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BIODIVERSITY RESEARCH

Landscape genetics of a specialized grasshopper inhabiting highly fragmented habitats: a role for spatial scale

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ABSTRACT

Aim The study of geographical discontinuities in the distribution of genetic variability in natural populations is a central topic in both evolutionary and conservation research. In this study, we aimed to analyse (1) the factors associated with genetic diversity at the landscape spatial scale in the highly specialized grasshopper *Mioscirtus wagneri* and (2) to identify the relative contribution of alternative factors to the observed patterns of genetic structure in this species.

Location La Mancha region, Central Spain.

Methods We sampled 28 populations of the grasshopper *M. wagneri* and genotyped 648 individuals at seven microsatellite loci. We employed a causal modelling approach to identify the most influential variables associated with genetic differentiation within a multiple hypothesis-testing framework.

Results We found that genetic diversity differs among populations located in different river basins and decreases with population isolation. Causal modelling analyses showed variability in the relative influence of the studied landscape features across different spatial scales. When a highly isolated population is considered, the analyses suggested that geographical distance is the only factor explaining the genetic differentiation between populations. When that population is excluded, the causal modelling analysis revealed that elevation and river basins are also relevant factors contributing to explaining genetic differentiation between the studied populations.

Main conclusions These results indicate that the spatial scale considered and the inclusion of outlier populations may have important consequences on the inferred contribution of alternative landscape factors on the patterns of genetic differentiation even when all populations are expected to similarly respond to landscape structure. Thus, a multiscale perspective should also be incorporated into the landscape genetics framework to avoid biased conclusions derived from the spatial scale analysed and/or the geographical distribution of the studied populations.

Keywords
Causal modelling, genetic diversity, genetic structure, historical barriers, landscape genetics, population fragmentation.

INTRODUCTION

The study of the distribution of genetic variability in natural populations is a central topic in both evolutionary and conservation biology (Lande, 1988; Frankham, 1996, 2003; Hedrick, 2001). Several factors can determine the degree of genetic structure observed in natural populations, including natural and sexual selection, the dispersal abilities of organisms, and the presence of historical and/or current barriers to gene flow (Cushman *et al.*, 2006; Ortego *et al.*, 2008; Wang & Summers, 2010). Population fragmentation is expected to particularly reduce gene flow in organisms with limited dispersal

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abilities and showing specific requirements for certain habitats which are geographically restricted and scattered in the landscape (Wang, 2009). Reduced gene flow may in turn decrease the genetic diversity of small populations, a process which can ultimately lead to the erosion of species evolutionary potential and reduce the long-term persistence of natural populations (Saccheri *et al.*, 1998; Frankham, 2005; Pertoldi *et al.*, 2007; Ortego *et al.*, 2009a). Thus, gene flow can have a profound influence on both the evolutionary trajectories of populations and their persistence in time and space (Wiens, 1997).

One of the main objectives in conservation genetics is identifying the factors determining population genetic structure to establish management practices aimed to preserve the genetic identity of populations while minimizing the risks associated with reduced gene flow (Hedrick, 2001). Landscape genetics is a relatively recent discipline conceived as a combination of landscape ecology and population genetics to study the influence of environmental and landscape features on gene flow (Manel *et al.*, 2003; Sork & Waits, 2010; see also Wang *et al.*, 2009). Landscape genetics can help to infer how individuals move across the landscape, particularly for organisms that are difficult to track using traditional capture–mark–recapture methods (Wang *et al.*, 2009). This discipline also offers valuable tools to identify cryptic historical barriers to gene flow that may have contributed to determining current patterns of population genetic structure (Pease *et al.*, 2009; Coulon *et al.*, 2010). Thus, landscape genetics can help to understand the environmental factors determining genetic discontinuities at different spatiotemporal scales, inferring contemporary individual movement (e.g. Cushman *et al.*, 2006) and identifying historical barriers shaping population divergence (e.g. Manel *et al.*, 2003; Pease *et al.*, 2009). This information can ultimately help to define management areas and evaluate the potential impacts of landscape disturbance on long-term species persistence (Leclerc *et al.*, 2008).

Here, we study the factors associated with genetic diversity and structure at the landscape scale in the grasshopper *Mioscirtus wagneri* (Kittary, 1859). *Mioscirtus wagneri* is a highly specialized organism exclusively inhabiting hypersaline low grounds with patches of *Suaeda vera*, the halophilic plant on which it depends for food (Cordero *et al.*, 2007). This species shows a highly fragmented distribution, and most of their populations have persisted in highly isolated and small pockets of suitable habitat (Ortego *et al.*, 2009b, 2010). These habitats are located along different river basins, generally around endorheic saline lagoons which in several cases are the remains of old river beds (Peinado, 1994; Valero-Garcés *et al.*, 2000). For this reason, the genetic structure of their populations is likely to be a consequence of both contemporary and historical processes that can be potentially identified using a landscape genetic approach (Manel *et al.*, 2003; Pease *et al.*, 2009; Sork & Waits, 2010).

To study the factors associated with the spatial genetic structure and variability in the grasshopper *M. wagneri*, we have sampled all currently known populations ($n = 28$) of this species in La Mancha region, central Spain, and typed 648 individuals at seven microsatellite loci. First, we study which landscape features are associated with genetic structure in this species to test the following predictions: (1) because of the distribution of the particular habitats used by this species, we predict that lowland areas along rivers have acted as corridors between populations, and this has led to higher gene flow within than between river basins; (2) if dispersal mainly occurs across lowland areas, we also expect that gene flow is higher within areas with similar elevational ranges. We also explore whether the relative contribution of these factors to genetic structure is stable over different spatial scales involving populations with contrasting degrees of genetic differentiation (Anderson *et al.*, 2010; Cushman & Landguth, 2010). To address these questions, we employ a causal modelling approach that uses partial Mantel tests between genetic and landscape resemblance matrices to identify the most influential variables associated with genetic structure within a multiple hypothesis-testing framework (Cushman *et al.*, 2006; Cushman & Landguth, 2010). Second, we study which factors contribute to explain the levels of genetic diversity observed in the studied populations. In particular, we expect (3) that genetic diversity is lower in more isolated populations showing reduced gene flow with other populations and (4) in basins including a small number of populations.

METHODS

Sampling and study area

During 2006–09, we sampled 28 populations of *M. wagneri* in La Mancha region, Central Spain. We are confident that these sampling localities cover all the populations of *M. wagneri* in La Mancha region, as several other potentially adequate habitats (i.e. saline/hypersaline lagoons and low grounds) have been extensively prospected without any record of the species. We collected 17–54 adult individuals per population, and the specimens were preserved whole in 1500 μL of 96% ethanol at −20 °C until needed for genetic analyses. Population code descriptions and further information on sampling localities are given in Table 1 and Fig. 1.

Microsatellite genotyping and basic statistics

We genotyped individuals using seven polymorphic microsatellite markers isolated and characterized from a genomic library of a *M. wagneri* specimen (Table 2; Aguirre *et al.*, 2010). We used NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) kits to extract and purify genomic DNA from a hind leg of each individual. 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 $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, NED or VIC) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf, Hamburg, Germany) thermal cycler. The PCR programme

Table 1 Geographical location and sample size of the 28 studied populations of *Mioscirtus wagneri* in hypersaline lagoons and saline grounds in La Mancha region.

Locality	*N*	Code	Latitude	Longitude	River basin	
					Main	Secondary
Saladar de Ocaña	20	OCA	39°58′59″ N	3°38′02″ W	Tajus	Tajus
Saladar de Huerta	20	HUE	39°50′02″ N	3°37′15″ W	Tajus	Tajus
Saladar de Villasequilla	20	VIL	39°53′06″ N	3°44′31″ W	Tajus	Tajus
Laguna del Cerrillo	20	CER	39°42′08″ N	3°18′13″ W	Guadiana	Cigüela
Laguna del Altillo	20	ALT	39°41′36″ N	3°18′06″ W	Guadiana	Cigüela
Laguna de Longar	20	LON	39°41′53″ N	3°19′25″ W	Guadiana	Cigüela
Laguna de la Albardiosa	20	ALB	39°39′56″ N	3°17′28″ W	Guadiana	Cigüela
Laguna de Navarredonda	21	NAV	39°38′40″ N	3°14′28″ W	Guadiana	Cigüela
Saladar de La Villa de Don Fadrique	54	FAD	39°38′02″ N	3°13′58″ W	Guadiana	Cigüela
Laguna Larga	20	LAR	39°36′16″ N	3°18′57″ W	Guadiana	Cigüela
Laguna de Tírez	20	TIR	39°32′21″ N	3°21′27″ W	Guadiana	Cigüela
Laguna de Peña Hueca	20	PEN	39°30′50″ N	3°20′29″ W	Guadiana	Cigüela
Laguna de Quero	43	QUE	39°29′58″ N	3°15′32″ W	Guadiana	Cigüela
Saladar de La Sangría	20	SAN	39°27′24″ N	3°18′08″ W	Guadiana	Cigüela
Laguna de Los Carros	20	CAR	39°28′14″ N	3°15′42″ W	Guadiana	Cigüela
Laguna de Pajares	21	PAJ	39°27′16″ N	3°12′21″ W	Guadiana	Cigüela
Laguna de Villafranca	20	VFR	39°28′21″ N	3°20′29″ W	Guadiana	Cigüela
Laguna de la Sal	20	LSA	39°26′15″ N	3°20′05″ W	Guadiana	Cigüela
Laguna de las Yeguas	20	YEG	39°25′12″ N	3°17′02″ W	Guadiana	Cigüela
Laguna de Cerro Mesado	20	CME	39°19′40″ N	3°16′18″ W	Guadiana	Cigüela
Laguna de Palomares	20	PAL	39°32′13″ N	3°10′02″ W	Guadiana	Cigüela
Laguna de La Laguna	20	LAG	39°32′21″ N	3°08′07″ W	Guadiana	Cigüela
Laguna de Salicor	52	SCO	39°27′56″ N	3°10′36″ W	Guadiana	Cigüela
Saladar de El Pedernoso	20	PED	39°29′21″ N	2°45′59″ W	Guadiana	Saona
Laguna de Alcahozo	20	ALC	39°23′23″ N	2°52′38″ W	Guadiana	Saona
Laguna de Manjavacas	18	MAN	39°24′26″ N	2°52′15″ W	Guadiana	Saona
Saladar de Las Mesas	22	MES	39°22′44″ N	2°46′16″ W	Guadiana	Saona
Las Tablas de Daimiel	17	DAI	39°10′03″ N	3°40′28″ W	Guadiana	Guadiana

used was 9-min denaturation at 95 °C followed by 35 cycles of 30 s at 94 °C, 45 s at the annealing temperature (Aguirre *et al.*, 2010) and 45 s at 72 °C, ending with a 5-min final elongation stage at 72 °C. Amplification products were run on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the genotypes were scored using GeneMapper 3.7 (Applied Biosystems).

Microsatellite genotypes were tested for departure from Hardy–Weinberg equilibrium within each population at each locus 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 equilibrium within each pair of loci and population using a likelihood-ratio statistic, whose distribution was obtained by a permutation procedure (Excoffier *et al.*, 2005).

Population genetic structure

We investigated population genetic structure among sampling locations by 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). To visualize the genetic relationship between the sampling localities, we performed a multivariate ordination using Pcagen (http://www2.unil.ch/popgen/softwares/pcagen.htm) with 10,000 randomization steps. We also analysed the spatial genetic structure using an individual-based approach as implemented in the program Structure (version 2.3.3; Pritchard *et al.*, 2000; Falush *et al.*, 2003; Hubisz *et al.*, 2009). The program Structure 2.3.3 is a Bayesian model-based clustering method which assigns individuals to populations based on their multilocus genotypes (Pritchard *et al.*, 2000; Falush *et al.*, 2003). For K population clusters, the program estimates the probability of the data $[\Pr(X|K)]$ and the probability of individual membership in each cluster using a Markov chain Monte Carlo (MCMC) method. We ran Structure assuming correlated allele frequencies and admixture (Pritchard *et al.*, 2000; Falush *et al.*, 2003) and using prior population information (Hubisz *et al.*, 2009). We conducted five independent runs for each value of K to estimate the true number of clusters with 10^6 MCMC cycles, following a burn-in period of 100,000 iterations. The simulated values of K ranged from 1 to 20. The number of populations best fitting the data set was defined using both log probabilities $[\Pr(X|K)]$ and ΔK, as described in the study by Evanno *et al.* (2005).

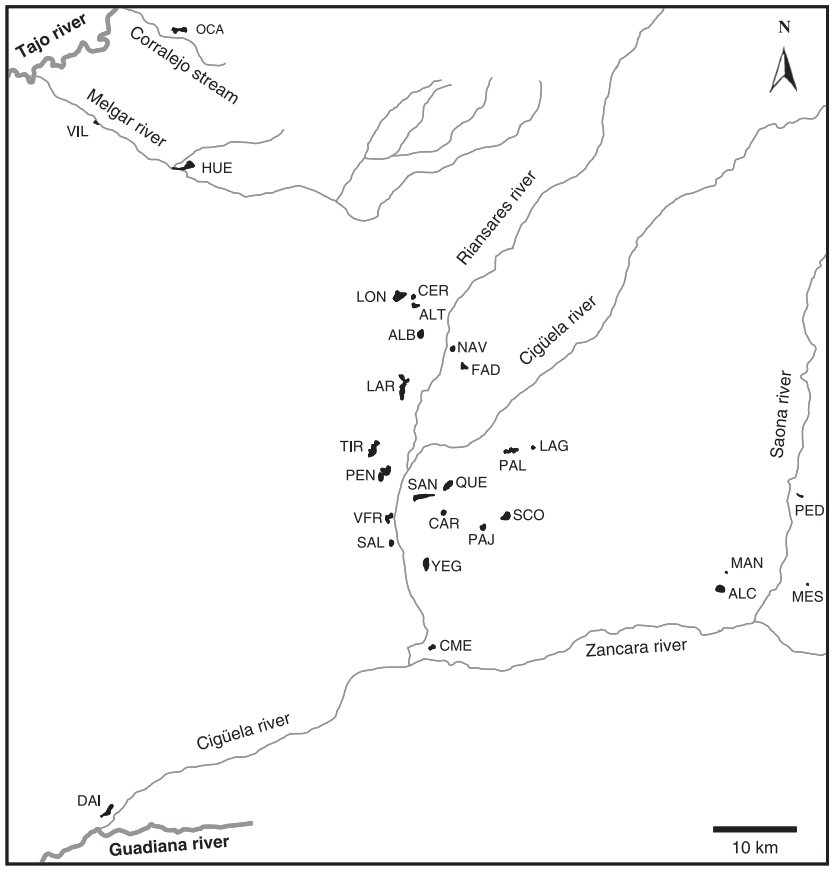

Figure 1 Map showing the spatial location of the saline/hypersaline patches (black areas) and main rivers (grey lines) in the study area. Population codes are described in Table 1.

Table 2 Microsatellite loci used to type the grasshopper *Mioscirtus wagneri*: number of alleles (K), allele size range in base pairs (bp), expected heterozygosity (H_E) and observed heterozygosity (H_O).

Locus	K	Allele size range (bp)	H_E	H_O
MwGTC8	19	140–176	0.80	0.74
MwGTD9	11	121–141	0.68	0.61
MwGTG12	19	415–477	0.74	0.69
MwGTA6	21	224–264	0.77	0.52
MwGTC12	13	297–390	0.62	0.67
MwGTC11	18	136–173	0.68	0.64
MwGATAB11	35	348–396	0.88	0.77

Genetic diversity

We used two metrics to estimate individual heterozygosity: (1) uncorrected heterozygosity (H_O), calculated as the proportion of loci at which an individual is heterozygous; (2) homozygosity by loci (*HL*), a microsatellite derived measure that improves heterozygosity estimates in most natural populations (Aparicio *et al.*, 2006). H_O and *HL* were calculated using Cernicalin, an Excel spreadsheet available on request. We analysed the patterns of individual heterozygosity using generalized linear mixed models (GLMMs) with a normal error structure and an identity link function implemented with the GLIMMIX macro of SAS 9.2 (SAS Institute, 2004). GLMMs allow analyses of data where the response variable is determined by both random and fixed effects. We considered four explanatory variables in the GLMMs: (1) population isolation, estimated as the average genetic differentiation of each population with all other populations in the study area (estimated on the basis of pairwise F_{ST} values reported in Table S1 in Supporting Information); (2) elevation; (3) the location of the population considering the main river basins in the study area; (4) the location of the population considering the secondary river basins in the study area. Average population differentiation and elevation were fitted as covariates. The main and secondary river basins were included as fixed factors always in separate models including different sets of the other explanatory variables. We fitted population identity as a random effect, allowing us to control for the potential non-independence of individual heterozygosity within sampling localities (Singer, 1998; Bolker *et al.*, 2009). The most parsimonious models were selected as having the lowest corrected Akaike's information criterion (AIC_c; Burnham & Anderson, 1998).

Landscape genetics and causal modelling

We considered the following four potential factors that could have influenced genetic differentiation between the studied populations of *M. wagneri*: (1) the geographical distance; (2)

the difference in population elevation; and the location of the population considering the (3) main and (4) secondary river basins in the study area (Table 1; Fig. 1). We calculated the matrices of Euclidean geographical distances and difference in elevation between pairs of sampling localities. We also calculated categorical matrices with 0s and 1s for pairs of sampling localities located in the same or different (main or secondary) river basin, respectively (e.g. Leclerc *et al.*, 2008; Quéméré *et al.*, 2010).

We employed a causal modelling approach to determine the environmental factors associated with genetic differentiation in the study system (Legendre, 1993; e.g. Cushman *et al.*, 2006; Richards-Zawacki, 2009; Wang & Summers, 2010; Quéméré *et al.*, 2010). Causal modelling uses a series of partial Mantel tests (Smouse *et al.*, 1986) to identify the landscape organizational model (i.e. combination of environmental factors) that best explains genetic differentiation. We considered 15 organizational models corresponding to all the patterns of causality among the four variables described previously (Table 3). Each model describes a hypothesis in which certain factors are associated with gene flow and carry a set of statistical predictions. A model is supported only if the entire set of hypotheses is supported (Legendre & Troussellier, 1988; Legendre, 1993; e.g. Wang & Summers, 2010; Quéméré *et al.*, 2010). We first computed simple and partial Mantel tests between the genetic distance matrix and the matrices corresponding to the different factors. Then, we compared the significant and non-significant tests to the expectations under the 15 organizational models (Table 3). All Mantel tests were performed using zt software with 10,000 permutations (Bonnet & Van de Peer, 2002; e.g. Wang & Summers, 2010).

RESULTS

Significant departures from HWE were observed in seven of 196 population–locus combinations after adjusting for multiple comparisons using sequential Bonferroni corrections (Rice, 1989). Such deviations involved the loci MwGTD9 (OCA and VIL localities), MwGTA6 (HUE and QUE localities) and MwGTC12 (FAD, YEG and DAI localities). We only found evidence of linkage disequilibrium between some pair of loci in FAD (MwGTG12-MwGTC12 and MwGTA6-MwGTC12), PAJ (MwGTD9-MwGTC12) and PAL (MwGTA6-MWGATAB 11) localities. Evidence of linkage disequilibrium and departures from HWE was not consistent across loci/populations. Thus, we used all genotypic data in subsequent analyses to avoid problems derived from considering different panels of microsatellite markers in analyses involving the different studied populations.

Genetic structure

Population scores from principal component analysis (PCA) were plotted on two axes (PC1 and PC2), which cumulatively explained 39.7% of the total genetic variability (PC1: 27.2%, $P < 0.001$; PC2: 12.5%, $P = 0.297$; Fig. 2). The eigenvalues of the first two principal axes were low (PC1: 0.13; PC2: 0.06), indicating that they are not explaining much of the observed genetic variability. Pairwise F_{ST} values indicated moderate to high levels of genetic differentiation, particularly between populations located in different river basins (see Table S1). On average, F_{ST} values between populations located within the same main basin (Tajus or Guadiana Rivers) were lower (mean = 0.06; range = −0.011 to 0.309) than the observed between populations located in different basins (mean = 0.13; range = 0.019–0.258). Similarly, F_{ST} values between populations located within the same secondary basin (Tajus, Cigüela, Saona or Guadiana) were lower (mean = 0.03; range = −0.011 to 0.191) than the observed between populations located in different secondary basins (mean = 0.116; range = 0.015–0.309). Structure analyses revealed an optimal clustering into 3–5 distinct groups (Fig. 3). Overall, the genetic structure of the study populations shows this general pattern: (1) the populations located in the Tajus river basin form a genetic cluster, although the VIL locality shows high levels of admixture with the populations located in the Cigüela river basin; (2) the populations located in Cigüela-Saona river basins present a high level of admixture and a north-west to south-east cline of genetic differentiation; (3) the isolated DAI locality shows high levels of differentiation and is genetically closer to the populations located in the Cigüela river basin than to those located in the Saona basin (Fig. 3; see also Table S1).

Genetic diversity

According to AIC_c, the model including the average genetic differentiation and the main river basin received the highest support considering any of the two heterozygosity estimates (i.e. H_O or *HL*) (Table 4; Figs 4 & 5). The next model with the highest support was that including the average genetic differentiation and the secondary river basin (Table 4; Figs 4 & 5). Post hoc Tukey tests showed that individual genetic diversity was significantly different between Tajus and Cigüela (H_O: $P = 0.010$; *HL*: $P = 0.017$), Tajus and Saona (H_O: $P < 0.001$; *HL*: $P < 0.001$), Cigüela and Saona (H_O: $P = 0.002$; *HL*: $P = 0.005$). However, there were no differences in heterozygosity between the single population located in the Guadiana basin and any of the other population groups ($P > 0.3$ in all cases; Fig. 5b). All other models had a difference in the AIC_c value > 3 and were thus not further considered in the discussion (Table 4; Burnham & Anderson, 1998).

Landscape genetics and causal modelling

When all the sample sites were considered, simple Mantel tests for the four analysed factors were highly significant ($P < 0.001$) and showed correlation values ranging between 0.46 and 0.77 (Fig. 6). The higher correlation values were obtained for the Euclidean distance between populations (Fig. 6). Causal modelling analyses based on partial Mantel

Table 3 Results from causal modelling analyses.

			Organizational models														
Partial Mantel test	*r*	*P*-values	1 D	2 E	3 M	4 S	5 D + E	6 D + M	7 D + S	8 E + M	9 E + S	10 M + S	11 D + E + M	12 D + E + S	13 D + M + S	14 E + S + M	15 D + E + S + M
(a) All populations																	
DG.E	+0.65 <	**0.001**	S	NS			S	S	S	NS	NS		S	S	S	NS	S
DG.M	+0.70 <	**0.001**	S		NS		S	S	S	NS		NS	S	S	S	NS	S
DG.S	+0.51 <	**0.001**	S			NS	S	S	S		NS	NS	S	S	S	NS	S
EG.D	+0.13	0.019	NS	S			S	NS	NS	S	S		S	S	NS	S	S
EG.M	+0.38 <	**0.001**		S	NS		S	NS		S	S	NS	S	S	NS	S	S
EG.S	+0.31 <	**0.001**		S		NS	S		NS	S	S	NS	S	S	NS	S	S
MG.D	−0.01	0.558	NS		S		NS	S	NS	S		S	S	NS	S	S	S
MG.E	−0.10	0.958		NS	S		NS	S		S	NS	S	S	NS	S	S	S
MG.S	+0.17	**0.005**			S	NS		S	NS	S	NS	S	S	NS	S	S	S
SG.D	+0.13	0.013	NS			S	NS	NS	S		S	S	NS	S	S	S	S
SG.E	+0.54 <	**0.001**		NS		S	NS		S	NS	S	S	NS	S	S	S	S
SG.M	+0.58 <	**0.001**			NS	S		NS	S	NS	S	S	NS	S	S	S	S
Model support rate			1.00	0.50	0.17	0.33	0.80	0.60	0.70	0.30	0.50	0.30	0.58	0.75	0.58	0.42	0.67
(b) Excluding Daimiel (DAI) population																	
DG.E	+0.58 <	**0.001**	S	NS			S	S	S	NS	NS		S	S	S	NS	S
DG.M	+0.56 <	**0.001**	S		NS		S	S	S	NS		NS	S	S	S	NS	S
DG.S	+0.45 <	**0.001**	S			NS	S	S	S		NS	NS	S	S	S	NS	S
EG.D	+0.23	**0.001**	NS	S			S	NS	NS	S	S		S	S	NS	S	S
EG.M	+0.14	**0.008**		S	NS		S	NS		S	S	NS	S	S	NS	S	S
EG.S	+0.38 <	**0.001**		S		NS	S		NS	S	S	NS	S	S	NS	S	S
MG.D	+0.20	**0.007**	NS		S		NS	S	NS	S		S	S	NS	S	S	S
MG.E	+0.15	**0.008**		NS	S		NS	S		S	NS	S	S	NS	S	S	S
MG.S	+0.36 <	**0.001**			S	NS		S	NS	S	NS	S	S	NS	S	S	S
SG.D	+0.17	**0.007**	NS			S	NS	NS	S		S	S	NS	S	S	S	S
SG.E	+0.51 <	**0.001**		NS		S	NS		S	NS	S	S	NS	S	S	S	S
SG.M	+0.49 <	**0.001**			NS	S		NS	S	NS	S	S	NS	S	S	S	S
Model support rate			0.50	0.50	0.50	0.50	0.60	0.60	0.60	0.60	0.60	0.60	0.75	0.75	0.75	0.75	1.00
(c) Only populations within the Cigüela-Saona river basin																	
DG.E	+0.62 <	**0.001**	S	NS			S		S		NS			S			
DG.S	+0.13	0.033	S			NS	S		S		NS			S			
EG.D	−0.23	0.999	NS	S			S		NS		S			S			
EG.S	−0.04	0.756		S		NS	S		NS		S			S			
SG.D	+0.35 <	**0.001**	NS			S	NS		S		S			S			
SG.E	+0.65 <	**0.001**		NS		S	NS		S		S			S			
Model support rate			0.50	0.00	–	1.00	0.17	–	0.83	–	0.50	–	–	0.50	–	–	–

D, Euclidean distance; E, elevation; M, main river basin; S, secondary river basin.

The statistical predictions for each partial Mantel test for each model are indicated as S (expected to be significant) and NS (not expected to be significant). Boxes where a particular test is not applicable for a specific model are represented in black. For each Mantel test, a period separates the main matrices on the left from the covariate matrix on the right (e.g. DG.E tests for the correlation between D and G controlling for E). *P*-values and correlation coefficients (*r*) for Mantel tests are indicated. Bold type means *P*-values are statistically significant after sequential Bonferroni correction. The grey boxes are a visual help to identify tests where the *P*-value matched the expected result of the test for each model. The black boxes identify tests where the *P*-value did not match the expected result of the test for each model. The support rate for each model is the proportion of supported hypotheses in relation to the total number of tested hypotheses.

tests revealed that only the model including the Euclidian geographical distance was fully supported (Table 3a). We also tested the same 15 hypothetical organizational models excluding the geographically isolated population of Tablas de Daimiel (DAI). In this case, we found that the model including all the analysed factors was fully supported (Table 3b). More detailed analyses only considering the populations located within the Cigüela-Saona river basins showed that the model including the location of the population in the secondary river basin was fully supported, although we also found strong support for the model including both the secondary river basin and the Euclidian geographical distance (Table 3c).

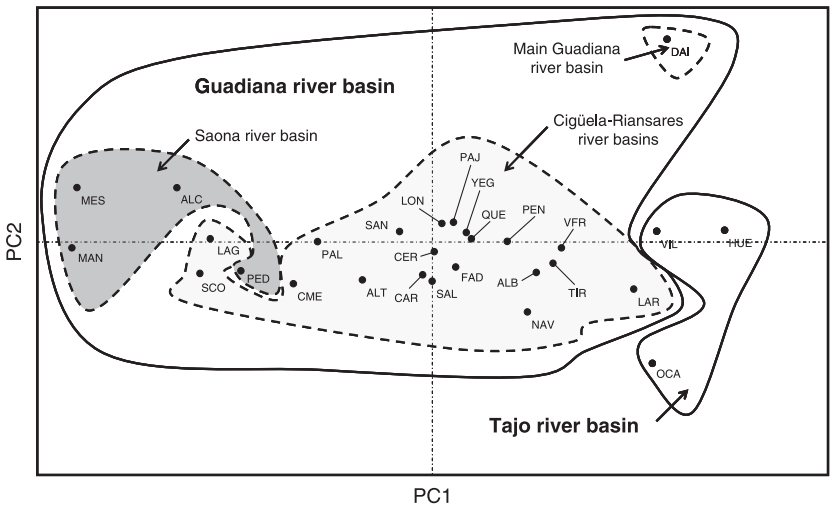

Figure 2 Principal component analysis (PCA) showing the genetic relationship between *Mioscirtus wagneri* sampling localities. Localities are grouped according to their main (Tajo, Guadiana; solid lines) and secondary (Tajo, Cigüela-Riansares, Saona, Guadiana; dashed lines) river basins. Only PC1 is statistically significant ($P < 0.001$).

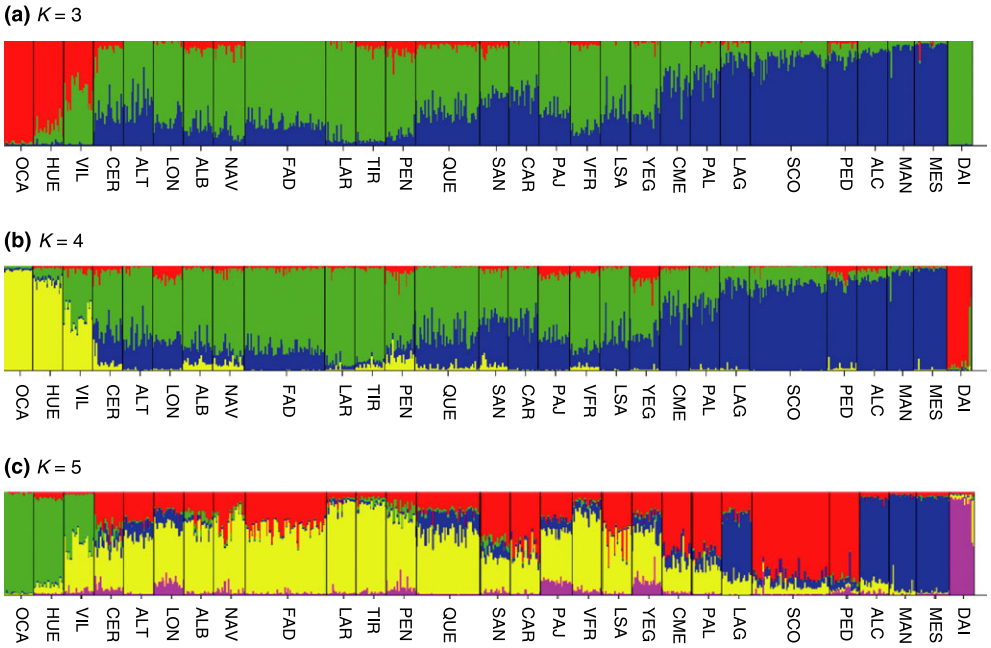

Figure 3 Results of genetic assignment based on Bayesian method implemented in the program STRUCTURE for (a) 3, (b) 4, and (c) 5 genetic clusters (K). Each individual is represented by a thin vertical line, which is partitioned into K-coloured segments that represent the individual's probability of belonging to the cluster with that colour. Black lines separate individuals from different populations. Population codes are described in Table 1.

DISCUSSION

We have found support to the prediction that river basins have acted as corridors between populations and that gene flow is higher within areas with similar elevational ranges. However, causal modelling analyses have also shown important variability in the relative influence of the studied landscape features across different spatial scales. When the highly isolated population of DAI is considered, the model only including the geographical distance between populations is fully supported (Table 3a). This indicates that geographical distance is the main factor determining the genetic differentiation between populations, suggesting an isolation-by-distance pattern of genetic structure (Slatkin, 1993; Hutchison & Templeton, 1999). However, when this highly differentiated population is excluded from the causal modelling analysis, we found that the model including all the studied factors is fully supported (Table 3b). DAI is a highly isolated population, showing a strong genetic differentiation with other populations and constituting a distinctive genetic cluster. Thus, its geographical isolation together with a remarkable genetic differentiation in this population may have contributed to reduce the relative importance and statistical signal of the other studied landscape features. These results suggest that the

Table 4 Model selection to assess the association between individual genetic diversity [estimated as homozygosity by loci (*HL*) and uncorrected heterozygosity (H_O)] and average genetic differentiation (F_{ST}) of each population with all other studied populations (G), elevation (E), and main (M) and secondary (S) river basin.

				H_O			*HL*		
Model no.	Model	*K*	*n*	AIC	AIC_c	Δi	AIC	AIC_c	Δi
1	M + G	2	648	−356.4	−356.3	0.0	−359.0	−359.0	0.0
2	S + G	2	648	−354.3	−354.3	2.0	−356.3	−356.2	2.8
3	G	1	648	−353.2	−353.1	3.2	−355.5	−355.5	3.5
4	S	1	648	−349.6	−349.6	6.7	−351.5	−351.5	7.5
5	E + G	2	648	−348.9	−348.9	7.4	−351.1	−351.1	7.9
6	E + M + G	3	648	−345.4	−345.4	10.9	−347.7	−347.6	11.4
7	E + S + G	3	648	−343.2	−343.2	13.1	−341.4	−341.4	17.6
8	M	1	648	−342.2	−342.2	14.1	−343.0	−343.0	16.0
9	E + S	2	648	−336.7	−336.7	19.6	−338.6	−338.5	20.5
10	E	1	648	−332.3	−332.3	24.0	−333.0	−333.0	26.0
11	E + M	2	648	−329.4	−329.3	27.0	−330.1	−330.0	29.0

AIC, Akaike's information criterion; AIC_c, corrected AIC value; Δi, difference in AIC_c value from that of the strongest model; *K*, number of parameters in the model; *n*, sample size.
Population identity was included as random effect in all the models.

spatial scale considered and the inclusion of outlier populations may have profound effects on the inferred patterns of gene flow even when all populations are expected to similarly respond to landscape structure (Leclerc *et al.*, 2008; see also Bull *et al.*, 2011).

After evaluating multiple competing hypotheses excluding the highly differentiated DAI population from causal modelling analyses, we found that gene flow was higher within than between main and secondary river basins. The studied populations are generally located around endorheic saline low grounds that in some cases are known to be old river beds or palaeocanals (Peinado, 1994; Valero-Garcés *et al.*, 2000). Currently, most of these habitats are still placed along active rivers, and these lowland areas may have acted as corridors favouring inter-population gene flow. This may also explain the significant effect of difference of elevation: populations located at different elevational ranges are expected to be separated by unfavourable habitats because saline and hypersaline habitats are usually located in low grounds where salt accumulation is favoured (Comin & Alonso, 1988; Valero-Garcés *et al.*, 2000). This result also supports previous studies indicating that elevation is an important obstacle for gene flow that produces population structure (Cushman *et al.*, 2006; Zalewski *et al.*, 2009). However, our study area is particularly flat and elevation of the study populations only ranges from 505 to 700 m. Thus, in the case of *M. wagneri*, the more important barrier to dispersal is expected to be the absence of adequate habitats in elevated areas rather than the topographic elevation or irregularity *per se*. This may also explain why we have not found support for the model including elevation at the lowest spatial scale only including the populations located within the Saona and Cigüela river basins (Table 3c). Elevation in this area only ranges from 638 to 700 m, and the very low variability of this parameter may explain the absence of significant statistical signal even if that variable is an important determinant of gene flow (Bull *et al.*, 2011).

Most of the studied populations are currently located in small pockets of suitable habitats highly isolated mainly because of the historical fragmented nature of inland saline environments in the study area (Peinado, 1994). Thus, the inferred pattern of genetic structure at the larger spatial scale considered (i.e. main/secondary river basins) is expected to reflect past gene flow rather than current dispersal movements that may be very infrequent considering that habitat connectivity between most studied populations is generally absent (Fig. 1) and that the dispersal potential of this species is very low. *Mioscirtus wagneri* virtually disappears a few metres beyond the patches where its host plant *S. vera* is present (P.J. Cordero, unpublished data), and a recent study carried out at a very fine spatial scale has also revealed restricted dispersal of this species within two populations only extending over < 2.5 km (Ortego *et al.*, 2011). Genetic structure is expected to have a substantial time-lag in its response to changes in gene flow, and we hypothesize that subtle genetic differentiation among some close populations may also be reflecting pre-fragmentation dispersal rather than contemporary gene flow (Cushman *et al.*, 2006). Most populations of this grasshopper show high local population sizes (> 1000 individuals; P.J. Cordero, unpublished data) which is expected to strongly decrease the rate of genetic divergence and may also have contributed to reduce differentiation at neutral markers among populations with scarce or disrupted gene flow (Wright, 1943; see also Cushman *et al.*, 2006). Alternatively, the low genetic differentiation observed between close populations could also have resulted from contemporary dispersal movements across unsuitable habitat patches that may be difficult to record if only a few individuals per generation are involved in inter-population gene flow.

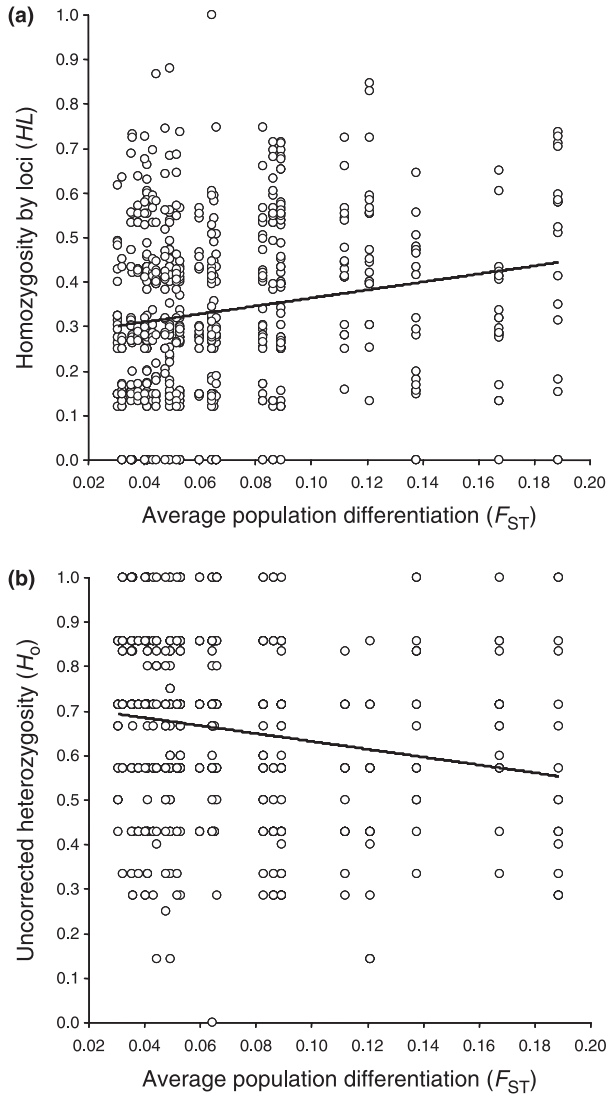

Figure 4 Relationship between (a) uncorrected heterozygosity (H_O) and (b) homozygosity by loci (*HL*) and average population differentiation (F_{ST}).

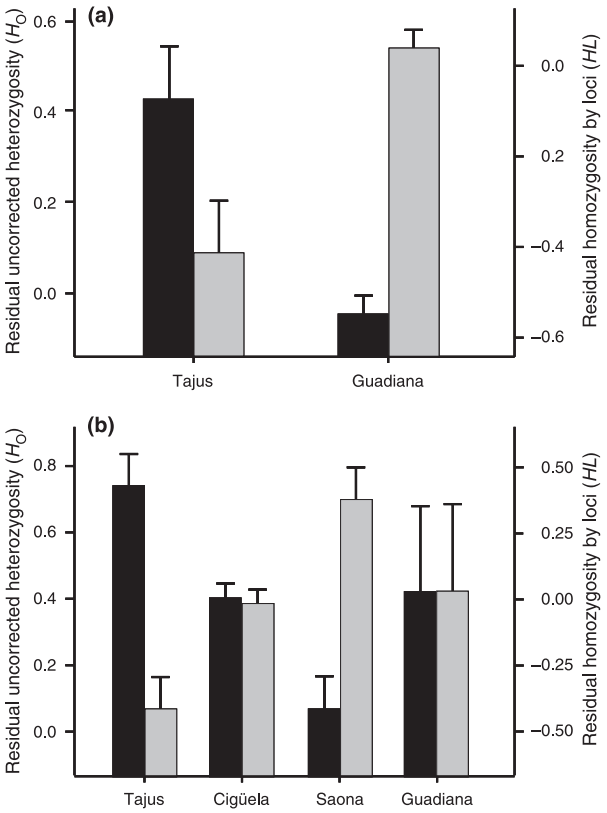

Figure 5 Uncorrected heterozygosity (H_O, black bars) and homozygosity by loci (*HL*, grey bars) for individuals collected in each of the (a) main and (b) secondary river basins located within the study area. Bars represent mean ± SE values. Both heterozygosity estimates are expressed as statistical residuals obtained after controlling for the effect of average population differentiation.

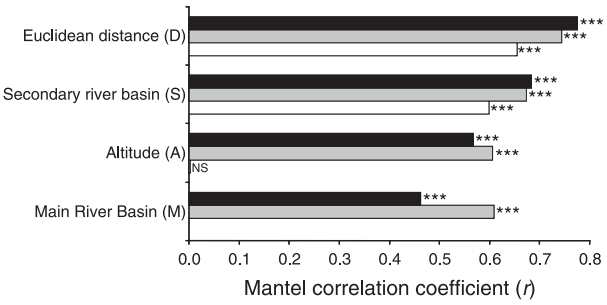

Figure 6 Results of simple Mantel tests for the different variables analysed including all populations (black bars), excluding Daimiel (DAI) population (grey bars) and only considering populations located within the Cigüela-Saona river basins (white bars). ***$P < 0.001$; NS: $P > 0.05$.

According to our predictions, we have found that the levels of genetic diversity are lower in highly isolated populations showing reduced gene flow (i.e. high average F_{ST} values) with other populations (Fig. 4). This result suggests that long-term isolation has probably eroded the genetic variability and supports a previous study performed at a large spatial scale (Ortego *et al.*, 2010). However, we did not find support to the hypothesis predicting lower levels of genetic variability in basins including a lower number of populations. Instead, we have found that genetic diversity is higher in the populations located in the Tajo basin which is only constituted by three isolated populations in contrast to the several populations located at the Guadiana basin (Figs 1 & 5a). It is similarly surprising the relatively high levels of genetic diversity observed in the highly isolated DAI population (Fig. 5b). These populations are highly differentiated (see F_{ST} values and STRUCTURE analyses), suggesting that the observed differences in genetic diversity may be consequence of historical processes rather than contemporary population dynamics. The genetic diversity in Saona basin was lower than the levels observed in the populations located along Cigüela basin. The Saona basin contains only four populations that are often located in small

habitat patches (e.g. MAN and MES; Fig. 1), and this may explain their lower diversity in comparison with the more widespread populations of *M. wagneri* within the Cigüela basin.

Our results have important implications for the conservation of the studied species and other co-distributed and similarly specialized organisms inhabiting inland saline environments. The patterns of genetic structure observed in relation to landscape features suggest that both river basins and elevation are important factors determining gene flow among populations. Thus, management practices aimed to restore the connectivity between close populations should consider the more adequate dispersal routes (i.e. lowlands/along river basins) revealed by causal modelling analyses at local spatial scales. Data about the genetic identity and variability of the studied populations should also be considered to guide future reintroduction or translocation programmes, which could help to minimize the risk associated with the reduced genetic diversity observed in some populations while preserving their genetic identity and potential local adaptations. Future studies analysing contemporary patterns of gene flow would help to reveal how individuals move across different habitats within populations and contribute to understand patterns of gene flow observed at different spatiotemporal scales. Our study also suggests that multiscale approaches should also be incorporated into the landscape genetics framework as it has previously been done in other fields of ecology (e.g. Ortego & Diaz, 2004; Illera *et al.*, 2010).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Pairwise population F_{ST} values. Values in bold are statistically significant ($P < 0.05$).

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