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Population genetics of *Mioscirtus wagneri*, a grasshopper showing a highly fragmented distribution

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Abstract

The genetic consequences of population fragmentation and isolation are major issues in conservation biology. In this study we analyse the genetic variability and structure of the Iberian populations of *Mioscirtus wagneri*, a specialized grasshopper exclusively inhabiting highly fragmented hypersaline low grounds. For this purpose we have used seven species-specific microsatellite markers to type 478 individuals from 24 localities and obtain accurate estimates of their genetic variability. Genetic diversity was relatively low and we detected genetic signatures suggesting that certain populations of *M. wagneri* have probably passed through severe demographic bottlenecks. We have found that the populations of this grasshopper show a strong genetic structure even at small geographical scales, indicating that they mostly behave as isolated populations with low levels of gene flow among them. Thus, several populations can be regarded as independent and genetically differentiated units which require adequate conservation strategies to avoid eventual extinctions that in highly isolated localities are not likely to be compensated for with the arrival of immigrants from neighbouring populations. Overall, our results show that these populations probably represent the 'fragments' of a formerly more widespread population and highlight the importance of protecting Iberian hypersaline environments due to the high number of rare and endangered species they sustain.

Keywords: genetic diversity, genetic structure, microsatellites, *Mioscirtus wagneri*, orthoptera, population fragmentation

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Introduction

Numerous species show highly fragmented distributions due to recent human induced habitat destruction, as a consequence of a natural patchy distribution of adequate habitats (e.g. oceanic islands) or due to historical climatic/geological events which have disjointed an originally continuous or more widespread population (Saunders *et al*. 1991; Lindenmayer & Fischer 2006). Small population size and isolation can result in increasing genetic differentiation and strong geographic structure of genetic variability due to limited connectivity with other populations and reduced gene flow (Martínez-Cruz *et al*. 2007; Coulon *et al*. 2008; Tzika *et al*. 2008). Further, these populations are also likely to suffer a considerable loss of genetic variation due to a combination of inbreeding and random genetic drift particularly when these processes are not compensated by the 'rescuing' effect of immigration (Frankham 1996; Vilà *et al*. 2003; Johnson *et al*. 2009). This can compromise the long-term viability of these populations if low genetic diversity diminishes their ability to respond to selection and adapt to novel and changing environmental conditions (Frankham 2005; Willi *et al*. 2006; Pertoldi *et al*. 2007). For these reasons, the genetic consequences of population fragmentation and isolation are major issues in conservation biology (Saunders *et al*. 1991; Frankham 1995; Fahrig 2002).

Inland hypersaline environments in the Western Mediterranean are habitats characterized by an extreme natural fragmentation (Abellán *et al*. 2007;

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Cordero *et al*. 2007; Muñoz *et al*. 2008). According to pollen records, these habitats were much more widespread during the Messinian saline crisis when different climatic/geological events resulted in the desiccation of most of the Mediterranean basin and allowed the expansion of fauna and flora adapted to the arid and saline environments predominating in that period (Blanco *et al*. 1997; Ribera & Blasco-Zumeta 1998; Blondel & Aronson 1999; Krijgsman *et al*. 1999). The Pliocene flooding of the Mediterranean basin with the last opening of the Gibraltar strait around 5.33 Ma together with Pleistocenic climatic change probably contributed to the progressive fragmentation of these habitats (Krijgsman *et al*. 1999; Valero-Garcés *et al*. 2000). Although these habitats are still predominant in certain areas of Central Asia and North Africa, inland hypersaline environments are much more fragmented in other regions where they are still present such as the Iberian Peninsula (Abellán *et al*. 2007; Cordero *et al*. 2007). This makes them threatened habitats of great conservation concern due to the high number of endemic species and relict populations they sustain (Ribera & Blasco-Zumeta 1998; Abellán *et al*. 2007). Although the patchy distribution of these inland hypersaline environments has been mainly driven by the natural and historical processes above mentioned, human activities have strongly contributed to their increasing fragmentation in recent times (Gómez *et al*. 2005; Cordero *et al*. 2007). Fortunately, most of these habitats have been protected or proposed for protection in recent years when environmental authorities have progressively focussed on their unique plant and animal communities (Muñoz 2007). Thus, these habitats and their associated communities offer an interesting model system to analyse a number of interesting questions on the genetic consequences of population fragmentation which also have direct implications for the conservation of these threatened habitats (Abellán *et al*. 2007; Muñoz *et al*. 2008; Ortego *et al*. 2009).

Mioscirtus wagneri (Orthoptera: Acrididae) is a highly specialized grasshopper exclusively inhabiting hypersaline low grounds with patches of *Suaeda vera*, the halophilic plant on which it exclusively depends for food (Cordero *et al*. 2007). This grasshopper shows a classical Mediterranean-Turanian disjunct distribution whose populations have probably become progressively fragmented from late Tertiary (Ribera & Blasco-Zumeta 1998; Ortego *et al*. 2009). Its current distribution pattern suggests the existence of an originally more widespread and expanding population during the Messinian saline crisis at the end of the Miocene (5.3 Mya; Krijgsman *et al*., 1999). Contraction of favourable habitats after the Messinian saline crisis probably reduced their populations which may have persisted through the Pleistocene to present days only constituting relict populations with different degree of isolation (Ribera & Blasco-Zumeta, 1998; Sanmartín, 2003). In contrast to North Africa and the Near East, Iberian hypersaline environments are less extensive and show a highly patchy distribution which results in extremely fragmented populations of *M. wagneri* (Cordero *et al*. 2007; Ortego *et al*. 2009). Thus, the Iberian populations of this grasshopper show an 'inland island-like' distribution consisting of several isolated populations with similar habitat requirements as many other co-distributed organisms (Ribera & Blasco-Zumeta, 1998). Accordingly, a recent study based on mitochondrial DNA (mtDNA) sequences has revealed a marked phylogeographical structure in this area of the species distribution range which suggests a complete lack of gene flow between main Iberian population clusters from the Early Pleistocene (Ortego *et al*. 2009). However, cryptic genetic structure at small spatial scales would have gone unnoticed and adequate analyses of genetic variability require the resolution offered by nuclear markers such as microsatellites (Aguirre *et al*. in press).

In this study we analyse the genetic variability and structure of the Iberian populations of *Mioscirtus wagneri*. For this purpose, we have used seven species-specific microsatellite markers to type 478 individuals from 24 localities and obtain accurate estimates of their genetic variability. In particular we tested the following predictions: (i) due to the fragmented and patchy distribution of the particular habitats required by *M. wagneri*, we would expect low gene flow and strong genetic differentiation and structure between most analysed populations/subpopulations. We would expect a pattern of genetic structure concordant with that obtained in a previous mtDNA-based study (Ortego *et al*. 2009), although we expected that the higher resolution of microsatellite markers in comparison with mtDNA sequences reveals cryptic genetic structure at smaller geographical scales; (ii) we also expected that genetic differentiation between populations increases with geographical distance (i.e. isolation by distance) due to (2.1) historical serial colonization/population fragmentation and genetic drift at large geographical scales (Ramachandran *et al*. 2005; Mills *et al*. 2007) and (2.2) migration-drift equilibrium at local scales (Hutchison and Templeton 1999); (iii) we predicted that population genetic variability increases with gene flow with other populations and decreases with spatial isolation; (iv) finally, the study populations are likely to show differential genetic signatures of population size reductions due to recent (human induced) and historical (climate change/geological events) factors contributing to the progressive contraction of the particular habitats required by the study species.

Methods

Sampling and study area

During 2006–2007, we sampled 24 populations of *M. wagneri*. We are confident these populations cover the entire species distribution range in the Iberian Peninsula, as several other potentially adequate habitats for *M. wagneri* (i.e. saline/hypersaline lagoons and low grounds) have been extensively prospected without any record of the species (Cordero *et al*. 2007; Ortego *et al*. 2009). We collected 11–31 adult individuals per population and specimens were preserved whole in 1500 μL ethanol 96% at −20 °C until needed for genetic analyses. Population code description and further information on sampling locations are given in Table 1.

Microsatellite genotyping

We genotyped individuals using seven polymorphic microsatellite markers isolated and characterized from a genomic library of a *M. wagneri* specimen from 'Las Yeguas' locality (MwGTC8, MwGTD9, MwGTG12, MwGTA6, MwGTC12, MwGTC11, MwGATAB11; Aguirre *et al*. in press). Probably due to the marked phylogeographical structure and genetic divergence among *M. wagneri* populations within the Iberian Peninsula (Ortego *et al*. 2009), some microsatellite loci did not amplify in certain populations strongly differentiated from the population from which the genomic library was constructed. Microsatellite GTC11 did not amplify in southwest populations (TIN, CAS) whereas GTA6 failed to do so in southwest (TIN, CAS) and northeast populations (UTX, SAL, CHI, GRA). Thus, we typed individuals at five to seven microsatellite markers depending on their population of origin. To make comparable data between populations with a different number of typed markers, all population genetic parameters and analyses (see below) were also performed only considering the five microsatellite loci (MwGTC8, MwGTD9, MwGTG12, MwGTC12, MwGATAB11) that worked in all populations. We used NucleoSpin Tissue

Table 1 Estimates of genetic variability for *Mioscirtus wagneri* populations. *N*, sample size; A_R, mean standardized allelic richness; A_{Priv}, mean number of private alleles; *M*-ratio, mean Garza-Williamson index. All statistics are shown for all typed loci at each locality and only considering the five loci that amplified in all populations

			All typed loci			Five shared loci		
Locality	Code	*N*	A_R	A_{Priv}	*M*	A_R	A_{Priv}	*M*
Northeast								
Pantano de Utxesa	UTX	20	5.67	0.99	0.53	5.31	0.98	0.50
Laguna Salada	SAL	20	3.96	0.22	0.62	3.74	0.27	0.59
Laguna de Chiprana	CHI	18	3.96	0.01	0.55	3.47	0.00	0.52
Laguna Salada Grande	GRA	20	4.88	0.21	0.79	4.33	0.22	0.78
Central–Southeast								
Saladar de Ocaña-Aranjuez	OCA	20	6.79	0.46	0.70	6.87	0.69	0.64
Saladar de Huerta	HUE	20	5.69	0.11	0.63	5.86	0.02	0.64
Saladar de Villasequilla	VIL	20	6.88	0.27	0.70	7.09	0.41	0.70
Laguna del Cerrillo	CER	20	5.83	0.00	0.65	5.49	0.00	0.66
Laguna del Altillo	ALT	20	5.64	0.00	0.67	5.33	0.00	0.70
Laguna de Longar	LON	20	5.66	0.09	0.57	5.25	0.14	0.57
Laguna Larga	LAR	20	5.48	0.00	0.61	5.23	0.00	0.62
Laguna de Tírez	TIR	20	6.13	0.00	0.69	5.72	0.00	0.73
Laguna de Peña Hueca	PEN	20	5.51	0.02	0.59	5.82	0.03	0.63
Laguna de Quero	QUE	23	5.68	0.00	0.66	5.34	0.01	0.66
Laguna de la Sal	LSA	20	5.42	0.12	0.68	5.23	0.18	0.67
Laguna de las Yeguas	YEG	20	5.55	0.10	0.63	5.47	0.15	0.60
Laguna de Salicor	SCO	31	4.63	0.08	0.59	4.54	0.03	0.64
Laguna de Alcahozo	ALC	20	4.09	0.00	0.56	3.74	0.00	0.53
Laguna de Manjavacas	MAN	18	3.89	0.00	0.57	3.77	0.00	0.58
Saladar de Cordovilla	COR	20	6.36	0.65	0.56	6.66	0.95	0.58
Saladar de Agramón	AGR	11	4.86	0.18	0.66	5.20	0.26	0.66
Saladar del Margen	MAR	20	6.58	0.68	0.60	6.34	0.88	0.59
Southwest								
Río Tinto	TIN	17	6.57	1.53	0.57	6.57	1.53	0.57
Castro Marin	CAS	20	4.18	0.53	0.60	4.18	0.53	0.60

(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, 1X reaction buffer (67 mM Tris–HCl, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tweenty-20, EcoStart Reaction Buffer, Ecogen), 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) thermal cycler. The PCR programme used was 9 min denaturing at 95 °C followed by 35 cycles of 30 s at 94 °C, 45 s at the annealing temperature (Aguirre *et al*. in press) 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 Analyser (Applied Biosystems) and genotypes were scored using GeneMapper 3.7 (Applied Biosystems).

Genetic diversity estimates

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). Measures of allelic richness (A_R) and number of private alleles (A_{Priv}) were standardized for sample size using the program HP-Rare (Kalinowski 2005).

A significant population size reduction (i.e. bottleneck) can produce distinctive genetic signatures in the distributions of alleles and expected heterozygosity (Cornuet & Luikart 1996; Luikart & Cornuet 1998; Garza & Williamson 2001). In populations that have experienced a significant reduction in size, the number of alleles is reduced faster than the heterozygosity because of the rapid loss of rare alleles. A heterozygosity excess relative to the level expected at mutation–drift equilibrium for the number of alleles present can then indicate a genetic bottleneck (Cornuet & Luikart 1996). Each study population was tested for heterozygosity excess in order to detect recent population bottlenecks using the program Bottleneck 1.2.02 (Cornuet & Luikart 1996; Piry *et al*. 1999). We run Bottleneck under the two-phase model (TPM) that is supposed to fit microsatellite evolution better than other methods (Dirienzo *et al*. 1994). We used 1000 replications, assuming 10% of the infinite allele model and 90% of the stepwise mutation model. Statistical significance was assessed with a two-tailed Wilcoxon signed rank test, the most powerful and robust statistics when using fewer than 20 polymorphic loci (Piry *et al*. 1999). We also investigated the distribution of the allele frequencies using the mode shift test also implemented in the program Bottleneck (Piry *et al*. 1999). For each locus and locality we calculated the *M*-ratio, defined as the ratio of the number of alleles to the range of allele sizes, a statistic that can detect reductions in population sizes (Garza & Williamson 2001). *M*-ratios were calculated as follows: $M = k/r$, the ratio of the number of alleles to the range of allele sizes, where k = the number of alleles and r = the number of possible alleles sizes between the smallest and the largest observed alleles (Garza & Williamson 2001). A declining population will have a smaller *M*-ratio than a stable one because k is expected to decrease faster than r in small populations due to genetic drift causing loss of rare alleles (Garza & Williamson 2001). We calculated mean *M*-ratios across all loci for each population and this mean value was compared with $M = 0.68$, the threshold value below which a population can reasonably be assumed to have undergone a reduction in population size (Garza & Williamson 2001).

Population genetic structure

We investigated population genetic structure among sampling 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). To visualize the genetic relationship among the sampling localities we performed a multivariate ordination using Pcagen (http://www.unil.ch/izea/softwares/pcagen.html) with 10 000 randomization steps. We also studied the spatial genetic structure using Structure 2.2, a Bayesian model-based clustering method which assigns individuals to populations based on their multilocus genotypes (Pritchard *et al*. 2000). Structure 2.2 accounts for genotype ambiguities such as the possible presence of null alleles (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, and 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 25 when pooling individuals from all localities and from 1 to 5 when using individuals from a subset of localities previously grouped into a single cluster based on the first global analysis. The number of populations best fitting the data set was defined both using log probabilities $[\Pr(X|K)]$ and ΔK, as described in

Evanno *et al*. 2005. Thus, the estimated number of subpopulations is taken to be the value of K at which $\Pr(X \mid K)$ plateaus.

The possible occurrence of an isolation-by-distance pattern was assessed comparing pairwise matrices of genetic (F_{ST}) and Euclidean geographical distances. For this purpose, we used IBDWS version 3.16, which performs a Mantel test and a Reduced Major Axis (RMA) regression analysis (Jensen *et al*. 2005). The significance of Mantel test was assessed by 10 000 randomizations of the genetic distance matrix. The test was one-tailed as only a positive correlation between geographical and genetic distances is expected (e.g. Gómez *et al*., 2007).

Results

Genetic diversity

A total of 167 alleles were observed across all populations of *M. wagneri* over the seven analysed loci. Average allelic richness (A_R) calculated considering all typed loci was strongly correlated with A_R calculated considering the five loci that amplified in all populations ($r = 0.969$; $P < 0.001$; Table 1). Similar correlations were observed for the number of private alleles (A_{Priv}; $r = 0.978$; $P < 0.001$) and *M*-ratios ($r = 0.915$; $P < 0.001$) calculated using the two different sets of marker loci (Table 1). A_R estimated considering all loci ranged from 3.89 to 6.88 alleles in MAN and VIL localities, respectively (Table 1). Only considering the five loci that amplified in all populations, average allelic richness ranged from 3.47 to 7.09 alleles in CHI and VIL localities, respectively (Table 1). With the exception of some localities in Central Spain, all populations exhibited private alleles (Table 1). Absence of private alleles in certain localities (CER, ALT, LAR, TIR, QUE, ALC, and MAN) from central Spain was probably due to the high number of sampled populations in this geographical area which showed relatively low levels of genetic differentiation among them (see below). This is likely to have reduced the probability of finding private alleles in this intensively sampled area. After adjusting for multiple comparisons, significant departures from HWE were observed in 6 out of 160 population-locus combinations. Such deviations involved the locus MwGTD9 (TIN locality), MwGTA6 (OCA, HUE, PEN, MAR localities) and MwGTC12 (TIN locality). We found no evidence of linkage disequilibrium among loci, indicating that the analysed markers can be treated as independent from each other.

Reduced inter-population gene flow and random genetic drift can erode genetic variability of isolated populations (Crow & Kimura 1970; Frankham 1996). Here, we estimate gene flow and population isolation: (i) calculating the distance to the nearest population; (ii) calculating average genetic differentiation of each locality with all other studied populations (estimated on the basis of pair-wise F_{ST} values; Table 2). Then, we explored the possible association between these two variables and standardized allelic diversity (A_R) to study the relationship between gene flow/population isolation and genetic variability. The precision of genetic diversity estimates could be different because sample sizes (i.e. the number of analysed individuals) used for their estimation slightly varied between localities. So, we used sample size to give observations different weights in a weighted least-squares analysis. Considering all studied populations, standardized allelic diversity (A_R) was negatively associated with average population genetic differentiation (Student's *t*-test; $t = -2.92$; $P = 0.008$; Fig. 1) but we found no significant correlation with population isolation (Student's *t*-test; $t = 0.404$; $P = 0.690$).

Wilcoxon signed-rank tests did not reveal any significant excesses of heterozygosities under the TPM, suggesting that the studied populations of *M. wagneri* have not experienced a recent and/or strong population bottleneck. Similar results were obtained when only the five loci that amplified in all populations were considered or using different percentage (20% and 30%) of multistep mutations in the TPM. The mode shift test neither showed any modal shift in allele frequency distribution characteristic of a population bottleneck. By contrast, *M*-ratios suggested that bottlenecks had occurred in several populations. Across all sites and loci, the *M*-ratio ranged from 0.53 to 0.79 including all typed loci and from 0.50 to 0.78 when only those loci that amplified in all populations were considered (Table 1). Only six populations (GRA, OCA, VIL, ALT, TIR and SAL) showed values higher than the critical value of 0.68 proposed by Garza and Williamson (2001) (Table 1).

Genetic structure

Population scores from Principal Component Analysis (PCA) were plotted on two axes (PC1 and PC2) which cumulatively explained 55.8% of the total genetic variability (PC1: 42.2%, $P = 0.005$; PC2: 13.8%, $P = 0.353$; Fig. 2). A similar pattern was obtained when only the five loci that amplified in all populations were considered (PC1: 49.4%, $P < 0.001$; PC2: 13.7%, $P = 0.037$). A more detailed analysis only considering the populations from La Mancha region (Central Iberia) showed three main clusters grouping north, central and south populations (see also STRUCTURE analyses below). In this case, population scores were also plotted on two principal axes (PC1 and PC2) which cumulatively explained

Table 2 Pair-wise population F_{ST} values calculated considering all microsatellite markers that amplified in the two populations involved in the pair-wise comparison (above the diagonal) and only considering the five loci that worked in all studied populations (below the diagonal). Values in bold are statistically significant ($P < 0.05$)

	UTX	SAL	CHI	GRA	OCA	HUE	VIL	CER	ALT	LON	LAR	TIR	PEN	QUE	LSA	YEG	SCO	ALC	MAN	COR	AGR	MAR	TIN	CAS
UTX	—	**0.097**	**0.139**	**0.106**	**0.251**	**0.284**	**0.260**	**0.302**	**0.315**	**0.302**	**0.284**	**0.274**	**0.292**	**0.292**	**0.305**	**0.299**	**0.392**	**0.371**	**0.408**	**0.213**	**0.318**	**0.192**	**0.118**	**0.195**
SAL	**0.065**	—	**0.149**	**0.095**	**0.294**	**0.321**	**0.292**	**0.336**	**0.344**	**0.335**	**0.321**	**0.307**	**0.324**	**0.326**	**0.335**	**0.325**	**0.418**	**0.389**	**0.432**	**0.215**	**0.335**	**0.249**	**0.169**	**0.216**
CHI	**0.143**	**0.150**	—	**0.058**	**0.299**	**0.326**	**0.307**	**0.336**	**0.349**	**0.319**	**0.317**	**0.312**	**0.331**	**0.332**	**0.321**	**0.325**	**0.428**	**0.397**	**0.441**	**0.258**	**0.357**	**0.264**	**0.231**	**0.337**
GRA	**0.090**	**0.100**	**0.052**	—	**0.240**	**0.284**	**0.266**	**0.301**	**0.302**	**0.278**	**0.278**	**0.271**	**0.288**	**0.289**	**0.287**	**0.278**	**0.391**	**0.351**	**0.401**	**0.216**	**0.287**	**0.193**	**0.162**	**0.263**
OCA	**0.281**	**0.315**	**0.328**	**0.257**	—	**0.188**	**0.114**	**0.133**	**0.131**	**0.146**	**0.135**	**0.122**	**0.145**	**0.131**	**0.159**	**0.118**	**0.215**	**0.201**	**0.229**	**0.127**	**0.196**	**0.173**	**0.191**	**0.307**
HUE	**0.290**	**0.336**	**0.360**	**0.294**	**0.170**	—	**0.069**	**0.097**	**0.135**	**0.110**	**0.112**	**0.141**	**0.101**	**0.092**	**0.099**	**0.102**	**0.191**	**0.191**	**0.227**	**0.152**	**0.225**	**0.243**	**0.190**	**0.296**
VIL	**0.282**	**0.319**	**0.349**	**0.293**	**0.101**	**0.069**	—	**0.042**	**0.070**	**0.082**	**0.032**	**0.049**	**0.039**	**0.018**	**0.050**	**0.047**	**0.140**	**0.142**	**0.175**	**0.131**	**0.216**	**0.198**	**0.189**	**0.282**
CER	**0.322**	**0.353**	**0.373**	**0.320**	**0.108**	**0.119**	**0.040**	—	−0.006	0.008	**0.023**	**0.021**	0.015	−0.008	−0.005	−0.003	**0.033**	**0.059**	**0.069**	**0.174**	**0.252**	**0.240**	**0.224**	**0.329**
ALT	**0.333**	**0.363**	**0.380**	**0.321**	**0.098**	**0.160**	**0.077**	−0.005	—	0.008	**0.032**	**0.022**	0.013	0.014	0.005	−0.002	**0.018**	**0.041**	**0.055**	**0.181**	**0.251**	**0.240**	**0.235**	**0.342**
LON	**0.328**	**0.358**	**0.352**	**0.298**	**0.127**	**0.131**	**0.091**	0.013	0.013	—	**0.043**	**0.049**	**0.027**	**0.032**	0.008	0.010	**0.065**	**0.059**	**0.080**	**0.178**	**0.243**	**0.232**	**0.230**	**0.340**
LAR	**0.315**	**0.347**	**0.357**	**0.305**	**0.124**	**0.126**	**0.035**	**0.022**	**0.035**	**0.054**	—	0.006	**0.026**	0.005	0.011	**0.021**	**0.090**	**0.099**	**0.158**	**0.169**	**0.241**	**0.221**	**0.214**	**0.315**
TIR	**0.306**	**0.336**	**0.353**	**0.301**	**0.112**	**0.154**	**0.062**	0.008	0.015	**0.052**	0.006	—	**0.018**	0.007	**0.028**	0.016	**0.073**	**0.078**	**0.119**	**0.157**	**0.227**	**0.207**	**0.211**	**0.314**
PEN	**0.285**	**0.319**	**0.335**	**0.283**	**0.092**	**0.099**	**0.037**	−0.002	0.008	0.016	0.009	0.004	—	0.009	0.014	0.011	**0.054**	**0.072**	**0.109**	**0.166**	**0.238**	**0.227**	**0.186**	**0.292**
QUE	**0.309**	**0.341**	**0.368**	**0.305**	**0.093**	**0.114**	0.009	−0.008	**0.024**	**0.046**	−0.001	−0.007	−0.001	—	−0.003	−0.005	**0.064**	**0.061**	**0.117**	**0.160**	**0.261**	**0.219**	**0.219**	**0.328**
LSA	**0.311**	**0.343**	**0.344**	**0.294**	**0.122**	**0.121**	**0.044**	−0.004	0.010	0.012	0.002	0.012	0.003	0.001	—	0.012	**0.046**	**0.085**	**0.118**	**0.176**	**0.262**	**0.245**	**0.226**	**0.327**
YEG	**0.317**	**0.344**	**0.358**	**0.297**	**0.089**	**0.122**	**0.050**	−0.002	0.001	0.017	**0.027**	0.009	0.001	−0.003	0.017	—	**0.033**	**0.038**	**0.068**	**0.162**	**0.228**	**0.214**	**0.217**	**0.319**
SCO	**0.397**	**0.423**	**0.448**	**0.398**	**0.173**	**0.218**	**0.135**	**0.033**	0.015	**0.068**	**0.076**	**0.039**	**0.041**	**0.074**	**0.054**	**0.029**	—	**0.049**	**0.067**	**0.260**	**0.350**	**0.327**	**0.308**	**0.402**
ALC	**0.401**	**0.425**	**0.446**	**0.388**	**0.185**	**0.228**	**0.166**	**0.072**	**0.053**	**0.074**	**0.114**	**0.084**	**0.074**	**0.081**	**0.108**	**0.047**	**0.050**	—	**0.032**	**0.252**	**0.324**	**0.287**	**0.311**	**0.407**
MAN	**0.447**	**0.473**	**0.508**	**0.447**	**0.224**	**0.278**	**0.210**	**0.088**	**0.063**	**0.096**	**0.188**	**0.131**	**0.110**	**0.150**	**0.148**	**0.083**	**0.065**	**0.038**	—	**0.284**	**0.361**	**0.321**	**0.378**	**0.467**
COR	**0.223**	**0.239**	**0.273**	**0.224**	**0.108**	**0.141**	**0.128**	**0.166**	**0.175**	**0.176**	**0.171**	**0.160**	**0.139**	**0.146**	**0.160**	**0.153**	**0.243**	**0.264**	**0.303**	—	**0.095**	**0.184**	**0.127**	**0.233**
AGR	**0.314**	**0.329**	**0.372**	**0.280**	**0.151**	**0.192**	**0.198**	**0.230**	**0.225**	**0.220**	**0.229**	**0.214**	**0.189**	**0.232**	**0.230**	**0.199**	**0.315**	**0.320**	**0.369**	**0.085**	—	**0.231**	**0.178**	**0.294**
MAR	**0.225**	**0.250**	**0.289**	**0.194**	**0.175**	**0.239**	**0.208**	**0.241**	**0.240**	**0.240**	**0.238**	**0.225**	**0.210**	**0.216**	**0.237**	**0.212**	**0.313**	**0.297**	**0.344**	**0.181**	**0.204**	—	**0.202**	**0.303**
TIN	**0.276**	**0.308**	**0.344**	**0.274**	**0.191**	**0.190**	**0.189**	**0.224**	**0.235**	**0.230**	**0.214**	**0.211**	**0.186**	**0.219**	**0.226**	**0.217**	**0.308**	**0.311**	**0.378**	**0.127**	**0.178**	**0.202**	—	**0.147**
CAS	**0.363**	**0.379**	**0.437**	**0.369**	**0.307**	**0.296**	**0.282**	**0.329**	**0.342**	**0.340**	**0.315**	**0.314**	**0.292**	**0.328**	**0.327**	**0.319**	**0.402**	**0.407**	**0.467**	**0.233**	**0.294**	**0.303**	**0.107**	—

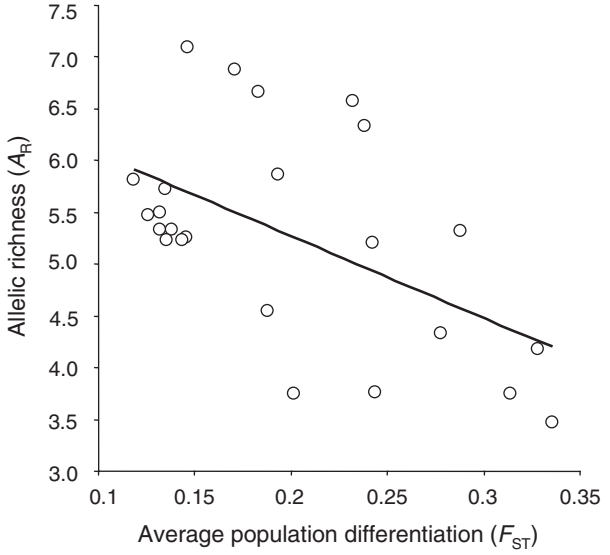

Fig. 1 Relationship between standardized allelic richness (A_R) and average population differentiation with all other studied populations.

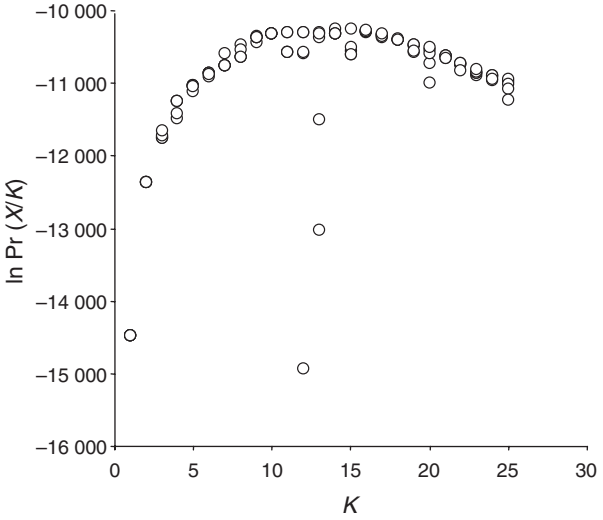

Fig. 3 Results of Bayesian clustering analysis for 478 *Mioscirtus wagneri* collected in 24 sample localities across the Iberian Peninsula. For each number of population clusters tested (*K*), Pr(*X* | *K*) is the probability of the data.

52.9% of the total genetic variability (PC1: 34.5%, $P = 0.001$; PC2: 18.4%, $P = 0.010$). Pairwise F_{ST} values indicated high levels of genetic differentiation and all non-significant pair-wise comparisons corresponded to some close localities from La Mancha region (Table 2). Accordingly, STRUCTURE analyses revealed the presence of a strong genetic structure within the Iberian Peninsula and indicated an optimal clustering into eight distinct groups (Figs 3 and 4a). One group corresponds to northeast populations and included UTX, SAL, CHI, and GRA localities. Another group was mainly constituted by OCA and HUE populations. A third–fourth cluster grouped most populations from central Iberia which became progressively differentiated southward into a fifth cluster particularly patent in MAN and ALC localities. Finally, the highly distant populations from COR-AGR, MAR, and CAS-TIN grouped in three other distinct clusters (Fig. 4a). STRUCTURE analyses based on the five loci that worked in all populations resulted in a similar pattern but some clusters revealed in the analysis considering all typed loci joined (data not shown). Finally, we re-analysed the data from the main clusters obtained in the above analyses to detect possible subtle genetic structure not revealed when all localities are pooled (e.g. Tzika *et al*. 2008). We found that some localities that appeared clustered in the global analysis including all populations split into different genetic clusters after such detailed STRUCTURE analyses (Fig. 4b). Northeast populations split into three clusters (UTX, SAL, and CHI-GRA) whereas COR-AGR and CAS-TIN populations also showed considerable genetic differentiation (Fig. 4b). By contrast, populations from La Man-

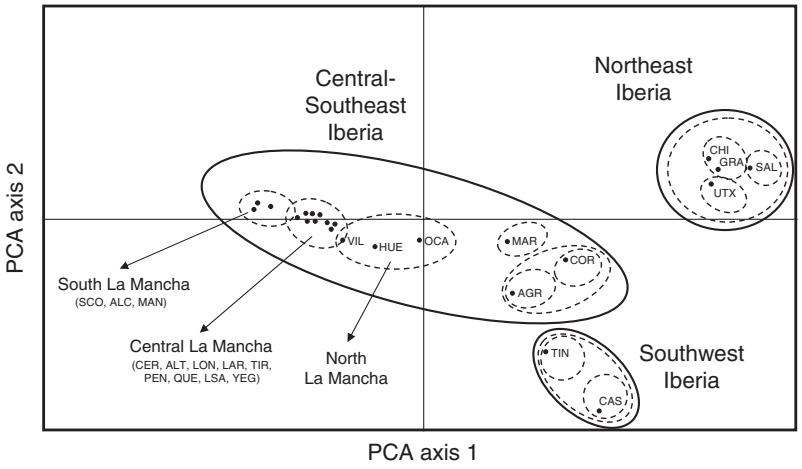

Fig. 2 PCA showing the genetic relationship among *Mioscirtus wagneri* sampling localities. Locations within each ellipse represent main population clusters identified on the basis of mtDNA haplotypes (solid ellipses; Ortego *et al*. 2009) and clusters/sub-clusters obtained from microsatellite-derived STRUCTURE analyses (dotted ellipses) (see Fig. 4).

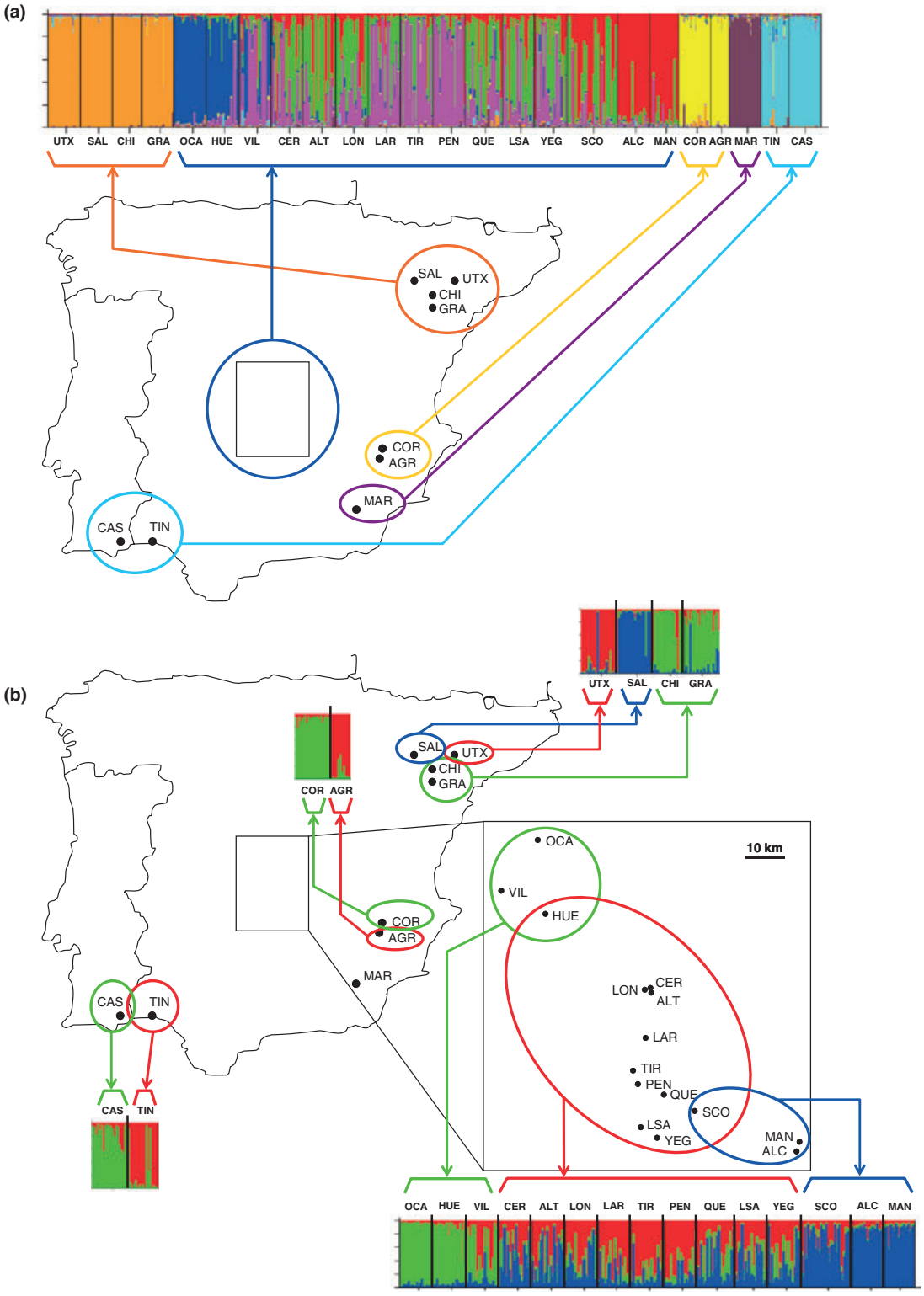

Fig. 4 Results of the genetic assignment of individual *Mioscirtus wagneri* based on the Bayesian method implemented in the program STRUCTURE. Panel (a) represents the output from a global analysis pooling data from all populations (with $K = 8$) whereas panel (b) shows the result derived from more detailed analyses focused on the clusters obtained in the global analysis. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour.

cha presented a pattern similar to the above reported for the global analysis considering all populations (Fig. 4b).

Isolation-by-distance analyses revealed a significant positive correlation between genetic and geographic distances ($Z = 284.19$, $r = 0.828$, one-sided $P < 0.001$ from 10 000 randomizations) and a value of $r^2 = 0.685$ for RMA regression analysis (Fig. 5). A similar pattern was obtained when only the five loci that worked in all populations were considered ($Z = 296.74$, $r = 0.839$, one-sided $P < 0.001$ from 10 000 randomizations; RMA $r^2 = 0.704$) We re-analysed the data only considering the intensively sampled populations from La Mancha (Central Spain) and we also found a highly significant positive correlation between genetic and geographic distances ($Z = 33.74$, $r = 0.606$, one-sided $P < 0.001$ from 10 000 randomizations; RMA $r^2 = 0.367$).

Discussion

In this study we have found that Iberian populations of *M. wagneri* show an 'inland island–like' distribution, with a strong spatial genetic structure and a marked population differentiation. STRUCTURE analyses considering all populations revealed eight genetic clusters corresponding with the main geographical areas which often split in other clusters after more detailed analyses. A previous study based on mitochondrial DNA (mtDNA) sequences showed a strong genetic structure at a large geographical scale, with three main clades corresponding with populations located in northeast, central–southeast and southwest Iberia which probably evolved in allopatry (Ortego *et al*. 2009). Although microsatellite and mtDNA phylogeographical analyses were mostly comparable, the pattern of genetic structure at finer spatial scales remained unresolved using mitochondrial gene sequences (Fig. 2). For instance, data on mtDNA showed a genetic uniformity for all central–southeast populations which has now resulted in six–seven genetic clusters. Thus, the obtained pattern of genetic subdivision revealed by nuclear markers suggests the presence of barriers to dispersal not only among the three genetic clusters (main geographical areas) obtained in the previous mtDNA based study but also at smaller spatial scales (Figs 2 and 4). Past natural habitat reduction is likely to be responsible for the general phylogeographical pattern observed at both mitochondrial and microsatellite markers, whereas subtle genetic differentiation observed at smaller spatial scales (e.g. within main population clusters; Fig. 4b) are more likely to have resulted from current anthropogenic habitat destruction (Ortego *et al*. 2009). As above mentioned, *M. wagneri* is a highly specialist grasshopper which has been exclusively found in hypersaline low grounds with patches of the halophilic plant *Suaeda vera* on which it entirely depends for food and refuge (Cordero *et al*. 2007; Ortego *et al*. 2009). The densities of this grasshopper sharply decline at low covers of *S. vera* and it virtually disappears few meters beyond the patches of this plant (P. J. Cordero, unpublished). Thus, both historical and recent fragmentation of hypersaline environments are likely to have decisively contributed to reduce gene flow between populations and would have favoured a rapid genetic differentiation at local spatial scales.

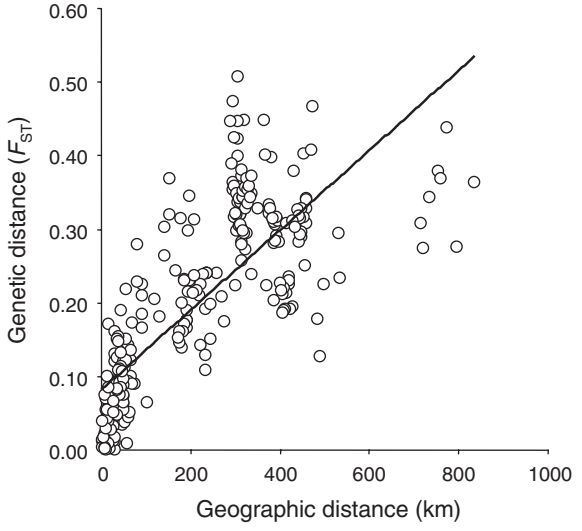

Fig. 5 Correlation between genetic (F_{ST}) and geographic distance for the Iberian populations of *Mioscirtus wagneri*. Reduced Major Axis (RMA) regression line is showed.

We have also found a highly significant pattern of isolation by distance which is difficult to interpret as indicative of migration–drift equilibrium in a system with such a marked genetic structure (Hutchison and Templeton 1999). Rather, the observed pattern is likely to have been generated through serial colonization from nearby populations and subsequent genetic drift, i.e. as consequence of persistent founder effects (Ramachandran *et al*. 2005; Mills *et al*. 2007). Alternatively, a historical pattern of isolation by distance in a supposedly continuous population could have persisted after a progressive habitat fragmentation despite strong genetic drift and differentiation. By contrast, the pattern of isolation by distance observed at smaller spatial scales (e.g. within La Mancha region) could be also compatible with a migration–drift equilibrium scenario as is suggested by the relatively high admixture levels revealed by STRUCTURE analyses in certain localities (Fig. 4b). The lower levels of genetic differentiation observed between close localities at such smaller spatial scale also suggest that gene flow and immigration from nearby popula-

tions may be relatively frequent. Thus, different scenarios could explain the patterns of isolation by distance observed at large and local spatial scales which are also likely to reflect progressive fragmentation at different temporal scales, i.e. past natural habitat reduction and recent human induced habitat fragmentation.

Genetic diversity was relatively low and the observed *M*-ratios suggested that several *M. wagneri* populations have probably passed through a population size reduction. This may be explained by the small-size patches of adequate habitats in most localities which often sustain small and discontinuous populations of this grasshopper. It is worth mentioning the case of Manjavacas locality which showed the lowest levels of both genetic diversity and *M*-ratios among all analysed populations. Manjavacas locality probably sustains the smallest population of *M. wagneri* here studied: we could only find it in a very small patch of *Suaeda vera* (lower than ½ hectare) remaining with a particularly low population density (P. J. Cordero, unpublished). However, despite the low *M*-ratio value observed in both this and several other localities we have found no significant heterozygote excess or shift in allele frequency distribution in any studied population. A population bottleneck is expected to have a long-lasting genetic signature on *M*-ratios whereas heterozygosity excess and allele frequency distribution are likely to recover relatively quickly (Garza & Williamson 2001). Thus, these tests are expected to provide evidence of population decline and recovery over different time scales and this may also help to explain the history of the studied populations (Garza & Williamson 2001). Our results suggest that the current genetic composition of the studied populations of *M. wagneri* has been primarily influenced by past population declines probably characterized by recurrent demographic bottlenecks during a progressive fragmentation of a supposedly more widespread population.

Standardized allelic diversity was negatively correlated with average genetic differentiation with all other populations, suggesting that long term isolation and reduced gene flow have probably contributed to erode genetic variability in certain populations (Brede *et al*. 2008). By contrast, we have found no significant association between genetic variability and population isolation. Population geographic isolation is likely to be a less accurate estimator of gene flow than observed average pair-wise F_{ST} values. This may explain why distance to the nearest population does not correlate with population genetic variability. Alternatively, such contrasting patterns may reflect population isolation at different time scales. Long-term isolation could have caused population genetic differentiation and reduced genetic variability, a pattern which may not be revealed by the current degree of geographical isolation estimated by distance to the nearest population. In any case, additional information on the species population dynamics is necessary to get a better understanding of the factors influencing current levels of genetic diversity. Further studies should include information on the area of favourable habitat available in each locality, systematic censuses and detailed data on population densities necessary to estimate current population sizes. This information together with analyses on fine-spatial scale genetic structure would help to determine the species dispersal potential and the current levels of gene flow both within and between neighbour populations (e.g. Double *et al*. 2005; Ortego *et al*. 2008a,b).

The results here reported have important implications for the conservation of both the study species and several other co-distributed organisms with similar habitat requirements. On one hand, data on spatial genetic structure indicate that several populations should be regarded as independent and genetically differentiated units which may show local adaptations to particular environmental conditions (Ortego *et al*. 2009). On the other hand, the strong genetic structure observed suggests that several studied populations mostly behave as isolated populations with low levels of gene flow between them. Particularly some isolated and geographically restricted populations (e.g. the genetic clusters of MAR or COR-AGR localities) probably represent the 'remains' of a formerly more widespread population that once extinct may not re-colonize (Frankham *et al*. 2002). Thus, this spatial population structure together with the small size of suitable patches makes these populations particularly vulnerable to human alterations. Although the small-size patches of these relict habitats have an important natural component, human activities have also contributed to their further fragmentation and size reduction in recent times. These habitats have been often fragmented through extensive ploughing whereas intensive livestock grazing and the input of fresh water or urban wastes have greatly contributed to alter their natural dynamics (Valero-Garcés *et al*. 2000; Cordero *et al*. 2007). Although most of these habitats are now protected, conservation strategies have frequently neglected their less conspicuous arthropod communities (Cordero *et al*. 2008). For instance, managers have often supported the input of water to certain hypersaline lagoons to favour popular avian communities, a practice that disrupts the natural hydric dynamics of these temporal wetlands and also results in a continuous flooding of the halophile vegetation ring used by *M. wagneri* and several other species exclusively inhabiting this habitat (Cordero *et al*. 2008).

In conclusion, our analyses indicate that Iberian populations of *M. wagneri* show a deep genetic structure

and strong differentiation even at small geographical scales. On the other hand, the studied populations show different levels of genetic diversity, some of them with particularly low variability and marked genetic signatures of past population size reductions. Overall, these results indicate that hypersaline environments constitute island-inlands of relict habitats which offer an interesting model system to study the genetic consequences of population fragmentation and isolation.

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