

# Genetic and morphological divergence among Gravel Bank Grasshoppers, *Chorthippus pullus* (Acrididae), from contrasting environments

Valerio Ketmaier · Heiko Stuckas · Julien Hempel ·  
Ingmar Landeck · Michael Tobler · Martin Plath ·  
Ralph Tiedemann

Received: 28 September 2009 / Accepted: 27 August 2010 / Published online: 14 September 2010  
© Gesellschaft für Biologische Systematik 2010

**Abstract** Gravel Bank Grasshopper (*Chorthippus pullus*) populations inhabit two contrasting environments, pebbly gravel banks with scarce vegetation cover in mountainous areas along the Alps and lowland grasslands dominated by Common Heather (*Calluna vulgaris*). Heath populations of *C. pullus* have been rediscovered only recently, and show a distribution scattered across Central Europe. The wings are

**Electronic supplementary material** The online version of this article (doi:10.1007/s13127-010-0031-1) contains supplementary material, which is available to authorized users.

V. Ketmaier (✉) · J. Hempel · R. Tiedemann  
Unit of Evolutionary Biology & Systematic Zoology,  
Institute of Biochemistry & Biology, University of Potsdam,  
Karl-Liebknecht-Straße 24–25, Haus 25,  
14476 Potsdam, Germany  
e-mail: ketmaier@uni-potsdam.de

H. Stuckas  
Senckenberg Naturhistorische Sammlungen Dresden,  
Museum of Zoology,  
Königsbrücker Landstraße 159,  
01109 Dresden, Germany

I. Landeck  
Research Institute for Post-Mining Landscapes Inc.,  
Brauhausweg 2,  
03238 Finsterwalde, Germany

M. Tobler  
Departments of Biology and Wildlife & Fisheries Sciences,  
Texas A & M University,  
2258 TAMU,  
College Station, TX 77843, USA

M. Plath  
Department of Ecology & Evolution,  
J.-W.-Goethe-University of Frankfurt,  
Siesmayerstraße 70a,  
60054 Frankfurt am Main, Germany

reduced in this species; thus, it has low potential for long-distance dispersal. We used sequence data on a newly developed non-coding nuclear marker from three gravel-bank and four heath populations to test whether grasshoppers from the two environments represent distinct lineages. Gravel-bank populations were studied in southern Germany (Bavaria), heath populations in eastern Germany (Brandenburg and Saxony) and Ukraine. We compared those genetic data with an analysis of variation in a suite of morphometric traits. Finally, we combined genetic and morphometric data to reconstruct a plausible scenario for the ecological shift observed in *C. pullus*. Our newly developed marker did not sort populations from contrasting environments in two monophyletic lineages. Nevertheless, we found a general lack of gene flow between the gravel-bank and heath populations. There was pronounced variation among populations in morphometric traits. That variation was partially partitioned by habitat type, and populations from the same habitat tended to be more similar than those from different habitats. Our data suggest that heath populations originated through northward expansion from multiple southern European refugia, and that the gravel-bank populations represent one of these sources. Patterns of genetic and morphometric divergence suggest that gravel-bank and heath populations may be in the process of incipient speciation.

**Keywords** *Chorthippus pullus* · Genetic divergence · Morphometrics · Nuclear DNA · Biogeography

## Introduction

Populations of a species inhabiting contrasting environments offer the opportunity to investigate patterns of

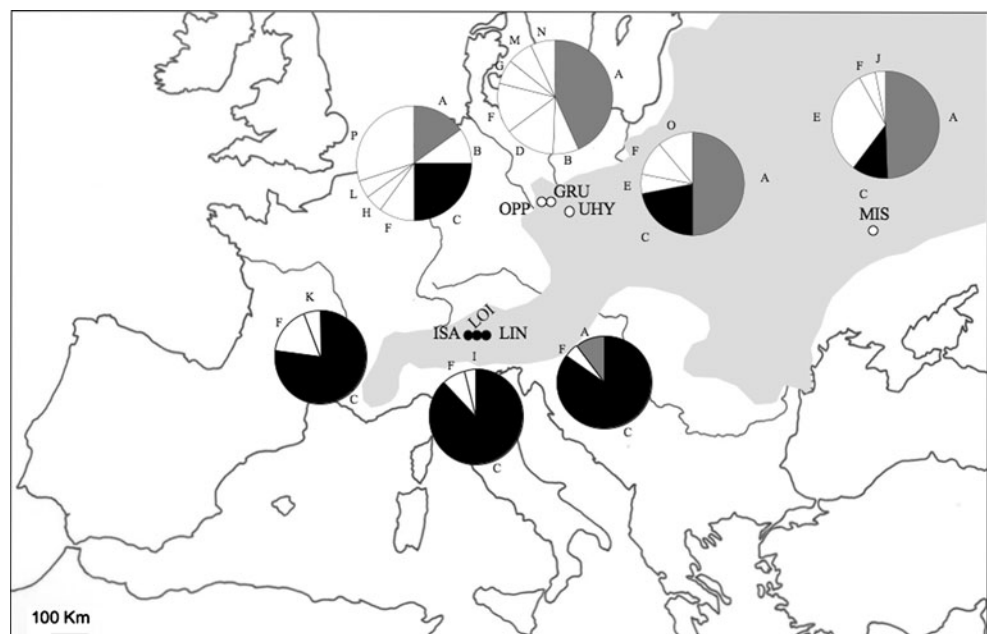
differentiation in morphological and genetic traits, to discern whether respective variations parallel each other and to detect ongoing processes of divergence. This differentiation is of particular importance when it involves physiological and ecological characters that may potentially act as reproductive isolating mechanisms (Schluter 2000). The presence of divergence in morphological traits among populations that differ ecologically points towards selection as a likely cause of the observed pattern. In particular, if selection is strong and populations have adapted to different conditions only recently, then the magnitude of divergence in morphological traits may not be matched by a similar degree of genetic divergence at selectively neutral loci, as the latter is often a secondary consequence driven by random genetic drift and selection on other traits that are genetically linked to the loci under consideration (Dieckmann et al. 2004).

Here, we examine the Gravel Bank Grasshopper, *Chorthippus pullus* (Philippi, 1830), populations of which show discrete ecological partitioning throughout the species' range, to determine the extent of morphometric and genetic divergence among them. *Chorthippus pullus* is a gomphocerine member of the family Acrididae and a congener to the well-known *C. parallelus*, a species that has become paradigmatic concerning patterns of postglacial recolonization in Europe (Hewitt 1999). Members of *C. pullus* are brachypterous, i.e. their wings are shorter than the bodies, with wing reduction more accentuated in females. The Gravel Bank Grasshopper is distributed along the Alps, in Central Europe, throughout the Balkans and eastward to the Caucasus (Fig. 1). It occurs in several scattered populations, with disjunct distribution (Fontana et al. 2004). As the common name suggests, the species

typically colonizes warm and dry habitats along sun-exposed pebbly riverbeds situated between 1,000 m and 1,250 m of altitude where it shows a clear preference for gravel banks with scarce (less than 10%) vegetation cover and sparse shrub succession. It is considered as endangered throughout most of its range (Fontana et al. 2004), with the major threats coming from alterations of riverbanks due to canalization, gravel extraction or the regulation of river flow regimes. These processes favor colonization by pioneer plants, which is usually coupled with the immigration of other grasshopper species (e.g. *Chorthippus biguttulus*) that out-compete *C. pullus* (Reich 1991). Given the apparently strict ecological requirements of the Gravel Bank Grasshopper, it was quite surprising to find the species in a few scattered locations in the Central European lowlands. The latter are characterized by a much denser vegetation cover as compared to gravel banks (40% and more) and are dominated by the Common Heather (*Calluna vulgaris*). Coniferous forests (*Pinus sylvestris*) with open waysides may also represent suitable lowland habitats for the species (Ingmar Landeck, pers. obs.).

Populations from these contrasting habitat types are likely to experience different selective pressures. In addition, the low potential for long-distance dispersal of the species and its discontinuous distribution give reason to suspect a high degree of genetic structuring (Reinhardt et al. 2005). We used a putatively non-coding nuclear DNA marker to test whether the populations from the different habitat types cluster in two monophyletic groups, and to assess the degree of genetic structuring within and among them. Gravel-bank populations were sampled along three different rivers in Bavaria (Germany). Heath populations

**Fig. 1** Geographic locations and genetic composition of the seven populations of the Gravel Bank Grasshopper included in the study. ISA, LOI and LIN (Bavaria, Germany) are gravel bank populations (solid circles); OPP, GRU (both Brandenburg, Germany), UHY (Saxony, Germany) and MIS (Ukraine) are heath populations (open circles). Population composition shown in pie-diagrams, with slice size proportional to frequency of corresponding allele (coded as in Table 2); the two most frequent alleles (A and C) represented in grey and black, respectively; all others in white. Shaded area is the species' range (Maas et al. 2002; I. Landeck unpublished data)



were studied at one Ukrainian and three German localities (two in Brandenburg and one in Saxony; Fig. 1). Next, we examined variation in a suite of morphometric traits that have proven informative in detecting divergence at the population level in *Chorthippus parallelus* (Tregenza et al. 2000). Ecological speciation theory predicts that divergent selection on traits is the highest between populations from contrasting environments (Nosil et al. 2005; Schluter 2000; Tobler et al. 2008).

So far, there was no information regarding the direction of the ecological shift, i.e. on whether the gravel-bank or the heath populations represent the ancestral form, nor on whether this shift occurred only once (from a common ancestral population) or several times independently (from multiple source populations). Phylogeographic studies have shown that the congener, *C. parallelus*, has recolonized Central Europe since the ice ages from different southern European refugia (Hewitt 1999). We therefore suspected that Bavarian gravel-bank populations might represent the ancestral form, from which the heath populations originated. Post-glacial expansions are typically associated with a loss of genetic variability in recolonizing populations, because the latter usually experience multiple founder events during this process (Taberlet et al. 1998). Here, we combined genetic and morphometric data to construct a plausible phylogeographic scenario for the Gravel Bank Grasshopper.

## Material and methods

### Gravel Bank Grasshopper sampling

Grasshoppers were collected from Germany (Bavaria, Saxony and Brandenburg) and Ukraine between July and September 2006 (Fig. 1). Gravel-bank populations were sampled along three Bavarian rivers, the Isar (ISA; Nature Reserve/Special Area of Conservation, districts Garmisch-Partenkirchen and Bad Tölz-Wolfratshausen;  $n=9$ ; 4 males, 5 females), the Loisach (LOI; Nature Reserve Ammergebirge, district Garmisch-Partenkirchen;  $n=12$ ; 7 males, 5 females), and the Linder (LIN; Nature Reserve Ammergebirge, district Garmisch-Partenkirchen;  $n=10$ ; 4 males, 6 females). Heath populations were studied at three German and one Ukrainian locality. The German sites were in Brandenburg (Oppelhai, OPP;  $n=10$ ; 5 males and 5 females. Grünhaus/Finsterwalde, GRU;  $n=9$ ; 7 males and 2 females) and in Saxony (Uhyst, UHY;  $n=10$ ; 2 males and 8 females); the Ukrainian site is Mezhyrich (MIS;  $n=21$ ; 10 males, 1 female, 10 juveniles, the latter included in molecular analysis only). In total, 82 individuals were sampled. Grasshoppers were collected by hand or using a net, preserved in 96% ethanol, and stored at 4°C.

### Genetics

Total genomic DNA was extracted from the femoral muscle of the right hind leg using the DNeasy Tissue Kit and the Animal Tissue Protocol (Qiagen). We initially used two individuals (one each from the LOI and LIN populations) and the primers for a non-coding nuclear DNA locus (Cpnl-1) in *C. parallelus* (Cooper and Hewitt 1993). PCRs were conducted in a total volume of 37.5  $\mu$ l and contained 1.5 U Taq DNA polymerase, 0.2  $\mu$ M of each deoxyribonucleotide triphosphate, 1  $\times$  PCR buffer with 1.5 mM MgCl<sub>2</sub> (all QBiogene), 200  $\mu$ M of each primer, and 2  $\mu$ l of DNA template. PCRs were performed in a Biometra T3000 thermocycler using the following conditions: an initial denaturation for 10 min at 94°C was followed by 30 cycles consisting of a denaturation step at 94°C for 45 s, an annealing step at 57°C for 45 s, and an extension at 72°C for 1 min, with a final extension at 72°C for 2 min.

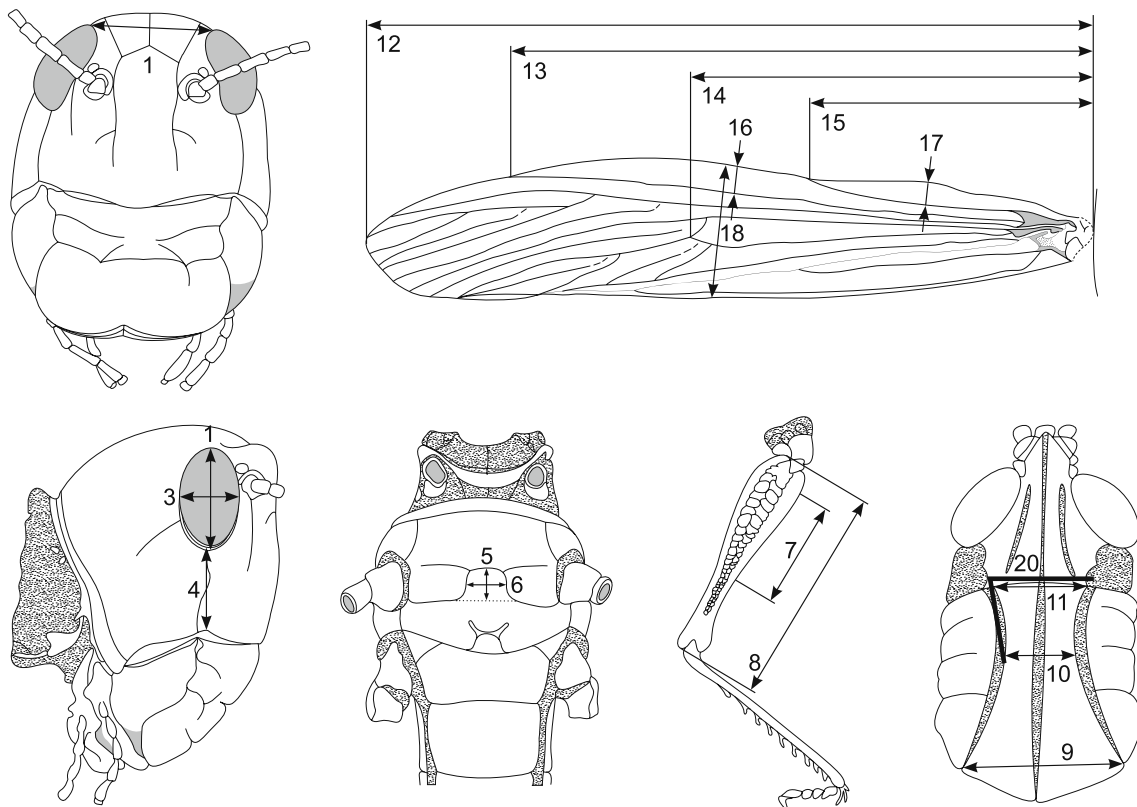
These PCRs produced amplicons of 470 bp. We cloned these PCR products using the TOPO vector of the TOPO TA-cloning kit (Invitrogen). A total of 52 transformant colonies were picked and amplified with T3/T7 primers. Twelve amplicons approximating the expected size were sequenced on an ABI 3100 automated sequencer with BigDye 3.1 chemistry (Applied Biosystems) following the manufacturer's protocol. Sequencing of the cloned PCR products yielded identical sequences in all cases. These were screened for homology with Cpnl-1 of *C. parallelus* as well as with other published sequences (including the seven complete genomes available for insects) using the BLAST algorithm (Altschul et al. 1997). The BLAST search found no similarity with any published coding genes with the Expect values (E) set at the default threshold of 10; aligning our sequences and Cpnl-1 sequences proved unfeasible. Based on the obtained sequences we therefore designed a new primer pair specific for this putative new *C. pullus* nuclear locus 1 (Cpunl-1; Cpunl-1 forward 5'-TAG GCT TCT AGC GAC GTC CAT GT-3' and Cpunl-1 reverse 5'-CCC TTG CCA TCT CCT CTC TCT GT-3'). This primer pair, which consistently produced amplicons of about 360 bp, was then used for genotyping 76 out of the 82 *C. pullus* individuals collected for the study (amplifications failed in 6 individuals). PCR and sequencing conditions were the same as above. The obtained sequences were edited and aligned with Sequencher 4.6 (Gene Codes Corp.) and BioEdit (Hall 1999). We paid particular attention to distinguishing heterozygous sites by adopting the criteria described in Cooper and Hewitt (1993), and we performed multiple sequencing in case of ambiguities. We used the Open Reading Frame (ORF) Finder (Rombel et al. 2002) to find all open reading frames in our sequences using the standard genetic code.

The genetic variability of populations was estimated by  $H_e$  (expected mean heterozygosity under Hardy-Weinberg equilibrium, HWE) and  $H_o$  (observed mean heterozygosity) with GENEPOP on the Internet (<http://genepop.curtin.edu.au/>). The same software was used to conduct a probability test for deviation from HWE with 1000 dememorization steps and 300 batches with 10,000 iterations each. Excess or deficit of heterozygotes was further evaluated by Wright's  $F_{IS}$  (Wright 1965), which was calculated using the Weir & Cockerham estimator ( $f$ ) (Weir and Cockerham 1984) as implemented in FSTAT (Goudet 1995). We used ARLEQUIN 3.0 (Excoffier et al. 2005) to calculate allele frequencies and gene diversity ( $h$ ) for each population. Levels of genetic diversity were tested by hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) using ARLEQUIN with 1000 permutations. The AMOVA design included three groups based on a geographical criterion (i.e. proximity; Bavaria vs. Brandenburg+Saxony vs. Ukraine). We used ARLEQUIN to calculate pairwise  $F_{ST}$  and nucleotide diversity ( $\pi_n$ ) values for all dyadic comparisons of *C. pullus* populations. Statistical significance of these values was assessed by 1000 permutations with sequential Bonferroni correction for multiple testing. To test for isolation by distance within the species, a Mantel test was performed in ARLEQUIN between pairwise geographical distances and  $F_{ST}$  values (Slatkin 1993).

We constructed a haplotype network based on the statistical parsimony method (Templeton et al. 1992) as implemented in the program TCS 1.13 (Clement et al. 2000). Gaps were treated as fifth-state data, and instances of haplotype looping were resolved using predictions from coalescent theory (Crandall and Templeton 1993; Posada and Crandall 2001). We also analyzed data phylogenetically by Neighbor Joining (NJ; Saitou and Nei 1987) and Maximum Likelihood (ML; Felsenstein 1981) in PAUP\* 4.0 $\beta$ 10 (Swofford 2002; heuristic searches, 100 random stepwise additions, TBR branch swapping algorithm). We used MODELTEST (Posada and Crandall 1998) to determine the best model of sequence evolution, and in the subsequent calculation of ML distances for NJ analyses. The robustness of the NJ and ML phylogenetic hypotheses was tested by 1000 bootstrap replicates (Felsenstein 1985).

### Morphometrics

Measurements of the lengths of 18 structures on the head, thorax, forewings, and hind legs (Fig. 2; measured on left side of body only) as well as of the angle between the front margin and left carina of the pronotum were made on 72 individuals (excluding the 10 juveniles from MIS). In addition we counted the number of pegs per mm on the inside of the hind femur. The peg row forms the stridulatory



**Fig. 2** Morphometric distances measured on each individual of *C. pullus* sampled for the study. For descriptions of numbered traits, see Table 1

file that the male rubs against its forewing to produce sound. Still, this character is also found in females, even though trait expression is weaker there than in males (Saldamando et al. 2005). Hence, we considered a total of 20 morphometric characters in both sexes. These traits are listed in Table 1 and depicted in Fig. 2. All measurements were made under a Leica DM LS 2 stereoscope with an eyepiece graticule; images were taken with a Leica DC 300 digital camera and processed with the IM 50 v.4.0 software (Leica). All statistical analyses were conducted with SPSS 16 (SPSS Inc.).

The morphometric traits were subjected to multivariate analysis of covariance (MANCOVA). Assumptions of multivariate normal error and of homogeneity of variances and covariances were met for all analyses performed.  $F$ -ratios were approximated using Wilks' lambda, effect strengths using partial eta squared ( $\eta_p^2$ ). We followed the same approach adopted for *C. parallelus* by using the femur length (FL) as a covariate to control for multivariate allometry (Butlin et al. 1991). 'Population' and 'sex' were included as independent variables because of the well-known sexual dimorphism in the species (Gwynne 1984; Vincent 2006). Since interaction terms were not significant ( $F_{114, 185.79} \leq 0.981$ ,  $P \geq 0.541$ ), only main effects were analyzed.

To provide another intuitive measure of effect strength, we further conducted a heuristic discriminant function analysis (DFA) to determine the percentage of specimens that could be correctly classified to the population of origin. To do so, we first removed the effects of sex and allometry by using residuals of a preparatory MANCOVA. In this MANCOVA, traits were used as dependent variables, 'femur length' as a covariate, and 'sex' as an independent variable.

We also investigated the relation between phenotypic differentiation among populations and their genetic distance, geographic distance, and differences in habitat types using a partial Mantel test with 2000 randomizations. We calculated pairwise phenotypic distance among populations. As for the DFA, we first removed the effects of sex and allometry with a preparatory MANCOVA and conducted all further calculations using residuals. Residual values of each trait were averaged for each population, and population averages were  $z$ -transformed to adjust for differences in scale among traits; after transformation, all data fit a Gaussian distribution with a mean of zero and a standard deviation of one. Based on the transformed population means, we calculated a matrix of Euclidean distances among all population pairs, which served as the dependent variables. Predictor matrices were based on pairwise genetic distances ( $F_{ST}$  values), geographic distances among sites (in kilometers; log-transformed), and habitat type (alpine gravel banks or lowland heath).

## Results

### Characteristics of the Cpunl-1 locus and sequence variation

The open reading frame search carried out on our sequences with the ORF finder revealed a maximum reading frame of 99 bp in the 3' end region of the analyzed fragment (33 amino acids). We then scanned GenBank with Protein Blast (BLAST suite; NCBI) in search of possible similarities with any proteins in the database, but this search did not produce significant matches.

By using the primer pair we had specifically developed for *C. pullus* we were able to amplify and directly sequence a 310 bp fragment from 76 individuals (amplifications failed in 6 individuals). After editing of sequences the final alignment was 300 bp long; sequences obtained for this study have been deposited in GenBank (accession Nos. HM565241–HM565256). The  $\chi^2$  test for base homogeneity indicated that base frequency distribution was homogeneous across populations ( $P=1.000$ ).

Frequencies of A's and C's were very similar (0.276 and 0.227, respectively) and slightly higher than those of G's ( $G=0.178$ ). Most of the variation was due to single base-pair substitutions. These are distributed among 10 polymorphic sites, nine of which undergo transitions. A single insertion of 3 nucleotides (CTC) was detected in the OPP population (haplotype P).

### Population-genetic structure

We identified both homo- and heterozygotes in our samples; this further supports the interpretation that Cpunl-1 is a single locus of nuclear origin. We could define a total of 16 alleles (haplotypes) the frequencies of which are shown in Table 2 and Fig. 1. The allele alignment is shown in the "Electronic Supplementary Material". The number of alleles per population ranged from 3 (ISA, LOI, and LIN; all gravel-bank populations) to 7 (OPP and GRU; both heath populations). All populations but LIN had from 1 (ISA, LOI, UHY, MIS) to 3 (OPP) private alleles. Alleles A and C were the most frequent, with frequencies of 0.25 and 0.43, respectively. Allele F was shared among all populations, but its frequency never exceeded 0.17 (ISA); C was the second most widespread allele, being present in all populations but one (GRU). The frequency of this allele shows a marked geographic variation; it was always  $\geq 78\%$  in the gravel-bank populations while it reaches a maximum of only 25% in heath populations (Fig. 1; Table 2).

Gene diversity ( $h$ ) is higher in the Brandenburg, Saxony and Ukrainian samples ( $0.670 \leq h \leq 0.867$ ) compared to the Bavarian ones ( $0.235 \leq h \leq 0.396$ ; Table 2). Tests for deviation from HWE were significant for three populations (OPP, GRU, and MIS). For OPP and MIS we obtained large, positive  $F_{ST}$

**Table 1** Mean and standard error (in parentheses) values for morphometric traits investigated in males (respective upper data) and females of *Chorthippus pullus*

Populations		Gravel bank			Heath			
Trait No.	Description	Bavaria			Brandenburg		Saxony	Ukraine
		ISA (n=9)	LOI (n=12)	LIN (n=10)	OPP (n=11)	GRU (n=9)	UHY (n=10)	MIS (n=21)
1	Width of forehead	0.89 (0.04)	0.94 (0.02)	0.92 (0.06)	1.10 (0.09)	0.98 (0.04)	0.82 (0.06)	1.08 (0.06)
		1.29 (0.08)	1.43 (0.04)	1.47 (0.06)	1.38 (0.03)	1.44 (0.07)	1.17 (0.05)	1.38 (–)
2	Length of compound eye	0.96 (0.02)	0.96 (0.02)	0.96 (0.04)	0.97 (0.05)	0.98 (0.03)	0.91 (0.01)	0.99 (0.03)
		1.36 (0.10)	1.28 (0.01)	1.32 (0.03)	1.29 (0.03)	1.29 (0.01)	1.42 (0.09)	1.26 (–)
3	Width of compound eye	1.53 (0.03)	1.43 (0.06)	1.51 (0.05)	1.69 (0.05)	1.54 (0.02)	1.55 (0.01)	1.48 (0.03)
		1.50 (0.12)	1.66 (0.03)	1.66 (0.10)	1.84 (0.01)	1.76 (0.01)	1.55 (0.08)	1.71 (–)
4	Sub-ocular groove	1.05 (0.03)	1.11 (0.07)	1.04 (0.03)	1.16 (0.03)	1.13 (0.02)	1.12 (0.01)	1.14 (0.02)
		1.34 (0.08)	1.16 (0.02)	1.31 (0.08)	1.26 (0.01)	1.25 (0.00)	1.30 (0.06)	1.30 (–)
5	Length of mesosternal inter-space	0.68 (0.03)	0.68 (0.02)	0.76 (0.04)	0.78 (0.03)	0.77 (0.04)	0.68 (0.08)	0.76 (0.03)
		1.10 (0.04)	1.04 (0.03)	1.09 (0.03)	1.12 (0.02)	1.22 (0.08)	1.13 (0.03)	0.97 (–)
6	Width of mesosternal inter-space	0.48 (0.02)	0.39 (0.02)	0.44 (0.02)	0.55 (0.03)	0.46 (0.03)	0.54 (0.01)	0.45 (0.02)
		0.72 (0.02)	0.64 (0.01)	0.64 (0.02)	0.73 (0.04)	0.74 (0.08)	0.71 (0.01)	0.56 (–)
7	Length of hind femur	8.73 (0.20)	8.48 (0.16)	8.86 (0.23)	9.65 (0.23)	9.81 (0.10)	9.7 (0.09)	9.07 (0.20)
		8.94 (0.56)	9.84 (0.31)	9.85 (0.28)	9.74 (0.75)	10.11 (–)	10.08 (0.07)	10.40 (–)
8	Length of stridulatory pegs	3.58 (0.51)	3.41 (0.11)	3.83 (0.22)	3.93 (0.21)	4.13 (0.07)	3.16 (0.16)	4.01 (0.13)
		4.05 (0.05)	3.99 (0.24)	4.16 (0.16)	4.51 (0.16)	5.23 (0.15)	4.52 (0.15)	4.83 (–)
9	Width of pronotum A	1.31 (0.04)	1.37 (0.07)	1.37 (0.08)	1.52 (0.06)	1.38 (0.03)	1.40 (0.01)	1.39 (0.03)
		1.84 (0.06)	1.73 (0.03)	1.80 (0.04)	1.76 (0.02)	1.76 (0.03)	1.81 (0.02)	1.78 (–)
10	Width of pronotum B	1.07 (0.03)	1.11 (0.04)	1.09 (0.04)	1.13 (0.05)	1.02 (0.03)	1.10 (0.17)	1.07 (0.03)
		1.42 (0.04)	1.39 (0.03)	1.39 (0.05)	1.29 (0.04)	1.31 (0.01)	1.28 (0.03)	1.34 (–)
11	Width of pronotum C	1.98 (0.13)	1.99 (0.02)	2.05 (0.04)	2.15 (0.13)	1.99 (0.01)	2.00 (0.09)	1.92 (0.05)
		2.70 (0.06)	2.70 (0.02)	2.82 (0.09)	2.54 (0.04)	2.57 (0.14)	2.64 (0.04)	2.44 (–)
12	Length of wing	8.54 (0.10)	8.71 (0.14)	9.58 (–)	8.91 (0.25)	9.27 (0.21)	8.75 (0.03)	8.56 (0.17)
		8.42 (0.38)	7.96 (0.21)	8.76 (0.19)	7.97 (0.24)	8.22 (0.01)	8.22 (0.30)	8.27 (–)
13	Length of costal field	7.64 (0.43)	8.18 (0.14)	9.04 (–)	8.36 (0.23)	8.55 (0.19)	8.29 (0.02)	8.03 (0.14)
		7.86 (0.38)	7.48 (0.20)	8.18 (0.12)	7.97 (0.25)	7.66 (0.19)	7.73 (0.27)	7.54 (–)
14	Size of wing stigma	5.18 (0.22)	5.66 (0.15)	5.78 (–)	5.88 (0.10)	5.97 (0.22)	5.88 (0.18)	5.70 (0.12)
		5.64 (0.39)	5.60 (0.16)	5.95 (0.13)	5.57 (0.30)	5.66 (0.17)	5.21 (0.32)	5.93 (–)
15	Length of precostal field	5.39 (0.02)	5.51 (0.24)	6.61 (–)	5.82 (0.22)	5.50 (0.05)	5.35 (0.23)	5.59 (0.19)
		6.18 (0.15)	5.81 (0.21)	6.76 (0.22)	5.95 (0.48)	5.71 (0.03)	6.35 (0.24)	6.20 (–)
16	Width of costal field	0.61 (0.03)	0.58 (0.04)	0.63 (–)	0.61 (0.07)	0.71 (0.01)	0.69 (0.01)	0.61 (0.03)
		0.38 (0.02)	0.35 (0.02)	0.43 (0.06)	0.39 (0.03)	0.52 (0.05)	0.44 (0.02)	0.37 (–)
17	Width of precostal field	0.32 (0.02)	0.32 (0.03)	0.39 (–)	0.41 (0.03)	0.40 (0.03)	0.40 (0.04)	0.39 (0.02)
		0.44 (0.07)	0.38 (0.003)	0.39 (0.02)	0.37 (0.07)	0.52 (0.05)	0.38 (0.03)	0.30 (–)
18	Width of wing	2.42 (0.06)	2.44 (0.09)	2.36 (–)	2.72 (0.13)	2.72 (0.05)	2.70 (0.01)	2.43 (0.07)
		2.34 (0.13)	2.11 (0.03)	2.39 (0.03)	2.38 (0.11)	2.56 (0.05)	2.27 (0.08)	1.81 (–)
19	Number of stridulatory pegs per mm	35.51 (4.91)	34.71 (2.76)	35.75 (3.04)	27.00 (2.00)	26.71 (1.66)	37.50 (7.50)	26.90 (1.80)
		30.60 (2.46)	25.60 (2.06)	24.33 (1.54)	22.2 (1.53)	20.50 (1.50)	22.88 (1.61)	22 (–)
20	Angle between front margin and left carina of pronotum [in °]	71.64 (3.02)	74.23 (1.72)	73.26 (1.05)	72.04 (1.44)	73.00 (0.21)	71.99 (1.55)	74.04 (0.92)
		73.00 (0.85)	71.18 (1.00)	70.41 (0.61)	73.82 (0.91)	71.88 (3.06)	70.26 (0.66)	75.56 (–)

Trait numbers 1–18 and corresponding parameter results [in mm] for linear measurements as in Fig. 2; population codes as in Fig. 1

**Table 2** Allele frequencies and genetic variability estimates

Populations	Gravel bank			Heath				Overall	
	Bavaria ISA (n=9)	LOI (n=12)	LIN (n=10)	Brandenburg OPP (n=10)	GRU (n=7)	Saxony UHY (n=9)	Ukraine MIS (n=19)		
Alleles									
A			0.10	0.15	0.43	0.50	0.50	0.25	
B				0.10	0.07			0.20	
C	0.78	0.88	0.85	0.25		0.22	0.11	0.43	
D					0.14			0.01	
E						0.06	0.32	0.08	
F	0.17	0.08	0.05	0.10	0.14	0.11	0.05	0.08	
G					0.07			0.01	
H				0.05				0.01	
I			0.04					0.01	
J							0.03	0.01	
K	0.06							0.01	
L				0.05				0.01	
M					0.07			0.01	
N					0.07			0.01	
O						0.11		0.01	
P				0.30				0.04	
Variability estimates	Bavaria ISA (n=9)	LOI (n=12)	LIN (n=10)	Bavaria pooled (n=31)	Brandenburg OPP (n=10)	GRU (n=7)	Saxony UHY (n=9)	E Germany pooled (n=26)	Ukraine MIS (n=19)
<i>h</i>	0.396	0.235	0.289	0.292	0.867	0.833	0.715	0.838	0.659
<i>H<sub>o</sub></i>	0.222	0.250	0.100	0.194	0.300	0.571	0.666	0.500	0.368
<i>He</i>	0.471	0.308	0.368	0.290	0.876	0.879	0.764	0.831	0.662
<i>P<sub>HWE</sub></i>	0.374	1.000	0.054	0.033	0.000	0.017	0.199	0.000	0.000
<i>f</i>	0.439	-0.065	0.654*	0.337	0.656*	0.314	0.068	0.403*	0.450*

*h*, *H<sub>o</sub>* and *He* are estimates of gene diversity, observed and expected mean heterozygosity under Hardy-Weinberg equilibrium, respectively; *P<sub>HWE</sub>* are probability values for significant departures from Hardy-Weinberg equilibrium; \* indicates statistical significance (*P*≤0.05)

**Table 3** Results from hierarchical analysis of molecular variance (AMOVA)

Hierarchy	Categories	% variation	Fixation indexes
Bavaria vs. Brandenburg + Saxony vs. Ukraine	among groups	43.3	$F_{CT}=0.29^*$
	among populations within groups	7.5	$F_{SC}=0.05^*$
	within populations	49.2	$F_{ST}=0.33^*$

\* $P \leq 0.05$ 

values (0.656 and 0.450,  $P \leq 0.05$ ) indicating that departures from HWE predictions are due to an excess of homozygotes. LIN also had a high  $f$  value (0.656;  $P_{HWE}=0.054$ ).

Table 3 reports the results of the AMOVA analysis. AMOVA detected a highly significant overall  $F_{ST}$  value (0.33); the  $F_{CT}$  value was comparably high (0.29). Among-group (i.e. among habitat types) and within-population levels together accounted for more than 90% of the detected variation, while only 7.5% of the variation were attributed to divergence among populations within the same habitat type.

Thirteen out of a total of twenty-one pairwise  $F_{ST}$  values were significant after sequential Bonferroni correction for multiple testing. All comparisons involving Bavarian vs. either Brandenburg/Saxony or Ukrainian populations ( $0.220 \leq F_{ST} \leq 0.510$ ) were statistically significant. No significant pairwise comparisons were observed within Bavaria ( $0.000 \leq F_{ST} \leq 0.003$ ) or Brandenburg+Saxony ( $0.010 \leq F_{ST} \leq 0.097$ ). Within heath populations, MIS (Ukraine) was significantly differentiated only from the westernmost Brandenburg population (OPP;  $F_{ST}=0.170$ ).  $F_{ST}$  values tended to increase with geographic distance (Mantel test,  $P=0.055$ ).

The lack of genetic divergence (i.e. insignificant  $F_{ST}$ ) among geographically proximate populations enabled us to collapse them into three groups, i.e. (1) Bavaria, (2) Brandenburg+Saxony, and (3) Ukraine. In this analysis, Bavarian, Brandenburg+Saxony and Ukrainian popula-

tions were significantly differentiated from one another ( $0.071 \leq F_{ST} \leq 0.492$ , Table 4). The same Table also shows the pairwise nucleotide diversity  $\pi_n$  among these groups. We found very low  $\pi_n$  values; these are significant for Bavaria vs. Brandenburg+Saxony, and for Bavaria vs. Ukraine.

The haplotype network structure (Fig. 3) revealed slight structuring by geographic origin of the samples and/or by habitat type, and a higher number of unique haplotypes in the heath populations. Haplotypes were generally only one mutational step away from each other, with the exceptions of haplotypes B (OPP and GRU) and P (exclusive to OPP); haplotype P showed a 3 bp insertion (see above). NJ and ML analyses produced largely unresolved trees (not shown); bootstrap analyses moderately supported only the node linking haplotypes B and P, both exclusive to heath populations (75–78% bootstrap values for NJ and ML, respectively).

#### Morphometrics

Table 1 lists the descriptive statistics for each of the 20 morphometric traits in the seven populations; values are given separately for males and females of each population. The morphometric analyses not only revealed a pronounced sexual dimorphism and significant allometric effects, but also detected pronounced differentiation among populations (Tables 5, 6). Traits driving differences among populations included width of forehead, width of mesosternal inter-space, length of stridulatory pegs, width of pronotum B, width of wings, width of pronotum C, length of pre-costal field, and length of wings [ordered from higher to lower eta squared ( $\eta_p^2$ ); Table 6]. Using DFA, over 85% of the specimens (compared to the expected 14.3% under a null hypothesis of no pattern) could be assigned to the population of origin based on the morphometric measurements alone (Fig. 4, Table 7).

The partial Mantel test explained 23.7% in pairwise phenotypic distances. Neither genetic distances (pairwise  $F_{ST}$  values;  $r=-0.179$ ,  $P=0.120$ ) nor geographic distances among populations ( $r=0.199$ ,  $P=0.167$ ) correlated significantly with phenotypic distances. However, we found a marginally significant effect of habitat type ( $r=0.407$ ,  $P=0.056$ ). As the separation along function 1 of the DFA suggests (Fig. 4; alpine gravel-bank populations on average

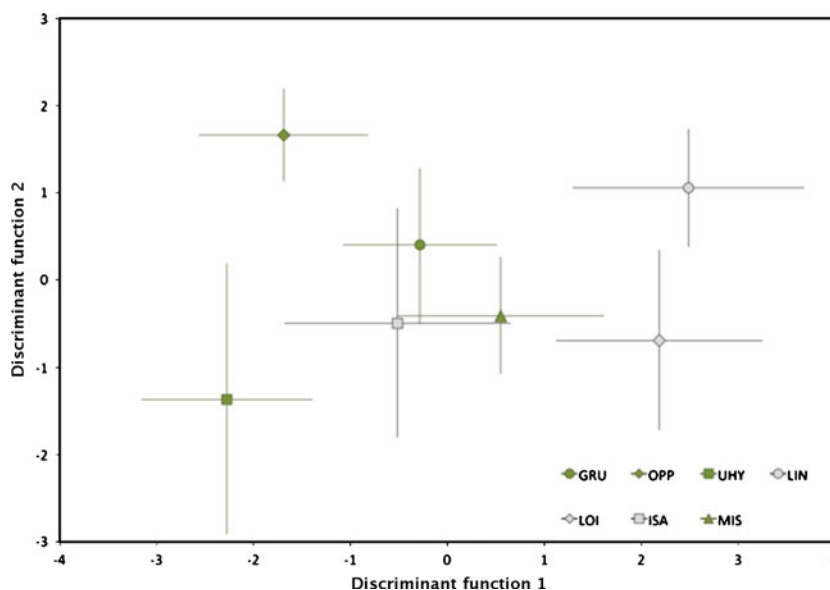
**Table 4** Pairwise  $F_{ST}$  (below table diagonal) and nucleotide diversity  $\pi_n$  (above diagonal) values; pooling into three major groups of populations (Bavaria, Brandenburg + Saxony, Ukraine) based on geographic proximity and the insignificance of pairwise within-group  $F_{ST}$  values (see text)

Populations	Region		
	Gravel bank	Heath	
Region	Bavaria	Brandenburg + Saxony	Ukraine
Bavaria	—	0.001*	0.001*
Brandenburg + Saxony	0.337*	—	0.001
Ukraine	0.492*	0.071*	—

Asterisks indicate statistical significance ( $P \leq 0.016$ ) after Bonferroni correction (1000 permutations)



**Fig. 4** Discriminant function plot (group centroids  $\pm$  SD for the first two discriminant functions) for the analysis presented in Table 7. Dark grey centroids: lowland heath populations; light grey centroids: alpine gravel bank populations



above figures nearly double the 2% uppermost value reported for coding, allegedly selectively not neutral, nuclear loci in *Drosophila* (Zhang and Hewitt 2003).

We identified between three and seven alleles per population, with all populations but one (LIN) showing at least one private allele. We also found pronounced differences in allele frequencies and genetic variability estimates between gravel bank and heath populations (Table 2; for detailed discussion of this issue, see the following text section). The locus thus exhibits a level of variability suitable to tracing processes at the population level (Zhang and Hewitt 2003).

#### Gravel bank and heath populations: distinct lineages?

We initially hypothesized that gravel-bank and heath populations could represent two distinct lineages. This would have been supported, had we found a spatial distribution of alleles sorted chiefly by habitat type. However, the network structure revealed that three out of the five haplotypes found in the gravel-bank populations are also present in the heath populations. Conversely, heath populations host a higher number of unique haplotypes. The structure of the network does not show a clear phylogeographic break between gravel-bank and heath populations; nonetheless, there was a significant divergence among habitat types with regard to allele frequencies, as indicated by significant pairwise  $F_{ST}$  values. The geographically most remote population (MIS; heath population) is significantly differentiated from all gravel-bank populations as well as from one heath population (OPP). This is the only case of significant divergence among populations within the same ecological grouping (i.e. habitat type). It is worth noting, however, that the  $F_{ST}$  value between MIS and OPP is 2–3 times smaller than the values between MIS and

gravel-bank populations. In summary, pairwise  $F_{ST}$  values were always consistently large when comparing populations from contrasting habitat types. A test for conformance to isolation-by-distance expectations only bordered significance. Such a slight pattern of isolation by distance is mostly due to alleles A and C, which show markedly different frequencies among populations (Fig. 1). The AMOVA results suggest that geographic distance is not the only force driving genetic divergence in the species, but more extensive sampling throughout the species' range is needed to properly address this issue. Although our study is based on a relatively small number of populations and individuals, the spatial distribution of Cpn1-1 haplotypes in *C. pullus* is clearly different from that obtained in *C. parallelus* (Cooper and Hewitt 1993), in which Cpn1-1 haplotypes could be sorted into two discrete geographic groupings, which in turn corresponded to two subspecies (*C. parallelus erythropus* in Spain, *C. parallelus parallelus* in France).

Our examination of multiple morphometric traits confirms the strong sexual dimorphism already reported for the species (Gwynne 1984; Vincent 2006), with females, on average, being larger than males and having shorter wings (relative to total body length) that are unsuitable for flying. In addition, morphometrics proved useful to assign individuals to the population of origin using discriminant function analysis (DFA), indicating distinct phenotypes in each population. At least a part of the phenotypic variation ( $\sim 20\%$  of the total variance) appears to be due to environmental conditions differing between heath and gravel-bank populations, as we found a marginally significant effect of habitat type in the partial Mantel test. It needs to be stressed, though, that no information is currently available on the heritability of these traits, relative to the effects of phenotypic plasticity. Still, morphometric divergence could be ascribed, at least in part,

to the major ecological shift considered here (river bank vs. heath). The actual selection pressures driving trait divergence remain to be studied in detail for most of the divergent traits investigated (see Butlin and Hewitt 1998 and Tregenza 2002 for natural and sexual selection affecting trait divergence). For example, the prozona (pronotum C) size is often associated with population divergence in grasshoppers (Tregenza 2002 and references therein) and has frequently been found positively associated with male mating success in a variety of insects ranging from bugs to beetles, including crickets (McLain and Boromisa 1987; Simmons et al. 1992; Vencl 2004). The trait appears to be an important character in male-male sexual competition: (1) it may indicate physical strength and, therefore, be correlated with fighting ability; and (2) the prozona can carry weaponry in other insects, suggesting that this part of the body can be involved directly in fighting (Hongo 2007; McLain and Boromisa 1987). It is tempting to speculate that local differences between *C. pullus* habitats determine differential strengths of (sexual) selection on the trait, as heath populations tend to have a higher density during the reproductive season than gravel-bank populations (Walther 2006; Ingmar Landeck, pers. obs.). In contrast, low population densities may relax sexual selection on male traits since, for instance, females may not be able to be choosy about their mates and male-male encounter rates are low.

#### Population genetics and phylogeography

Genetically, gravel-bank populations are considerably less polymorphic than heath populations. The contrasting ecological conditions experienced by these two groups of populations coupled with the historical processes that might have affected them on the medium to long time scale (see next paragraph) may account for these differences. In particular, mountain gravel banks are subjected to sudden and unpredictable changes, especially in the spring (massive floods due to snowmelt) and summer (thunderstorms). These phenomena coincide with the emergence and reproduction times of *C. pullus* (Schädler and Stadler 2000; Schwarz 1998), and should cause massive losses of individuals and associated pronounced random genetic drift. In contrast, heath probably provides more stable habitat, and populations should be less prone to fluctuations in population size (Hongo 2007). Additionally, heaths are regularly clear-felled for maintenance purposes. This creates potential corridors that would facilitate gene flow among populations (Hongo 2007). In contrast, chances for consistent movements of individuals among mountain populations inhabiting different gravel banks are virtually zero, as this would involve the crossing of mountain ridges or long-distance travel through unsuitable habitat along river valleys, both of which are unlikely scenarios for a species with very low potential for long-

distance dispersal (Maas et al. 2002; Reich 1991; Reinhardt et al. 2005). Deviations from HWE were more pronounced in the heath populations but they were also detected in the pooled gravel-bank populations. Given the low and generally non-significant  $F_{ST}$  values detected at the within-group level, as well as the lack of a clear isolation-by-distance pattern, and following Slatkin's (1993) generalizations on the genetic drift-gene flow equilibrium, we hypothesize that neither gravel-bank nor heath populations have reached an equilibrium between genetic drift and gene flow. However, given the aforementioned ecological differences between the two habitats, we also hypothesize that genetic drift is predominant in gravel-bank populations, while gene flow is the main force shaping the genetic architecture of heath populations. Testing this hypothesis would require acquiring genetic data (ideally based on multiple nuclear loci; i.e. microsatellites) for a similar number of populations sampled over the same geographical scale from each of the two contrasting environments.

From a historical perspective, the higher diversity in the heath populations might suggest that they are ancestral relative to the Alpine populations. This would be at odds with the pattern of post-glacial northward recolonization of Europe unanimously revealed in comparative phylogeographies of taxonomically unrelated taxa, including *C. parallelus* (Hewitt 1999; Taberlet et al. 1998). We have no southern European samples other than those from the Alps, thus we cannot explicitly test this scenario. However, allele A shows a clear East-West cline in Ukrainian and German populations and is present at a very low frequency only in the easternmost Alpine population (LIN). This allele could belong to a lineage that expanded north- and westward from a southeastern refugium, which is impossible to identify here but conceivably located in the Balkan region according to the species range (Fig. 1). Allele C shows a reverse pattern, being the most common allele in the gravel-bank populations and becoming infrequent in the heath populations. Moreover, its central position in the haplotype network suggests that it is probably ancestral. Thus it is well possible that this haplotype originates from an Alpine refugial lineage. The higher diversity of heath populations may be the result of a secondary contact between allopatrically evolved lineages that have met in the area, each of which was carrying unique haplotypes.

#### Conclusions

Our sequence data identified highest genetic divergence among *C. pullus* populations from different habitat types although populations with different ecologies are not reciprocally monophyletic. Our data also suggest a significant lack of gene flow between the two variants. It is interesting to note that ecological speciation assumes an initial phase where natural selection drives divergence in

phenotypic traits. Sexual selection would then reinforce the direction of natural selection due to mating preferences for the same traits (Schluter 2000). Also, under such a scenario levels of morphological divergence and gene flow are correlated (Lu and Bernatchez 1999). Disentangling these factors and processes is the greatest challenge in any study of ecological speciation. Our present data, although not conclusive, contain at least some evidence pointing in that direction. First, we have found a significant phenotypic differentiation among populations, even though differences were not just along the axis gravel bank-heath. Second, levels of gene flow are generally low except for nearby populations. We hypothesize that gravel bank and heath populations represent a case of incipient segregation by expansion to new habitat types. We are aware that the hypothesized scenario will require further, more in-depth testing. At the same time, we do believe that this study opens

intriguing perspectives and may represent a useful starting point for future investigations in this endangered species.

**Acknowledgements** This study would not have been possible without the help of G. Waeber (Ökologisch-Faunistische Arbeitsgemeinschaft, ÖFA; Schwabach). We also wish to thank R. Weid (Obere Naturschutzbehörde; Munich) for valuable suggestions, J. Voith (Bayerisches Landesamt für Umweltschutz, LfU; Augsburg) for his support during the early stages of the project, and E. Beyer (Regierung von Oberbayern, Sachgebiet 55.1 Naturschutz; Munich) for granting us permission to sample in the Bavarian reserves. J. Zettel (University of Bern, Switzerland) and P. Detzel (Gruppe für ökologische Gutachten Stuttgart/ Hochschule für Wirtschaft und Umwelt; Nürtingen) provided useful bibliographic information. M. Fischer (Senckenberg Naturhistorische Sammlungen Dresden) drew Fig. 2. O. Bininda-Emonds and K. Klass provided comments that improved the manuscript substantially. Finally, we wish to thank K. Moll and A. Schneider for technical assistance in the laboratory. M. Tobler was supported by Swiss National Science Foundation (SNF); additional financial support came from the University of Potsdam.

## Appendices

**Table 6** Between-subject effects of MANCOVA

Source	Dependent variable	<i>df</i>	<i>F</i>	<i>P</i>	$\eta_p^2$
Population	Length of stridulatory pegs	6	4.184	0.002	0.313
	Number of stridulatory pegs per mm	6	2.007	0.080	0.180
	Width of pronotum A	6	1.331	0.259	0.127
	Width of pronotum B	6	3.897	0.003	0.298
	Width of pronotum C	6	2.657	0.025	0.225
	Angle between front margin and left carina of pronotum	6	1.354	0.250	0.129
	Length of wing	6	2.293	0.048	0.200
	Width of wing	6	3.491	0.005	0.276
	Length of precostal field	6	1.867	0.103	0.169
	Length of costal field	6	2.306	0.047	0.201
	Width of precostal field	6	1.989	0.083	0.178
	Width of costal field	6	2.072	0.071	0.184
	Size of wing stigma	6	1.170	0.336	0.113
	Length of compound eye	6	0.793	0.580	0.080
	Width of compound eye	6	3.404	0.006	0.271
	Sub-ocular groove	6	0.475	0.824	0.049
	Width of forehead	6	6.509	<0.001	0.415
	Width of mesosternal inter-space	6	6.381	<0.001	0.410
	Length of mesosternal inter-space	6	1.510	0.192	0.141
	Sex	Length of stridulatory pegs	1	28.500	<0.001
Number of stridulatory pegs per mm		1	12.198	0.001	0.182
Width of pronotum A		1	145.678	<0.001	0.726
Width of pronotum B		1	76.189	<0.001	0.581
Width of pronotum C		1	138.221	<0.001	0.715
Angle between front margin and left carina of pronotum		1	1.418	0.239	0.025
Length of wing		1	20.500	<0.001	0.272
Width of wing		1	26.139	<0.001	0.322

**Table 6** (continued)

Source	Dependent variable	<i>df</i>	<i>F</i>	<i>P</i>	$\eta_p^2$
Allometry	Length of precostal field	1	6.057	0.017	0.099
	Length of costal field	1	20.767	<0.001	0.274
	Width of precostal field	1	1.551	0.218	0.027
	Width of costal field	1	73.268	<0.001	0.571
	Size of wing stigma	1	1.778	0.188	0.031
	Length of compound eye	1	80.406	<0.001	0.594
	Width of compound eye	1	8.918	0.004	0.140
	Sub-ocular groove	1	8.309	0.006	0.131
	Width of forehead	1	74.792	<0.001	0.576
	Width of mesosternal inter-space	1	130.352	<0.001	0.703
	Length of mesosternal inter-space	1	147.609	<0.001	0.729
	Length of stridulatory pegs	1	4.881	0.031	0.082
	Number of stridulatory pegs per mm	1	4.562	0.037	0.077
	Width of pronotum A	1	5.299	0.025	0.088
	Width of pronotum B	1	11.477	0.001	0.173
	Width of pronotum C	1	12.148	0.001	0.181
	Angle between front margin and left carina of pronotum	1	0.155	0.695	0.003
	Length of wing	1	0.334	0.566	0.006
	Width of wing	1	0.601	0.441	0.011
	Length of precostal field	1	0.013	0.908	<0.001
	Length of costal field	1	0.097	0.757	0.002
	Width of precostal field	1	1.389	0.244	0.025
	Width of costal field	1	0.345	0.559	0.006
	Size of wing stigma	1	1.304	0.259	0.023
	Length of compound eye	1	5.458	0.023	0.090
	Width of compound eye	1	0.493	0.486	0.009
	Sub-ocular groove	1	0.751	0.390	0.013
	Width of forehead	1	14.247	<0.001	0.206
	Width of mesosternal inter-space	1	13.394	0.001	0.196
	Length of mesosternal inter-space	1	1.766	0.189	0.031

**Table 7** Discriminant function analysis of *Chorthippus* morphology

Trait	Function					
	1	2	3	4	5	6
Length of stridulatory pegs	-0.321	-0.186	0.542	0.394	-0.063	-0.145
Number of stridulatory pegs per mm	0.008	-0.309	0.248	0.035	-0.446	0.028
Width of pronotum A	-0.861	-0.003	-0.972	-0.062	0.201	0.301
Width of pronotum B	0.385	-0.187	-0.421	0.436	-0.632	0.009
Width of pronotum C	1.045	0.055	1.234	-0.854	0.117	0.064
Angle between front margin and left carina of pronotum	0.180	0.247	0.366	0.124	-0.360	0.300
Length of wing	0.277	-0.154	1.153	-0.100	-0.977	1.420
Width of wing	-0.321	0.342	-0.215	-0.444	-0.259	-0.506
Length of precostal field	-0.402	-0.287	-0.633	0.181	0.188	0.804
Length of costal field	-0.241	0.939	-0.623	-0.658	1.100	-1.124

Table 7 (continued)

	Function					
	1	2	3	4	5	6
Width of precostal field	0.388	-0.117	0.408	0.483	0.048	0.147
Width of costal field	0.268	-0.156	0.576	0.322	0.315	0.403
Size of wing stigma	0.481	-0.133	-0.196	0.549	0.038	-0.408
Length of compound eye	0.181	0.074	0.022	0.207	0.577	-0.111
Width of compound eye	-0.345	0.961	-0.006	0.097	0.493	-0.120
Sub-ocular groove	-0.166	0.623	0.219	0.211	0.199	0.350
Width of forehead	0.864	0.544	-0.020	0.230	0.002	-0.050
Width of mesosternal inter-space	-0.761	0.149	-0.167	-0.193	-0.350	0.034
Length of mesosternal inter-space	-0.363	-0.080	0.325	0.214	-0.160	0.152
Standard coefficient						
Canonical correlation	0.874	0.733	0.670	0.631	0.553	0.454
Eigenvalue	3.250	1.163	0.814	0.663	0.440	0.260
% variance explained	49.3	17.7	12.4	10.1	6.7	3.9
Chi-square	195.870	123.536	84.961	55.190	29.771	11.556
<i>df</i>	114	90	68	48	30	14
<i>P</i>	<0.001	0.011	0.080	0.221	0.477	0.642

## References

- Abercrombie, L. G., Anderson, C. M., Baldwin, B. G., Bang, I. C., Beldade, R., Bernardi, G., et al. (2009). Permanent genetic resources added to Molecular Ecology Resources database 1 January 2009–30 April 2009. *Molecular Ecology Resources*, *9*, 1375–1429.
- Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402.
- Butlin, R. K., & Hewitt, G. M. (1998). Genetics of behavioural and morphological differences between parapatric subspecies of *Chorthippus parallelus* (Orthoptera: Acrididae). *Biological Journal of the Linnean Society*, *33*, 233–248.
- Butlin, R. K., Ritchie, M. G., & Hewitt, G. M. (1991). Comparison among morphological characters in the *Chorthippus parallelus* hybrid zone (Orthoptera: Acrididae). *Philosophical Transactions of the Royal Society B*, *334*, 297–308.
- Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, *9*, 1657–1659.
- Cooper, S. J. B., & Hewitt, G. M. (1993). Nuclear DNA sequence divergence between parapatric subspecies of the grasshopper *Chorthippus parallelus*. *Insect Molecular Biology*, *2*, 185–194.
- Crandall, K. A., & Templeton, A. R. (1993). Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics*, *134*, 959–969.
- Dieckmann, U., Doebeli, M., Metz, J. A., & Tautz, D. (2004). *Adaptive speciation*. Cambridge: Cambridge University Press.
- Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, *131*, 479–491.
- Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin ver 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics*, *1*, 47–50.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, *17*, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*, 783–791.
- Fontana, P., Tirello, P., & Buzzetti, F. M. (2004). The *Chorthippus* of the pebbly river-beds (*Glyptobothrus pullus*) in Italy: conservation and first protection actions (Orthoptera, Acrididae). *Atti Accademia Roveretiana degli Agiati*, *4*, 57–70.
- Goudet, J. (1995). FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity*, *86*, 485–486.
- Gwynne, D. T. (1984). Sexual selection and sexual differences in Mormon Crickets (Orthoptera: Tettigoniidae, *Anabrus simplex*). *Evolution*, *38*, 1011–1022.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Research*, *41*, 95–98.
- Hewitt, G. M. (1999). Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, *68*, 87–112.
- Hongo, Y. (2007). Evolution of male dimorphic allometry in a population of the Japanese horned beetle *Trypoxylus dichotomus septentrionalis*. *Behavioral Ecology and Sociobiology*, *62*, 245–253.
- Lu, G. Q., & Bernatchez, L. (1999). Correlated trophic specialization and genetic divergence in sympatric lake whitefish ecotypes (*Coregonus clupeaformis*): support for the ecological speciation hypothesis. *Evolution*, *53*, 1491–1505.
- Maas, S., Detzel, D., & Staudt, A. (2002). *Gefährdungsanalyse der Heuschrecken in Deutschland*. Bonn: Bundesamt für Naturschutz.
- McLain, D. K., & Boromisa, R. D. (1987). Male choice, fighting ability, assortative mating and the intensity of sexual selection in the Milkweed Longhorn Beetle, *Tetraopes tetraophthalmus*

- (Coleoptera, Cerambycidae). *Behavioral Ecology and Sociobiology*, 20, 239–246.
- Nosil, P., Vines, T. H., & Funk, D. J. (2005). Perspective: reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution*, 59, 2256–2263.
- Posada, D., & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitutions. *Bioinformatics*, 14, 817–818.
- Posada, D., & Crandall, K. A. (2001). Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, 16, 37–45.
- Reich, M. (1991). Grasshoppers (Orthoptera, Saltatoria) on alpine and pre-alpine riverbanks and their use as indicators for natural floodplain dynamics. *Regulated Rivers: Research and Management*, 6, 333–339.
- Reinhardt, K., Köhler, G., Maas, S., & Detzel, P. (2005). Low dispersal ability and habitat specificity promote extinctions in rare but not in widespread species: the Orthoptera of Germany. *Ecography*, 28, 593–602.
- Rombel, I. T., Sykes, K. F., Rayner, S., & Johnston, S. A. (2002). ORF-FINDER: a vector for high-throughput gene identification. *Gene*, 282, 33–41.
- Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406–425.
- Saldamando, C. I., Miyaguchi, S., Tatsuta, H., Kishino, H., Bridles, J. R., & Butlin, R. K. (2005). Inheritance of song and stridulatory peg number divergence between *Chorthippus brunneus* and *C. jacobsi*, two naturally hybridizing grasshopper species (Orthoptera, Acrididae). *Journal of Evolutionary Biology*, 18, 703–712.
- Schädler, M., & Stadler, J. (2000). Verbreitung und Lebensraum des Kiesbankgrashüpfers *Chorthippus pullus* in Sachsen. *Articulata*, 15, 7–15.
- Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford: Oxford University Press.
- Schwarz, W. (1998). Wanderverhalten und Aktionsraum adulter *Chorthippus pullus* in einer Wildflusslandschaft bei Salzburg. *Linzer Biologische Beiträge*, 30, 605–611.
- Simmons, L. W., Teale, R. J., Maier, M., Standish, R. J., Bailey, W. J., & Withers, P. C. (1992). Some costs of reproduction for male Bush Crickets, *Requena verticalis* (Orthoptera, Tettigonidae). Allocating resources to mate attraction and nuptial feeding. *Behavioral Ecology and Sociobiology*, 31, 57–62.
- Slatkin, M. (1993). Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, 47, 264–279.
- Swofford, D. (2002). *PAUP\*: Phylogenetic Analysis Using Parsimony\* (and other methods) ver. 4.1*. Sunderland: Sinauer Associates.
- Sword, G. A., Senior, L. B., Gaskin, J. F., & Joern, A. (2007). Double trouble for grasshopper molecular systematics: intra-individual heterogeneity of both mitochondrial 12S-valine-16S and nuclear internal transcribed spaces ribosomal DNA sequences in *Hesperotettix viridis* (Orthoptera: Acrididae). *Systematic Entomology*, 32, 420–428.
- Taberlet, P., Fumagalli, L., Wust-Saucy, A. G., & Cosson, J. F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, 7, 453–464.
- Templeton, A. R., Crandall, K. A., & Sing, C. F. (1992). A cladistic analysis of phenotypic associations and haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, 132, 619–633.
- Tobler, M., DeWitt, T. J., Schlupp, I., García de León, F. J., Herrmann, R., Feulner, P. G. D., et al. (2008). Toxic hydrogen sulfide and dark caves: phenotypic and genetic divergence across two abiotic environmental gradients in *Poecilia mexicana*. *Evolution*, 62, 2643–2659.
- Tregenza, T. (2002). Divergence and reproductive isolation in the early stages of speciation. *Genetica*, 116, 291–300.
- Tregenza, T., Pritchard, V. L., & Butlin, R. K. (2000). Pattern of trait divergence between populations of the Meadow Grasshopper, *Chorthippus parallelus*. *Evolution*, 54, 574–585.
- Ustinova, J., Achmann, R., Cremer, S., & Mayer, F. (2006). Long repeats in a huge genome: microsatellite loci in the grasshopper *Chorthippus biguttulus*. *Journal of Molecular Evolution*, 62, 158–167.
- Vencl, F. V. (2004). Allometry and proximate mechanisms of sexual selection in *Photinus* fireflies, and some other beetles. *Integrative and Comparative Biology*, 44, 242–249.
- Vincent, S. E. (2006). Sex-based divergence in head shape and diet in the Eastern Lubber Grasshopper (*Romalea microptera*). *Zoology*, 109, 331–338.
- Walther, D. (2006). *Habitatpräferenz und Populationsstruktur des Kiesbank-Grashüpfers Chorthippus pullus (Philippi 1830) (Orthoptera, Acrididae) an zwei Standorten im Pfynwald (Schweiz, VS)*. Diploma thesis. Bern: University of Bern, Faculty of Science.
- Weir, B. S., & Cockerham, C. C. (1984). Estimating *F*-statistics for the analysis of population structure. *Evolution*, 38, 1358–1370.
- Wright, S. (1965). The interpretation of population structure by *F*-statistics with regard to systems of mating. *Evolution*, 19, 395–420.
- Zhang, D. X., & Hewitt, G. M. (2003). Nuclear analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, 12, 563–584.