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Discordant patterns of genetic and phenotypic differentiation in five grasshopper species codistributed across a microreserve network

JOAQUÍN ORTEGO,* VICENTE GARCÍA-NAVAS,† VÍCTOR NOGUERALES‡ and PEDRO J. CORDERO‡

*Department of Integrative Ecology, Estación Biológica de Doñana, EBD-CSIC, Avda. Américo Vespucio s/n, E-41092, Seville, Spain, †Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057, Zurich, Switzerland, ‡Grupo de Investigación de la Biodiversidad Genética y Cultural, Instituto de Investigación en Recursos Cinegéticos – IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, E-13005, Ciudad Real, Spain

Abstract

Conservation plans can be greatly improved when information on the evolutionary and demographic consequences of habitat fragmentation is available for several codistributed species. Here, we study spatial patterns of phenotypic and genetic variation among five grasshopper species that are codistributed across a network of microreserves but show remarkable differences in dispersal-related morphology (body size and wing length), degree of habitat specialization and extent of fragmentation of their respective habitats in the study region. In particular, we tested the hypothesis that species with preferences for highly fragmented microhabitats show stronger genetic and phenotypic structure than codistributed generalist taxa inhabiting a continuous matrix of suitable habitat. We also hypothesized a higher resemblance of spatial patterns of genetic and phenotypic variability among species that have experienced a higher degree of habitat fragmentation due to their more similar responses to the parallel large-scale destruction of their natural habitats. In partial agreement with our first hypothesis, we found that genetic structure, but not phenotypic differentiation, was higher in species linked to highly fragmented habitats. We did not find support for congruent patterns of phenotypic and genetic variability among any studied species, indicating that they show idiosyncratic evolutionary trajectories and distinctive demographic responses to habitat fragmentation across a common landscape. This suggests that conservation practices in networks of protected areas require detailed ecological and evolutionary information on target species to focus management efforts on those taxa that are more sensitive to the effects of habitat fragmentation.

Keywords: generalist species, genetic diversity, genetic structure, phenotypic divergence, population fragmentation, population genetics, specialist species

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Introduction

Habitat destruction and fragmentation are major threats to global biodiversity (Noss & Csuti 1994; Lindenmayer & Fischer 2006). Extensive clearing of natural vegetation for agriculture and large-scale farming have dramatically modified landscapes over centuries (Blondel & Aronson 1999; Fahrig 2002). As a result of this process, many species have become extinct and others persist in highly fragmented or isolated habitat patches. These remnant populations often sustain small effective population sizes, which can increase vulnerability to demographic stochasticity and reduce genetic diversity and evolutionary potential to respond to environmental changes and diseases (Saunders *et al.* 1991; Willi *et al.*

Correspondence: Joaquín Ortego, Fax: +34 954 621 125; E-mail: joaquin.ortego@csic.es

2006). In the long term, these processes can compromise population viability and lead to local extinctions, particularly when dispersal from other population sources is absent or limited (Saccheri *et al.* 1998; Spielman *et al.* 2004; Frankham 2005). For these reasons, understanding the ability of organisms to respond to habitat fragmentation and disperse among populations is a major concern for conservation biologists (Saunders *et al.* 1991). These fragmented populations also constitute an ideal 'natural' laboratory to study the evolutionary consequences of population isolation, analyse spatial variation in selective regimes, and disentangle the relative role of gene flow and local evolutionary pressures on spatial patterns of adaptation (Richardson *et al.* 2014; e.g. Bonal *et al.* 2012; Pickup *et al.* 2012; Willi & Hoffmann 2012; Phillipsen & Lytle 2013; Zhao *et al.* 2013).

Molecular markers able to resolve patterns of genetic variability at fine spatial and temporal scales, integrated with novel analytical approaches, have proven to be a powerful tool to infer species responses to habitat fragmentation, particularly in organisms for which dispersal movements are difficult to track for different technical reasons (Lange *et al.* 2010; Quéméré *et al.* 2010). Most studies evaluating the effects of habitat fragmentation are focused on a single species, an approach that can certainly provide key information to guide management practices for the target species (e.g. Wang 2009). However, reserve networks are generally intended to protect several organisms that are likely to be affected by habitat fragmentation in diverse and complex ways (Lange *et al.* 2010; Callens *et al.* 2011). For this reason, data on population genetic diversity and structure across multiple codistributed species can inform whether at least some of them can be managed jointly or which one(s) are more vulnerable to habitat fragmentation and require particular attention (DiLeo *et al.* 2010; Callens *et al.* 2011). Conservation plans can be greatly improved when information on the consequences of habitat fragmentation is available for several species, but so far only a relatively small number of studies on population and landscape genetics have employed a multispecies comparative approach (e.g. DiLeo *et al.* 2010; Lange *et al.* 2010; Callens *et al.* 2011; Aparicio *et al.* 2012; Habel *et al.* 2013; Phillipsen *et al.* 2015).

Combined with genetic information, data on phenotypic variation can help to infer patterns of local adaptation to divergent natural selection regimes (Merilä & Crnokrak 2001; McKay & Latta 2002; e.g. Leinonen *et al.* 2006; Oneal & Knowles 2013; García-Navas *et al.* 2014). Empirical and theoretical work suggests that local adaptation can evolve when the effect of selection is sufficiently strong to counter the homogenizing effect of gene flow, a phenomenon that can potentially occur at any spatiotemporal scale depending on the relative strength of both processes (Richardson *et al.* 2014). For these reasons, the opportunity for evolutionary change and local adaptation is likely to be higher in organisms with limited dispersal capacity and increased population fragmentation (Willi *et al.* 2007; Willi & Hoffmann 2012). The study of phenotypic variation and local adaptation also has important implications from a conservation standpoint and can help to guide conservation agendas aimed to preserve not only species but also the idiosyncratic evolutionary trajectories of their different populations (Fraser & Bernatchez 2001; Moritz 2002). The study of patterns of phenotypic divergence in species assemblages may reveal either the signature of convergent evolutionary responses to shared environment (e.g. to predators or microclimate) or evidence divergent sources of selection, which can inform on whether co-occurring taxa are affected by similar evolutionary pressures (e.g. Ingley *et al.* 2014) or whether these are different or largely decoupled (e.g. Lowe *et al.* 2012). Comparing phenotypic divergence across multiple species can also help to understand whether the evolution of local adaptations is more frequent in taxa experiencing a higher degree of habitat fragmentation than in those inhabiting more continuous habitats and expected to be less prone to population subdivision. This has important implications for the management of focal species of conservation concern: strong phenotypic divergence indicative of local adaptation processes would call for actions aimed to preserve the evolutionary particularities of individual populations, whereas management practices intended to promote dispersal and population connectivity would be advisable in the absence of local adaptation (Ouborg *et al.* 2010 and references therein). However, with the exception of a study that compared phenotypic divergence between two species of codistributed salamanders (Lowe *et al.* 2012), no study has yet integrated phenotypic and genotypic data across multiple co-occurring species to understand the evolutionary consequences of habitat fragmentation and its implications for guiding conservation actions.

Orthoptera have been often found to be highly sensitive to landscape alterations in terms of genetic diversity and structure (Keller *et al.* 2013a; Gauffre *et al.* 2015; Ortego *et al.* 2015), phenotypic variation (Heidinger *et al.* 2010; Gomez & Van Dyck 2012) and extinction risk (Reinhardt *et al.* 2005). Some studies have also shown that certain species are more susceptible than others to suffer the negative effects of habitat fragmentation (Reinhardt *et al.* 2005; Lange *et al.* 2010; Keller *et al.* 2013b), which suggests that ecological assemblages of orthopterans are a good model system to study the impacts of human-driven habitat alterations across multiple species with contrasting life history traits (Lange

et al. 2010). In this study, we set out to analyse whether the extent and spatial patterns of phenotypic and neutral genetic diversity and structure differ among species that inhabit a common landscape but show contrasting life histories, particularly in terms of preferences for habitats that have experienced a different degree of fragmentation (Fig. 1). To address this question, we used as a study system an assemblage of five grasshopper species codistributed across a singular microreserve network located in Central Spain (Figs 1 and 2). The study sites have been protected in recent years due to their unique plant and animal communities associated with their characteristic saline/hypersaline lagoons and lowlands (Cirujano-Bracamonte & Medina-Domingo 2002; Cordero *et al.* 2007; Cordero & Llorente 2008). Although the patchy distribution of these inland saline environments is mostly the result of natural and historical processes, land clearing for agriculture has strongly contributed to their increased fragmentation and the destruction of many other natural habitats of the region such as esparto grass formations (Ortego *et al.* 2012a, 2015). The five focal study species have important differences in dispersal-related morphology (body size and wing length; e.g. Reinhardt *et al.* 2005; Heidinger *et al.* 2010; Butler 2012; Gomez & Van Dyck 2012; Levy & Nufio 2015), degree of habitat specialization, and extent of fragmentation of their respective habitats in the study region, factors that we expect to have a significant impact on their patterns of genetic and phenotypic variability and structure (Fig. 1a; see Materials and methods for a detailed description of the study species). Even though all of the studied taxa show some differences in at least one of the above-mentioned traits, they can be broadly classified into two main groups: small–medium species with preferences for microhabitats that have experienced a considerable degree of fragmentation and medium–large generalist species occupying both natural habitats and agricultural lands (Fig. 1a). Using this system and genotypic and phenotypic data for each species and population, we tested whether taxa that are highly host/habitat specific and linked to highly fragmented habitats show stronger genetic and phenotypic structure than codistributed generalist species inhabiting a heterogeneous but continuous matrix of suitable habitat.

We first analyse the patterns of genetic and phenotypic variability for each studied species and test whether such patterns differ in magnitude and spatial congruence among taxa. Second, we explore the underlying mechanism shaping phenotypic divergence to determine whether it is primarily driven by selection or random genetic drift (e.g. Palo *et al.* 2003; Saether *et al.* 2007; Lowe *et al.* 2012). Specifically, we hypothesize (i) stronger genetic and phenotypic divergence in small–medium species showing preferences for highly fragmented microhabitats due to their limitations to disperse among distant suitable habitat patches, which ultimately can increase the opportunity for the evolution of local adaptations. We also hypothesize that medium–large-body size generalist species inhabiting continuous habitats have (ii) higher levels of genetic diversity and lower variance in genetic diversity across populations as a consequence of widespread gene flow and an ephemeral impact of local demographic dynamics. According to the contrasting life histories and degree of habitat fragmentation among the studied taxa (Fig. 1), we hypothesize (iii) that spatial patterns of genetic and phenotypic variability and structure are not congruent across most of the studied species, but we expect higher resemblance in small–medium species with higher degree of habitat fragmentation due to their more similar responses to the parallel large-scale destruction of their natural habitats.

Materials and methods

Study species

We selected five grasshopper species that co-occur in most of the studied microreserves and show contrasting life history traits and degree of habitat fragmentation in the region, factors that we hypothesize to impact their spatial patterns of genetic and phenotypic variation (Fig. 1a). All the studied species belong to the family Acrididae and are short-horned, winged grasshoppers with a 1-year generation time. All the studied species are native to the study area and distributed in many other adjacent areas from the Iberian Peninsula and the western Mediterranean region (Llucià-Pomares 2002 and references therein). *Mioscirtus wagneri* (Kittary, 1859) (subfamily: Oedipodinae) (hereafter, Mw) has a small body size (♂: 14–16 mm; ♀: 19–22 mm) and is a highly specialized grasshopper (Fig. 1). In the Iberian Peninsula, this species exclusively inhabits saline and hypersaline lowlands with patches of shrubby sea-blite (*Suaeda vera*), the halophilic plant on which it depends for food (Ortego *et al.* 2012a). In the study area, the habitat of this species is highly fragmented due to both its limited natural extension and large-scale land clearing for agriculture in the region (Ortego *et al.* 2010). As a result, the populations of this species only persist in small and highly isolated patches of suitable habitat restricted to a few saline lowlands scattered across the landscape (see fig. 1 in Ortego *et al.* 2012a for a map showing available habitats of Mw within the study area). In this sense, previous studies have revealed that this species shows a very deep genetic structure at different spatiotemporal scales (Ortego *et al.* 2009, 2010,

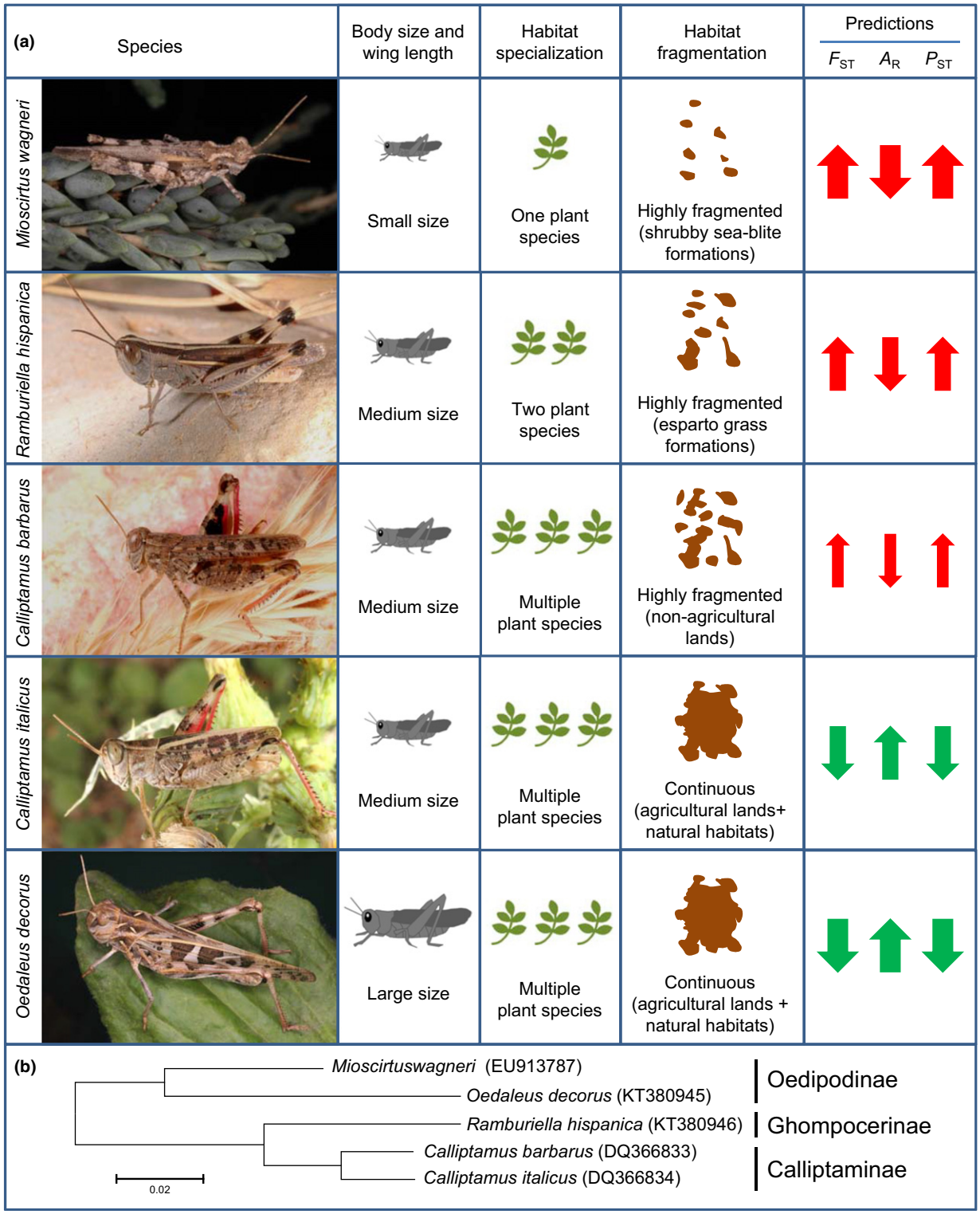

Fig. 1 (a) Characteristics of the five studied species in terms of body size and wing length, habitat specialization, and degree of fragmentation of their respective habitats in the study area (photographs by Pedro J. Cordero). The five species are codistributed and were sampled across a microreserve network located in La Mancha region, Central Spain. The last column indicates the predicted patterns of genetic differentiation (F_{ST}), genetic diversity (A_R) and phenotypic differentiation (P_{ST}) for each studied species; (b) maximum-likelihood tree based on partial sequences of the 16S mitochondrial gene showing the phylogenetic relationships among the five studied species. GenBank accession numbers (in parentheses) and subfamilies for each species are also indicated.

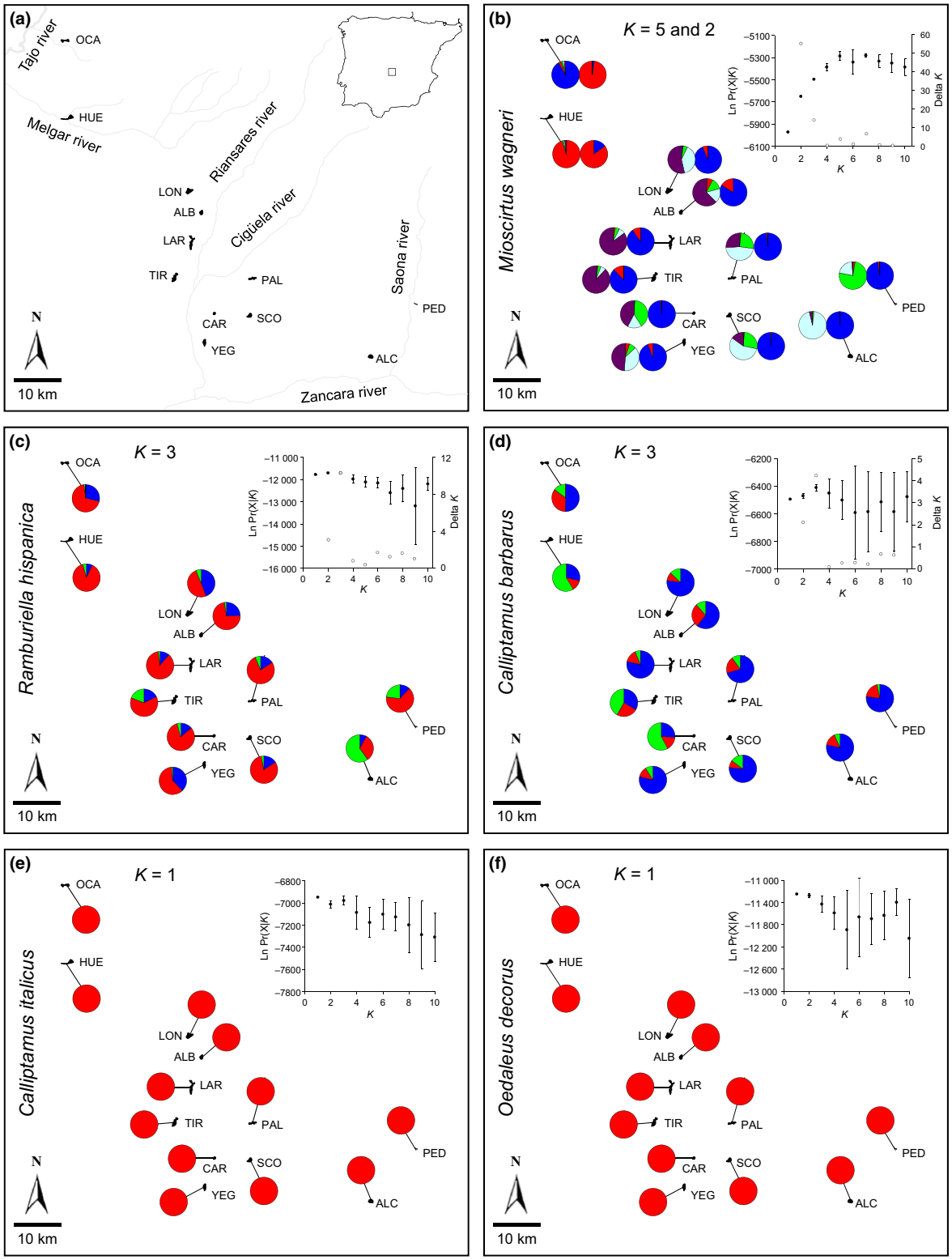

Fig. 2 (a) Geographical location of sampling sites and (b–f) genetic assignment of populations for each species based on the Bayesian method implemented in the program STRUCTURE. The admixture proportions generated by STRUCTURE for each species were represented using pie charts, with each colour indicating a different genotypic cluster. Insets show the mean (±SD) log probability of the data [ln Pr($X \mid K$)] over 10 runs (left axis, black dots and error bars) for each value of K and the magnitude of ΔK as a function of K (right axis, open dots). Population codes are described in Table 1.

2011, 2012a). *Ramburiella hispanica* (Rambur, 1838) (subfamily: Gomphocerinae) (hereafter, Rh) is a specialized and medium-sized (♂: 17–23 mm; ♀: 25–30 mm) grasshopper that in the study area is restricted to semi-natural vegetation areas covered with the esparto grasses *Lygeum spartum* and *Stipa tenacissima* (P. J. Cordero & J. Ortego, personal observation) (Fig. 1a). Suitable habitats of this species are also highly fragmented and have suffered a considerable reduction in parallel with the contraction experienced by the habitats occupied by Mw due to extensive land clearing for agriculture (Ortego *et al.* 2015). However, remnant habitats of Rh are more connected than those of Mw given that Rh occupies all patches where Mw is present plus many others not devoted to agriculture and covered with esparto grass formations (see fig. 1 in Ortego *et al.* 2015 for a map showing available habitats of Rh within the study area). *Calliptamus barbarus* (Costa, 1836) (subfamily: Calliptaminae) (hereafter, Cb) is a medium-sized (♂: 13–21 mm; ♀: 19–31 mm) and generalist grasshopper that feeds on many grass species (Blanchet *et al.* 2012a,b and references therein) (Fig. 1a). In the study area, this species is ubiquitous in any patch of semi-natural vegetation but absent in agricultural areas (P. J. Cordero & J. Ortego, personal observation). The habitat of Cb is highly fragmented, but in a lesser extent than in the two previous species as it occupies many nonagricultural habitat patches where the specific plant formations required by Mw and Rh are not present. Thus, the specific habitats of Mw are embedded within those habitat patches occupied by Rh, which in turn are embedded within those larger patches inhabited by Cb (Fig. 1a). *Calliptamus italicus* (L., 1758) (subfamily: Calliptaminae) (hereafter, Ci) is a medium-sized (♂: 14–25 mm; ♀: 22–33 mm) and generalist grasshopper species found in both semi-natural habitat patches and agricultural systems (Fig. 1a). This species has been reported to be an occasional agricultural pest (Blanchet *et al.* 2012a,b and references therein). *Oedaleus decorus* (Germar, 1826) (subfamily: Oedipodinae) (hereafter, Od) is a large-size (♂: 18–24 mm; ♀: 25–38 mm) generalist grasshopper (Fig. 1a) (all measurements according to Harz 1975). This species is declining or has become extinct in some European countries (see Kindler *et al.* 2012 and references therein), but it is common in our study area and can be found at high densities in most semi-natural habitat patches, field margins and agricultural systems (P. J. Cordero & J. Ortego, personal observation).

To illustrate the phylogenetic relationships among our study species, we built a phylogenetic tree in the program MEGA 6.06 using a maximum-likelihood method and GTR + I + γ as substitution model (Tamura *et al.* 2013). We used sequences of a segment of the 16S rRNA mitochondrial gene (459–463 bp) obtained in our laboratory (for Mw, Rh and Od) as described in Ortego *et al.* (2009) or retrieved from the GenBank (for Cb and Ci) (Fig. 1b). New sequences were deposited in the GenBank with accession numbers KT380945–KT380946. Figure 1b shows that the study species are not phylogenetically clustered according to the three main studied factors, indicating that similarities among species in body size and the degree of habitat specialization and susceptibility to fragmentation are independent of their phylogenetic relationships.

Study sites and sampling

The study was carried out in 12 localities from La Mancha region, Central Spain (~2500 km^2; Table 1; Fig. 2a). Population code descriptions and further information on sampling sites are given in Table 1. During 2006–2013, we aimed to sample in each locality ~20 adult specimens of each studied species (Mw: $n = 242$; Rh: $n = 234$; Cb: $n = 204$; Ci: $n = 219$; Od: $n = 221$; Table 1). We intended to sample an equal number of males and females in each locality, but sample sizes are often male-biased due to the difficulties in capturing females at some sites for some species (Table 1). Identification of *Calliptamus* species based on morphological characters is challenging for females, so we only sampled males for the two studied species of this genus (Blanchet *et al.* 2012a,b). Two species (Ci and Od) were not present in OCA locality. Another species (Cb) was very scarce in HUE locality, and we were only able to collect three specimens despite intensive sampling effort in the area (Table 1). In 10 localities, all the species could be collected in sufficient numbers (≥8 specimens) to perform population genetic analyses (Table 1). Most comparisons across species reported in the Results section refer to these 10 populations. All specimens were preserved in 1500 μL of 96% ethanol at −20 °C until needed for genetic analyses.

Microsatellite genotyping

We used NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) kits or a salt extraction protocol (Aljanabi & Martinez 1997) to purify genomic DNA from a hind leg of each individual. We used 5–12 microsatellite markers to genotype each sampled individual from the different species (Mw: Aguirre *et al.* 2010; Rh: Aguirre *et al.* 2014; Cb and Ci: Blanchet *et al.* 2010a; Od: Berthier *et al.* 2008; see Table S1, Supporting information). Amplifications were conducted in 10-μL reaction volumes containing ~5 ng of template DNA, 1× reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer; Ecogen, Madrid, Spain), 2 mM

Table 1 Geographical location of the 12 microreserves from La Mancha region considered in this study and sample sizes (number of males/females in parentheses; only males were collected for Cb and Ci) and genetic variability (A_R) for each studied species

				Sample size					Allelic richness (A_R)				
Locality	Code	Latitude	Longitude	Mw	Rh	Cb	Ci	Od	Mw	Rh	Cb	Ci	Od
Saladar de Ocaña	OCA	−3.630508	39.985445	20 (9/11)	20 (12/8)	20	—	—	5.68	8.02	9.96	—	—
Saladar de Huerta	HUE	−3.617103	39.838697	20 (10/10)	18 (15/3)	3	20	20 (11/9)	5.18	8.15	—	8.83	9.96
Laguna de Longar	LON	−3.321046	39.700548	20 (10/10)	20 (10/10)	20	20	18 (8/10)	5.02	7.91	9.78	8.79	9.39
Laguna de La Albardiosa	ALB	−3.288700	39.658024	20 (12/8)	20 (10/10)	17	20	19 (10/9)	5.42	7.51	9.69	8.40	10.60
Laguna Larga	LAR	−3.317164	39.609088	20 (16/4)	19 (11/8)	20	20	19 (15/4)	4.67	8.06	10.01	8.53	10.44
Laguna de Tírez	TIR	−3.354411	39.546603	20 (10/10)	19 (9/10)	8	17	22 (10/12)	5.38	7.93	8.48	9.00	10.27
Laguna de Palomares	PAL	−3.172344	39.535906	20 (10/10)	20 (10/10)	19	22	22 (13/9)	4.30	7.98	9.73	8.51	10.01
Laguna de Los Carros	CAR	−3.262528	39.472016	20 (10/10)	19 (9/10)	18	19	20 (10/10)	4.43	7.95	9.13	8.99	9.78
Laguna de Las Yeguas	YEG	−3.281576	39.418396	20 (10/10)	20 (10/10)	19	21	20 (10/10)	5.07	8.08	10.18	8.94	10.01
Laguna de Salicor	SCO	−3.173809	39.470083	22 (15/7)	20 (14/6)	20	20	22 (11/11)	4.03	8.18	10.07	9.22	10.01
Laguna de Alcahozo	ALC	−2.875947	39.391585	20 (10/10)	19 (15/4)	20	20	20 (10/10)	3.63	7.14	9.82	8.34	9.92
Saladar de El Pedernoso	PED	−2.767518	39.491164	20 (10/10)	20 (17/3)	20	20	19 (9/10)	3.45	8.43	9.44	8.85	10.34

Mw, *Mioscirtus wagneri*; Rh, *Ramburiella hispanica*; Cb, *Calliptamus barbarus*; Ci, *Calliptamus italicus*; Od, *Oedaleus decorus*; A_R, standardized allelic richness.

$MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The PCR cycling profile used was 9 min denaturing at 95 °C followed by 40 cycles of 30 s at 94 °C, 45 s at the annealing temperature (see Table S1, Supporting information) and 45 s at 72 °C, ending with a 10 min final elongation stage at 72 °C. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and genotypes were scored using GENEMAPPER 3.7 (Applied Biosystems).

Microsatellite genotypes were tested for departure from Hardy–Weinberg equilibrium at each locus within each sampling population and species using an exact test (Guo & Thompson 1992) based on 900 000 Markov chain iterations as implemented in the program ARLEQUIN 3.1 (Excoffier *et al.* 2005). We also used ARLEQUIN 3.1 to test for linkage disequilibrium between each pair of loci for each population and species sampled using a likelihood-ratio statistic, whose distribution was obtained by a permutation procedure (Excoffier *et al.* 2005). We applied sequential Bonferroni corrections to account for multiple comparisons (Rice 1989).

Genetic diversity

For each species and population, we calculated allelic richness (A_R) standardized for the smallest sample size using the rarefaction method implemented in the program HP-RARE (Kalinowski 2005) and observed heterozygosity (H_O) using FSTAT (Goudet 1995). A_R and H_O were highly correlated across populations in all the studied species (Pearson rank correlations, Mw: $r = 0.967$; Rh: $r = 0.924$; Cb: $r = 0.823$; Ci: $r = 0.852$; Od: $r = 0.968$; all Ps < 0.01) and for simplicity we only used A_R as an estimate of population genetic diversity in subsequent analyses. We first compared genetic diversity among species using a one-way ANOVA. Then, we analysed the correlation of genetic diversity across populations between all pairs of species using Pearson rank correlations. A significant positive correlation of population genetic diversity in two species would suggest that their populations have similarly responded to the different factors (e.g. habitat fragmentation and genetic bottlenecks) affecting local levels of genetic diversity. Finally, we used Levene's tests to analyse whether variance in population genetic diversity is similar among the studied species. A high variance in genetic diversity among populations of a given species would indicate that its populations are differentially impacted by the demographic phenomena affecting local levels of genetic diversity. In contrast, if a species shows levels of genetic diversity that are similar across all its populations (i.e. low variance), this would imply that all of them are subjected to comparable demographic dynamics and/or that differences are ephemeral due to the homogenizing effects of gene flow. All statistical analyses were performed in SPSS 19.0.

Genetic structure

We investigated population genetic structure among sample locations calculating pairwise F_{ST} values and testing their significance with Fisher's exact tests after 10 000 permutations as implemented in ARLEQUIN 3.1 (Excoffier *et al.* 2005). Critical P-values for pairwise tests of allelic differentiation were determined using a sequential Bonferroni adjustment (Rice 1989). We calculated global F_{ST} values across all populations in FSTAT 2.9.3 and 95% confidence intervals (95% CI) were estimated by bootstrapping over loci (10 000 randomizations; Goudet 1995). Finally, we analysed patterns of genetic structure using the Bayesian Markov chain Monte Carlo clustering analysis implemented in the program STRUCTURE 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009). STRUCTURE assigns individuals to K populations based on their multilocus genotypes. We ran STRUCTURE assuming correlated allele frequencies and admixture and using prior population information (Hubisz *et al.* 2009). We conducted 10 independent runs for each value of $K = 1$–10 to estimate the 'true' number of clusters with 200 000 MCMC cycles, following a burn-in step of 100 000 iterations. The number of populations best fitting the data set was defined both using log probabilities [$\Pr(X|K)$] (Pritchard *et al.* 2000) and the ΔK method (Evanno *et al.* 2005), as implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012). We used CLUMPP to align multiple runs of STRUCTURE for the optimum K value using the Greedy algorithm (Jakobsson & Rosenberg 2007).

Concordance of spatial patterns of genetic structure

We analysed congruent patterns of genetic structure in two different ways. First, we assessed the correlation between genetic distance matrices (F_{ST}, calculated as described above) of all species pairs using classical Mantel tests. We also used partial Mantel tests to remove any confounding effects of geographical distance (i.e. isolation by distance) (e.g. Morgan *et al.* 2011; Widmer *et al.* 2012). All Mantel tests were performed using ZT software with 10 000 permutations (Bonnet & Van de Peer 2002). Second, we performed Procrustes rotation tests to analyse the degree of congruence between multivariate population allele frequency data of all species pairs (Jackson 1995; Peres-Neto & Jackson 2001; e.g. Widmer *et al.* 2012). In a first step, we

summarized variation in population allele frequencies for each species using mean-centred principal component analyses (PCAs) as implemented in R 3.0.3 (R Core Team 2012) package ADEGENET 1.4.1 (Jombart 2008). Next, we performed a Procrustes rotation to rotate the raw principal component matrices of the first three axes for each pair of species using the 'procuste' function in R 3.0.3 package ADE4. Procustean rotations were scaled to unit variance to obtain the more scale-independent and symmetric descriptive statistic 'Procrustes sum of squares' (m^2). Finally, we performed a PROTEST analysis (Jackson 1995) to test the significance of the similarity between the genetic matrices of each pair of species using the 'procuste.rtest' function with 9999 iterations in ADE4.

Phenotypic divergence

We studied the underlying factors shaping phenotypic variation examining the levels of quantitative divergence based on phenotypes (P_{ST}). P_{ST} (or 'phenotypic' Q_{ST}) is analogous to Q_{ST}, a measure of differentiation in quantitative genetic traits and the equivalent of F_{ST} for morphological characters (Spitze 1993). P_{ST} is used as a proxy for Q_{ST} when the required quantitative genetic information cannot be estimated and it is not possible to disentangle genetic variation among populations from environmental variation (e.g. in field studies; Raeymaekers *et al.* 2007; Brommer 2011). Here, we focused on body size, a morphological trait that typically exhibits a substantial additive genetic basis (Mousseau & Roff 1987; Merilä & Crnokrak 2001). The calculation of P_{ST} values allowed us to study patterns of phenotypic divergence across the different studied species that differ considerably in body size (Fig. 1) and for which simple Euclidean distance between population mean values of body size (e.g. Ortego *et al.* 2012b) is not directly comparable. Phenotypic differentiation was only studied in adult males, as females were not available for some species (Cb and Ci) as described above. For all individuals, we measured femur length to the nearest 0.1 mm using a stereoscopic microscope Leica S8 APO and the software LAS version 2.8.1. This morphological trait provides a good estimate of overall body size in grasshoppers and is highly correlated with estimates of body size based on other morphological traits (Ortego *et al.* 2012b). Global and pairwise P_{ST} values for all population pairs were estimated as

$$P_{ST} = \left[\frac{c}{h^2}\sigma^2_{GB}\right] / \left[\frac{c}{h^2}\sigma^2_{GB} + 2\sigma^2_{GW}\right],$$

where the scalar c expresses the additive genetic proportion of differences between populations (i.e. the proportion of the total variance that is presumed to be due to additive genetic effects across populations), h^2 is the assumed additive genetic proportion of differences between individuals within populations (narrow sense 'heritability'), σ^2_{GB} is the observed between-population variance component and σ^2_{GW} is the observed within-population variance component. Given the unknown magnitude of c and h^2 (whose ratio determines the accuracy of the approximation of Q_{ST} by P_{ST}), we computed P_{ST} values by varying the c and h^2 parameters (c/h^2 range: 0.1–2.0). The reported P_{ST} values are those obtained assuming $c = h^2 = 0.5$. These values were chosen given that the heritability estimate of male body size in the grasshopper *Chorthippus brunneus* has been previously reported to be 0.48 (Butlin & Hewitt 1986), which means that environmental and nonadditive genetic effects account for about half of the observed phenotypic variation. We assumed the proportion of variation due to additive genetic effects across populations c equals the proportion within population h^2 (i.e. $c/h^2 = 1$), which is a biologically realistic assumption (Brommer 2011). P_{ST} estimates did not change much when considering other more conservative scenarios ($c < h^2$) and provided analogous results (data not shown). Confidence intervals (CI) were estimated from 1000 bootstrap replicates using the 'boot' package (Ripley 2015) in R (R Development Core Team 2012).

The relationship between genetic (F_{ST}) and morphological (P_{ST}) differentiation across populations was analysed using Mantel tests. If genetic and phenotypic population divergence are positively correlated, this would imply that genetic drift has played an important role on phenotypic divergence. In contrast, if genetic and phenotypic divergence are decoupled this would suggest that phenotype is plastic or, in the case of highly heritable traits such as body size (Mousseau & Roff 1987), controlled by local selection (e.g. Leinonen *et al.* 2006; Lehtonen *et al.* 2009). These comparisons therefore serve as a gauge of the likely overall importance of genetic drift vs. local adaptation in body size variation. We analyse congruent patterns of phenotypic differentiation across the studied species assessing the correlation between phenotypic distance matrices (P_{ST}) of all species pairs using Mantel tests. We also used partial Mantel tests to remove any confounding effects of geographical distance (see previous section for details on Mantel and partial Mantel tests).

Results

Microsatellite data

All microsatellite markers were highly polymorphic across most populations and species, with 8–56 alleles per locus (Table S1, Supporting information). After

applying sequential Bonferroni corrections to compensate for multiple statistical tests, only two loci (RhA113 and RhC1) from Rh consistently deviated from HWE across all the studied populations and were excluded from further analyses (Table S1, Supporting information). We did not find any evidence of genotypic linkage disequilibrium at any pair of loci in any population and species (exact tests; all *P*s > 0.05).

Genetic diversity

A_R for each species and population is indicated in Table 1. Considering only the 10 localities where all the five species were collected, we found that A_R differed significantly among taxa (one-way ANOVA: $F_{4,45} = 223.99$, $P < 0.001$; Fig. 3). Post hoc Tukey tests showed that A_R was significantly different between all species pairs (all *P*s < 0.003) with the exception of the comparison involving Cb and Od ($P = 0.223$; Fig. 3). A_R increased in the order Mw < Rh < Ci < Cb < Od and was not significantly correlated across populations between any pair of species after sequential Bonferroni correction for multiple testing (all *P*s > 0.05). Finally, variance in population A_R significantly differed among species (Levene's test: $F_{4,45} = 2.78$, $P = 0.038$) and post hoc analyses indicated that only pairwise comparisons involving Mw were significant (Mw–Rh: $P = 0.025$; Mw–Ci = $P = 0.013$; Mw–Od = $P = 0.029$) (Fig. 3). In all comparisons, Mw had higher variance in A_R than the other species (Fig. 3). Analyses including all populations (Fig. 3) or using H_O as an estimate of population genetic diversity provided analogous results (data not shown).

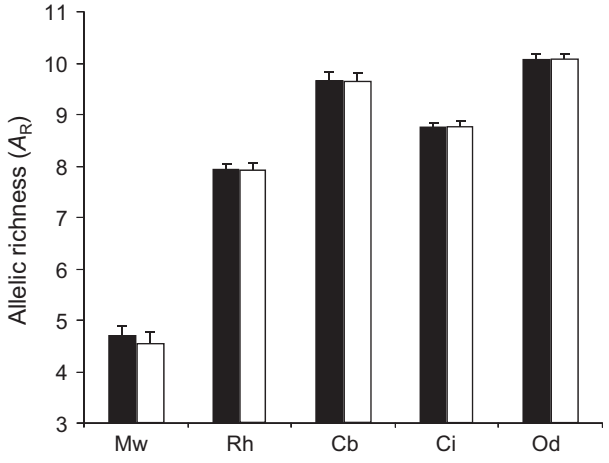

Fig. 3 Allelic richness (A_R) (mean ± SE) for each studied species (Mw = *Mioscirtus wagneri*; Rh = *Ramburiella hispanica*; Cb = *Calliptamus barbarus*; Ci = *Calliptamus italicus*; Od = *Oedaleus decorus*), including all populations (black bars) or only the 10 populations where all the taxa were sampled (white bars).

Genetic structure

Global F_{ST} values were significantly higher in specialist Mw than in all the other studied species (nonoverlapping 95% CI), but did not differ among Rh, Cb, Ci and Od (Fig. 4a). Considering only the 10 localities where all the taxa were collected, global F_{ST} values decreased in the order Mw ($F_{ST} = 0.055$, 95% CI: 0.044–0.066) > Rh ($F_{ST} = 0.017$, 95% CI: 0.012–0.023) > Od ($F_{ST} = 0.015$, 95% CI: 0.011–0.018) > Ci ($F_{ST} = 0.011$, 95% CI: 0.004–0.019) > Cb ($F_{ST} = 0.011$, 95% CI: 0.004–0.017) (Fig. 4a; see also Table S2, Supporting information, for pairwise F_{ST} values). Pairwise population comparisons provided analogous results (Fig. 4a; Table S2, Supporting information). STRUCTURE analyses considering all populations indicated a maximum

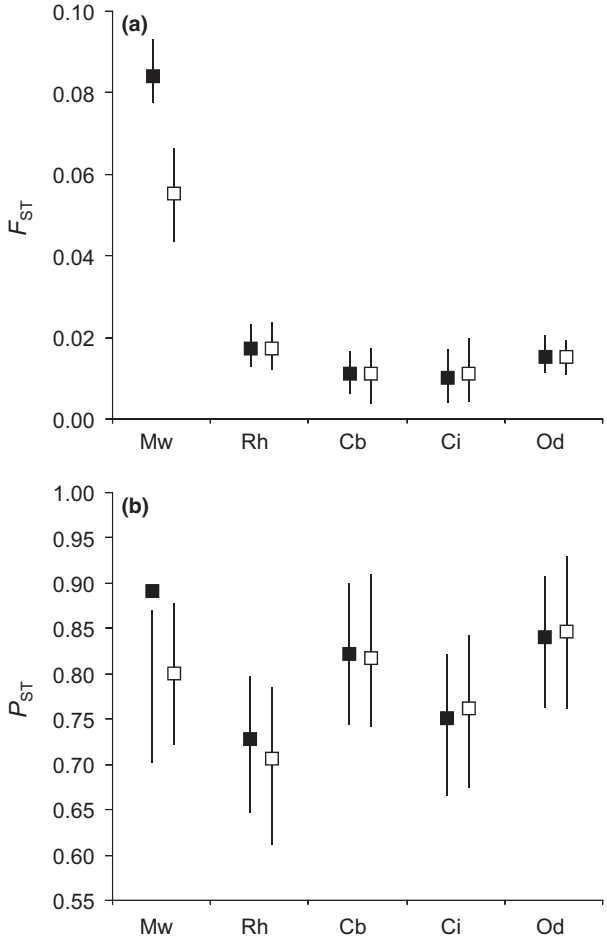

Fig. 4 Global (a) F_{ST} and (b) P_{ST} values and 95% confidence intervals for each studied species (Mw = *Mioscirtus wagneri*; Rh = *Ramburiella hispanica*; Cb = *Calliptamus barbarus*; Ci = *Calliptamus italicus*; Od = *Oedaleus decorus*), including all populations (black squares) or only the 10 populations where all the taxa were sampled (white squares).

value of $Pr(X|K)$ for $K = 5$ in Mw, $K = 3$ in Rh and Cb, and $K = 1$ in Ci and Od (Fig. 2). The Evanno *et al.* (2005) method indicated an optimal value of $K = 2$ for Mw and $K = 3$ for Rh and Cb (Fig. 2). STRUCTURE analyses considering only the 10 localities where all the taxa were collected, indicated an optimal value of $K = 3$ for Mw, $K = 2$ for Rh and Cb and $K = 1$ for Ci and Od.

Concordance of spatial genetic structure across species

Genetic and geographical distances were positively correlated in Mw ($r = 0.674$, $P < 0.001$), Rh ($r = 0.320$, $P = 0.017$) and Ci ($r = 0.303$, $P = 0.022$), but not in Cb ($r = 0.138$, $P = 0.177$) or Od ($r = 0.166$, $P = 0.128$). Mantel tests showed that genetic distance matrices were correlated between Mw and Ci ($P = 0.0048$), Rh and Ci ($P = 0.0026$), and Cb and Od ($P = 0.0017$) after controlling for multiple testing. However, only the correlation between genetic distance matrices of Mw and Ci remained significant after controlling for geographical distances in partial Mantel tests ($P = 0.004$; Table 2a). Procrustes rotations on the PCA matrices and PROTEST analyses showed no significant correlation of population allele frequencies in any species pair (all Ps > 0.1 and all $m^2 > 0.6$; Table 2c).

Table 2 Correlation coefficients (r) for Mantel test between (a) genetic (F_{ST}) and (b) phenotypic distance (P_{ST}) matrices of each species pair (below the diagonal) and for partial Mantel test controlling for geographical distance (above the diagonal); (c) Procrustes sum of squares (m^2) from PROTEST analyses

	Mw	Rh	Cb	Ci	Od
(a) Mantel and partial Mantel tests (r) for F_{ST}					
Mw	—	−0.083	0.125	**0.268**	0.040
Rh	0.158	—	−0.126	0.329	0.265
Cb	0.184	−0.075	—	0.004	0.422
Ci	**0.394**	**0.395**	0.046	—	0.281
Od	0.141	0.301	**0.435**	0.315	—
(b) Mantel and partial Mantel tests (r) for P_{ST}					
Mw	—	0.142	0.320	0.036	0.001
Rh	0.126	—	0.146	−0.078	0.112
Cb	0.284	0.162	—	0.017	0.121
Ci	0.025	−0.070	0.031	—	0.234
Od	0.026	0.091	0.080	0.216	—
(c) PROTEST analyses (m^2)					
Mw	—				
Rh	0.854	—			
Cb	0.894	0.704	—		
Ci	0.920	0.639	0.731	—	
Od	0.608	0.942	0.874	0.833	—

Values in bold are statistically significant after sequential Bonferroni correction ($\alpha = 0.05$).

Phenotypic divergence

All species showed very high levels of phenotypic differentiation (Fig. 4b; see Table S2, Supporting information). Global P_{ST} values did not differ among species (overlapping 95% CI; Fig. 4b). P_{ST} values were not correlated with genetic (F_{ST}) or geographical distance matrices in any species (all $r < 0.20$, all Ps > 0.11). Similarly, P_{ST} and F_{ST} distance matrices were not correlated in any species after controlling for geographical distance in partial Mantel tests (all $r < 0.07$, all Ps > 0.32). Considering only the 10 localities where all the taxa were collected, we found that P_{ST} values were not correlated between any species pair after sequential Bonferroni correction. No comparison was significant after controlling for geographical distances in partial Mantel tests (all Ps > 0.05; Table 2b). After controlling for multiple testing, average population femur length was correlated only between Mw and Cb ($r = 0.805$, $P = 0.005$). Finally, variance in population femur length differed significantly among species (Levene's test: $F_{4,45} = 7.87$, $P < 0.001$) and post hoc analyses indicated that significant pairwise comparisons involved Mw–Rh ($P = 0.025$), Mw–Od ($P = 0.001$), Rh–Od ($P = 0.016$), Cb–Od ($P = 0.013$) and Ci–Od ($P = 0.003$). In all comparisons, Od had higher variance in femur length than the other species and Rh had higher variance than Mw.

Discussion

Our analyses supported the hypothesis predicting that species with preferences for highly fragmented microhabitats show stronger patterns of genetic structure, harbour lower levels of within-population genetic diversity and have higher variance of among-population genetic diversity than codistributed generalist taxa inhabiting a continuous matrix of suitable habitat. This pattern was particularly marked for the small and highly specialist Mw, which inhabits extremely fragmented habitats and probably has a scarce capacity to disperse among isolated patches of suitable habitat (Fig. 1a). However, we did not find support for the hypothesis predicting that phenotypic divergence is more marked among species linked to highly fragmented microhabitats, neither did we find support for congruent patterns of phenotypic and genetic variability among any studied species, indicating that the studied taxa show idiosyncratic evolutionary (i.e. distinct patterns of phenotypic divergence) and demographic (i.e. contrasting levels of genetic diversity and structure) trajectories even though they share a common landscape.

Data on genetic structure indicate strong differences among taxa, with the specialist Mw showing a much higher genetic differentiation than the other species

studied (Fig. 4a). Mw is a small and highly specialist grasshopper that in the study area exclusively inhabits patches with shrubby sea-blite formations, the plant on which it depends exclusively for food (Cordero *et al.* 2007). These life history traits and the high fragmentation of its particular habitats are likely to have strongly limited interpopulation gene flow in this species and lead to strong genetic subdivision (King & Lawson 2001; Blanchet *et al.* 2010b; DiLeo *et al.* 2010; Lange *et al.* 2010; Keller *et al.* 2013b). The remarkable population genetic differentiation of Mw in contrast to the other species studied puts into a comparative context the deep genetic structure at landscape (Ortego *et al.* 2012a) and phylogeographic scales (Ortego *et al.* 2009) previously reported in this specialist grasshopper and highlights the extraordinary isolation of most of its populations. The other species studied here inhabit continuous habitats (Ci, Od), show a much lower degree of fragmentation of their specific habitats in the region (Rh, Cb) or have larger body/wing sizes (Rh, Cb, Ci, Od), factors that can explain their increased population connectivity and weak genetic differentiation (DiLeo *et al.* 2010; Lange *et al.* 2010). In the study area, the specialist grasshopper Rh inhabits semi-natural habitat patches occupied by two different host plant species (Ortego *et al.* 2015). These habitats also show a high fragmentation but are more widespread and connected than those occupied by Mw, which is exclusively restricted to small patches of saline and hypersaline lowlands (Ortego *et al.* 2012a, 2015). A higher habitat connectivity, together with the larger body size of Rh, can result in the actual level of habitat fragmentation being insufficient to strongly limit gene flow among populations (Lange *et al.* 2010; Keller *et al.* 2013b). This can explain why, contrary to our predictions, Rh shows a shallow genetic structure that is comparable to that reported in the generalist and more widespread studied species (Fig. 4a).

Explicit analyses to test congruent patterns of genetic structure have been employed in comparative phylogeography (e.g. Borer *et al.* 2012; Widmer *et al.* 2012), but such approaches have only rarely been used to compare the spatial distribution of genetic variation among codistributed species at the landscape scale (Fortuna *et al.* 2009). Our analyses of spatial congruence of genetic structure indicate that not only the degree of genetic differentiation, but also the spatial distribution of genetic variation strongly differs among the studied species. This incongruence between taxa may reflect differences in the spatial location of species-specific barriers to dispersal (Goldberg & Waits 2010; Frantz *et al.* 2012; Richardson 2012). However, the subtle genetic structure observed in most studied species is also likely to have strongly reduced the power to detect any concordance between population genetic distances or multivariate allele frequencies across the studied species. Contrary to our predictions, the species inhabiting highly fragmented natural habitats (Mw, Rh and Ci) did not show a significant spatial congruence in the distribution of genetic variation. Despite these three species having suffered a parallel drastic reduction of their suitable natural habitats, remnant nonagricultural lands and esparto grass formations occupied by Cb and Rh, respectively, are more common than the highly restricted habitats of Mw, which can explain the lack of congruence in the patterns of genetic differentiation among these codistributed species that a priori were expected to be severely impacted by habitat fragmentation (Ortego *et al.* 2012a, 2015).

Comparative analyses of genetic diversity indicate that the studied species also show contrasting responses to the different factors shaping within-population levels of genetic variability (Lange *et al.* 2010; Aparicio *et al.* 2012). In absolute terms, genetic diversity was lower in specialist than in generalist species (Fig. 3), which suggests that population fragmentation in the former (particularly in Mw) has resulted in higher genetic drift due to low local effective population sizes and more frequent population bottlenecks (Lange *et al.* 2010; Habel & Schmitt 2012; Keller *et al.* 2013b). In relative terms, we found that within-population levels of genetic diversity were not correlated across populations in any species pair. This suggests that gene flow, habitat fragmentation and local demographic dynamics affect each species in very different ways despite the fact that they share a common landscape (Lange *et al.* 2010; Aparicio *et al.* 2012). Mw also had higher variance in genetic diversity among populations than most of the other studied taxa, which indicates that the different populations of this species experience more contrasting demographic dynamics (Ortego *et al.* 2012a). The higher population connectivity in the other species (Figs 2 and 4a) may result in demographic changes (e.g. bottlenecks, and arrival of immigrants) that are only ephemerally reflected in local levels of variability due to the homogenizing effects of gene flow, and this leads to similar patterns of genetic diversity across all their populations (Lange *et al.* 2010).

Phenotypic divergence was comparably strong across all the studied taxa (global $P_{ST} > 0.7$; Fig. 4b), but was not correlated with population genetic divergence or geographical distances in any species. This implies that body size is not merely controlled by gene flow and drift and points to an important role of local adaptation in determining interpopulation differences in the studied trait (Leinonen *et al.* 2006; Lehtonen *et al.* 2009; Lowe *et al.* 2012). Phenotypic divergence was not

correlated between any pair of species, indicating that they do not show convergent evolutionary responses to their common environment (Lowe *et al.* 2012; Ingley *et al.* 2014). The contrasting body sizes and life histories of the studied species may be the result of different selective pressures brought about by contrasting communities of predators and interspecific interactions (Basolo & Wagner 2004; Berger *et al.* 2006; Ingley *et al.* 2014). Thus, different ecological pressures causing selection are likely to have decoupled the evolutionary responses of the different studied species (Lowe *et al.* 2012; Richardson *et al.* 2014).

Conclusions and implications for conservation

Our study highlights that habitat fragmentation can have very different demographic and evolutionary consequences even among closely related organisms (Short & Caterino 2009; Olsen *et al.* 2011). The studied generalist species inhabiting more continuous habitats (Ci and Od) present a low degree of genetic differentiation and, contrary to our hypothesis, these patterns are similar in absolute terms to those found in some taxa experiencing a high degree of habitat fragmentation in the study area (Rh and Cb; Fig. 4a). Only Mw shows a much higher degree of genetic differentiation than the other taxa (Fig. 4a), which suggests that only the extreme habitat fragmentation experienced by this species is above the threshold that remarkably disrupts interpopulation gene flow and considerably reduces local levels of genetic diversity. Our results support previous studies suggesting that basic data on life history traits and habitat specialization and fragmentation can help to anticipate species demographic responses and patterns of genetic divergence (DiLeo *et al.* 2010; Lange *et al.* 2010; Keller *et al.* 2013b; Phillipsen *et al.* 2015), but they also indicate that it is complicated to obtain accurate predictions about the degree of habitat fragmentation beyond of which population genetic structure and diversity are affected due to complex interactions among multiple influential factors (Lange *et al.* 2010; Callens *et al.* 2011; Keller *et al.* 2013b).

Our multispecies comparative approach can help to (i) determine baseline levels of genetic and phenotypic variation for taxa that are expected to maintain well connected populations (e.g. high-mobility and generalist species with a low degree of habitat fragmentation), (ii) identify the most (e.g. Mw) and least vulnerable (e.g. Rh and Cb) species among those that have experienced a considerable fragmentation of their respective habitats, and (iii) focus future research efforts on other taxa that may be affected by similar threats to those species with which they share similar habitats and life history traits and that have been identified to be more vulnerable (e.g. low-mobility species linked to hypersaline lowlands; Cordero & Llorente 2008). In view of our results, we suggest that biodiversity conservation in networks of protected areas requires detailed ecological and evolutionary information on several taxa with different habitat requirements and life history traits to identify target species that are more sensitive to the effects of habitat fragmentation and would gain more benefits from management practices aimed to improve population connectivity, increase the size and quality of appropriate habitat within each fragment, and maintain the idiosyncratic evolutionary trajectories of those populations presenting strong local adaptations (Rouget *et al.* 2006; Ouborg *et al.* 2010; Habel & Schmitt 2012; Habel *et al.* 2013). In more general terms, our multispecies comparative study offers a useful approach to identify the proximate causes of genetic and phenotypic variation in natural populations and can guide future research aimed to assess the impacts of habitat fragmentation across multiple codistributed species for which little information is available and that may show very different responses to the alterations affecting their common landscape. Overall, our study highlights the importance of inferring the evolutionary and demographic processes behind genetic and phenotypic patterns and offers a comprehensive framework to identify the mechanistic factors that may be compromising the long-term viability of natural populations and, ultimately, develop conservation agendas putting into practice the most efficient management solutions.

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J.O. and P.J.C. conceived the study. J.O. and V.G.-N. designed the study and analysed the data. J.O. wrote the manuscript. J.O., V.N. and P.J.C. collected the samples.

Data accessibility

DNA sequences: GenBank Accession nos. KT380945–KT380946.

Phenotypic and genotypic data; DNA sequence alignments; phylogenetic tree file; input files for STRUCTURE

analyses; input files for Mantel tests in ZT software: Dryad doi: 10.5061/dryad.3nr2f.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Microsatellite loci used to genotype each studied species.

Table S2 Pairwise population F_{ST} and P_{ST} values for each studied species.