



Variation in complex courtship traits across a hybrid zone between grasshopper species of the *Chorthippus albomarginatus* group

VARVARA VEDENINA*

Institute for Information Transmission Problems, Russian Academy of Sciences, Bolshoy Karetny per. 19, Moscow 127994, Russia

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Closely related grasshopper species of the *Chorthippus albomarginatus* group are notable for their extremely complex courtship songs, accompanied by a visual display. Two species of this group, *Ch. albomarginatus* and *Ch. oschei*, were previously shown to hybridize in a wide mosaic hybrid zone in Ukraine and Moldova. In this paper, variation in five courtship song characters, one character of visual display and the number of stridulatory pegs were analysed across the hybrid zone to estimate selection against hybrids and strength of assortative mating. Comparison of cline width and position across the hybrid zone showed concordant and coincident clines in four traits, such as three song characters and one morphological character, and discordant and non-coincident clines in two other song characters and the character of visual display. Concordance of clines in different characters suggests an equal strength of selection acting on underlying loci. Increase of variance and covariance between phenotypic traits at the cline centre could more likely result from assortative mating than from selection against hybrids. Most pairwise cases showed the highest covariance for the *oschei*-like, than for the *albomarginatus*-like hybrid populations. This indicates that introgression of the *oschei* genes into the *albomarginatus* genome is stronger than vice versa, and may be evidence of the movement of the hybrid zone in favour of *Ch. albomarginatus*. Analysis of associations between phenotype and local vegetation showed that mosaic structure of the hybrid zone is explained to a great extent by habitat–phenotype associations. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 275–291.

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INTRODUCTION

Hybrid zones have been documented in a broad range of different organisms and are suggested to be excellent ‘natural laboratories’ in which to study the characters and processes involved in divergence and speciation (Barton & Hewitt, 1985, 1989; Harrison, 1986, 1993). Hybrid zones can take several forms, from large areas of overlap to narrow contact zones. In most frequent cases referring to the tension zone model, the frequencies of the different alleles or

phenotypic traits form smooth gradients, or clines, across the contact zone, maintained by a balance between dispersal and selection against hybrids. Tension zones typically consist of a cluster of parallel clines with coincident centres and concordant widths. Such hybrid zones that have a tendency to be narrow in comparison with dispersal of the organism may effectively maintain isolation between diverged forms (e.g. Barton & Hewitt, 1985; Barton & Gale, 1993).

However, a substantial deviation from the smooth clines may occur in the case of non-random dispersal or survival of genotypes in relation to variation in a local habitat. Hybrid zones with such deviations have been described as mosaic hybrid zones (Harrison &

*E-mail: vedenin@iitp.ru

Rand, 1989). Mosaic hybrid zone models predict the persistence of parental populations throughout the hybrid zone in ecologically divergent patches that are to a great degree relative to the mean dispersal distance of the organism (Harrison, 1990). In such mosaic hybrid zones, the clines for different traits may be of different width. For example, in the hybrid zone between *Bombina* toad species, some quantitative traits show similar cline widths, whereas others are discordant (Nürnberger *et al.*, 1995). The discordance arises because the traits are not inherited additively or because they are the subject of various selection pressures. The authors favoured explanation is the second suggestion. The same conclusion is made for the hybrid zone between the grasshopper species *Chorthippus brunneus* and *Ch. jacobsi*, in which the cline width for the song characters is significantly narrower than for the stridulatory peg number (Bridle & Butlin, 2002). In some mosaic zones, deviations from a smooth cline may be explained by habitat-genotype effects (e.g. Harrison & Rand, 1989; MacCallum *et al.*, 1998), whereas in other cases, habitat variation explains only a small amount of phenotypic variation (Bridle, Baird & Butlin, 2001; Bridle & Butlin, 2002).

The extent to which cline width and cline position vary for different quantitative characters depends not only on the direct selection on the traits, but also on the associations between the traits that represent the underlying associations between sets of loci, or linkage disequilibrium (LD) (Barton & Gale, 1993). Measures of LD from data on different traits should reflect the homogeneity of changes in genome composition across the cline. LD can be calculated from covariances in quantitative traits (Nürnberger *et al.*, 1995). In a hybrid zone characterized by low selection and high recombination rate of loci, a very modest increase in LD and phenotypic variance would be expected close to the centre of the zone as a result of introgression. Similarly, if a trait is under strong selection or assortative mating is strong, the trait will exhibit a bimodal distribution and therefore will have a large variance and LD in the centre of the zone (Bridle & Butlin, 2002; Gay *et al.*, 2008).

In most studies of hybrid zones, the neutral markers are used to measure cline width and LD (Kohlmann & Shaw, 1991; Dod *et al.*, 1993; Hare & Avise, 1996; MacCallum *et al.*, 1998; Marshall & Sites, 2001; Steinmetz, Johannesen & Seitz, 2004; Sequeira *et al.*, 2005; Sotka & Palumbi, 2006; Yanchukov *et al.*, 2006; Yannic, Basset & Hausser, 2008; Kawakami *et al.*, 2009). A comparison of variation in neutral markers with variation in phenotypic traits allows identification of which traits are involved in reproductive isolation and may allow estimation of the levels of selection. Unlinked neutral alleles can

pass easily across the clines (neutral diffusion), unless there is widespread strong selection, which leads to widespread LD and thus indirect selection and a barrier to gene flow at neutral loci (Mallet *et al.*, 1990; Szymura & Barton, 1991; Nürnberger *et al.*, 1995; Butlin, 1998; Rohwer, Bermingham & Wood, 2001; Dasmahapatra *et al.*, 2002; Ross & Harrison, 2002; Alexandrino *et al.*, 2005; Takami & Suzuki, 2005; Grahame, Wilding & Butlin, 2006).

However, there are several difficulties using clines based on phenotypic traits (Nürnberger *et al.*, 1995; Gay *et al.*, 2008). First, non-additive genetic variance for phenotypic traits may cause distortion of phenotypic clines in comparison with the underlying allelic clines. Second, pleiotropy may give rise to phenotypic covariance in hybrid populations, which can be wrongly interpreted as linkage disequilibrium.

In the current study, distribution of complex phenotypic characters across the hybrid zone is analysed between two European grasshopper species *Chorthippus albomarginatus* and *Ch. oschei*. These species form a mosaic hybrid zone approximately 200 km wide on the territory of Ukraine and Moldova (Vedenina & von Helversen, 2003). The two species differ extremely in their complex courtship songs and, in addition, *Ch. oschei* demonstrates a characteristic visual display during courtship (von Helversen, 1986; Vedenina & von Helversen, 2009). Despite the song parameters having been relatively roughly scored by Vedenina & von Helversen (2003), it was found that intermediate phenotypes were in the minority in all hybrid localities studied, whereas males singing the parental songs dominated. A genetic analysis of the courtship song differences between *Ch. albomarginatus* and *Ch. oschei* showed a simple polygenic inheritance for two song characters, but an unusual type of inheritance for most song characters studied (Vedenina, Panyutin & von Helversen, 2007b). This type of inheritance was suggested to involve duplicate pairs of loci and elaborate interactions between non-allelic loci. In contrast, inheritance of the stridulatory peg number differences was consistent with a simple polygenic additive model.

The purpose of the current work was to study selection against hybrids in the *albomarginatus/oschei* hybrid zone, and investigate which traits are under stronger selection. First, the cline width and position for seven phenotypic characters across the *albomarginatus/oschei* hybrid zone are analysed. Among these characters, there are five courtship song traits, one character of courtship visual display and one morphological character, the stridulatory peg number. To estimate the cline distortion, the distribution of phenotypes within hybrid and allopatric sites is compared with distribution of the F₁ hybrid phenotypes. Second, the change in variance and cova-

riance between the traits across the hybrid zone is analysed. Covariance between traits is calculated separately for the allopatric sites and hybrid sites of the two types, *albomarginatus*- and *oschei*-like. This shows whether covariance is attributable to pleiotropy or linkage disequilibrium. Comparison of covariance in hybrid sites of different types also allows us to suggest the dynamics of the hybrid zone. Finally, associations between phenotype and local vegetation are analysed for 16 sites to check whether a mosaic structure of the hybrid zone is because of the habitat heterogeneity or any other reasons.

MATERIAL AND METHODS

COLLECTING AND SONG RECORDING

The specimens analysed in the current paper were collected in various localities of the contact zone in Ukraine and Moldova during the summer seasons of 1997–2007. In addition, the specimens from allopatric populations in Germany, Russia, Bulgaria and Hungary were analysed as well (Fig. 1, Table 1). Of 35 sampling localities, 17 sites were reported upon earlier (Vedenina & von Helversen, 2003).

To record the courtship song, a male was placed nearby a female. Courtship song recordings were made from 376 males. Most of the song recordings (from 253 males) were made in the laboratories of the Zoological Institute in Erlangen and the Institute for Information Transmission Problems in Moscow. During stridulation of the males studied in laboratory, both the sound and the movements of the hind legs were recorded with a custom-built opto-electronic device (details in Vedenina & von Helversen, 2003; Vedenina *et al.*, 2007b). The sampling rate was 2 kHz for recording the stridulatory movements and 100 kHz for the sound recordings. The ambient temperature near a singing male was 30–32 °C. The lesser number of the song recordings (from 123 males) was made in the field with cassette recorders; the upper frequency limit was 14 kHz. During the field recordings, the ambient temperature near a singing male varied within a range of 24–42 °C. The recordings were analysed with the TurboLab 4.0 program (Bressner Technology, Germany). Courtship behaviour was also studied using a Sony DCR-TRV 355E digital video camera; the video signals were transferred to a PC for analysis of the visual display.

The numbers of stridulatory pegs on the inner side of the hind femora were counted using a MBS-9 light microscope at 56× magnification. Morphological analysis was conducted in 470 males.

ANALYSIS OF COURTSHIP SONGS

In the present study, a more comprehensive song analysis was conducted than in the previous paper

(Vedenina & von Helversen, 2003), when parameters were roughly scored. In contrast to the previous statistical data, significant differences have been found in some parameters of the songs recorded at the same localities in different years (Mann–Whitney *U*-test: $P < 0.05$ for sites 14, 15, 17 and 33). The data obtained from the different years were combined in some figures; however, they were separately analysed. In regression analysis, the results were slightly different depending on whether these data were put together or not.

As the temperature near a singing male in the field varied within a wide range, only relative temporal parameters were used for the song analysis. Analysis of transitions from a pure *albomarginatus* song to a pure *oschei* song through intermediate variants helped us to reveal homologous song elements (Vedenina & von Helversen, 2003; Vedenina *et al.*, 2007b). For the current song analysis, only those characters that significantly differed in the parental songs were measured: the number of A/B pairs between C elements; relative loudness of A and B elements; relative duration of B, B1 and A1 elements in comparison with the C element (Figs 2, 3). The duration of the C element was shown to differ insignificantly in the parental songs. In addition to temporal song characters, one parameter of visual display accompanying the courtship song was evaluated. The strength of the stroke with the hind tibiae in the beginning of the C element was measured as the maximal angle between the femur and tibia (Figs 2, 3).

The mean value of each song trait for each individual male was determined from 3–5 cycles of the courtship song; one cycle corresponded to a period of the song element C (Fig. 3). All measurements were log-transformed. To further visualize and clarify the song variations between different populations, a principal component analysis (PCA) was applied to all five song parameters studied. All statistical analyses were performed using Statistica version 6 for Windows (StatSoft, Tulsa, OK, USA).

CLINE ANALYSIS

To illustrate a degree of similarity of clines in different characters, the clines were fitted with a Loess smoothing function and superimposed. To compare width and placement of different clines, the regression methodology suggested by Barton & Hewitt (1985) and Kruuk (1997) was used. If the clines in two characters have identical widths and central placements, regressing one character on another will give a straight line; if a cline in one character is displaced in space relative to another, then the quadratic term will be significant; if a cline in one character is narrower or wider than in another, the cubic term will

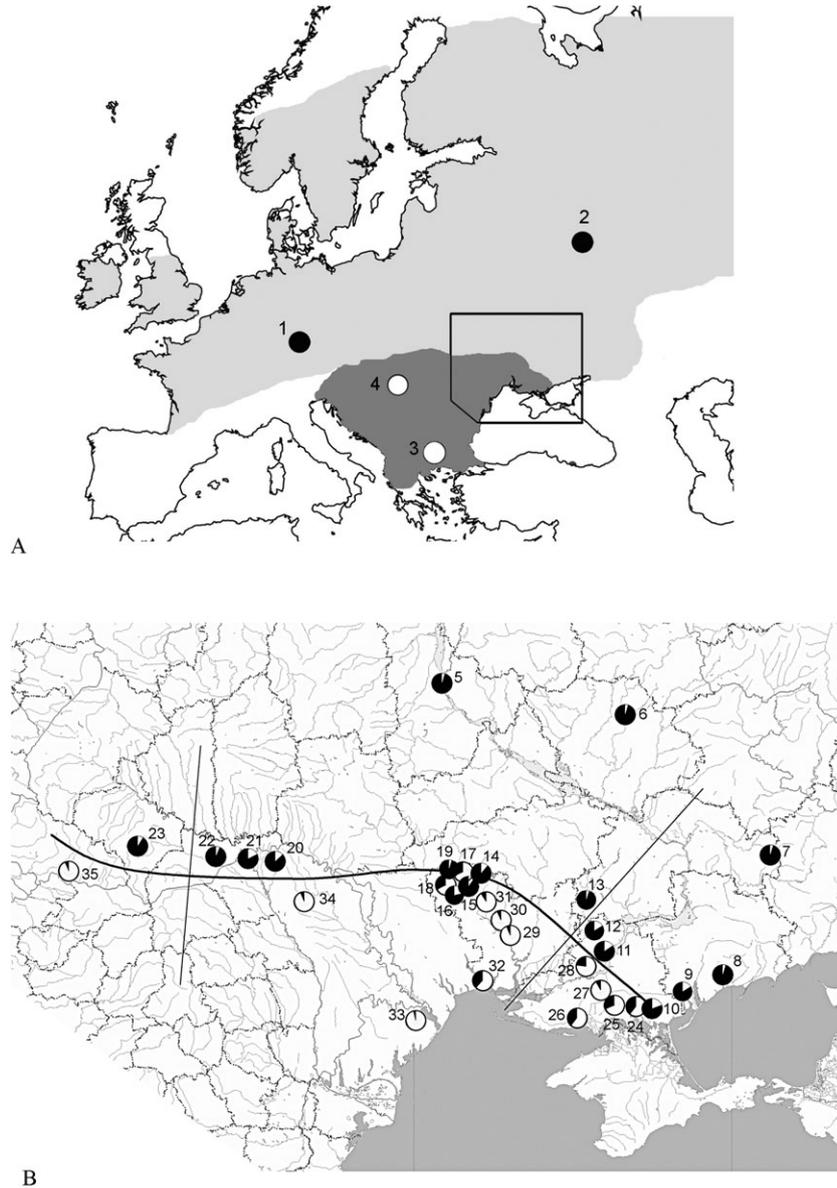


Figure 1. Maps showing: (A) the distribution of *Chorthippus albomarginatus* and *Ch. oschei* in Europe with sampling localities of pure species [*albomarginatus* (black circles), *oschei* (white circles)] and contact zone (square); (B) the hybrid sites on the territory of Ukraine and Moldova, where pie charts represent mean song phenotypes based on PC1 [*albomarginatus*-song phenotypes (filled), *oschei*-song phenotypes (open)]; heavy line indicates approximate centre of the hybrid zone; thin lines are transects used to plot character transitions (clines) shown in Figures 6 and 7.

be significant. This indirect approach allows avoiding problems associated with any spatial analysis; for example, when one should measure the geographic distances across a mosaic hybrid zone. To reduce the number of pairwise analyses entrained by seven characters, each character was regressed on the peg number. These regression analyses show if individual clines are displaced in space or differ in width from the cline in the peg number.

ESTIMATING VARIANCE AND COVARIANCE BETWEEN DIFFERENT CHARACTERS

Phenotypic variance is expected to highly increase in the centre of a hybrid zone when a trait exhibits a bimodal distribution. To compare changes in variances across the *albomarginatus/oschei* hybrid zone, only the localities with a sampling number more than five were used. Measures of LD may solve the question of how strong is the selection against hybrids and

Table 1. Sampling localities in the hybrid zone between *Chorthippus albomarginatus* and *Ch. oschei* shown in Figure 1

No.	Locality	Geographical coordinates	Number of males analysed acoustically	Number of males studied morphologically
1	Germany: Bavaria, 13 km N of Effeltrich	49°40'N, 11°15'E	20	20
2	Russia: S of Moscow	55°37'N, 37°32'E	16	22
3	Bulgaria: 50 km SW of Plovdiv, Lake Batak	41°57'N, 24°13'E	11	11
4	Hungary: province Békés, 5km NW of Battonya	46°19'N, 20°58'E	17	28
5	Ukraine: Kiev	50°25'N, 30°30'E	5	11
6	U: Poltava region, 24 km NE of Mirgorod	50°01'N, 33°57'E	12	21
7	U: Donetsk region, 25 km W of Krasnoarmeisk	48°21'N, 36°50'E	9	12
8	U: Zaporozh'je region, 40 km E of Melitopol	46°43'N, 35°50'E	12	20
9	U: Zaporozh'je region, 40 km SW of Melitopol	46°35'N, 35°08'E	6	10
10	U: Kherson region, 18 km E of Novotroitzkoe	46°23'N, 34°34'E	4	11
11	U: Kherson region, 22 km NE of Berislav	47°01'N, 33°36'E	2	2
12	U: Kherson region, 10 km NE of Velikaya Alexandrovka	47°23'N, 33°23'E	6	12
13	U: Dnepropetrovsk region, 15 km SW of Krivoy Rog	47°46'N, 33°15'E	7	9
14	U: border between Nikolaev and Kirovograd regions, 35 km NE of Pervomaisk	48°07'V, 31°18'E	26	29
15	U: Nikolaev region, 6 km E of Pervomaisk, Migija, meadow no. 1	48°01'49"N, 30°56' 31"E	32	35
16	U: Nikolaev region, 6 km E of Pervomaisk, Migija, meadow no. 3	48°01'53"N, 30°56' 02"E	10	10
17	U: Nikolaev region, 6 km E of Pervomaisk, Migija, meadow no. 2	48°01'47"N, 30°55' 33"E	30	26
18	U: Nikolaev region, Pervomaisk	48°03'N, 30°53'E	9	9
19	U: Nikolaev region, 11 km N of Pervomaisk	48°08'N, 30°48'E	7	8
20	Moldova: Edinetz region, Halahora de Sus	48°15'N, 27°11'E	2	2
21	Moldova: surroundings of Lipkani	48°15'N, 26°49'E	9	9
22	U: 10 km S of Chernovtsy	48°16'N, 26°06'E	3	9
23	U: Ivano–Frankovsk region, Mikulichin	48°25'N, 24°37'E	2	5
24	U: Kherson region, 7 km NW of Novotroitzkoe	46°23'N, 34°17'E	5	5
25	U: Kherson region, 15 km NE of Chaplinka	46°25'N, 33°43'E	7	8
26	U: Kherson region, 15 km E of Skadovsk	46°08'N, 33°06'E	12	17
27	U: Kherson region, 15 km E of Novaja Majachka	46°33'N, 33°29'E	10	10
28	U: Kherson region, 7 km NE of Berislav	46°52'N, 33°26'E	11	10
29	U: Nikolaev region, 13 km NW of Novaya Odessa	47°23'N, 31°36'E	14	17
30	U: Nikolaev region, 15 km SE of Voznesensk	47°28'N, 31°29'E	10	12
31	U: Nikolaev region, 9 km NW of Voznesensk	47°35'N, 31°11'E	9	9
32	U: Nikolaev region, beach of Tiligul'sky liman	46°40'N, 31°11'E	10	12
33	U: Odessa region, 20 km N of Belgorod-Dnestrovsky	46°17'N, 30°08'E	16	20
34	Moldova: Beltz	47°46'N, 27°54'E	9	12
35	U: Zakarpat'je region, 4 km NW of Tjachev	48°02'N, 23°31'E	6	7

whether the introgression occurs close to the centre of the hybrid zone. LD can be estimated from covariance between two quantitative traits, assuming that populations do not differ significantly from the Hardy–Weinberg equilibrium (Nürnberg *et al.*, 1995). However, as an unusual inheritance was docu-

mented for most song characters in *Ch. albomarginatus* and *Ch. oschei*, which assumed elaborate interactions between non-allelic loci (Vedenina *et al.*, 2007b), calculation of LD for these traits may yield the distorted results. At the same time, covariance between phenotypic traits may result either from LD

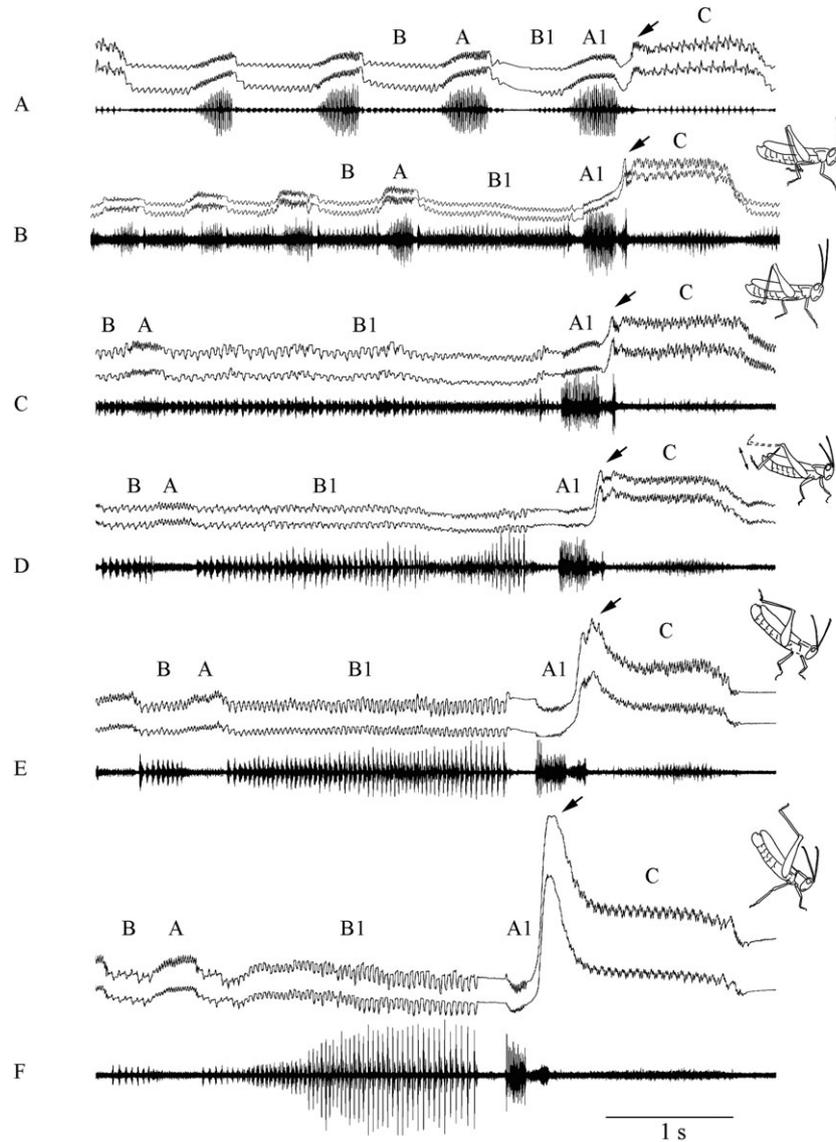


Figure 2. Transition from the courtship song of pure *Chorthippus albomarginatus* (A) to the song of pure *Ch. oschei* (F), through intermediate variants of the natural hybrids (B–E). A, B, B1, A1 and C are different song elements. In each case, two upper traces are leg-movement recordings, the low trace is the sound recording. The highest positions of the hind legs and abdomen are shown on the drawings on the right-hand side; the moments when they occur are marked with arrows.

or pleiotropy. This assumption is checked by calculation covariance between the traits in allopatric and hybrid populations. In this case, it is important that variance in allopatric populations is not equal to zero, which means that relevant loci with pleiotropic effects are polymorphic in these populations.

VEGETATION ANALYSIS

To examine if habitat–phenotype associations are responsible for the mosaic structure of the

albomarginatus/oschei hybrid zone, the correlation between phenotypic traits and vegetation data was studied in 16 localities. The vegetation data implied the number of plant species and the degree of xerophily. The latter value was calculated after identifying and classifying each plant species as more common for wet, medium or dry habitat. Abundance of each plant species was estimated in ranks from 1 to 3. Rank 1 indicated rare occurrence, rank 2 indicated common occurrence and rank 3 indicated dominance in locality.

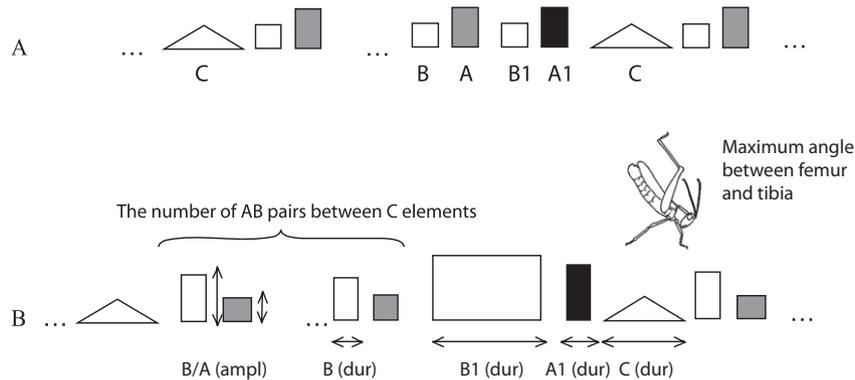


Figure 3. Schemes of the courtship songs of *Chorthippus albomarginatus* (A) and *Ch. oschei* (B). A, B, B1, A1 and C are different song elements. Five temporal parameters [the number of A/B pairs, B/A amplitude ratio (ampl), relative duration (dur) of B, B1, and A1 elements in comparison with C element] and one parameter of visual display (maximal angle between femur and tibia) used for the song analysis are shown in (B).

RESULTS

PRINCIPAL COMPONENT ANALYSIS OF THE SONG PARAMETERS

To visualize and clarify the song differences between different hybrid populations, a PCA was applied to all five song parameters studied. Not only the natural populations, but also the F_1 hybrids obtained from the crosses between the specimens from the German and Bulgarian populations and studied earlier (Vedenina *et al.*, 2007b), were included in PCA. The contribution of most characters to the first principal component (PC1) was larger than to the second principal component (PC2) (Fig. 4). However, most of the characters also contributed sufficiently to PC2, and one song parameter, B1 duration, contributed most of all to PC2. Taking into account the unusual inheritance pattern found for some characters (Vedenina *et al.*, 2007b), PC1 reflects the degree of the hybrid intermediacy, whereas PC2 reflects the hybrid novelty. The PC1 scores of some F_1 hybrids were intermediate between the scores of *Ch. albomarginatus* and *Ch. oschei* from allopatric populations, whereas the scores of most F_1 hybrids overlapped with the *Ch. albomarginatus* scores. In contrast, the PC2 scores of F_1 hybrids were different from the scores of both allopatric species, thus demonstrating the new hybrid features. Figure 4 shows that natural hybrids split into two groups: *albomarginatus*-like and *oschei*-like groups. To a large degree, natural hybrids overlapped with allopatric specimens, but some natural hybrids fell into the cluster of the F_1 hybrids. In general, intermediate phenotypes from hybrid populations were in the minority.

SONG-PEG VARIATION AMONG SITES

Plots of the song PC1 and the peg number variation at ten hybrid and two allopatric sites demon-

strate the distribution pattern of the phenotypes (Fig. 5). From these plots, it is evident that either an *albomarginatus*-like or *oschei*-like phenotype dominated at every site. Phenotype distribution was typically unimodal; however, there were sites where one parental type dominated and another type occurred in an extreme minority (for example, at sites 14, 15 and 18). Therefore, hybrid localities will be further subdivided into two types: *albomarginatus*-like and *oschei*-like sites. The sites situated close to each other (approximately 1 km apart) could be characterized by different phenotype distribution, as at sites 15 and 17 (Table 1, Fig. 5). At some localities, samples from different years differed significantly, as at sites 14 and 17 (Fig. 5). Comparison of the samples from different years reveals the shifts to the parental phenotypes at PC1: at site 14, there was the shift to the *albomarginatus* phenotype ($N = 6$ from 1997 and $N = 12$ from 1999, Mann-Whitney U -test: $U = 4$, $P = 0.003$), whereas at site 17, there was the shift to the *oschei* phenotype ($N = 11$ from 1998 and $N = 10$ from 2005, $U = 9$, $P = 0.001$).

COMPARISON OF CLINES IN DIFFERENT CHARACTERS

Transects used for the cline analysis were drawn to pass through the hybrid zone perpendicular to the line of an approximate centre of the zone (Fig. 1). This line was drawn between the most closely situated sites with different, *albomarginatus*-like and *oschei*-like, phenotypes (sites 15 and 16, 11 and 28, 10 and 24). Both transects were put together to plot the clines; allopatric localities were also included in the cline analysis.

In Figure 6, the clines for various characters look different. Three characters, such as B/A amplitude, duration of B1 element and femur/tibia angle, have more stepped clines than other song characters and the peg number. The clines for most characters have

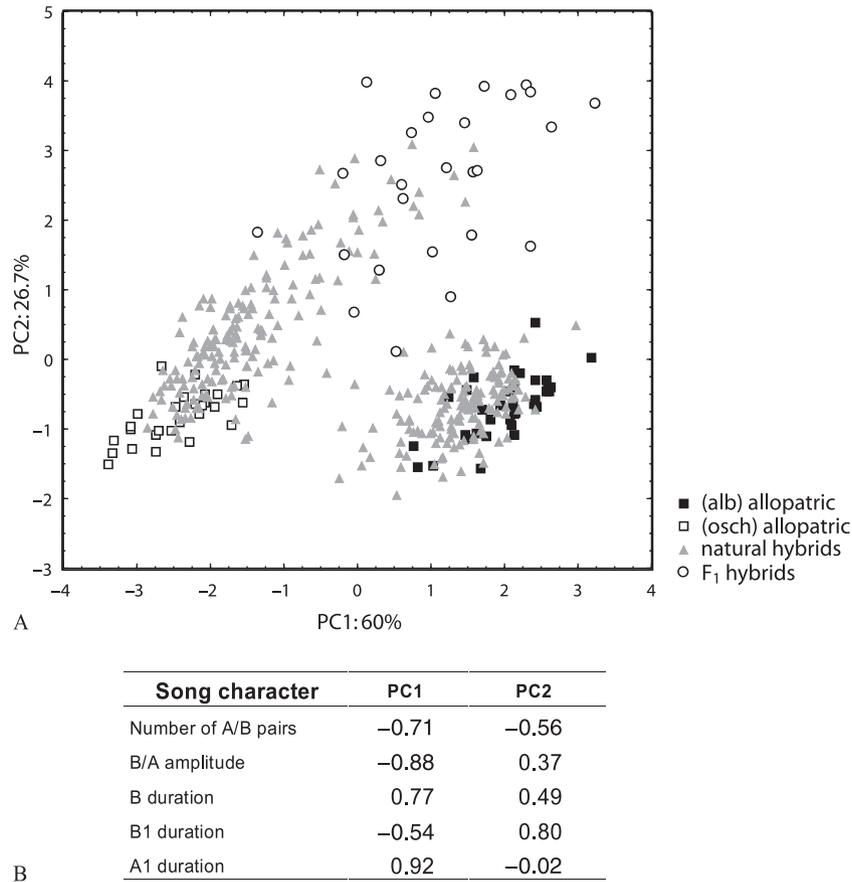


Figure 4. Results of a principal component analysis (PCA) based on five song characters: (A) PC1 and PC2 scores of allopatric populations of *Chorthippus albomarginatus* (alb) and *Ch. oschei* (osch), F₁ hybrids and natural hybrids from the contact zone; (B) loadings of different characters into PC1 and PC2.

a more or less sigmoid shape, whereas the cline in the B1 duration is shaped unusually, with one tail falling away non-exponentially.

To characterize central placement and width of the clines, the transect-independent regression method was used (Barton & Hewitt, 1985; Kruuk, 1997). Regressions for the five song characters and for the visual display character against the peg number are given in Table 2. For the three characters (number of AB pairs, duration of B and A1 element), neither quadratic nor cubic terms were significant, which indicates concordant and coincident clines with the cline in the peg number. Three other characters (B/A amplitude, duration of B1 element and femur/tibia angle) had the significant quadratic and cubic terms. However, if the significantly different data obtained from various years in localities 14, 15, 17 and 33 were separately analysed, one character, B/A amplitude, showed different results: an insignificant quadratic term and a hardly significant cubic term (Table 2). This implies that the cline difference in centre and width was highly pronounced in only two characters:

duration of B1 amplitude and femur/tibia angle. As the comparison of the clines presented in Figure 6 shows, these clines were narrower than the cline in the peg number and were displaced to the *albomarginatus*-side of the hybrid zone.

COMPARISON OF THE NATURAL AND F₁ HYBRIDS

The inheritance pattern of the individual characters should be taken into account for interpretation of the cline behaviour. For some characters, such as B/A amplitude, duration of A1 element and the peg number, a usual polygenic inheritance was previously found (Vedenina *et al.*, 2007b). The values of these characters in F₁ hybrids were nearly intermediate between the corresponding values of the parental species (Fig. 7A, B). Thus, the midpoint phenotypes found in the hybrid sites could be interpreted as F₁ hybrids, whereas parental-like phenotypes could be suggested as backcrosses. For other song characters, such as the number of A/B elements and duration of B and B1 elements, the unusual inheritance involving

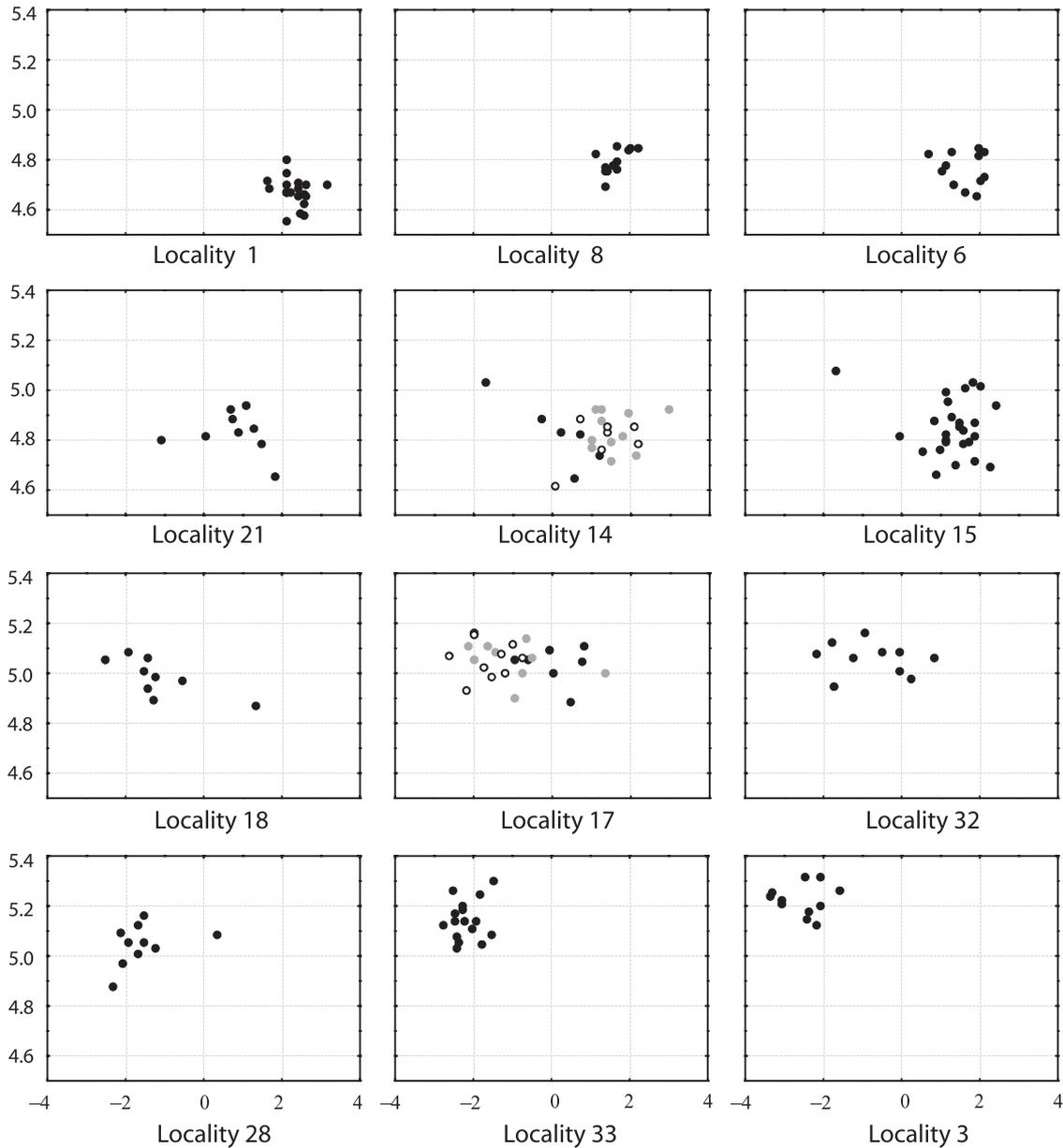


Figure 5. First principal component (PC1) of the song principal component analysis (PCA) (x -axis) and the peg number (y -axis) for the allopatric site of *Ch. albomarginatus* (locality 1), the allopatric site of *Ch. oschei* (locality 3) and ten hybrid localities (see Fig. 1 and Table 1). For localities 14 and 17, samples from different years are indicated: for locality 14, samples from 1997 (black circles); from 1999 (grey circles); from 2007 (white circles); for locality 17, samples from 1998 (black circles); from 2000 (grey circles); from 2005 (white circles).

duplicate pairs of loci was found. These characters had the extreme values in F_1 hybrids, i.e. the mean value of the hybrid trait was significantly larger than that in the parent with the largest value, or significantly smaller than that in the parent with the smallest value (Vedenina *et al.*, 2007b). Therefore, in the hybrid zone, not only the midpoint phenotypes, but also parental-like phenotypes and those with the extreme values of the characters, could appear the F_1 hybrids (Fig. 7C, D). For the character of

visual display, i.e. the angle between femur and tibia, the Mendelian inheritance with full dominance of the *albomarginatus*-parent was previously found (Vedenina *et al.*, 2007b). In the hybrid zone, F_1 hybrids could be undistinguished by this character from *Ch. albomarginatus* or backcrosses to *Ch. albomarginatus*; at the same time, the midpoint phenotypes were definitely not F_1 hybrids, but either F_2 hybrids or, most likely, backcrosses to *Ch. oschei* (Fig. 7E).

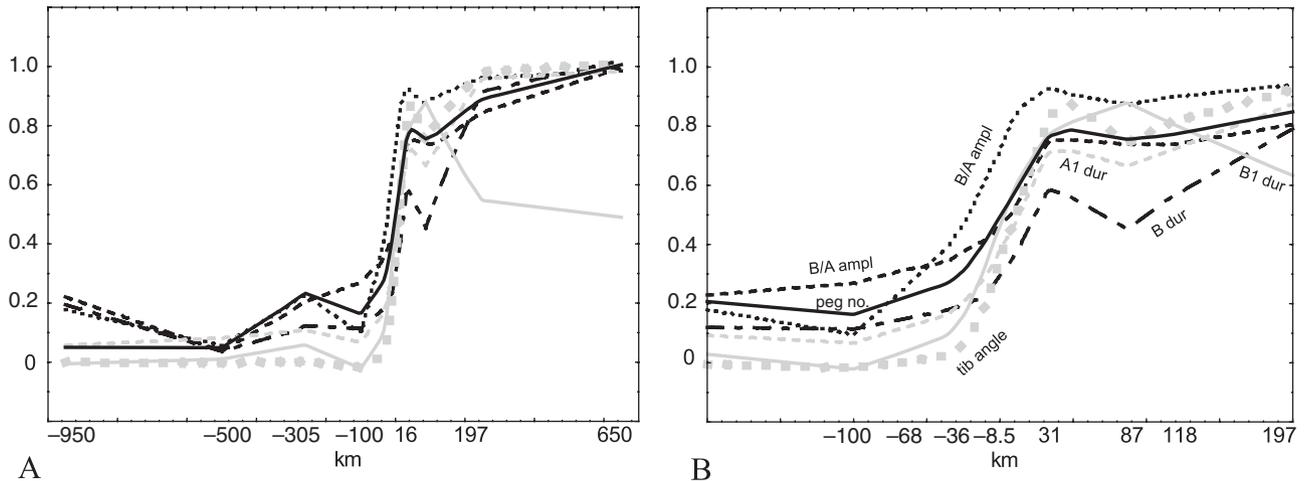


Figure 6. Clines for five song characters, for the femur/tibia angle and for the peg number across the *albomarginatus/oschei* hybrid zone at two different scales (A, B). Each curve is fitted with a Loess smoothing function. The very left point is the allopatric site of *Chorthippus albomarginatus*, the very right point is the allopatric site of *Ch. oschei*. All measurements are log_e-transformed and scaled along the 0–1 axis. tib, tibia angle; ampl, amplitude; dur, duration.

Table 2. Statistics for the regression analysis of all characters studied in the *albomarginatus/oschei* hybrid zone against the peg number

Character	b^2 (P)	b^3 (P)
Number of AB pairs	-2.278 (0.229) -3.132 (0.169)	2.005 (0.108) 2.600 (0.083)
B/A amplitude	3.739 (0.041) 3.167 (0.096)	-2.781 (0.021) -2.469 (0.049)
Duration of B element	-0.377 (0.849) -1.123 (0.613)	1.234 (0.343) 1.780 (0.224)
Duration of B1 element	8.679 (< 0.0001) 8.151 (< 0.0001)	-6.857 (< 0.0001) -6.550 (< 0.0001)
Duration of A1 element	2.640 (0.061) 2.160 (0.140)	-1.349 (0.139) -1.029 (0.276)
Angle between femur and tibia	6.351 (0.0006) 6.329 (0.0007)	-3.965 (0.001) -3.977 (0.001)

Quadratic and cubic terms with P -values are shown.

Bold type indicates significant values.

Second values for each character are calculated when significantly different data obtained from different years in localities 14, 15, 17 and 33 are not put together.

CHANGES IN VARIANCE AND COVARIANCE BETWEEN CHARACTERS ACROSS THE HYBRID ZONE

Comparison of variances across the hybrid zone showed the lower values of variance in the extreme phenotypes than in the intermediate phenotypes for the three song characters (the number of AB pairs, duration of A1 and B elements) and for the peg number (Fig. 8). Two song characters, B/A amplitude and dura-

tion of B1 element, exhibited, however, an increase in variance for the extreme values. For the latter trait, this does not mean the increase in the variance at the cline end, but is the consequence of the unusual inheritance pattern, when hybrids demonstrated extreme character values in comparison with the pure species (Fig. 7C). The changes in variance were comparable with the bell shape for A1 duration and, to a lesser degree, for the number of AB pairs and B duration. For B/A amplitude, B1 duration and the peg number, variance at the cline centre was not consistently high across all sites. The site numbers shown in Figure 8 for the two characters, the peg number and B1 duration, indicate that the increase in variance was found for some hybrid sites but not for the others.

Nevertheless, when the sampling sites were divided into four classes (allopatric *Ch. albomarginatus*, *albomarginatus*-like hybrids, *oschei*-like hybrids and allopatric *Ch. oschei*), the comparison of variance for all traits revealed the higher values in hybrid than in pure populations (Table 3). Similar results were obtained with covariance: of 21 trait pairs, 18 showed the higher covariance in hybrid than in pure populations. However, it is necessary to note that the covariance was not very high for all pairwise combinations (correlation coefficient did not exceed 0.5). Most (14) pairwise cases showed the highest covariance for the *oschei*-like hybrid populations, whereas only three pairwise cases showed the highest covariance for the *albomarginatus*-like populations (Table 3). In three trait pairs (the number of AB pairs vs. B/A amplitude, B duration vs. B/A amplitude, B/A amplitude vs. the peg number), estimates for the pure *albomarginatus* populations were relatively high and comparable with

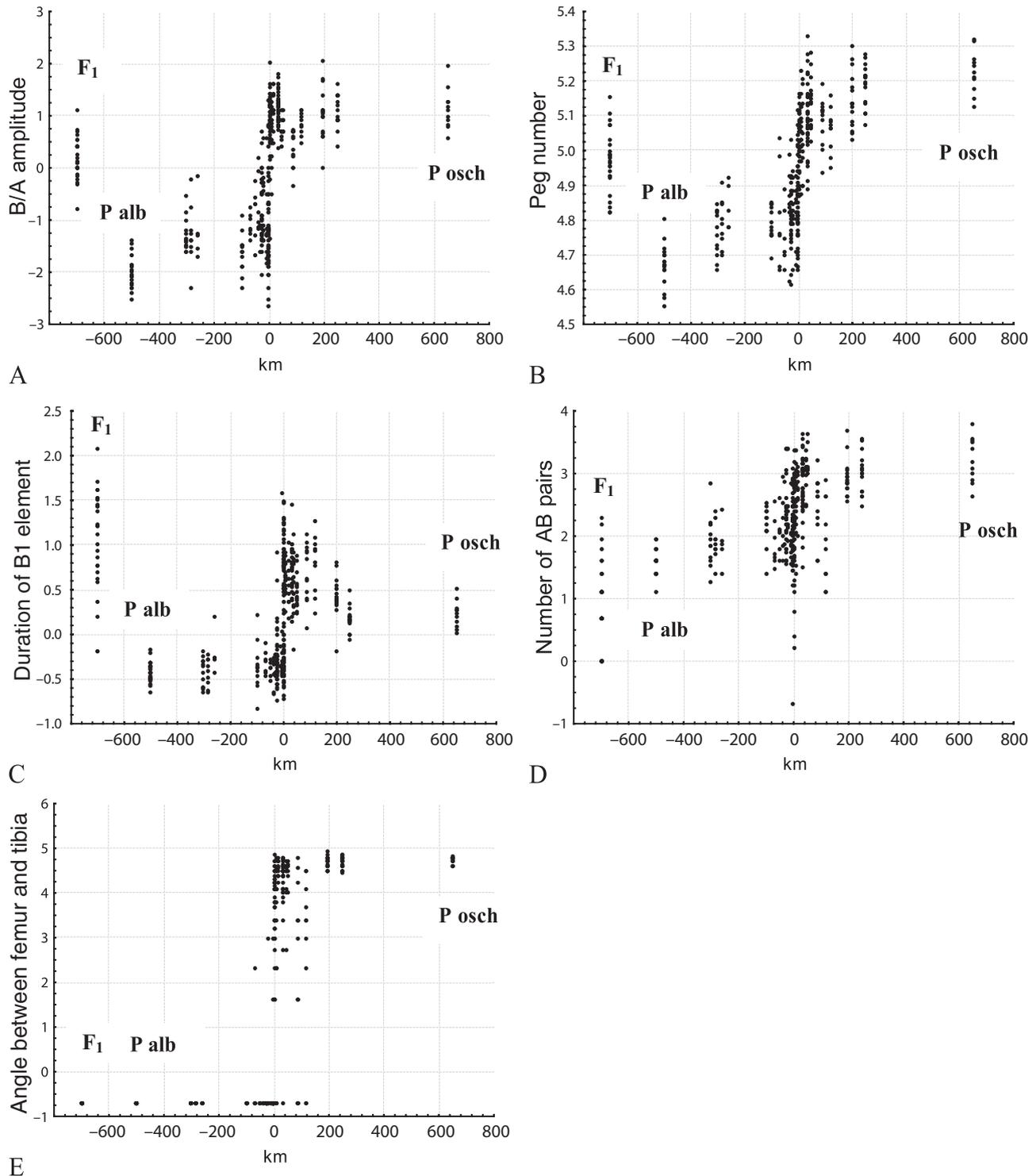


Figure 7. Clines for some characters compared with the distribution of F₁ hybrids between the parental species from the German (P alb) and Bulgarian (P osch) populations. The values of B/A amplitude (A) and the peg number (B) were intermediate in F₁ hybrids; the values of B1 duration (C) were larger in F₁ hybrids than in both parental species; the values of the number of AB pairs (D) had the same value in F₁ hybrids as in their *albomarginatus* parent or smaller than in both parental species; the character of visual display, i.e. the femur/tibia angle (E) had the same value in F₁ hybrids as in their *albomarginatus* parent. Each point is the individual value. All measurements are log_e-transformed.

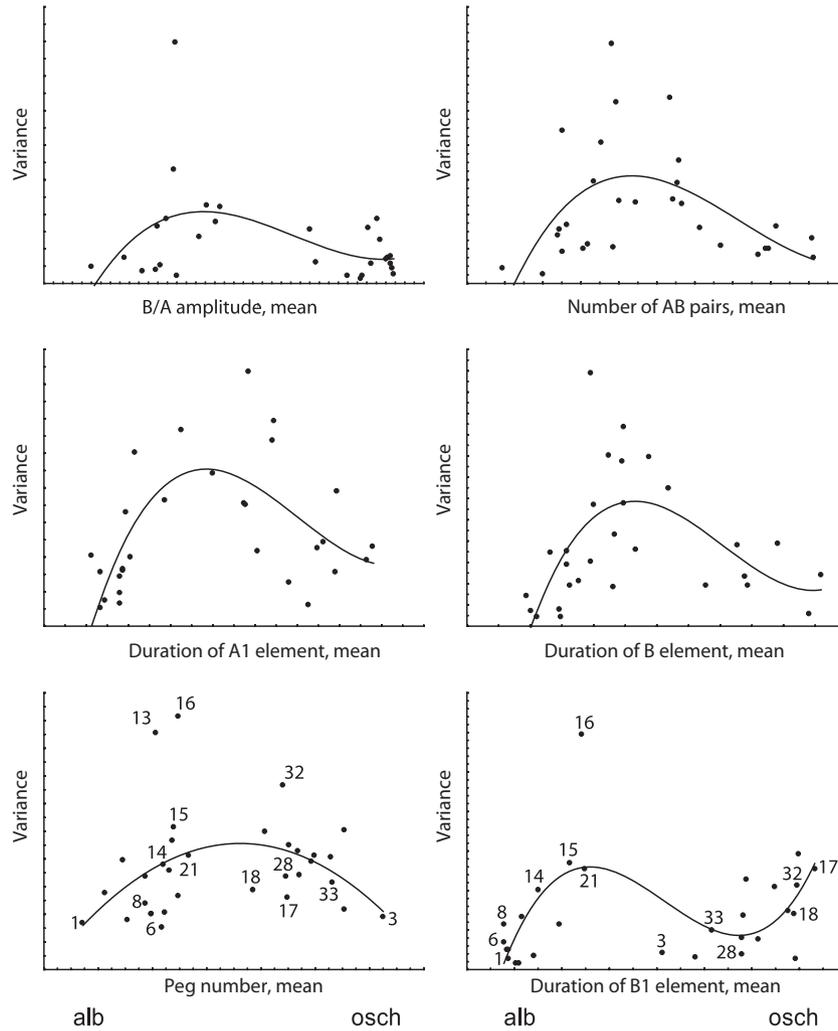


Figure 8. Variance for the five song characters and the peg number. In each graph, *albomarginatus* phenotypes are on the left, *oschei* phenotypes are on the right. Site numbers shown on the low graphs are as in Figures 1 and 5. Only sites represented by more than five individuals are shown.

those for the *albomarginatus*-like hybrids. However, as covariance in pure sites was not higher than in hybrid sites, pleiotropy seems unlikely. As a whole, the highest covariance was observed in the *oschei*-like populations for almost all characters except for B/A amplitude; the latter character showed the highest covariance for five of six combinations in the *albomarginatus*-like sites.

ASSOCIATIONS BETWEEN VARIATION IN PHENOTYPIC TRAITS AND VEGETATION

Associations were studied for the five song characters and the peg number. Correlation was relatively high between almost all phenotypic traits and the number of the plant species per site, varying within a range of 0.54–0.84 ($0.0001 < P < 0.03$). Similarly, most phenotypic traits correlated with the degree of xerophily:

coefficient R varied within a range of 0.5–0.58 ($0.02 < P < 0.05$). The non-significant correlations were only obtained between the vegetation data and the two song characters, B1 duration and the number of AB pairs. All these data evidence relatively high associations between the habitat and the phenotype. Correlation between phenotypic traits and the degree of xerophily suggests that *Ch. albomarginatus* prefers more wet meadows and *Ch. oschei* occurs in more dry habitats.

DISCUSSION

SELECTION AGAINST HYBRIDS

The comparison of cline width and position across the hybrid zone between *Ch. albomarginatus* and *Ch. oschei* showed concordant and coincident clines in

Table 3. Estimates of variance (on the diagonal) and covariance (above the diagonal) for five song characters, angle between femur and tibia and the peg number in the *albomarginatus/oschei* hybrid zone

	Number of AB pairs	B/A amplitude	B duration	B1 duration	A1 duration	Peg number	Femur/tibia angle
Δz	4.477	4.699	2.005	2.403	2.485	0.795	5.635
Number of AB pairs	<i>0.112</i> <i>0.249</i> <i>0.401</i> <i>0.120</i>	0.106 0.107 0.049 0.019	-0.047 -0.032 -0.145 -0.019	-0.005 -0.011 -0.073 0.003	0.001 -0.019 -0.102 -0.032	0.004 0.006 0.013 0.001	0 -0.004 0.312 0.012
B/A amplitude		<i>0.203</i> <i>0.486</i> <i>0.220</i> <i>0.110</i>	-0.051 -0.059 -0.021 -0.016	0.002 0.093 0.008 -0.005	0.001 -0.041 -0.025 -0.009	0.010 0.006 0.00001 -0.002	0 0.039 0.282 0.001
B duration		<i>0.052</i> <i>0.053</i> <i>0.143</i> <i>0.038</i>		0.018 0.003 0.063 0.003	0.005 0.031 0.061 0.022	-0.004 -0.001 -0.006 -0.003	0 -0.013 -0.198 -0.001
B1 duration			<i>0.032</i> <i>0.116</i> <i>0.097</i> <i>0.019</i>		0.009 -0.007 0.038 -0.003	-0.001 0.009 -0.003 0.001	0 0.019 -0.048 0.005
A1 duration				<i>0.022</i> <i>0.051</i> <i>0.097</i> <i>0.047</i>		-0.000005 -0.004 -0.006 -0.002	0 -0.008 -0.151 -0.005
Peg number						<i>0.005</i> <i>0.010</i> <i>0.008</i> <i>0.004</i>	0 0.005 0.017 0.001
Femur/tibia angle							<i>0</i> <i>0.232</i> <i>2.139</i> <i>0.013</i>

For each character combination, the estimates are given in the order: allopatric *albomarginatus* (first value, $N = 36$), *albomarginatus*-like hybrids (second value, $N = 146$), *oschei*-like hybrids (third value, $N = 149$), and allopatric *oschei* (fourth value, $N = 28$). The italic numbers are estimates of variance. Δz -values indicate the maximum trait difference across the transect. See text for details.

four traits (three song and one morphological characters) and discordant and non-coincident clines in three traits (two song characters and one character of visual display). Discordance of clines in different characters may evidence that some characters are under stronger selection than others. The cline analysis made in the current paper might indicate that traits, such as B/A amplitude, the duration of B1 element and the character of visual display, are under stronger selection than other characters, if the number of underlying loci is similar. However, for B/A amplitude, difference in cline width and position was hardly significant. For another song character, B1 duration, a non-additive inheritance was previously found. The mean value of the hybrid trait was significantly larger than that in the parent with the largest trait value, or significantly smaller than that in the parent with the smallest value. The extreme mean values for this parameter were also found in F_2 hybrids (Vedenina *et al.*, 2007b). Thus, one cannot exclude a distortion of the cline in the duration of the B1 element in comparison with the cline in the peg number, the character with an additive inheritance. Comparison of the natural and F_1 hybrids shows that such distortion does exist. For the character of visual display, the Mendelian inheritance with full dominance of the *albomarginatus*-parent was previously found. This means that the cline distortion in this character may also exist. As pattern of inheritance is known to be different for various traits of *Ch. albomarginatus* and *Ch. oschei*, the concordance of clines may only suggest an equal strength of selection acting on underlying loci, but different strengths of selection acting on the traits.

POSSIBLE CAUSES OF CHANGE IN VARIANCE AND COVARIANCE ACROSS HYBRID ZONE

Higher values of variance and covariance in hybrid than in pure populations may suggest either strong selection against hybrids or strong assortative mating between parental phenotypes. In our previous behavioural experiments, females of both species demonstrated a strong assortative mating (80–90% preference for conspecific males). Comparison of the parental and hybrid viability revealed a reduced hatching and increased larval mortality in F_1 and F_2 hybrids. At the same time, F_1 hybrid females did not show a lower selectivity in mate choice experiments than the females of the parental species (Vedenina, Kulygina & Panyutin, 2007a). Thus, assortative mating is suggested to be a more likely cause of increasing variance and covariance in the hybrid populations than selection against hybrids. If so, assortative mating appeared to be weaker than could be expected from the results of the behavioural

experiments. The highest significant correlations varied within the range of $0.5 < R < 0.58$, and they were only found for three pairwise combinations as the number of AB pairs vs. B/A amplitude and the duration of B element and the duration of B element vs. duration of A1 element.

Most pairwise cases showed the highest covariance in the *oschei*-like, than in the *albomarginatus*-like populations. For the character of visual display, the strength of such difference could be overestimated, as, in pure *albomarginatus* populations, variance is equal to zero. But even after excluding this trait from the analysis, most trait pairs still show higher covariance in the *oschei*-like populations. As there was no evidence of pleiotropy, the covariance could be created by linkage disequilibrium and therefore one could suggest a stronger assortative mating in the *oschei*-like sites than in the *albomarginatus*-like sites.

In the previous behavioural experiments, the females of *Ch. albomarginatus* showed less selectivity than the females of *Ch. oschei* (Vedenina *et al.*, 2007a). In mate choice experiments, the *albomarginatus* females did not discriminate between conspecific and hybrid males, whereas the *oschei* females in 90% cases mated with conspecific males. F_1 hybrid females behaved in the same way as the *albomarginatus* females: they did not distinguish the *albomarginatus* males from the hybrid ones, but significantly less frequently mated with the *oschei* males. In addition, we found an asymmetry in the hybrid viability depending on the direction of crosses. The hybrid offspring obtained from the *oschei* females demonstrated a lower survival than the offspring obtained from the *albomarginatus* females. These results are in concordance with the data obtained in the current study. As the data obtained evidence that the assortative mating is stronger in the *oschei*-like sites than in the *albomarginatus*-like sites, one can suggest the movement of the hybrid zone in favour of *Ch. albomarginatus*.

MOSAIC STRUCTURE OF THE HYBRID ZONE

In our previous study of the *albomarginatus/oschei* hybrid zone, a mosaic structure of the contact zone was suggested on the grounds of the rough estimations (Vedenina & von Helversen, 2003). In the current paper, a more detailed analysis of phenotypes and the study of vegetation confirmed our previous suggestion. According to our data, *Ch. albomarginatus* prefers inhabiting rather moist meadows, whereas *Ch. oschei* more likely occurs in the dryer habitats. Almost all European species of the *Ch. albomarginatus* group were shown to occur in meadows and pastures with domination of meso- and hydrophilic cereals (Vedenina & von Helversen, 2009). The excep-

tions are known for some northern populations of *Ch. oschei*, which can be found in the Hungarian Puszta and the Ukrainian steppes, representing the vast plains with a dry and hot summer season.

In many mosaic hybrid zones, parental populations occur in ecologically divergent patches. For example, the cricket species *Gryllus firmus* and *G. pennsylvanicus* hybridizing in North America prefer the habitats with different types of soil (Harrison & Rand, 1989; Ross & Harrison, 2002), whereas two species of *Bombina* toads hybridizing in Eastern Europe prefer either ponds or puddles (MacCallum *et al.*, 1998; Yanchukov *et al.*, 2006). In the *Bombina* hybrid zone, an active habitat preference is suggested to contribute to the structure of the hybrid zone. The above examples contrast with the mosaic hybrid zone between the grasshopper species *Ch. brunneus* and *Ch. jacobsi* in Spain (Bridle & Butlin, 2002). In this zone, the habitat variation explained only a small amount of phenotypic variation. Meanwhile, the distribution of genotypes was unimodal at some sites and bimodal at others. Bailey, Thomas & Butlin, (2004) suggest that the two parental genotypes are maintained in complete sympatry despite some hybridization. In the current study of the hybrid zone between *Ch. albomarginatus* and *Ch. oschei*, some sites showed the distributions close to unimodal with some intermediate forms; however, no locality with a pure bimodal distribution was found. There were the sites where one parental type dominated and another type was represented by a single specimen. The existence of such sites can be only explained by occasional dispersal of single individuals into a strange, closely situated habitat. This may be a reason for the relatively weak assortative mating suggested from analysis of change in variance and covariance. When one of the species is in the minority, females of the rare species could easily hybridize with the males of common species.

Habitat preference may promote an increase of the hybrid zone width. According to the model of the secondary contact zone suggested by Grabovsky (1995), an assortative mating in ecologically segregating forms results in the increase of the hybrid zone width if the hybrids are viable. Contrary to ecologically equivalent forms, the forms preferring different habitats can be easily distributed over a wide zone of sympatry, where the new zones of local hybridization appear. Thus, the barriers to gene flow between the two differentiated forms may become weaker and even a substantial mixing of the two gene pools may be expected in future. However, the *albomarginatus/oschei* hybrid zone deviates from this model in some aspects. First, the hybrid offspring showed the reduced fitness in comparison with the parental forms: the hybrids demonstrated the reduced viability and hybrid males lost when competing with the

parental forms for the females (Vedenina *et al.*, 2007a). Second, the habitat preferences in the *albomarginatus/oschei* hybrid zone were shown not to be very strict, as the correlation between phenotypic traits and the degree of the habitat xerophily was approximately 0.5. Thus, despite that the reproductive isolation between *Ch. albomarginatus* and *Ch. oschei* is incomplete, a rather restricted introgression which would not result in the homogenizing of the two forms can be suggested.

ARE ALL TRAITS STUDIED UNDER STRONG SEXUAL SELECTION?

In gomphocerine grasshoppers, the song is supposed to be the most important component of reproductive isolation. This, however, refers more to a calling song, which is used for a long-range communication. Long-range acoustic cues mainly provide species-specific identification and ease of localization (Gerhardt, 1991; Fitzpatrick & Gray, 2001; Vedenina, 2005; Heller, 2006). The calling song has to be reliably detected against the background of other simultaneously singing species. Moreover, the fine temporal elements of the song may be lost because of reverberations and scattering. Thus, in acoustic long-range systems it is easier to extract information about the species of a signaller than about the quality of a signaller. In contrast, during a close-range communication, the quality of a signaller may be easier and more directly evaluated, and therefore courtship may play an important role in the intraspecific mate choice and a lesser role in the interspecific discrimination (Fitzpatrick & Gray, 2001; Gray, 2005; Heller, 2006). In many species of gomphocerine grasshoppers, several courting males may sing nearby a female, which may facilitate the female choice and favour the competition among males similar to a 'lek'-situation. The evolution of female preferences driving males to extremely elaborate behavioural patterns has been discussed by von Helversen & von Helversen (1994).

Contrary to the courtship songs, the calling songs of *Ch. albomarginatus* and *Ch. oschei* are very similar and relatively simple (Vedenina & von Helversen, 2009). On the one hand, the similarity of their calling songs can be one of the factors favouring hybridization between these species. On the other hand, one can suggest that all characters of the courtship behaviour studied in *Ch. albomarginatus* and *Ch. oschei* are to a great extent the subject of sexual selection (Andersson, 1994). This may be a reason why selection against hybrids appeared to be not very strong, especially on some characters, such as the duration of the B1 element. In *Ch. oschei*, the B1 element is one of the most conspicuous elements of

the courtship song. According to the 'good genes' hypothesis of sexual selection (Zahavi, 1987; Maynard Smith, 1991), the females testing the quality of a male must prefer the most costly features of the signal. Production of a very loud B1 element probably requires much energy from a singing male and thus may be used by a female as an indicator of vigour. In some hybrid songs, the B1 element is of a much higher duration than in the *oschei* song, which may be also a sign of a good male quality. Superiority of some individual songs possessing such a feature might be expected to result in differential introgression of the relevant alleles.

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