together because there are frequently strong correlations between characters within sets. To compare divergence in all aspects of a character set simultaneously, a discriminant function analysis is performed, creating a set of discriminant function axes. The first discriminant function axis explains a certain proportion of the variance between populations in that trait, and each subsequent axis describes a proportion of the remaining variance. A separate ANOVA is conducted on each discriminant function axis, dividing the total variance according to the different hypotheses. Because the discriminant axes are uncorrelated with one another, an overall value for the proportion of between-population variance explained by a given hypothesis can be calculated by combining the results from each ANOVA on the separate axes. Combining axes is achieved by multiplying the proportion of the total variance explained by each axis by the proportion of the variance explained by each hypothesis in the ANOVA using that axis, and then adding these values together for all axes.

The significance of any difference in the proportion of the variance explained by dividing populations into groups according to a hypothesis, as opposed to the residual between-population variance, can be tested by calculating *F*-values by dividing explained and unexplained variances. Because each set of contrasts has to be orthogonal, each must be composed of two-way splits, and these must be chosen so that the different hypotheses do not confound one another. Some characters were not measured in all populations. The orthogonal contrasts that are combined to address each hypothesis are given in Table 1.

To examine the long-term refugia hypothesis we first determine how much of the total between-population variance is explained by comparing the Spanish populations (5, 10) with all other populations (this contrast is referred to as ''refugia (a)''). We then compare the Italian population (11) with Greek populations (7, 12) and those derived from the Greek refuge (1, 2, 3, 4, 6, 7, 8, 9, 12), ignoring those from Spain (this contrast is referred to as ''refugia (b)''). Adding the proportions of the total variance explained by these two comparisons gives us the proportion of the total between-population variance that is explained by dividing all populations according to the refugium in which their lineage was isolated. A similar set of contrasts allows us to examine the other hypotheses in a similar way.

To compare founder populations with stable populations we combine two comparisons: (1) the refugial Greek populations (7, 12) with their descendants in the rest of Europe (1, 2, 3, 4, 6, 8, 9); and (2) within the descendant group, we further contrast those descendants of the Greek refuge that are relatively distant from the refuge (populations 3, 4) with those nearer Greece (populations 1, 2, 6, 8, 9). Therefore, we are allowing for the possibility that there is variation in the extent

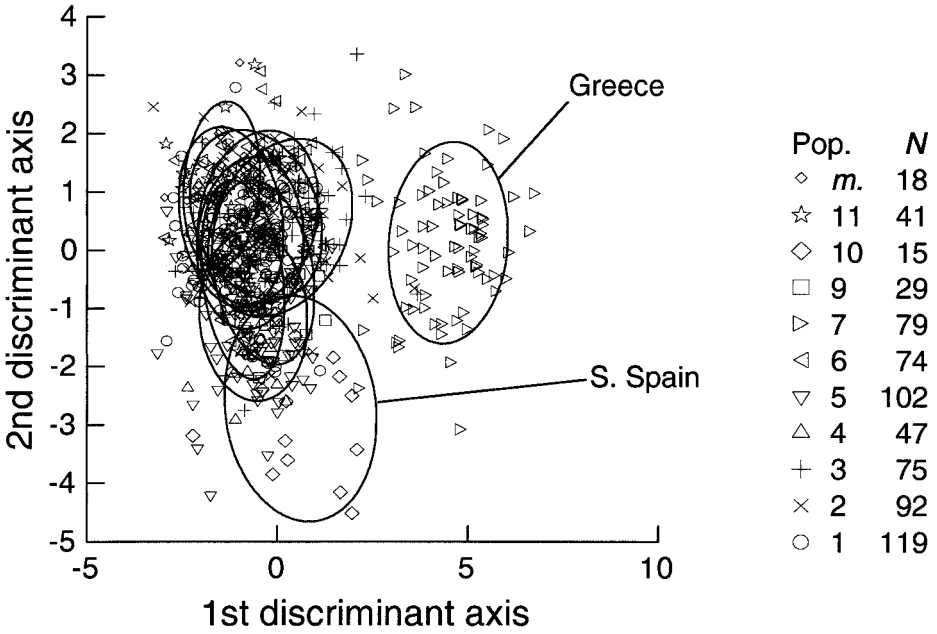

FIG. 2. Female morphology. Scores from first and second axes of discriminant function analysis of 11 populations (as labeled in Fig. 1; *m., Chorthippus montanus*). Ellipses are standard deviations centered on the sample means. Their orientation is determined by the covariance between first and second discriminant axes.

to which populations have been subject to founder events, with populations 1, 2, 3, 4, 6, 8, 9 grouped together when compared with populations 7 and 12, but split up when comparing populations 3 and 4 with populations 1, 2, 6, 8, 9.

To examine possible adaptations to altitude, while controlling for latitude, we compare three pairs of populations, one at high and one at low altitude: within Spain (5 vs. 10), within Greece (7 vs. 12), and within the descendants of the Greek refuge remaining in southern Europe (1 vs. 9).

To compare populations sympatric with *C. montanus* with allopatric populations we compare populations 2 and 6 with similar (low-altitude) populations in the same area of southern Europe (1, 8).

The different numbers of populations used in the different contrasts does not bias the ability of the hypothesis to explain variance between populations because the null hypothesis is that all comparisons contribute equally to the total variance. A comparison between A and B has the same expected variance as a comparison between C + D + E with F + G + H because the extra comparison included in the groups can both increase and decrease the between-group variance. To illustrate this point using female morphology as an example, we conducted ANOVAs assigning population designations arbitrarily and using either all possible permutations or 900 separate ANOVAs, whichever was smaller. This confirmed that the mean percentage of the variance explained by each hypothesis and the corresponding *F*-values were the same (all comparisons explained 12.7 ± 0.8% of total between population variance, all $F_{1,3} = 1.5 \pm 0.9$, $P > 0.29$).

RESULTS

Morphology

There is strong sexual dimorphism in all populations, with almost no overlap between sexes within populations in the morphological characters that we measured. A multivariate analysis of variance (MANOVA), with population and sex as factors and the four morphological characters measured in both sexes as dependent variables, confirms the difference between the sexes ($F_{4,1235} = 607$, $P < 0.001$). There are also large differences between populations in morphology (MANOVA, effect of population, $F_{40,4684} = 84.8$, $P < 0.001$) and interactions between population and sex ($F_{40,4684} = 2.40$, $P < 0.001$), showing that the pattern of differences between the sexes varies between populations. The two populations with the largest body size (measured in terms of pronotum length) are the most southerly, but there is not a strong effect of latitude on body size (regressions of latitude vs. mean pronotum length, males $r^2 = 0.22$, $F_{1,9} = 3.74$, $P = 0.09$; females $r^2 = 0.231$, $F_{1,9} = 4.00$, $P = 0.08$). To examine the nature of the differences between populations, we conducted discriminant function analyses, treating the two sexes separately. Plots of each individual's discriminant function score on the first and second discriminant axes are presented in Figures 2 and 3. It is apparent that in females (Fig. 2), the Greek population (7) differs from all the other populations in both sexes, as does the southern Spanish population (10). Among males (Fig. 3), where populations are better separated because of the additional stridulatory peg characters, these same two populations are distinct from other areas, as are the northern Spanish population (5) and to a lesser extent the English population (4). It is also clear that *C. montanus* (*m*) can be distinguished from all but the English and northern Spanish populations of *C. parallelus* on the basis of this analysis.

Table 2 gives standardized discriminant function coefficients for the first and second discriminant axes (DAs) in the analyses of male and female morphology. These coefficients quantify the contribution of each variable to the discriminant

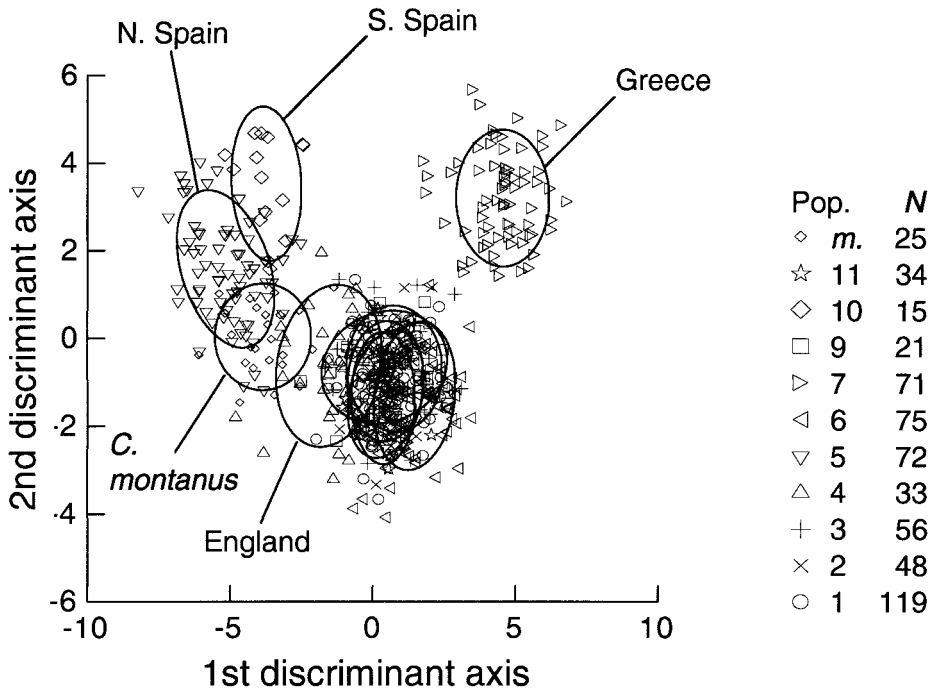

FIG. 3. Male morphology. Scores from first and second axes of discriminant function analysis of 11 populations (as labeled in Fig. 1; *m.*, *Chorthippus montanus*). Ellipses are standard deviations centered on the sample means. Their orientation is determined by the covariance between first and second discriminant axes.

function, controlling for other independent variables entered in the equation. It is clear that for both sexes several characters contribute to the separation. In females the first DA is mainly a measure of overall size, with the second being a measure of the ratios between characters. In males, the first DA is dominated by peg number, whereas the second is predominantly a measure of size.

Cuticular Composition

There are differences between the sexes and populations in the proportions of the various chromatographic peaks, each representing one or more compounds (MANOVA, using sex and population as factors and log contrasts of the 13 peaks: effect of sex $F_{13,\,872} = 24.325$, $P < 0.001$; effect of population $F_{169,8098} = 10.26$, $P < 0.001$; interaction between sex and population $F_{169,8098} = 2.2$, $P < 0.001$). Univariate tests on both sexes for each of the 14 chromatographic peaks (Fig. 4) indicate that all identified peaks contribute to the differences between populations (minimum $F_{13,\,438} = 3.109$, $P < 0.001$), although peaks may not be entirely independent of one another. The relative contributions of the log contrasts of individual peaks to the major differences between the populations in separate analyses for the two sexes are shown in Table 3. The relative importance of the different peaks in separating populations is correlated in males and females. A Pearson correlation of the absolute values of the standardized discriminant function scores for each peak from the first three DAs (which can be combined because they are independent of one another) reveals a strong correlation between the sexes ($r = 0.432$, df = 37, $P = 0.006$). Figure 5 illustrates the pattern of differences between populations and sexes, showing the mean first discriminant function scores from an analysis combining both sexes and discriminating only between populations. The variation in the pattern of sex differences among populations is as expected from the significant interaction found in the MANOVA. The sexes do not differ in how much variation they exhibit between populations: taking the sexes separately, MANOVAs for variation in peak composition between populations produce similar *P*-values (males $F_{169,4051} = 6.799$, $P < 0.001$; females $F_{169,3977} = 6.336$, $P < 0.001$).

TABLE 2. Standardized discriminant function coefficients for first and second discriminant axes (DA) in discriminant function analyses of male and female morphology.

	Males		Females	
Morphological feature	1st DA	2nd DA	1st DA	2nd DA
Prozona	0.52	0.37	0.50	0.39
Metazona	−0.18	0.33	−0.01	−1.20
Femur length	0.54	0.12	0.58	0.88
Femur width	−0.01	0.15	0.07	−0.48
Peg number	−0.74	0.51	—	—
File length	−0.33	0	—	—
% variance explained	70	25	77	15

Calling-Song

There are significant differences between populations in the temporal structure of male calling-songs (MANOVA between *C. parallelus* populations $F_{28,\,899} = 3.34$, $P < 0.001$). Data for male calling-song parameters and univariate *F*-tests (one-way ANOVA) for differences between populations for individual song characters are presented in Table 3. These

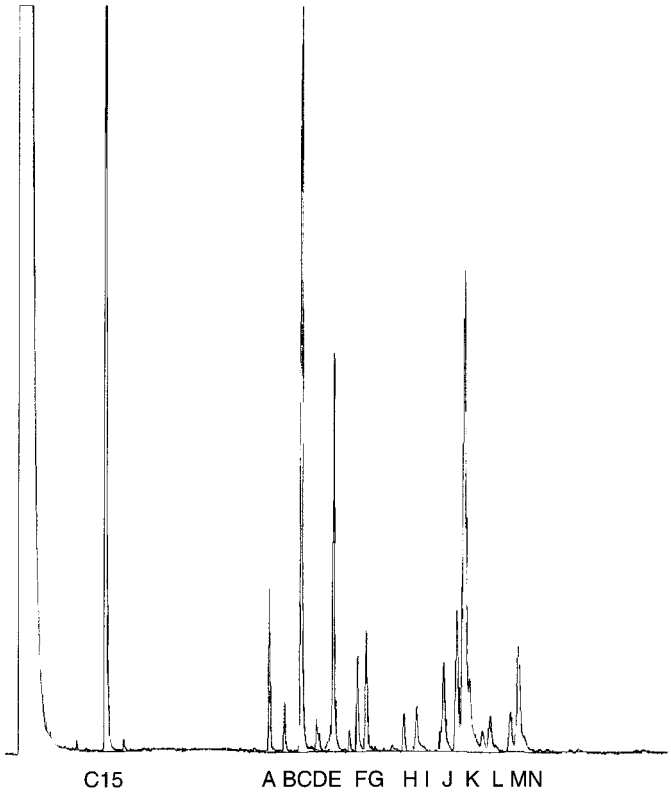

FIG. 4. A typical gas chromatograph trace from a male *Chorthippus parallelus*. Each peak represents a different class of hydrocarbon or other lipid, with longer and more complex molecules appearing further to the right. Letters are peak classifications used in analyses; C15 is pentadecane, which was added as an internal standard.

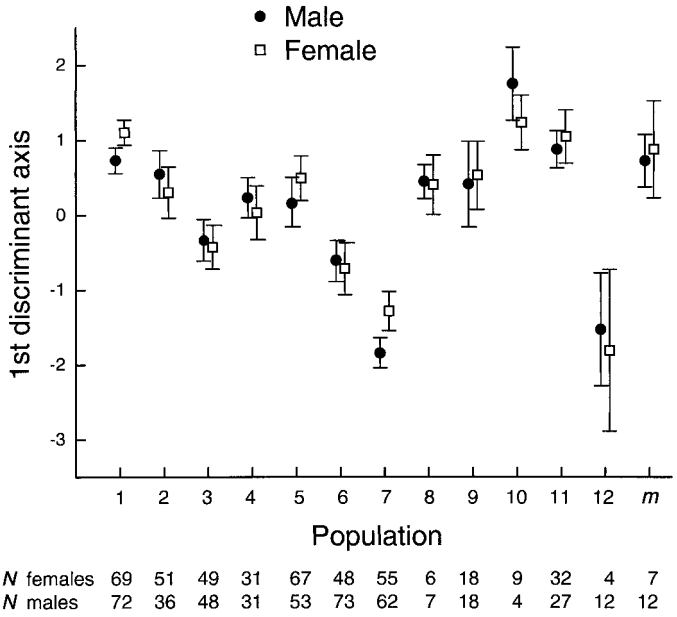

FIG. 5. Cuticular composition (populations as numbered in Fig. 1). Mean discriminant function scores ± 95% confidence limits for the first discriminant axes (which explains 37% of the variance between populations). Sample sizes for both sexes are given below each population.

analyses reveal that there are significant differences between populations in syllable number and echeme length, but no overall significant differences in syllable length and echeme interval. The discriminant function coefficients presented in the same table indicate that the populations are most reliably separated by a vector that is dominated by the contrast between syllable number and syllable length, mainly because this combination separates the Italian population (11) from the rest. Mean scores for the first discriminant function axis from an analysis of all those *C. parallelus* populations for which calling-song data were collected are presented in Figure 6. *Chorthippus montanus* was excluded from this analysis because its calling song is so different from that of *C. parallelus* (Table 3) that the analysis would be dominated by the difference between the two species, rather than the differences between *C. parallelus* populations. It is clear from this analysis that there are less striking differences between populations in male song than there are in cuticular composition. The most divergent group is the Italian population (11); but the English population (4) also differs from the others. From *t*-tests of song parameters for these populations versus all other populations, it is evident that males from the Italian

TABLE 3. Standardized discriminant function coefficients (see legend to Table 1) for first, second, and third disciminant axes (DA) in analyses of male and female cuticular composition. The three peaks with the largest contributions to each DA are given in bold.

	Males			Females		
Peak	1st DA	2nd DA	3rd DA	1st DA	2nd DA	3rd DA
A	0.171	0.078	**0.661**	**0.433**	0.316	**0.434**
B	0.074	**−0.454**	−0.252	−0.059	−0.127	−0.171
C	**0.423**	**−0.461**	**−0.534**	**−0.635**	**0.757**	−0.379
D	−0.200	−0.346	−0.256	0.04	−0.384	0.37
E	0.048	−0.356	0.128	−0.118	−0.379	0.097
F	0.328	0.206	0.269	0.283	0.294	0.154
G	0.120	0.162	0.337	−0.076	−0.266	**−1.277**
H	**−0.605**	**−0.377**	0.196	−0.397	**−0.434**	**0.498**
I	0.320	−0.313	0.185	**0.500**	−0.194	0.08
J	**−0.592**	0.027	0.061	−0.415	0.188	0.216
L	−0.241	−0.160	0.002	0.019	0.023	−0.045
M	−0.237	0.333	**−0.450**	−0.126	**0.392**	0.186
N	0.391	0.330	−0.289	0.233	0.321	0.069
% variance explained	36	23	12	29	24	17

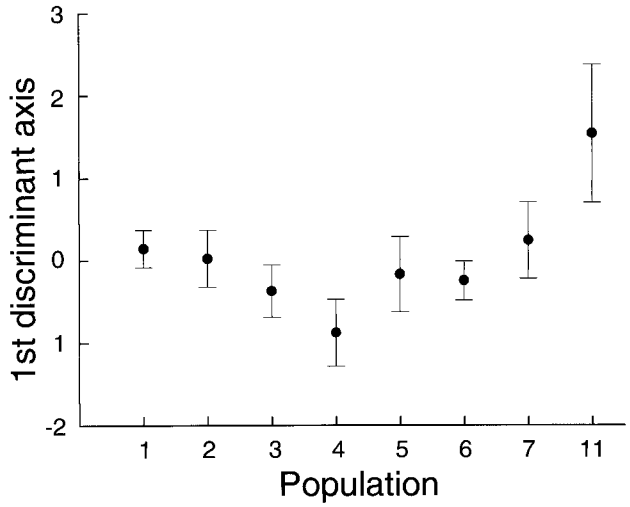

FIG. 6. Male calling-song (populations as numbered in Fig. 1). Mean discriminant function scores ± 95% confidence limits for the first discriminant axes (which explains 71% of the variance between populations). See Table 4 for sample sizes.

population have shorter syllables ($t_{258} = 3.94$, $P = 0.0001$) and more syllables per echeme ($t_{258} = 3.89$, $P = 0.0001$) than those from other populations. Individuals from the English population (4) also sing more syllables per echeme ($t_{258} = 3.71$, $P = 0.0003$) and have longer intervals between echemes ($t_{258} = 2.08$, $P = 0.039$) than those in other populations. Otherwise, there are no significant differences in song parameters between the Italian or English populations and the other populations.

Comparing Hypotheses for Divergence

The percentages of between-population variance explained by the various hypotheses are shown in Table 5. It is clear that both dividing populations into descendants of the different refugia and dividing them according to likelihood of having experienced founder events explains a large proportion of total population variance in all the traits we examined. In contrast, dividing populations according to environmental differences in terms of altitude or sympatry with a closely related species fails to explain a significant amount of the variation in any trait.

DISCUSSION

Morphology

There is considerable variation between populations in all of the morphological traits studied. Furthermore, there are differences in the pattern of sexual dimorphism between populations, and different morphological traits show the greatest differences between populations in the two sexes. In males, the most important trait separating populations is the ratio of overall size to the number of pegs on the hind femur. In females, overall size is also important, as is the ratio of metazona length to overall size.

Chorthippus montanus can be distinguished from most populations of *C. parallelus* on the basis of morphology using our analyses, despite exclusion of the two morphological characters known to be most different between the species: ovipositor length and wing length (Reynolds 1980). For males, the only populations of *C. parallelus* showing significant morphological overlap with *C. montanus* are those from northern Spain (5) and England (4), which are allopatric to *C. montanus*.

Cuticular Composition

Our analysis of cuticular composition reveals striking levels of variation between populations. As with the other traits, because all populations were reared under identical conditions in the laboratory, these differences cannot be ascribed to differences in diet or other such environmental differences. Even populations descended from the same refuge and remaining in geographic proximity show significant levels of differentiation. This is illustrated in Figure 5 by the differences seen between populations 1, 2, and 9, all of which occur within 100 km of each other in the Massif Central in France. Despite differences between many populations,

TABLE 4. Male calling-song measures (mean ± SE). All values are corrected for temperature (standardized to 30°C; see text). *N*, sample size; SN, syllable number per echeme; EL, echeme length/sec; SL, syllable length/sec; EI, echeme interval/sec. $F_{7,252}$, univariate *F*-test for differences between populations in individual parameters. DFC, discriminant function coefficient, standardized by within-population variances, for first axis of a discriminant function analysis. The DFC represents the relative weighting and relationship between parameters in distinguishing between populations. Both analyses excluded *Chorthippus montanus* (*m*).

Pop.	*N*	SN	EL	SL	EI
1	73	16.1 ± 0.3	2.4 ± 0.1	0.16 ± 0.004	8.1 ± 0.3
2	34	15.8 ± 0.4	2.4 ± 0.1	0.16 ± 0.006	8.1 ± 0.4
3	25	14.9 ± 0.4	2.2 ± 0.1	0.15 ± 0.007	6.7 ± 0.5
4	24	13.7 ± 0.7	2.1 ± 0.2	0.16 ± 0.009	6.3 ± 0.7
5	20	14.7 ± 0.6	2.2 ± 0.2	0.15 ± 0.008	6.7 ± 1.4
6	43	15.1 ± 0.3	2.3 ± 0.1	0.16 ± 0.006	8.3 ± 0.4
7	23	15.5 ± 0.5	2.3 ± 0.1	0.16 ± 0.004	7.1 ± 0.4
11	18	17.8 ± 0.7	2.2 ± 0.2	0.12 ± 0.008	6.7 ± 0.5
m	12	13.3 ± 0.4	3.4 ± 0.3	0.26 ± 0.017	12.3 ± 1.2
[1]$F_{7,252}$		6.29**	5.36**	1.00[ns]	2.34[ns]
[1]DFC		2.50	−0.84	−1.54	0.59

** $P < 0.001$ after sequential Bonferroni adjustment.
[ns] $P > 0.05$ after sequential Bonferroni adjustment.
[1] Analysis excluding *C. montanus*.

broader patterns are still apparent, notably that the two Greek populations have a distinct cuticular lipid composition compared to other populations. In addition to high levels of differentiation between populations, there are strong differences between the sexes, as observed by Neems and Butlin (1994). There are also interactions between population and sex, indicating that the pattern of sexual differences varies among populations. These population-sex interactions can also be seen in Figure 5: Some populations show greater differences between the sexes, demonstrating that in these populations there are differences between the sexes that are similar to the differences found between populations (because the analysis maximizes between-population variance). In general, there is a similar level of interpopulation variation in cuticular composition in both males and females, indicating that it is not under different selection pressures in males and females, as might be expected if it were under sexual selection.

All the gas chromatography peaks we measured contribute to the differences between populations, although some peaks vary more between sites than others. There is a weak correlation between the sexes in the relative contribution of the different peaks to our ability to distinguish between populations. This finding that in both sexes some classes of cuticular lipid vary more between populations supports the idea that some cuticular lipids are more free to vary between populations than others, possibly because different compounds have different roles. For instance compounds involved in waterproofing may be more constrained than those used in chemical communication.

Calling-Song

There are clear differences between populations in the temporal structure of their calling-songs, particularly in the number of syllables per echeme and the duration of each echeme. These differences support the findings of Dagley et al. (1994) that there are differences between populations of *C. parallelus* on a Scottish Island, an island in the English Channel, and in the Pyrenees. However, our findings differ from those of Dagley et al. (1994), who found that syllable number did not differ between populations, whereas syllable length varied the most between populations. Similarly, syllable length differed more between populations than did other calling-song parameters on either side of the Pyrenean hybrid zone (Butlin and Hewitt 1985a). These observations contrast with our finding and that of Flanagan (1997), who studied differences between populations on either side of the Italian Alps, that syllable number and echeme length differ most between populations. However, it is worth noting that although syllable length does not differ significantly between populations according to a univariate ANOVA, discriminant function analysis indicates that syllable length can be used in discriminating between populations. This indicates that there may be interactions between syllable length and other song parameters, and that these interactions are consistently different between populations, even though syllable length alone is not a reliable discriminator. These differences in which parameters vary between populations suggest that all the aspects of calling-song studied have the potential to evolve independently.

Comparison of song parameters between populations (Table 4) reveals that the two populations taht are most divergent from the general structure of the calling-song are the refugial Italian population (11) and the English population (4) near the extreme of the colonization route from the Balkan refuge. Calls of individuals from the Italian population (11) have on average 1.7 more syllables per echeme than those in the nearest population (1) and also have distinctly shorter syllables (0.12 sec compared with 0.15–0.16 sec). Males from England (4) have calls with the shortest echeme lengths and echeme intervals and a lower number of syllables per echeme than the nearest population (5). The different evolutionary histories of these populations suggest that neither long periods of isolation in refugia nor repeated founder events are essential for differences in signaling traits to evolve, although either process may tend to drive such divergence.

The lack of significant differences in calling-song between Spain (5) and France (1, 2, 3) is interesting, because populations of *C. parallelus* inhabiting the Iberian Peninsula are designated as a separate subspecies, *C. p. erythropus,* whereas those in the rest of Europe are known as *C. p. parallelus* (Reynolds 1980). Clinal changes in calling-song characteristics, particularly decreasing syllable length and echeme length in north-south transects through the Col de la Quillane in the eastern Pyrenees are well documented (Butlin and Hewitt 1985a; Butlin 1989). The lack of differences between our Spanish and French populations suggests that the calling-song patterns characterized as belonging to the Spanish subspecies may only be typical of certain populations within the Iberian Peninsula. These differences evolving within such a limited geographical area might be regarded as evidence that isolation in separate refugia is not necessary for major divergence. Alternatively, on the basis of nuclear DNA sequence variation, it has been suggested that there may have been more than one glacial refuge within the Iberian Peninsula (Cooper et al. 1995). Therefore, our northern Spain population (5) might have had a long-term evolutionary history independent of those populations examined in the Col de la Quillane, which is 400 km away in the Eastern Pyrenees.

Testing Competing Hypotheses for the Causes of Divergence

There are essentially two approaches to investigating what drives divergence between populations: comparisons between pairs of closely related species and analysis of geographic variation within species. The former approach is exemplified by Coyne and Orr's (1989, 1997) examination of genetic distance and reproductive isolation within the genus *Drosophila.* This study reveals a strong correlation between genetic distance and postmating isolation, as expected if genetic incompatibility is a side effect of general genetic divergence. For premating isolation, however, the picture is different. Particularly among sympatric species-pairs, there is frequently substantial premating isolation, even between closely related species, suggesting that such isolation has arisen under strong selection. This selection might be due to selection for assortative mating resulting from hybrid dysfunction (reinforcement; Butlin 1989; Butlin and Tregenza 1997) or to adaptation of mating signals to different habitats

(particularly if closely related sympatric species are more likely than allopatric species to live in different habitats, as a consequence of ecological displacement). Studies of planthoppers (Claridge et al. 1985), drosophilids (Ritchie and Gleason 1995), frogs (Ryan and Rand 1993), and grasshoppers (Meyer and Elsner 1996; see further examples and detailed discussion by Butlin and Tregenza 1998) have all found patterns of mating signal divergence that are independent of general genetic divergence.

A disadvantage of interspecific studies is that it is difficult to distinguish divergence during speciation from divergence after speciation—when populations no longer exchange genes. To be sure that we are observing patterns of divergence associated with speciation itself, we need to examine patterns of differences between populations within a single species. This has been done for very few taxa. In the salamander *Desmognathus ochrophaeus* (Tilley et al. 1990) and the túngara frog, *Physalaemus pustulosus* (Ryan et al. 1996), both genetic distance and the strength of assortative mating between pairs of populations are related to geographic distance. This correlation between divergence and distance, unlike the pattern between species of *Drosophila* (Coyne and Orr 1989, 1997), suggests that divergence is the result of gradual genetic differentiation in allopatry. In contrast, the rapid evolution of differences in mating signals between races of the brown planthopper, *Nilaparvata lugens* (Butlin 1996), and races of *Drosophila willistoni* (Gleason and Ritchie 1998) suggest that selection on traits contributing to reproductive isolation or rapid genetic drift due to a population bottleneck are responsible for divergence. In the case of the brown planthopper, the absence of sufficiently strong mate selection by females indicates that divergence is the result of genetic drift (Butlin 1996; see also Butlin and Tregenza 1998), whereas in *D. willistoni* sexual selection cannot be ruled out.

For *C. parallelus*, it is difficult to meaningfully examine the effects of simple geographic distance on divergence. Features such as mountain ranges and seas render the shortest distance between populations a poor measure of true colonization routes.

It is clear from Table 5 that both the refugial hypothesis and the founder-effect hypothesis provide powerful explanations for divergence between populations. In all cases, except male cuticular composition, both hypotheses explain a substantially greater proportion of the variance than that left unexplained. This result strongly suggests that long periods of isolation, and processes associated with range expansion (including repeated founder events) are both important factors in driving genetic divergence between populations. Given that these two sources of divergence have had very different time spans over which to accumulate (100,000–500,000 yr for divergence in allopatry versus 10,000 yr for founder effects; Hewitt 1996), it is striking that the founder hypothesis explains as much variance as the refugial hypothesis.

Sympatry with a close relative and altitude are only two of numerous possible environmental differences between populations and it is impossible to rule out the existence of other differences that could drive divergence. However, the lack of phenotypic differences between populations differing in these significant respects weakens the likelihood that adaptation to different environments is an important factor driving divergence in this species.

There are clear differences between the sexes in the relative importance of the refugial or founder hypotheses. In females, the founder hypothesis explains most of the variance in morphology, whereas the refugial hypothesis explains more of the variance in cuticular composition. In males the pattern is reversed. It is tempting to speculate that this sex difference is the result of relaxed sexual selection on males resulting from founder events (Kaneshiro 1989), because this hypothesis predicts greater effects of low population density on sexual signals (such as cuticular pheromones and male calling-song) than on nonsexually selected traits, such as morphology. However, this hypothesis does not explain why female morphology differs so much between populations distant from their refugia and their ancestors; it is more parsimonious to assume that the same effects could equally have driven divergence in male cuticular composition. Further-

TABLE 5. Percentage of between-population variance explained by dividing populations according to different hypotheses for divergence (see Materials and Methods). Refugia (a) compares populations from Spain with all other populations; refugia (b) compares the Italian population with Greek populations and those derived from the Greek refuge. Founder (a) compares the refugial Greek populations with their descendants in the rest of Europe; founder (b) compares descendants of the Greek refuge that are relatively distant from the refuge with those nearer Greece. Altitude compares three pairs of neighboring populations, one at high and one at low altitude. Sympatry compares populations sympatric with *Chorthippus montanus* with altitudinally similar allopatric populations in the same area of southern Europe. Unexplained is the percent among-population variance not explained by any of the planned comparisons (hypotheses). *P*-values are for comparison between the unexplained variance and the among-population variance explained by a particular hypothesis.

	Morphology				Calling-song		Cuticular composition			
	Female		Male		Male		Female		Male	
Hypothesis	% of variance explained	$F_{1,3}$	% of variance explained	$F_{1,3}$	% of variance explained	$F_{1,5}$	% of variance explained	$F_{1,2}$	% of variance explained	$F_{1,5}$
Refugia (a)	9	5.1*	46	19.6*	6	1.2	10	2.2*	19	7.6
Refugia (b)	5	2.6	1	0.6	46	8.7	11	2.4*	19	7.4
Founder (a)	71	39.1**	36	15.1*	8	1.4	30	6.7*	17	6.9*
Founder (b)	2	1.4	1	0.6	25	4.8	9	1.9	8	3.0
Altitude	1	0.7	2	0.8	—		5	1.2	12	4.8
Sympatry	6	3.2	6	2.5	5	0.9	8	1.9	5	2.1
Unexplained	5		7		10		27		20	

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

more, there are no obvious differences between signal traits and morphology in terms of which aspects of evolutionary history are associated with divergence. This suggests that divergence associated with range expansion is not the result of relaxed sexual selection, because this would predict greater changes in signaling traits. In general, despite characterizing more than a thousand individuals from 13 populations, the statistical power of the analyses available to us is still relatively weak, making it difficult to accurately assess the significance of the differences between the sexes and which hypothesis best explains variation between populations.

As with all attempts to gain insights into the evolutionary processes important in driving divergence, it is difficult to assess the relevance of the patterns we observe in *C. parallelus* for other species. It is possible that the causes of speciation are different in every case (e.g., reproductive character displacement is known to occur in some species; see Howard 1993; Butlin 1995). The only way to assess the generality of our findings is through more studies comparing divergence between populations with known evolutionary histories. The degree of differentiation associated with speciation varies between cases (Butlin and Tregenza 1998; Magurran 1998), making the extent to which observed patterns of divergence may subsequently translate into speciation events impossible to assess. Nevertheless, it is clear that both long periods of allopatry and events associated with rapid range expansion, notably the likelihood of repeated founder events, can lead to divergence between populations. Thus, both are likely to be important in driving speciation and the generation of diversity.

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