Divergence in the calling songs between sympatric and allopatric populations of the southern wood cricket *Gryllus fultoni* (Orthoptera: Gryllidae)

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Abstract

In the eastern United States the wood cricket *Gryllus fultoni* (Orthoptera: Gryllidae) occurs in sympatry with *G. vernalis* in an area between eastern Kansas and west of the Appalachian Mountains. Calling songs were recorded from 13 sympatric and allopatric localities. Both field and laboratory recordings showed that chirp rate (CR) and pulse rate (PR) overlapped extensively between allopatric populations of *G. fultoni* and sympatric populations of *G. vernalis*; by contrast, there was little or no overlap in these variables between sympatric populations of these two species. Divergence in PR and CR between the two species was thus greater in areas of sympatry than in areas of allopatry. Our field and laboratory studies of *G. fultoni* calling songs thus demonstrate the pattern expected of character displacement and support the genetic assumptions of this hypothesis. Other possible explanations for the sympatric divergence such as ecological character displacement and clinal variation are discussed.

Introduction

Geographical variation in communication systems provides information about possible selective forces that have shaped the variation and a trace of recent evolutionary history (Gerhardt & Huber, 2002). However, identifying selective forces is notoriously difficult because of the intensive effort required for adequate representation of geographical variation over broad areas and the uncertainty of identifying the relevant environmental and ecological factors. This problem is particularly acute in studying character displacement, which is a pattern that certain characters of closely related species are more dissimilar in sympatry than in allopatry (Brown & Wilson, 1956).

Character displacement has remained a controversial subject since its inception. Many studies have been justifiably criticized because they have not considered alternative hypotheses for the observed patterns of divergence in characters (Grant, 1972; Butlin, 1995;

Noor, 1999; Adams & Rohlf, 2000). Studies of character displacement usually involve comparisons of traits in question between allopatric and sympatric populations of closely related species. When the traits in question are more divergent in areas of sympatry than in areas of allopatry, the divergence is often explained as arising from selection against costly interspecific mating in the case of reproductive character displacement or from selection against interspecific resource competition in the case of ecological character displacement. However, other factors can also cause such a pattern of divergence, including developmental effects and clinal variation (Grant, 1972; Butlin, 1995; Noor, 1999).

As pointed out by Gabor & Ryan (2001), studies of character displacement, both reproductive and ecological, have often relied on a few sample populations from sympatry and allopatry. Because chance effects often arise when sample size is small, apparent differences between sympatric species may result from sampling errors (see Simberloff & Boecklen, 1981; Boecklen & NeSmith, 1985). Therefore, it is critical to demonstrate character displacement not only independent of environmental differences between allopatry and sympatry but also beyond chance expectations.

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Identifying selective forces responsible for the pattern of divergence in characters between allopatric and sympatric populations provides support for hypotheses about how divergence arises and is maintained. In reproductive character displacement, such forces are the elimination of unfit hybrids by natural selection, a process termed reinforcement (Dobzhansky, 1940; Howard, 1993). When closely related species come into secondary contact, hybrid offspring may have lower fitness than offspring from within-taxon matings. Then hybrids will be selected against, and the reproductive fitness of the parents is significantly reduced compared with parents that mate with partners of the same taxon. The predicted outcome of such reinforcement (*sensu* Blair, 1955) is reproductive character displacement in which diverged characters between sympatric species reduce or eliminate interspecific mating.

The importance of reinforcement and reproductive character displacement has been controversial because of theoretical difficulties (Felsenstein, 1981; Paterson, 1982; Barton & Hewitt, 1985; Spencer *et al.*, 1986; Butlin, 1989) and lack of empirical evidence (Walker, 1974a; Veech *et al.*, 1996). However, recent theoretical developments using genetic models suggest that reproductive character displacement could occur under a wide variety of conditions (Liou & Price, 1994; Kelly & Noor, 1996; Servedio & Kirkpatrick, 1997; Kirkpatrick & Servedio, 1999; Kirkpatrick, 2000). These theoretical conclusions have been supported by recent empirical evidence for reproductive character displacement (see Table 1 in Gabor & Ryan, 2001; Gerhardt, 1999; Höbel & Gerhardt, 2003).

Ecological character displacement, like reproductive character displacement, is expected to evolve in response to an interaction between sympatric taxa (Brown & Wilson, 1956; Grant, 1972). The difference is that in ecological character displacement the interaction consists of competition over resources rather than mating mistakes. Competition leads to divergence in the resources used or the ways that the resources are acquired, e.g. different bill sizes or shapes in birds (see Schluter *et al.*, 1985). The evolution of reproductive isolation is seen as a by-product of genetic divergence between populations occupying different habitats. However, it is difficult to discern selection arising from ecological competition from selection arising from assortative mating, and few studies have examined the possible role of ecological factors in reproductively isolating traits between two sympatric taxa (Noor, 1999).

In crickets, developmental conditions can affect the expression of male calling songs and female mate preferences. When recorded at standard temperatures, males reared in high temperature regimes produced calling songs with higher chirp rates (CRs) and carrier frequencies in *Allonemobius fasciatus* (Olvido & Mousseau, 1995) and with higher pulse rates (PRs) in *Gryllus rubens* (Walker, 2000) than males reared in low temperature

regimes. Grace & Shaw (2004) found that changes in male calling songs and female preference functions induced by developmental temperatures were matched in a Hawaiian cricket *Laupala cerasina*. That is, female crickets reared in warmer conditions preferred songs with faster PRs produced by males reared in warmer conditions. These studies suggest that when temperature is consistently different between allopatric and sympatric habitats during development, divergence in calling song characters between allopatric and sympatric cricket populations can be generated without interactions with sympatric species.

Besides reproductive or ecological character displacement or developmental effects, environmental factors such as clinal variation may explain the geographical variation in reproductively isolating traits. Clinal variation presumably reflects adaptation to progressive spatial patterns of variation in environments that may differ in temperature, seasonality, elevation and other factors (Brown & Lomolino, 1998). There is evidence that clinal variation in body size in frogs can influence call characteristics that may in turn affect assortative mating within populations. In the Puerto Rican treefrog *Eleutherodactylus coqui*, for example, male body size increased as elevation increased. Because body size was negatively correlated with the fundamental frequency of each note in the advertisement call, elevation explained the variation in fundamental frequency (Narins & Smith, 1986).

Here, we document geographical variation in calling songs among sympatric and allopatric populations of two wood crickets *Gryllus fultoni* (Alexander) and *G. vernalis* Blatchley (Orthoptera: Gryllidae). In eastern United States these two species occur together in an area between eastern Kansas and west of the Appalachian Mountains. Alexander (1957) first noted qualitative differences in calling songs between allopatric and sympatric populations of *G. fultoni*. We recorded calling songs in 13 localities encompassing allopatric and sympatric zones. The patterns of geographical variation in calling songs of allopatric and sympatric populations of *G. fultoni* were examined in light of the alternative explanations outlined above. Calling songs recorded in the field may be influenced by unknown environmental differences between allopatric and sympatric populations during development or adult period. Thus, we also reared allopatric and sympatric *G. fultoni* crickets from eggs to adults under standard laboratory conditions, and calling songs were recorded from these lab-reared crickets. If no divergence was found in the calling songs of the lab-reared crickets, any observed differences in calling songs are likely to have been caused by environmental differences between allopatric and sympatric localities. However, if divergence in calling songs persists even after the common garden rearing, the divergence almost certainly has a heritable basis, one of the key assumptions of character displacement (Arthur, 1982; Schluter & McPhail, 1992; Higgie *et al.*, 2000).

Methods

Study populations

Gryllus fultoni occurs throughout the eastern United States from eastern Kansas to the Atlantic Coast and from southern Indiana to northern Florida (Fig. 1). The sympatric zone between *G. fultoni* and *G. vernalis* ranges from eastern Kansas to west of the Appalachian Mountains and from southern Indiana to north of the Appalachian Mountains. *Gryllus vernalis* ranges north from the sympatric zone. The geographical distributions of *G. fultoni* and *G. vernalis* shown in Fig. 1 are based on Alexander (1968), Walker (2001) and D. Weissman (personal communication). However, further studies are required to refine the ranges, because the peripheral limits of the distributions of both species are unclear.

Both *G. fultoni* and *G. vernalis* are nymphal-diapause species (Alexander, 1957) and are univoltine, except in north Florida, where *G. fultoni* has a partial second generation with the adults occurring in August and September (Walker, 1974b). Both species are strictly micropterous in the field and have a narrower head than pronotum (Alexander, 1957). Because of similar life history and morphology, *G. fultoni* and *G. vernalis* are regarded as closely related species (D. Weissman, personal communication). The phylogenetic relationship based on DNA genes revealed that the *Gryllus* species in eastern North America may be divided into four clades that were characterized by difference in life history traits between clades (Huang *et al.*, 2000). Unfortunately, *G. vernalis* was not incorporated into this phylogeny, and the phylogenetic relationship between *G. fultoni* and *G. vernalis* still needs to be determined.

Geographical coordinates, recording dates, and habitats of state parks where we conducted field recordings are described in Fig. 1 and Table 1. Both *G. fultoni* and *G. vernalis* were found in a large body of contiguous forests. In eastern United States, such contiguous forests are usually found in state parks where field recordings of this study were conducted. We often failed to find wood crickets in state parks whose forests were fragmented. Both species were apparently absent in high mountains, and the Appalachian Mountains may serve as a barrier
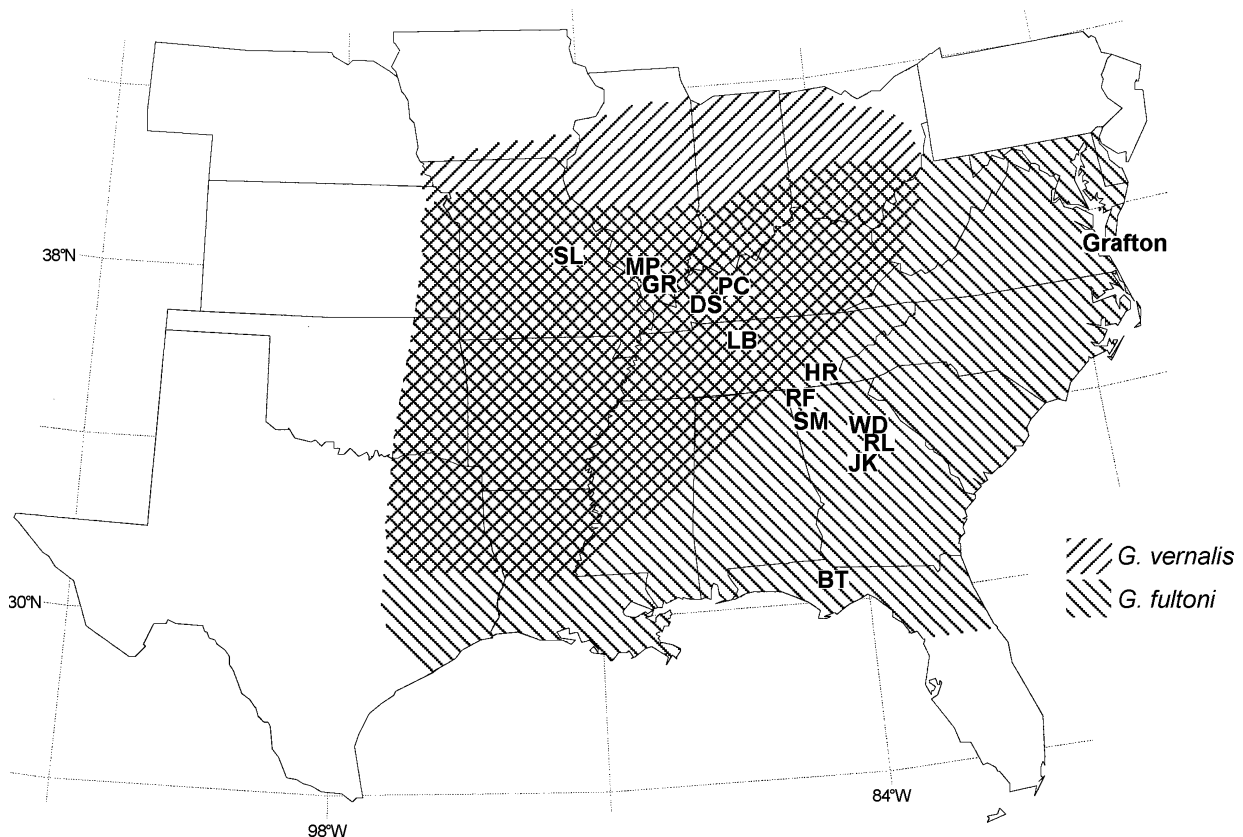

Fig. 1 Geographical distributions of *Gryllus fultoni* and *G. vernalis*. The right- and left-hatched areas denote distributions of *G. fultoni* and *G. vernalis*, respectively. The cross-hatched area represents the sympatric zone. Calling songs of *G. fultoni* were recorded in seven allopatric localities: BT, JK, RL, WD, SM, RF and HR. Calling songs of both *G. fultoni* and *G. vernalis* were recorded in six sympatric localities: LB, DS, PC, GR, MP and SL. See Table 1 for locality abbreviation. Doherty & Callos (1991) also described a *G. fultoni* population near Grafton, Virginia.

Locality	n	City, state	Latitude	Longitude	Elevation	Habitat
Allopatry						
BT	5	Bristol, Florida	30°34.1′N	84°56.9′W	95 m	Leaf litter
JK	20	Jackson, Georgia	33°10.5′N	84°0.9′W	165 m	Leaf litter
RL	5	Rutledge, Georgia	33°40.1′N	83°36.3′W	189 m	Leaf litter
WD	20	Winder, Georgia	33°58.6′N	83°43.6′W	271 m	Leaf litter
SM	27	Summerville, Georgia	34°26.7′N	85°20.7′W	200 m	Leaf litter
RF	12	Rising Fawn, Georgia	34°50.0′N	85°28.9′W	547 m	Leaf litter
HR	10	Harrison, Tennessee	35°10.2′N	85°7.1′W	216 m	Leaf litter
		Grafton, Virginia*	37°33.5′N	76°28.2′W	30 m	N/A
Sympatry						
LB	11	Lebanon, Tennessee	36°4.6′N	86°18.3′W	201 m	Grass
DS	14	Dawson Springs, Kentucky	37°4.4′N	87°39.3′W	202 m	Grass
PC	31	Park City, Kentucky	37°6.7′N	86°3.8′W	217 m	Grass
GR	13	Goreville, Illinois	37°32.1′N	88°58.8′W	177 m	Grass
MP	14	Murphysboro, Illinois	37°47.3′N	89°22.7′W	124 m	Grass
SL	9	Sullivan, Missouri	38°12.1′N	91°6.3′W	240 m	Grass

Table 1 Recording localities of *Gryllus fultoni*. Geographical coordinates and elevations of localities were drawn from DeLorme Topo USA (version 3.0). Depending on where the crickets occurred, habitat was classified as either leaf litter or grassy habitat.

n, the total number of recordings for each locality.

*The Grafton, Virginia, population was from Doherty & Callos (1991).

between allopatric and sympatric *G. fultoni* populations. The highest elevation at which we found crickets was 547 m in our study (Table 1).

In preliminary studies, we extensively searched for both *G. fultoni* and *G. vernalis* in the border areas in Tennessee and northern Georgia but failed to find any *G. fultoni* that were intermediate in morphology between sympatric and allopatric populations (Y. Jang, unpublished data). To adequately sample calling songs of both *G. fultoni* and *G. vernalis* in each state park, we randomly ordered hiking trails and then checked for calling songs starting from the top-ranked trails. We then tried to record all cricket calling songs heard along about the first 150 m of each trail. This approach worked well for sampling calling songs of allopatric males of *G. fultoni* and sympatric males of *G. vernalis*. However, *G. fultoni* was relatively uncommon in sympatric localities, and these crickets mostly occurred at forest edges. Accordingly, we tried to record calling songs of all sympatric *G. fultoni* near sites where we recorded calling songs of sympatric *G. vernalis*. Calling songs of *G. fultoni* were recorded at seven allopatric localities, and calling songs of both *G. fultoni* and *G. vernalis* were recorded at six sympatric localities (Table 1). The total number of recordings for each locality is shown in Table 1, and one male cricket contributed to only one recording.

For each locality where calling songs of *G. fultoni* were recorded, we classified the habitat as either leaf litter or grassy (Table 1). This research followed the forest classification of Kricher & Morrison (1998) and was conducted in Southern Mixed Pine-Oak, Mixed Appalachian, Oak-Hickory and Oak-Hickory-Pine forests. Oak and hickory trees were often found in all localities, and acorns and hickory nuts were usually obvious among the leaf litter on the forest floor. In addition, the leaf-litter habitat usually occurred in heavily shaded areas. By contrast, grassy habitats were exposed, had no tree cover, and usually lacked acorn or hickory nuts on the ground.

Recordings and analyses of calling songs

Males produced calling songs from underneath fallen leaves or in grasses or from the top of fallen leaves (Y. Jang, unpublished data). Singing activity increased in both species around dusk, with a gradual decrease towards midnight. Field recordings were made between dusk and midnight on rainless nights. Calling songs were recorded with a tie-clip microphone (RadioShack Corp., Fort Worth, TX, USA; model 33-3013; frequency response: ±3 dB from 70 to 16 000 Hz), which was placed as close to the singing male as possible. Output from the microphone was fed into a Sony PCM-M1 DAT recorder (Sony, Tokyo, Japan) with a sampling rate of 44.1 kHz. Singing crickets were recorded for about 30 s. During each recording night, ambient temperature was continuously monitored every 30 s using a HOBO data logger (model H08-004-02; Onset Computer Corp., Pocasset, MA, USA) that was placed 5 cm above the ground near recording sites. Cricket signals were analysed using CANARY 1.2.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA) installed on a Macintosh computer. Frequencies below 1 kHz were filtered out to reduce background noise. We examined five consecutive chirps from each male for statistical analyses.

The definitions of calling song characters used in this paper follow Bennet-Clark (1989). The calling songs of *G. fultoni* and *G. vernalis* consist of repeated chirps, each having usually three pulses (Fig. 2). Pulse duration (PD) is defined as the time between the start and the end of a pulse. Pulse period is the time between the start of one pulse and the start of the subsequent pulse. PR is the inverse of the pulse period. Chirp period is the time
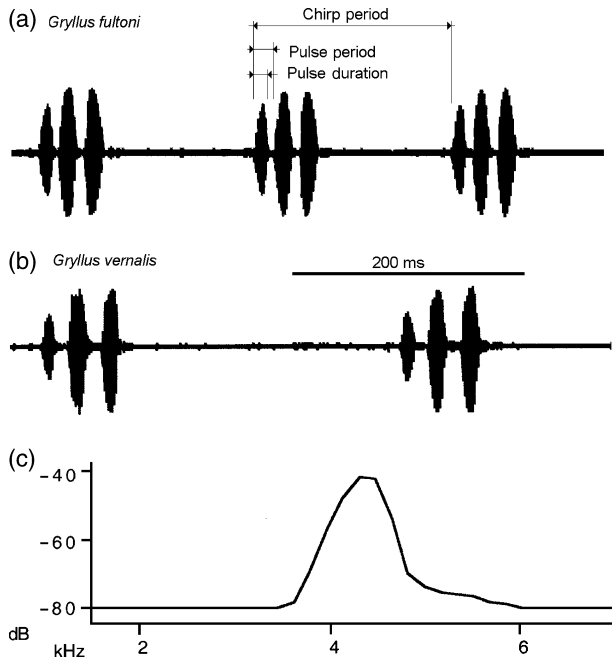
Fig. 2 Oscillograms of *Gryllus fultoni* (a) and *G. vernalis* (b) calling songs and the spectrum (c) of a *G. fultoni* chirp. Calling songs of both *G. fultoni* and *G. vernalis* consist of repeating chirps, each having usually three pulses. See the main text for descriptions of calling song characters.

between the start of one chirp and the start of the subsequent chirp. CR is the inverse of the chirp period. Carrier frequency (CF) refers to the frequency with the most acoustic energy.

Species identification

Determining patterns of calling song characters between *G. fultoni* and *G. vernalis* requires species identification independent of calling song characters. After recording, we visually inspected calling males and used a combination of body coloration and size to identify the species (Alexander, 1957). If identification of callers was not reliably determined in the field, we examined them further in the lab. Males were put on white papers in a brightly illuminated condition, and their body coloration and size were determined. This method identified with clarity all but two samples from DS and SL each. The analyses of calling songs showed that CRs (5.91, 5.14 s^{-1} at 23 °C; see Table 2) and PRs (49.80, 46.76 s^{-1} at 23 °C) of these two males were well within the ranges of *G. fultoni*. We excluded calling songs of these two individuals from further statistical analyses because of the conflict between the species identification method and values of calling songs. Based on calling songs, we believe that the coloration of these two individuals resulted from the natural variation rather than being hybrids or intermediate forms. Unlike these two individuals above, calling songs of intermediate forms or hybrids are expected of intermediate between calling songs of parent populations or parent species (see Walker, 2000).

Laboratory rearing and calling song recording

Adults of *G. fultoni* were caught in the field in 1999 and 2002, and the progeny of these crickets were reared from eggs to adults in the lab. The populations of *G. fultoni* sampled for laboratory rearing consisted of JK (35), RL (23), RF (27), HR (15), LB (32), DS (27),

Table 2 Regression coefficients of calling song characters against temperature for allopatric *Gryllus fultoni*, sympatric *G. fultoni* and sympatric *G. vernalis* populations.

Character	Slope ± SE	H$_0$: slope = 0 (t, P)		R^2	$\bar{X}$ ± SD at 23 °C
Allopatric *G. fultoni* (N = 99)					
CR	0.198 ± 0.029	6.7	<0.001	0.327	4.112 ± 0.763 s^{-1}
PR	1.949 ± 0.207	9.4	<0.001	0.477	43.794 ± 5.483 s^{-1}
PD	−0.366 ± 0.097	−3.8	<0.001	0.128	12.086 ± 2.575 ms
CF	33.344 ± 7.991	4.2	<0.001	0.152	4183 ± 211 Hz
Sympatric *G. fultoni* (N = 92)					
CR	0.247 ± 0.020	12.1	<0.001	0.620	5.119 ± 0.542 s^{-1}
PR	2.752 ± 0.165	16.7	<0.001	0.756	48.748 ± 4.387 s^{-1}
PD	−0.239 ± 0.080	−3.0	0.004	0.089	12.523 ± 2.144 ms
CF	35.679 ± 7.567	4.7	<0.001	0.198	4297 ± 201 Hz
Sympatric *G. vernalis* (N = 62)					
CR	0.108 ± 0.019	5.4	<0.001	0.348	2.733 ± 0.455 s^{-1}
PR	1.342 ± 0.095	14.2	<0.001	0.771	31.334 ± 2.264 s^{-1}
PD	−0.526 ± 0.145	−3.6	0.001	0.179	14.160 ± 3.478 ms
CF	28.104 ± 10.291	2.7	0.008	0.111	4441 ± 246 Hz

Calling songs of both *G. fultoni* and *G. vernalis* were recorded in the field, and see Table 1 and Fig. 1 for collecting localities. Values of mean and standard deviation for each calling song character adjusted to 23 °C using these regression equations are also shown.
Slope ± SE, regression coefficient ± standard error; $\bar{X}$ ± SD, mean ± standard deviation; CR, chirp rate; PR, pulse rate; PD, pulse duration; CF, carrier frequency.

PC (24), GR (19) and SL (22). The parentheses above represent the number of field-caught females used for egg collection. Crickets were reared in plastic bins (33 × 50 × 29 cm) with holes on the side for ventilation. Both juvenile and adult crickets were provided *ad libitum* with cricket chow, lettuce and shelter. All crickets were maintained at 23 ± 1 °C and with 14 : 10 h light : dark photoperiod during development and recording.

Calling songs were recorded in a temperature-controlled anechoic chamber (3 × 3 × 2 m). Virgin male crickets were randomly drawn from each population for recording. A male cricket was housed in a container (12 × 12 × 9 cm) with a screened lid. Crickets were allowed to acclimate to the temperature for at least 30 min. Males aged between 7 and 40 days were recorded during the scotophase. A thermocouple probe (model 450-AKT; Omega Engineering, Inc., Stamford, CT, USA) placed near the recording cricket was used to note the exact temperature. The temperature of the anechoic chamber was maintained at 23 ± 1 °C.

A Sennheiser microphone (ME 66 shotgun head + K6 powering module, frequency response: 50–20 000 Hz ± 2.5 dB) was placed 58 cm directly above the container. Output from the microphone was fed into a Sony PCM-M1 DAT recorder operating at a sampling rate of 44.1 kHz. Singing crickets were recorded for 1 min. Cricket signals were analysed using RAVEN 1.1 (Cornell Laboratory of Ornithology) installed on a PC. We examined at least 12 consecutive chirps for statistical analyses.

Statistical analyses for field recordings

We used analysis of covariance (ANCOVA) to test whether allopatric and sympatric populations of *G. fultoni* differed in their calling songs. The basic ANCOVA model included a covariate, 'temperature', and two independent variable, 'locality' and 'zone'. Locality was nested within zone. Zone indicated whether a population was allopatric or sympatric. Following Sokal & Rohlf (1995), we tested the assumption of slope parallelism before testing homogeneity of Y-intercepts. We also conducted least-square regression analyses for three groups: (1) allopatric populations of *G. fultoni* and (2) sympatric populations of *G. fultoni* and (3) sympatric populations of *G. vernalis*. All populations in each zone, allopatric or sympatric, were pooled together, and their calling song characters were regressed against temperature. Examination of residual and normality plots showed no obvious heterosedasticity or nonlinearity of residuals in any of the regression analyses. In separate analyses, we conducted ANCOVA to assess the possibility that clinal variation explained the geographical distribution of calling song properties. The predictor variable was 'zone', and the covariates were 'temperature', 'latitude', 'longitude' and 'elevation'.

Statistical analyses for laboratory recordings

We also used ANCOVA to test whether allopatric and sympatric *G. fultoni* populations differed in calling songs. The basic ANCOVA model included two covariates, 'age' and 'temperature', and three independent variables, 'individual', 'locality' and 'zone'. Individual was nested within locality, which in turn nested within zone. We recorded some crickets twice on different days to assess within-individual variability, which was the error term in ANCOVA. The number of total recordings and the number of unique individuals for each locality are shown in Table 5.

The analyses of field recordings showed that allopatric populations located near the sympatric zone were intermediate in values of some calling song characters. Thus, in a separate ANCOVA analysis 'zone' was divided into three categories: far allopatric (JK, RL), near allopatric (RF, HR) and sympatric (LB, DS, PC, GR, SL) localities.

Results

Divergence in calling song characters in *G. fultoni*

Using ANCOVA, we first examined the distributions of calling songs recorded in the field to see whether calling song characters differed between allopatric and sympatric *G. fultoni* populations. To test the ANCOVA assumption of slope parallelism (see Sokal & Rohlf, 1995), the interaction term, between temperature and zone, was added to the basic ANCOVA model (see Methods). The interaction between temperature and zone was significant for PR ($F_{1,177} = 5.111$, $P < 0.05$, Fig. 3b); regression slopes of allopatric and sympatric PRs converged as temperature decreased. CR ($F_{1,177} = 1.756$, $P = 0.187$), PD ($F_{1,177} = 1.757$, $P = 0.187$) and CF ($F_{1,177} = 0.044$, $P = 0.834$) did not exhibit this interaction, and further procedures of ANCOVA were conducted on these characters. Results of ANCOVA showed that allopatric and sympatric populations of *G. fultoni* differed significantly in CR ($F_{1,11} = 18.498$, $P = 0.001$) and CF ($F_{1,11} = 7.453$, $P < 0.05$), but not in PD ($F_{1,11} = 0.001$, $P = 0.972$) after temperature adjustment. Therefore, CR and CF between allopatric and sympatric populations of *G. fultoni* were significantly different throughout the recorded temperatures. The difference in PR between allopatric and sympatric *G. fultoni* populations was temperature-dependent.

Next, we compared the distributions of calling songs of both *G. fultoni* and *G. vernalis* to see whether the differences in calling songs characters may be used for reproductive isolation between the two species. Although the ANCOVA result was significant for CF, the distributions of CF overlapped among all three groups: sympatric *G. fultoni*, allopatric *G. fultoni* and sympatric *G. vernalis* populations (Fig. 3d). Such overlap was also evident in PD (Fig. 3c). Thus, reproductive isolation based solely on PD and CF is unlikely between *G. fultoni* and *G. vernalis* in sympatry.
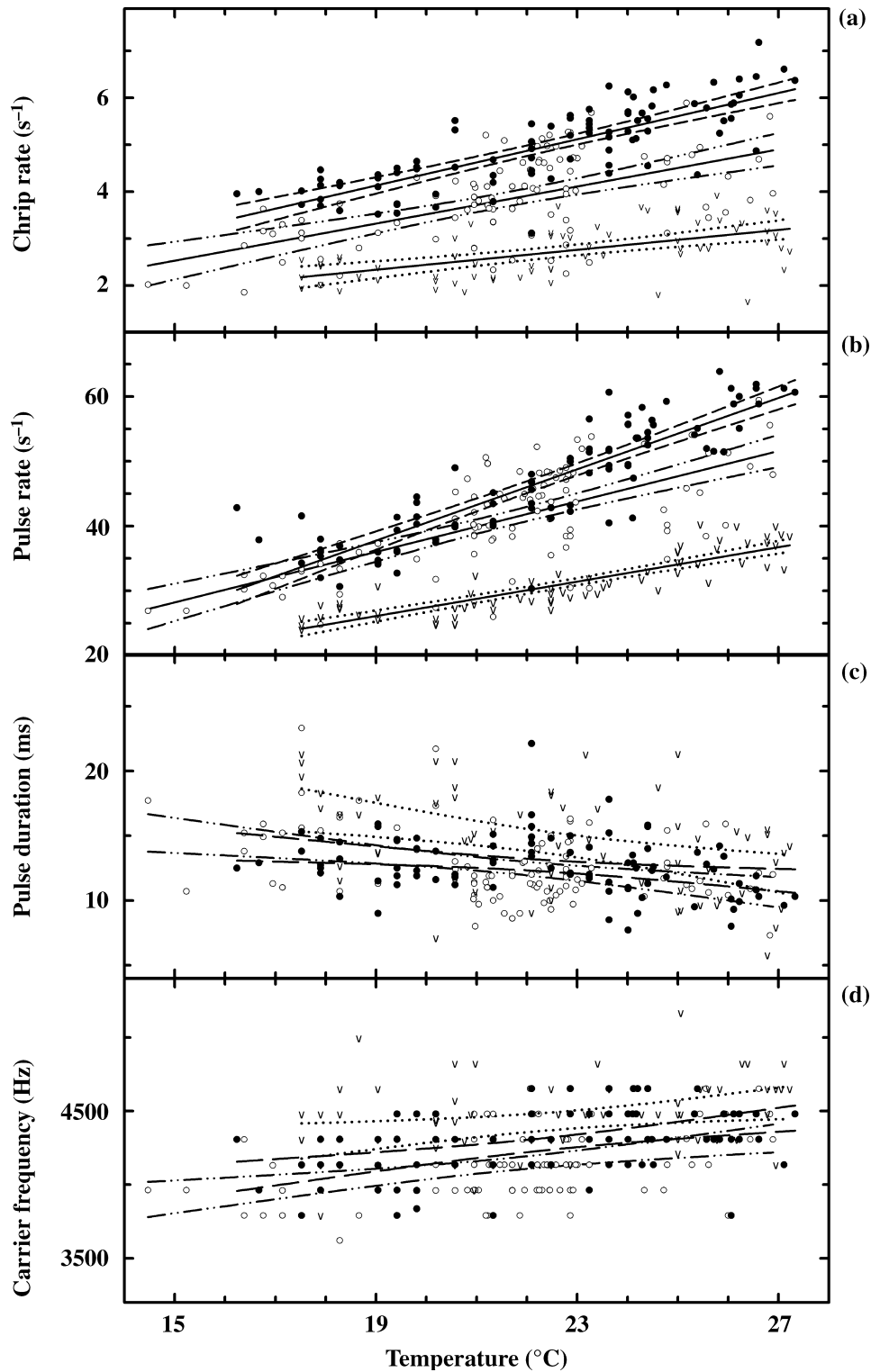
Fig. 3 Regression analyses of calling song characters against temperature for the field study. Least-square linear regressions and confidence intervals (95%) were fitted separately to allopatric *Gryllus fultoni* (○,—·—··, *n* = 99), sympatric *G. fultoni* (●, — — — —, *n* = 92) and *G. vernalis* (v, ···, *n* = 62) populations. Values of regression coefficients are shown in Table 2. Only confidence intervals are shown in (c) and (d) to avoid congestion.

The difference in CR between allopatric and sympatric populations of *G. fultoni* showed the pattern expected of character displacement, i.e. greater differences vis-à-vis *G. vernalis* in sympatric populations (Fig. 3a and Table 2). Indeed, the distributions of CR showed little or no overlap between *G. fultoni* and *G. vernalis* in sympatry. Distributions of PR also showed little overlap between *G. fultoni* and *G. vernalis* in sympatry (Fig. 3b and Table 2). Therefore, if female mate preferences are based on CR and PR, interspecific matings are unlikely throughout the recorded temperatures between *G. fultoni* and *G. vernalis* in sympatry.

Clinal variation

Because CR and PR had a potential for being used as a reproductively isolating mechanism between *G. fultoni* and *G. vernalis* in sympatry, the clinal variation analyses were focused on these two variables. Spatial variables significantly interacted with zone especially for CR and PR (Table 3), and no further procedures of ANCOVA were conducted. Instead, the relationships between spatial variables and calling song characters were graphically inspected. Calling song characters were regressed against recorded temperatures, and then unstandardized residuals (ε) of calling song characters were plotted against spatial variables (Fig. 4). The interaction between zone and longitude for CR may be characterized by a quadratic equation (Fig. 4a; x = longitude; β_x = 10.51, SE = 1.669, t = 6.3, $P < 0.001$; β_{x^2} = −0.059, SE = 0.010, t = −6.2, $P < 0.001$). As longitude changed, most of the change in CR occurred in the allopatric zone, whereas CR changed relatively little in the sympatric zone.

All three spatial variables significantly interacted with PR (Table 3). Like CR, PR changed rapidly with latitude (Fig. 4c; x = latitude; β_x = 29.13, SE = 6.261, t = 4.7, $P < 0.001$; β_{x^2} = −0.394, SE = 0.089, t = −4.4, $P < 0.001$) and longitude (Fig. 4b; x = longitude; β_x = 64.60, SE = 13.981, t = 4.6, $P < 0.001$; β_{x^2} = −0.366,

SE = 0.080, t = −4.6, $P < 0.001$) from remote allopatry to the near-allopatric zone; PR changed little with latitude and longitude in the sympatric zone. The relationship between elevation and PR was unclear. Thus, the distributions of CR and PR may be explained by spatial variables, especially in the allopatric zone.

Habitat

In all allopatric localities, *G. fultoni* was associated with forest habitats (Table 1). That is, they occurred in the leaf litter throughout the forest. In contrast, sympatric populations of *G. fultoni* were found in grasses at the forest edge. The apparent shift from forest habitat to grassy habitat was consistent with predictions of ecological character displacement. Although *G. fultoni* was associated with the grassy habitat, forest dwelling *G. vernalis* was still located within a few metres of *G. fultoni* in all sympatric localities. In fact, we often found males of one species and females of the other species within a few metres in most sympatric localities. Thus, different habitat use did not physically separate some individuals of these two species in sympatric localities.

Analyses of laboratory recordings

In contrast to the field data, there were no significant interactions between temperature and zone ($P \leq 0.156$) for all calling song characters because of the narrow temperature range, 2 °C, that we used for recording. There were also no interactions between age and zone ($P \leq 0.188$) for any calling song character. Temperature significantly affected CR, PR, and CF, and age significantly affected only PR (Table 4). Individuals differed significantly in all calling song characters (Table 4). This substantial variation among individual crickets is a prerequisite for sexual selection on male calling song characters.

Table 3 Results of ANCOVA examining the relationship between *Gryllus fultoni* calling song characters and spatial variables such as latitude, longitude and elevation.

	Chip rate	Pulse rate	Pulse duration	Carrier frequency
Model	$F_{9,181}$ = 57.1, $P < $ **0.001**	65.7, <**0.001**	5.9, <**0.001**	8.6, <**0.001**
Temperature	$F_{1,181}$ = 186.8, $P < $ **0.001**	363.2, <**0.001**	25.4, <**0.001**	38.5, <**0.001**
Latitude	$F_{1,181}$ = 0.7, P = 0.400	0.0, 0.875	0.5, 0.483	0.2, 0.651
Longitude	$F_{1,181}$ = 43.1, $P < $ **0.001**	15.9, <**0.001**	0.0, 0.916	0.4, 0.535
Elevation	$F_{1,181}$ = 0.0, P = 0.935	0.1, 0.809	0.0, 0.968	4.0, **0.046**
Zone	$F_{1,181}$ = 36.1, $P < $ **0.001**	30.2, <**0.001**	2.3, 0.130	3.3, 0.072
Zone × temperature	$F_{1,181}$ = 4.4, P = **0.037**	10.6, **0.001**	2.5, 0.113	0.1, 0.711
Zone × latitude	$F_{1,181}$ = 1.0, P = 0.318	13.4, <**0.001**	7.8, **0.006**	0.0, 0.966
Zone × longitude	$F_{1,181}$ = 22.1, $P < $ **0.001**	5.9, **0.016**	0.6, 0.448	2.9, 0.092
Zone × elevation	$F_{1,181}$ = 0.3, P = 0.569	5.6, **0.019**	2.5, 0.112	2.3, 0.127
R^2	0.740	0.766	0.227	0.299

The response variables were chirp rate, pulse rate, pulse duration and carrier frequency. The predictor variable was zone, and the covariates were temperature, latitude, longitude and elevation. Calling songs of both *G. fultoni* and *G. vernalis* were recorded in the field, and see Table 1 and Fig. 1 for collecting localities (n = 191). Values in boldface are *P* values < 0.05.
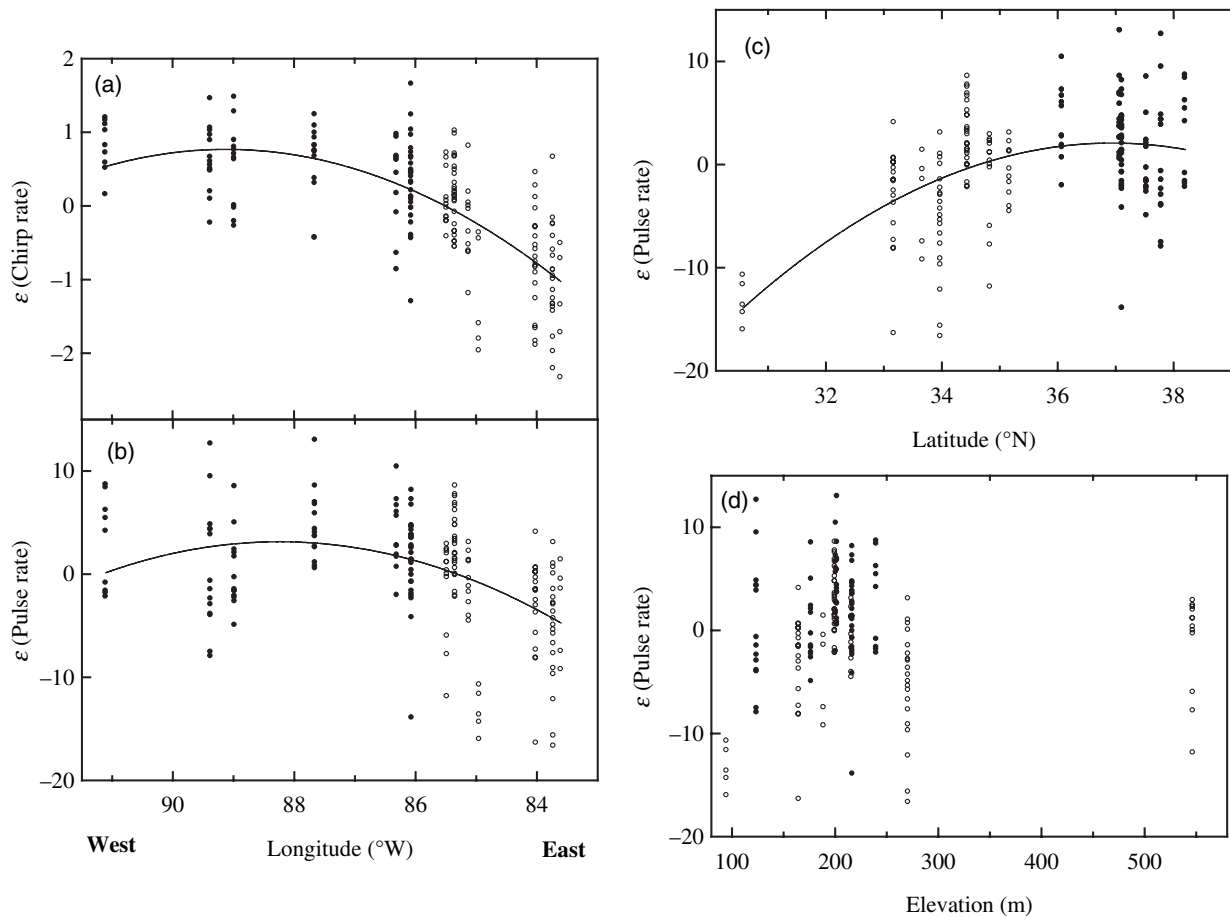
Fig. 4 Spatial variations among *Gryllus fultoni* populations in chirp rate (a) and pulse rate (b, c, d) for the field study. We included only chirp rate and pulse rate for graphical analyses, because these two characters had a potential for being used as a reproductive isolating mechanism between *G. fultoni* and *G. vernalis* in sympatry. Calling song characters were regressed against recorded temperatures, and then unstandardized residuals (ε) of calling song characters were plotted against spatial variables. Solid circles represent the sympatric populations ($n = 92$), and open circles denote the allopatric populations ($n = 99$). Curves are obtained from quadratic regression analyses (see text).

We estimated the effect of locality on calling songs by dividing the mean square of locality by the combined mean square values of individual and error. Locality had a significant effect for CR ($F_{7,209} = 5.294$, $P < 0.001$), PR ($F_{7,209} = 3.396$, $P < 0.01$) and CF ($F_{7,209} = 4.556$, $P < 0.001$), but not for PD ($F_{7,209} = 0.145$, $P = 0.855$). Likewise, we estimated the effect of zone by dividing the mean square of zone by the combined mean square values of locality, individual and error. Zone had a significant effect for CR ($F_{1,7} = 8.244$, $P < 0.05$) but not for PR ($F_{1,7} = 1.085$, $P = 0.332$), PD ($F_{1,7} = 0.869$, $P = 0.382$), or CF ($F_{1,7} = 1.501$, $P = 0.259$).

In general, the patterns of calling song characters between allopatric and sympatric populations were very similar in both field and laboratory data. Furthermore, the divergence in CR observed in this study was consistent with the prediction of character displacement. That

is, CR was higher in sympatric populations of *G. fultoni* than in allopatric populations of *G. fultoni* in relation to *G. vernalis* CRs. Unlike the field data, no significant divergence was detected in PR between allopatric and sympatric populations reared in the laboratory. Because of the common rearing environment and statistical control of environmental covariates, the difference in CR between allopatric and sympatric populations of *G. fultoni* populations was unlikely to have been caused by environmental or chance effects.

Far allopatric vs. near allopatric vs. sympatric populations

The basic ANCOVA model was run with three zones: far allopatric, near allopatric and sympatric. In the field data, CR ($F_{2,10} = 18.498$, $P < 0.001$) and CF ($F_{2,10} = 7.453$,

Table 4 Nested analyses of covariance on calling song characters for the laboratory study ($n = 326$). The ANCOVA model includes two covariates, age and temperature, and three independent variables, zone, locality and individual. Individual was nested within locality, which in turn nested within zone. Some individuals were recorded twice to understand the within-individual variations.

Source	d.f.	MS	F	P
Chirp rate				
Age	1	0.37	2.04	0.156
Temperature	1	2.18	11.89	0.001
Zone	1	27.71	151.24	<0.001
Locality	7	2.83	15.43	<0.001
Individual	209	0.35	1.92	<0.001
Error	106	0.18		
Pulse rate				
Age	1	45.80	5.97	0.016
Temperature	1	108.59	14.16	<0.001
Zone	1	117.42	15.32	<0.001
Locality	7	83.64	10.91	<0.001
Individual	209	16.96	2.21	<0.001
Error	106	7.67		
Pulse duration				
Age	1	3.00	1.28	0.261
Temperature	1	0.099	0.04	0.838
Zone	1	10.97	4.67	0.033
Locality	7	4.05	1.72	0.112
Individual	209	6.24	2.65	<0.001
Error	106	2.35		
Carrier frequency				
Age	1	35 752	3.22	0.76
Temperature	1	79 774	7.18	0.009
Zone	1	956 998	86.12	<0.001
Locality	7	520 851	46.87	<0.001
Individual	209	103 218	9.29	<0.001
Error	106	11 112		

$P = 0.010$) differed significantly among all three zones. PD ($F_{2,10} = 0.001$, $P = 0.999$) was not significant, and PR was not analysed due to the significant interaction between temperature and zone. In the laboratory data, CR ($F_{2,6} = 26.842$, $P < 0.001$) still differed significantly among all three zones. Zone was not significant for PR ($F_{2,6} = 4.109$, $P = 0.075$), PD ($F_{2,6} = 0.440$, $P = 0.663$) and CF ($F_{2,6} = 0.925$, $P = 0.447$). However, pairwise comparisons showed that the significant differences in PR were between far allopatric and near allopatric populations and between far allopatric and sympatric populations (Tables 5 and 6). That is, PRs of the near allopatric populations were closer to those of the sympatric populations than to those of the far allopatric populations. In addition, carrier frequencies were significantly different among all three zones. Therefore, the data suggest the recognition of three distinct zones based on differences in calling song characters.

Discussion

Results of both field and laboratory analyses showed that sympatric populations of *G. fultoni* had significantly higher CRs than did the allopatric populations of *G. fultoni* (Fig. 3a and Table 5). Sympatric and allopatric populations of *G. fultoni* also produced calling songs with different PRs, but this difference was temperature-dependent (Fig. 3b). In the laboratory study, PR of far allopatric populations significantly differed from those of sympatric and near allopatric populations in *G. fultoni*. Because of these differences, the distributions of CR and PR usually did not overlap between sympatric populations of these two species. Such divergence in CR and PR may provide a basis for premating isolation between *G. fultoni* and *G. vernalis* in sympatry. Furthermore, the divergence in CR and PR was unlikely to have been caused by environmental differences between far allopatric and sympatric *G. fultoni* populations, because crickets in the laboratory study were raised from eggs to adults under the same standard conditions. Rather, the divergence in calling songs almost certainly has a genetic basis.

Locality	n_1	n_2	Chirp rate (s^{-1})	Pulse rate (s^{-1})	Pulse duration (ms)	Carrier frequency (Hz)
Far allopatric						
JK	44	32	2.954 ± 0.075	40.661 ± 0.487	11.184 ± 0.270	4072.212 ± 18.551
RL	52	29	3.072 ± 0.068	44.237 ± 0.437	11.144 ± 0.242	4319.290 ± 16.647
Near allopatric						
RF	76	43	3.739 ± 0.063	46.273 ± 0.407	11.432 ± 0.225	4124.838 ± 15.483
HR	11	10	3.555 ± 0.138	44.776 ± 0.890	11.457 ± 0.493	3997.427 ± 33.900
Sympatric						
LB	19	13	4.384 ± 0.115	45.799 ± 0.743	11.686 ± 0.412	4485.116 ± 28.302
DS	60	44	4.030 ± 0.077	44.529 ± 0.497	10.960 ± 0.275	4263.517 ± 18.908
PC	18	14	4.005 ± 0.121	46.189 ± 0.786	10.685 ± 0.435	4214.152 ± 29.921
GR	24	17	4.016 ± 0.094	46.193 ± 0.606	10.063 ± 0.335	4035.557 ± 23.065
SL	22	16	3.816 ± 0.111	44.656 ± 0.721	10.853 ± 0.399	4327.507 ± 27.452

Table 5 Estimated marginal means (±SE) of calling song characters for the laboratory study.

The means of calling song characters were evaluated at mean values of covariates (age = 19.13 days, temperature = 22.88 °C) in ANCOVA. See Methods for more information about the ANCOVA model and for the abbreviations of localities. n_1 indicates the total number of recordings for each locality, whereas n_2 denotes the number of unique individuals for recording.

Table 6 Pairwise comparisons of calling song characters for the laboratory study ($n = 326$). *Gryllus fultoni* populations were grouped into three zones: far allopatric, near allopatric and sympatric zones. The population means of calling song characters were estimated based on ANCOVA (see Tables 4 and 5). Each cell contains the mean difference of two groups (first row – second row) ± standard error and its significance level.

	Far allopatric vs. near allopatric	Near allopatric vs. sympatric	Far allopatric vs. sympatric
Chirp rate	−0.694 ± 0.072, <0.001	−0.331 ± 0.074, <0.001	−1.026 ± 0.062, <0.001
Pulse rate	−3.630 ± 0.464, <0.001	0.788 ± 0.477, 0.101	−2.842 ± 0.399, <0.001
Pulse duration	−0.272 ± 0.257, 0.292	0.586 ± 0.264, 0.029	0.314 ± 0.221, 0.158
Carrier frequency	89 ± 17.7, <0.001	−156 ± 18.1, <0.001	−67 ± 15.2, <0.001

The possibility that the observed divergence in calling song characters between allopatric and sympatric populations which occurred due to sampling error is very unlikely in *G. fultoni*. The field recordings of *G. fultoni* calling songs were based on 13 allopatric and sympatric populations; the calling songs of nine of these populations were further subjected to analysis after laboratory rearing. Analyses of calling songs showed that differences in calling song characters between allopatric and sympatric populations were consistent in field and laboratory recordings. The parts of the geographical range of *G. fultoni* that we sampled may be divided into three zones based on calling song characters: far allopatric, near allopatric and sympatric zones. CR and PR were fastest in sympatric populations, lowest in far allopatric populations and intermediate in near allopatric populations (Tables 5 and 6). The existence of intermediate values of calling song characters is probably explained by gene flow between sympatric and allopatric populations. Computer simulations suggest that despite such gene flow, reinforcement is still possible (Servedio & Kirkpatrick, 1997; Servedio, 2000).

We now discuss the possible explanations for how divergence in calling song characters between allopatric and sympatric populations of *G. fultoni* might have arisen under the scenarios of reproductive character displacement, ecological character displacement, developmental effect, or clinal variation.

Reproductive character displacement

Because of the similar calling song structure (Fig. 2), the possibility of heterospecific mating exists if the identifying properties of the calling songs of the two species are not sufficiently different for recognition by females. We have shown that values of CR and PR are statistically distinguishable and hardly overlap in sympatric areas, whereas the values of allopatric populations of *G. fultoni* overlap broadly with the values typical of *G. vernalis*. Thus, the divergence in CR and PR between allopatric and sympatric populations of *G. fultoni* is consistent with the pattern expected of reproductive character displacement. However, the divergence in calling songs alone does not necessarily result in premating isolation between the two taxa. Playback experiments using both single-stimulus and two-stimuli designs revealed that

females from sympatric and near allopatric populations were significantly more discriminating against stimuli whose parameters are representative of *G. vernalis* calling songs or intermediate between *G. fultoni* and *G. vernalis* calling songs than were females from far allopatric populations (Y. Jang and H.C. Gerhardt, unpublished data). Thus, the divergence in calling songs and discrimination by sympatric females may contribute to effective premating isolation between *G. fultoni* and *G. vernalis* in sympatry.

Reinforcement, as the selective mechanism for reproductive character displacement, relies on selection against hybrids in areas of sympatry, driving the evolution of premating isolation after secondary contact. We failed to find hybrids of *G. fultoni* and *G. vernalis* in the field. Alexander (1968) indicated that *G. fultoni* and *G. vernalis* produced hybrids in some mating attempts. However, we could not produce hybrids between *G. fultoni* and *G. vernalis* in hybridization experiments in a laboratory condition. The hybridization experiments consisted of 17 *G. fultoni* ♂ × *G. vernalis* ♀ pairs and 14 *G. fultoni* ♀ × *G. vernalis* ♂ pairs, and none of these pairs produced instars (Y. Jang, unpublished data). Alexander (1957) also obtained no offspring from 4 *G. fultoni* ♂ × *G. vernalis* ♀ pairs and 4 *G. fultoni* ♀ × *G. vernalis* ♂ pairs, whereas all intraspecific matings produced offspring. Accordingly, more work on hybridization rates and hybrid success is needed to determine whether reinforcement is playing a role in the divergence of CR and PR in *G. fultoni*. If the existence of strong post-mating isolating mechanisms is confirmed, then our results could also be interpreted as an example of reproductive character displacement as defined by Butlin (1989), who uses this term to describe both the geographical pattern and the process if there is little or no gene flow between the interacting taxa.

Ecological character displacement

The apparent shift from forest habitats in the allopatric zone to grassy habitats in the sympatric zone by *G. fultoni* is best explained by ecological character displacement in response to the presence of forest dwelling *G. vernalis*. Habitat separation between closely related species in the sympatric zone lessens competition and should be favoured by natural selection. Furthermore, ecological

selection against hybrids can enhance reproductive isolation through reinforcement (Kirkpatrick, 2001).

The habitat differences between allopatric and sympatric populations of *G. fultoni* were first noted by Alexander (1957). In North Carolina and Virginia, where *G. vernalis* does not occur, *G. fultoni* is found in leaf litter in deciduous forests; whereas, in Ohio, Indiana and Illinois, *G. fultoni* occurs in hillsides, old fields, or prairie openings near forests, and the forest habitat is occupied by *G. vernalis* (Alexander, 1957). He also speculated that calling song difference between allopatric and sympatric populations of *G. fultoni* may be associated with differences in ecology.

How might calling song divergence result from habitat separation? Diverging traits shaped by the process of ecological character displacement are usually related to food consumption. Examples include divergence in feeding morphology in two Plethodontid salamander species (Adams & Rohlf, 2000), beak size differentiation in Darwin's finches *Geospiza* spp. (Schluter *et al.*, 1985) and body size differentiation related to food preference in the threespine sticklebacks, *Gasterosteus aculeatus* (Schluter & McPhail, 1992; Schluter, 1994; Nagel & Schluter, 1998). In *G. fultoni*, feeding morphology or other characteristics including behaviour and physiology may have differentiated under selection for local habitats and may be indirectly related to divergence in CR and PR between areas of allopatry and sympatry.

Clinal variation and developmental effect

Chirp rate and PR varied clinally, especially among allopatric populations of *G. fultoni* (Fig. 4 and Table 3). Clinal variation usually indicates that some environmental factors such as temperature, precipitation, or season length vary with latitude or longitude, and these factors, in turn, influence expression of calling songs in crickets. Recent findings that the expression of both male calling songs and female mate preferences depends on developmental temperature (Olvido & Moussear, 1995; Walker, 2000; Grace & Shaw, 2004) suggest the possibility of clinal effects on calling songs in our studies. Divergence in calling song characters may thus arise during development from different temperature regimes between areas of allopatry and sympatry. In all cases where developmental effects on calling songs were studied rates of calling song characters were faster at high rearing temperatures than at low rearing temperatures (Olvido & Moussear, 1995; Walker, 2000; Grace & Shaw, 2004). If we assume that southern populations of *G. fultoni* would be subjected to warmer temperatures than northern populations during the months when crickets develop, then this explanation does not apply to the results of our study. That is, CR and PR were slower in southern (warmer) populations than in northern (cooler) populations. Of course it is also possible that the developmental effect of temperature on temporal properties of calling songs is the opposite of what has been found so far.

Alternatively, habitat segregation between *G. fultoni* and *G. vernalis* in sympatry may cause divergence in *G. fultoni* calling songs via developmental effects. Grassy habitats at the edge of forests may be warmer than inside forests. Thus, sympatric populations of *G. fultoni* may experience higher temperatures throughout development than allopatric populations of *G. fultoni*, resulting in the production of higher CR and PR in sympatric localities than in allopatric localities.

Although the field data of calling song characters suggest that divergence may be clinal in nature, our laboratory data also indicate that divergence in calling songs of *G. fultoni* was not caused by environmental differences between allopatric and sympatric localities. Heritable variation in isolating characters is a necessary condition for both reproductive and ecological character displacement, whereas it is not required in the clinal variation hypothesis. Our analyses of calling songs in lab-reared *G. fultoni* suggest that the divergence in calling songs could have evolved under an evolutionary process such as reproductive or ecological character displacement.

The best way to test the clinal variation scenario is to study east–west or north–south transects in which both allopatric and sympatric populations of *G. fultoni* populations are sampled within narrow zones of latitude or longitude, respectively. Doherty & Callos (1991) studied an allopatric population of *G. fultoni* in Grafton, Virginia (Fig. 1), whose latitude was similar to those of sympatric populations. However, calling songs of the Grafton population more closely resembled those of allopatric populations than those of sympatric populations. Therefore, the Grafton data do not support the clinal variation scenario, but more transect studies are needed to clarify the relationship between clinal factors and calling song characters.

Further studies

Identifying the selective forces across populations that influence calling song characteristics may be difficult, but this approach can provide valuable insights into the recent evolutionary history of these populations. Although our study shows patterns of divergence in CR and PR between allopatric and sympatric populations of *G. fultoni*, identifying the processes that drove this divergence is always problematic. Furthermore, the hypotheses discussed above are not mutually exclusive. Divergent selection may initiate reproductive isolation between populations in different habitats, but at later stages natural selection might be involved during reinforcement of premating isolation (Turelli *et al.*, 2001). When different habitats lie geographically adjacent to each other, habitats and clines are inherently related. Therefore, not only does each hypothesis need to be

critically tested, but alternative hypotheses should be rejected to determine the selective force(s) for divergence in calling song characters in *G. fultoni*.

Acknowledgments

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