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Phylogeography of the Iberian populations of *Mioscirtus wagneri* (Orthoptera: Acrididae), a specialized grasshopper inhabiting highly fragmented hypersaline environments

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Phylogeographic studies from western Palaearctic have generally focused on species able to disperse and track their emerging suitable habitats after the last ice age. However, data on species whose biogeographical histories differ from this bulk of Palaearctic fauna are scarce. This is clearly the case of some specialized organisms inhabiting inland hypersaline environments, which are likely to have had a wider distribution range during the late Tertiary and may have persisted through the Pleistocene to the present day only constituting relict populations. In this study, we use partial sequences from two mitochondrial genes [16S rRNA (16S) and cytochrome oxidase subunit II (COII)] to investigate the phylogeography of the Iberian populations of *Mioscirtus wagneri* (Orthoptera: Acrididae), a specialized grasshopper exclusively inhabiting hypersaline low grounds. Our results show that *M. wagneri* exhibits a marked phylogeographical structure, forming three main clades which correspond with populations located in north-east, central–south-east and south-west Iberia. These geographical areas did not share any haplotype, indicating that gene flow between them is absent. Nested clade analyses revealed that these lineages have probably evolved in allopatry and data on sequence divergence suggest population fragmentation from the Early Pleistocene. Overall, these results provide a broader perspective on the contribution of historical climate/geological events to biogeographical patterns of organisms currently forming relict populations of great conservation concern. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **97**, 623–633.

ADDITIONAL KEYWORDS: allopatric fragmentation – conservation genetics – genetic diversity – mtDNA.

INTRODUCTION

Phylogeographic analyses are crucial to understand current patterns of species distribution and have also been proved to be a powerful complementary tool to infer historical climate and geological processes (Hewitt, 2000; Emerson, 2002; Schmitt, 2007). Conservation biology also benefits from this relatively recent discipline: the recognition of evolutionary significant units within species is of major importance to guide management strategies (Vogler & Desalle, 1994; Palsboll, Berube & Allendorf, 2007). Phylogeographic studies have been mainly focused on analysing the role of Pleistocene climatic oscillations in current patterns of species distribution, inferring refugia and postglacial distributional changes in organisms generally able to disperse and track their emerging suitable habitats after the last ice age

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(Hewitt, 1996, 2000, 2004; Schmitt, 2007). However, other species may have suffered very different historical population dynamics. This seems to be the case of numerous specialized organisms inhabiting highly fragmented inland saline environments and certain steppe areas which usually show extreme disjunct distributions across the Mediterranean basin and central Asia (Ribera & Blasco-Zumeta, 1998). These organisms, particularly those showing low dispersal potential, are likely to have had a wider distribution range during the late Tertiary, probably after population expansions during the Messinian salinity crisis at the end of the Miocene (5.3 Mya; Krijgsman *et al.*, 1999), and they may have persisted through the Pleistocene to the present day only constituting relict populations (Ribera & Blasco-Zumeta, 1998; Sanmartin, 2003).

In the Iberian Peninsula, inland saline environments are rare habitats distributed in small-sized patches generally located within a number of endorheic basins (Comin & Alonso, 1988). The high richness of rare and endemic species usually found in these environments, together with their reduced population sizes and associated demographic instability, often makes them threatened habitats of great conservation concern (Ribera & Blasco-Zumeta, 1998; Diogo *et al.*, 1999; Abellán *et al.,* 2007). Thus, the study of the phylogeography of these particular organisms is not only relevant for a better understanding on an interesting biogeographical pattern, but also from an applied perspective, as identifying significant management units is necessary to design appropriate conservation strategies (Abellán *et al.,* 2007; Pérez-Collazos & Catalán, 2007; Muñoz *et al.*, 2008). However, the population genetics of these species has been rarely studied, with some recent exceptions involving endemic taxa with small range distributions (Diogo *et al.*, 1999; Abellán *et al.,* 2007; Pérez-Collazos & Catalán, 2007) and zooplanktonic organisms (Gómez, Carvalho & Lunt, 2000; Gómez *et al.*, 2007; Muñoz *et al.*, 2008). The latter are frequently submitted to long passive dispersal and current phylogeographical patterns and genetic structures are the result of recent colonization events, with strong founder effects and monopolization processes (Gómez *et al.*, 2000, 2007; Muñoz *et al.*, 2008). These patterns may differ greatly from that expected in organisms with a lower dispersal potential, which present distributions probably reflect relict populations persisting to the present day rather than post-Pleistocene re-colonizations (Ribera & Blasco-Zumeta, 1998).

In this study, we use partial sequences from two mitochondrial genes to investigate the phylogeography and genetic diversity of the Iberian populations of *Mioscirtus wagneri* (Kittary, 1859) (Orthoptera: Acrididae), a highly specialized grasshopper exclusively inhabiting hypersaline low grounds. This species shows a classical Mediterranean–Turanian disjunct distribution and in the Iberian Peninsula it only occupies hypersaline environments with patches of *Suaeda vera*, the halophilic plant on which it depends for food (Cordero, Llorente & Aparicio, 2007). In contrast with other co-distributed organisms with higher colonization abilities, its low dispersal capacity has probably determined that geographically distant populations have progressively become unconnected from the late Tertiary (Ribera & Blasco-Zumeta, 1998). Our aim is using this grasshopper as a model system to shed light on the phylogeography history of species inhabiting these relict habitats and drawing general conclusions on the processes determining the current distribution of inland saline ecosystems in the Iberian Peninsula.

MATERIAL AND METHODS

Sampling and study area

During 2006–2007, we sampled 24 populations of *M. wagneri* (Fig. 1; Table 1). 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; P. J. Cordero, unpubl. data). We analysed 2–12 adult individuals per population (Table 1) and specimens were preserved whole in 1500 µL ethanol 96% at −20 °C until needed for genetic analyses.

DNA extraction, PCR and sequencing

We used NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) kits to extract and purify genomic DNA from a hind leg of each individual. Segments (totalling 744 bp) of two mitochondrial genes, 16S rRNA (16S) and cytochrome oxidase subunit II (COII), were amplified using polymerase chain reaction (PCR). Primers 16Sar (5′-CGCCTGTTTAACAAAAACAT-3′) and 16Sbr (5′-CCGGTCTGAACTCAGATCACGT-3′) (Simon *et al.*, 1994) were used to amplify a 484-bp region of the 16S gene. A 260-bp fragment of the COII gene was amplified using the primers C2-J-3400 (5′-ATTGGACATCAATGATATTGA-3′) and C2-N-3661 (5′-CCACAAATTTCTGAACATTGACCA-3′) (Simon *et al.*, 1994). Approximately 5 ng of template DNA was amplified in 25-µL reaction volume containing 1× reaction buffer [67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer, Ecogen], 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 µM of each primer and 0.1 U of *Taq* DNA

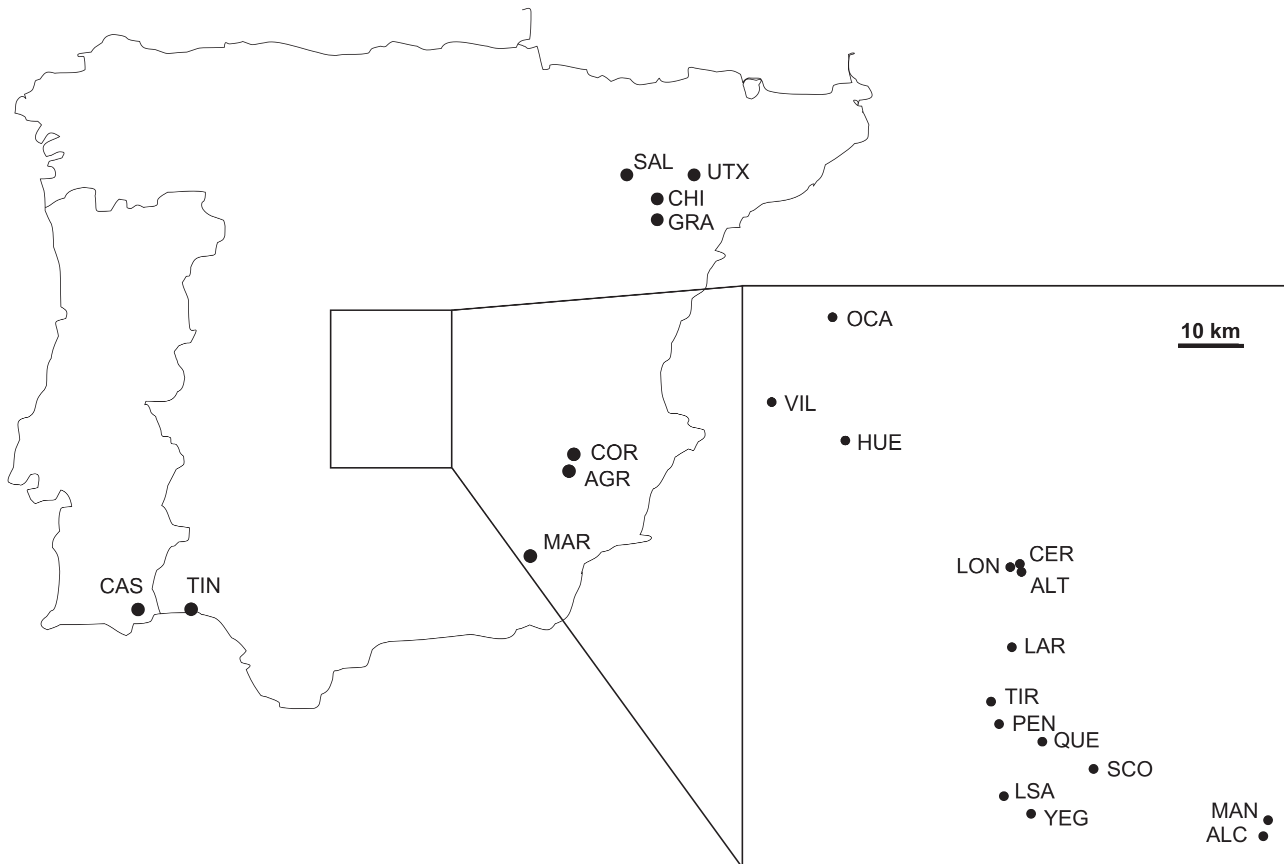

Figure 1. Sampling sites of *Mioscirtus wagneri* in the Iberian Peninsula. Population codes are described in Table 1.

EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf, Hamburg, Germany) 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 (16S: 60 °C; COII: 50 °C) and 45 s at 72 °C, ending with a 5-min final elongation stage at 72 °C. PCR products were purified using NucleoSpin Extract II (Macherey-Nagel) kits and bidirectionally sequenced on an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were edited and aligned using the program BIOEDIT (Hall, 1999). All sequences have been deposited in GenBank (accession numbers EU913775–EU913788).

Phylogenetic analyses of mitochondrial haplotypes

Maximum likelihood (ML) phylogeny was constructed in PAUP using a general time-reversible (GTR) substitution model and empirical base frequencies (Swofford, 1998). Supplementary was a minimum evolution (ME) phylogeny built using the software MEGA version 3.1 with a Kimura 2-parameter distance matrix (Kumar *et al.*, 2008). Node support in both ML and ME phylogenetic analysis was tested using 1000 bootstrap replications. Phylogenetic trees were rooted using *Podisma sapporensis* (Orthoptera: Acrididae) as the outgroup (accession numbers, 16S: AB213376; COII: AB213340).

Genetic variability and population structure

We calculated nucleotide diversity (π) and haplotype diversity (h) using DNASP version 4.0 (Rozas & Rozas, 1999). All analyses of population genetic structure were performed only considering those populations with five or more individuals sequenced (e.g. Gómez *et al.*, 2007). Tajima's D statistic (Tajima, 1989) was computed in ARLEQUIN version 3.11 (Excoffier, Laval & Schneider, 2005) to test for selective neutrality; its significance was estimated by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation adapted from Hudson (1990). Genetic differentiation between populations was determined by calculating pairwise F_{ST} values and testing their significance by 10 000 permutations using ARLEQUIN.

Table 1. Geographical location and sample size of the 24 studied populations of *Mioscirtus wagneri* in hypersaline lagoons and saline grounds of the Iberian Peninsula

Locality	Province	Code	Latitude	Longitude	Sample size
Northeast					
Pantano de Utxesa	Lleida	UTX	41°29′N	0°30′E	8
Laguna Salada	Zaragoza	SAL	41°30′N	0°43′W	9
Laguna de Chiprana	Zaragoza	CHI	41°14′N	0°11′W	8
Laguna Salada Grande	Teruel	GRA	41°02′N	0°12′W	6
Central–southeast					
Saladar de Ocaña–Aranjuez	Toledo	OCA	39°58′N	3°38′W	10
Saladar de Huerta	Toledo	HUE	39°50′N	3°37′W	10
Saladar de Villasequilla	Toledo	VIL	39°53′N	3°44′W	11
Laguna del Cerrillo	Toledo	CER	39°41′N	3°18′W	10
Laguna del Altillo	Toledo	ALT	39°41′N	3°18′W	10
Laguna de Longar	Toledo	LON	39°41′N	3°19′W	10
Laguna Larga	Toledo	LAR	39°36′N	3°18′W	9
Laguna de Tírez	Toledo	TIR	39°32′N	3°21′W	9
Laguna de Peña Hueca	Toledo	PEN	39°31′N	3°20′W	10
Laguna de Quero	Toledo	QUE	39°29′N	3°15′W	2
Laguna de la Sal	Toledo	LSA	39°26′N	3°20′W	12
Laguna de las Yeguas	Toledo	YEG	39°25′N	3°17′W	10
Laguna de Salicor	Ciudad Real	SCO	39°27′N	3°10′W	5
Laguna de Alcahozo	Ciudad Real	ALC	39°23′N	2°52′W	10
Laguna de Manjavacas	Cuenca	MAN	39°24′N	2°52′W	10
Saladar de Cordovilla	Albacete	COR	38°33′N	1°38′W	3
Saladar de Agramón	Albacete	AGR	38°24′N	1°37′W	5
Saladar del Margen	Granada	MAR	37°38′N	2°34′W	10
Southwest					
Río Tinto	Huelva	TIN	37°13′N	6°54′W	10
Castro Marin	Algarve	CAS	37°14′N	7°30′W	10

The pattern of population genetic structure was analysed using a spatial analysis of molecular variance as implemented by SAMOVA version 1.0 (Dupanloup, Schneider & Excoffier, 2002). This method identifies the optimal grouping option (K) for the data by maximizing the among-group component (F_{CT}) of the overall genetic variance. We defined the number of population (K) and ran 100 simulated annealing processes. We simulated different numbers of populations, ranging from $K = 2$ to $K = 10$, to determine the best population clustering option. Based on sequence divergence rates described for other arthropods of ~0.8% per Myr for 16S gene (Hewitt, 1996; Sturmbauer, Levinton & Christy, 1996; Page, Humphreys & Hughes, 2008) and ~2.0%/Myr for the more rapidly evolving COII gene (Brown, George & Wilson, 1979; Brower, 1994; Lunt, Ibrahim & Hewitt, 1998), we estimated the split time between the main lineages described.

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 3.15, which performs a Mantel test and a reduced major axis (RMA) regression analysis (Jensen, Bohonak & Kelley, 2005). The significance of the 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 was expected (e.g. Gómez *et al.*, 2007).

NESTED CLADE ANALYSIS

A parsimony network for all haplotypes was constructed following the procedure of Templeton, Crandall & Sing (1992) as implemented in TCS version 1.21 (Clement, Posada & Crandall, 2000) with the default setting of 95% parsimony connection limit. Association between nested clades and geographic distribution was evaluated in GeoDis version 2.5 (1 000 000 permutations; Posada, Crandall & Templeton, 2000). The inference key (updated 11 November 2005) modified from Templeton (1998) was used to establish the potential biological causes of these associations.

RESULTS

Phylogenetic relationships between *M. wagneri* populations

We obtained sequences for both analysed genes from 207 individuals, recovering five haplotypes for 16S and nine for COII (Table 2). The trees inferred for each gene separately were very similar (Fig. 2). In both cases, the phylogenetic relationships between the three main geographical areas were not well resolved and, in the case of the COII-derived tree, the haplotypes from central–south-east and south-west populations were not monophyletic (Fig. 2). However, the phylogenetic tree did improve when it was built on the concatenated fragments of both genes. When both genes were put together we obtained a total of 12 distinct haplotypes. Both ML and ME trees retrieved a very similar tree topology and supported the monophyly of the haplotypes found in the three main geographical areas with per cent bootstrap values over 50% (Fig. 3A). Further, ME tree also revealed the presence of two monophyletic clades in the Iberian Peninsula, corresponding with populations located in north-east and central–south geographical areas (Fig. 3A). By contrast, the ML tree retrieved three main clades but did not resolve any phylogenetic relationships among them.

Nucleotide diversity

Tajima's D did not differ significantly from the expectation under neutrality in any population or population group (north-east, central–south-east, south-west) ($P > 0.05$ in all cases). Estimates of intrapopulation genetic diversity are given in Table 2. Each population contained an average of 1.13 haplotypes for the 16S gene (range 1–2) and 1.46 for the COII gene (range 1–3). Thirteen out of the 24 populations completely lacked genetic diversity at the

Table 2. Estimates of genetic diversity for the 24 studied populations of *Mioscirtus wagneri* based on mitochondrial data; π (standard deviation), nucleotide diversity; h (standard deviation), gene diversity

Population code	π ($\cdot 10^2$)	h	Haplotypes 16S (n)	Haplotypes COII (n)
Northeast				
UTX	0.06 (0.03)	0.68 (0.01)	MW2 (5), MW3 (3)	MW9 (1), MW10 (7)
SAL	0.03 (0.02)	0.22 (0.17)	MW2 (9)	MW10 (8), MW11 (1)
CHI	0.00 (0.00)	0.00 (0.00)	MW2 (8)	MW10 (8)
GRA	0.12 (0.05)	0.60 (0.21)	MW2 (4), MW3 (2)	MW9 (1), MW10 (5)
Central–southeast				
OCA	0.03 (0.01)	0.20 (0.15)	MW4 (10)	MW8 (1), MW12 (9)
HUE	0.00 (0.00)	0.00 (0.00)	MW4 (10)	MW12 (10)
VIL	0.00 (0.00)	0.00 (0.00)	MW4 (11)	MW12 (11)
CER	0.06 (0.02)	0.47 (0.13)	MW4 (10)	MW7 (3), MW12 (7)
ALT	0.05 (0.02)	0.36 (0.16)	MW4 (10)	MW7 (2), MW12 (8)
LON	0.00 (0.00)	0.00 (0.00)	MW4 (10)	MW12 (10)
LAR	0.03 (0.02)	0.22 (0.17)	MW4 (9)	MW7 (1), MW12 (8)
TIR	0.00 (0.00)	0.00 (0.00)	MW4 (9)	MW12 (9)
PEN	0.00 (0.00)	0.00 (0.00)	MW4 (10)	MW12 (10)
QUE	0.00 (0.00)	0.00 (0.00)	MW4 (2)	MW12 (2)
LSA	0.04 (0.02)	0.30 (0.15)	MW4 (12)	MW7 (2), MW12 (10)
YEG	0.06 (0.02)	0.47 (0.13)	MW4 (10)	MW7 (3), MW12 (7)
SCO	0.00 (0.00)	0.00 (0.00)	MW4 (5)	MW12 (5)
ALC	0.00 (0.00)	0.00 (0.00)	MW4 (10)	MW12 (10)
MAN	0.00 (0.00)	0.00 (0.00)	MW4 (10)	MW12 (10)
COR	0.00 (0.00)	0.00 (0.00)	MW4 (3)	MW12 (3)
AGR	0.00 (0.00)	0.00 (0.00)	MW4 (5)	MW12 (5)
MAR	0.00 (0.00)	0.00 (0.00)	MW4 (10)	MW12 (10)
Southwest				
TIN	0.05 (0.03)	0.38 (0.18)	MW1 (10)	MW6 (8), MW13 (1), MW14 (1)
CAS	0.05 (0.02)	0.36 (0.16)	MW1 (8), MW5 (2)	MW6 (10)

Data for π and h are presented combining DNA sequence information from the two mitochondrial genes analysed (16S and COII). Haplotypes for 16S and COII genes (as described in the GenBank) recovered in each sampling site are indicated. The number of individuals with the same haplotype in each population is shown in parentheses.

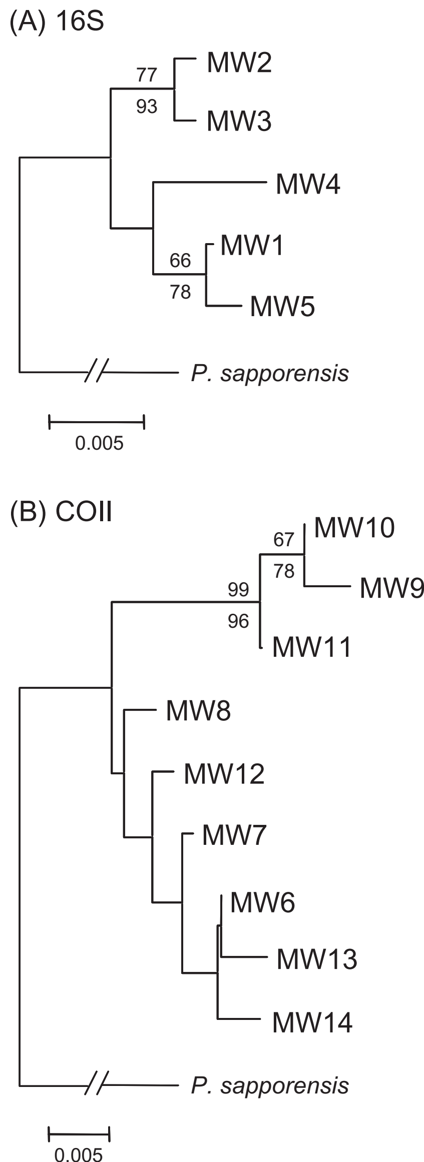

Figure 2. Phylograms showing the relationships between *Mioscirtus wagneri* haplotypes for the 16S and COII mitochondrial genes. Per cent bootstrap support in the maximum likelihood (ML, above branches) and minimum evolution (ME, below branches) analyses are shown (only bootstrap support values over 50% are shown). The tree was rooted with sequences from *Podisma sapporensis*.

studied DNA fragments. We explored possible geographical trends of genetic diversity. The precision of genetic diversity estimates could be different because sample sizes (i.e. the number of analysed individuals) used for their estimation varied between localities. So, we used sample size to give observations different weights in a weighted least-squares analysis. Combining sequence information from the two analysed genes, we found no pattern of genetic diversity with latitude (π: Student's t-test = 0.376; $P = 0.711$; h: $t = 0.559$; $P = 0.582$) or longitude (π: Student's t-test = –0.187; $P = 0.853$; h: Student's t-test = –0.008; $P = 0.994$). Further, genetic diversity was not associated with the distance to the nearest population (π: $t = 0.307$; $P = 0.762$; h: Student's t-test = 0.580; $P = 0.568$), an estimate of population isolation. Similar results were obtained after analysing estimates of nucleotide diversity for both gene fragments separately (data not shown).

Genetic structure

Considering sequence information from both mitochondrial genes combined, we found that in each geographical area there was a single dominating haplotype (north-east: H1; central–south-east: H7; south-west: H9). One haplotype (H8) was also relatively frequent in La Mancha (central Spain), whereas the others were generally only carried by a small number of individuals (< 5) (Fig. 3B). Populations from these three different geographic areas did not share any haplotype, suggesting that gene flow between them is absent. Accordingly, the comparison of the population pairwise F_{ST} values indicated high levels of population differentiation. In general, all populations were significantly differentiated from all others except those within the same geographical area (Table 3). SAMOVA analyses indicated that F_{CT} values sharply increased between $K = 2$ and $K = 3$, while all values for $K \geq 3$ were very similar. Thus, $K = 3$ is the best population clustering option, supporting the north-east, central–south-east and south-west groups, according with the tree major clades of the phylogenetic tree. The proportion of the overall variance explained by differences among these three groups was 97.80% ($F_{CT} = 0.98$), whereas differences between populations ($F_{SC} = 0.08$) and within populations ($F_{ST} = 0.98$) explained an additional 0.17 and 2.03% of the overall variance, respectively. All variance components were highly significant ($P < 0.001$). Based on sequence divergence rates described for other arthropods, we can estimate the split time between lineages of north-east and south-west areas around the Early Pleistocene (16S: ~1.30 Myr; COII: ~1.15 Myr). Similarly, central–south-east/north-east divisions were also estimated to have occurred in the Early Pleistocene (16S: ~1.28 Myr; COII: ~1.15 Myr), whereas central–south-east and south-west clades have probably diverged more recently (16S: ~1.02 Myr; COII: ~0.38 Myr).

Isolation-by-distance analyses between *M. wagneri* populations revealed a significant positive correlation between genetic and geographic distances ($Z = 597.66$, $r = 0.761$, one-sided $P < 0.001$ from 10 000 randomizations) and a value of $r^2 = 0.579$ for RMA regression

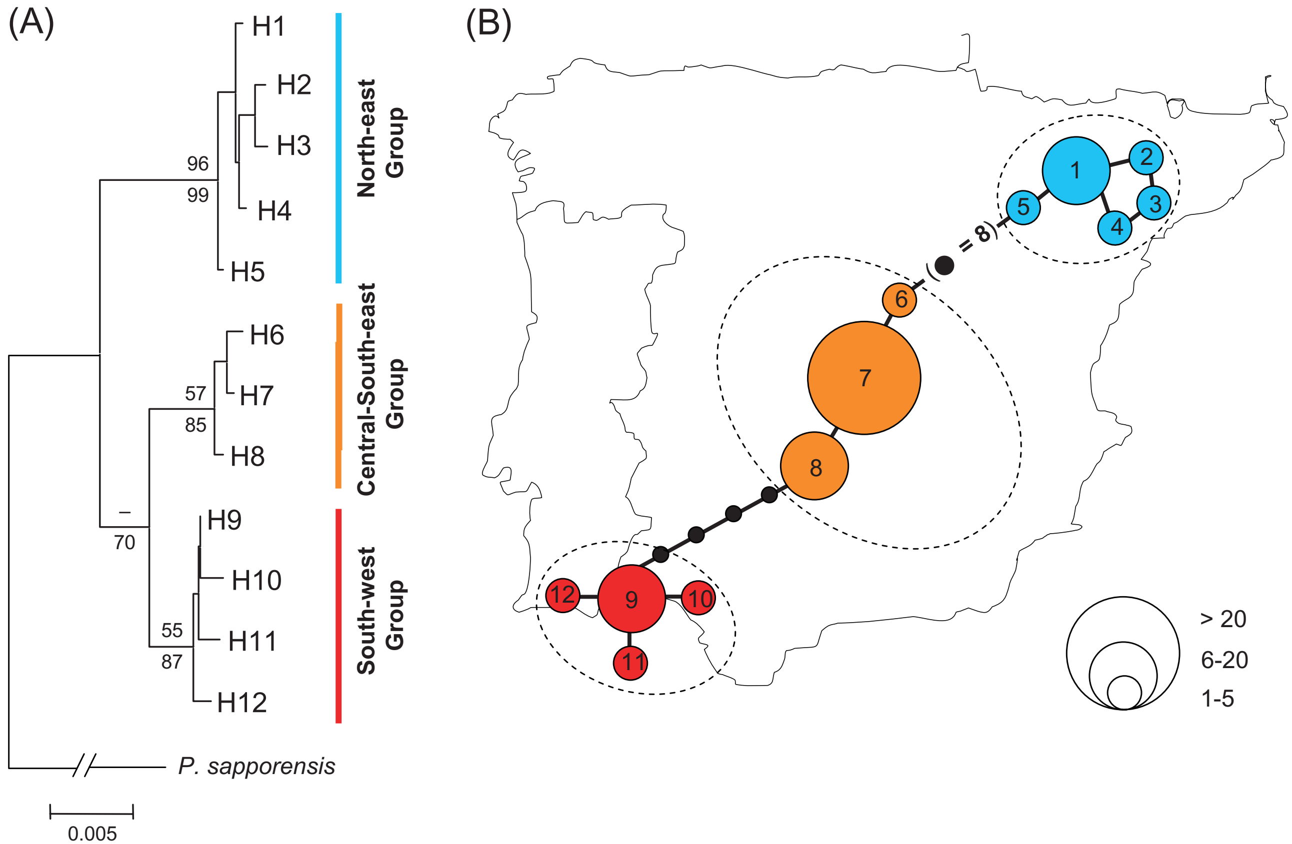

Figure 3. A, phylogram showing the relationships between *Mioscirtus wagneri* haplotypes (combining sequence information from the 16S and COII genes). Per cent bootstrap support in the maximum likelihood (ML, above branches) and minimum evolution (ME, below branches) analyses are shown (only bootstrap support values over 50% are shown). The tree was rooted with sequences from *Podisma sapporensis*. B, networks obtained for *M. wagneri* haplotypes using statistical parsimony. Circles represent haplotypes and numbers indicate haplotype code. Each connection is a single mutation step with black circles representing inferred haplotypes. Size of circles is proportional to the number of individuals with that haplotype. Colours in the online version (blue: north-east; orange: central–south-east; red: south-west) and ellipses identify geographical areas.

analysis. Similar results were obtained when sequence information from both 16S and COII genes were analysed separately (16S: $Z = 571.10$, $r = 0.774$, $P < 0.001$; RMA $r^2 = 0.599$; COII: $Z = 587.68$, $r = 0.758$, $P < 0.001$; RMA $r^2 = 0.575$).

Nested clade analysis

The haplotype network provided by TCS is shown in Figure 3B. It was used to construct a nested design, which was structured into four first-level and two second-level clades (Fig. 4). Analyses using GeoDis revealed that two clades (including total cladogram) showed significant geographical–genetic associations (Table 4). According to NCA inference key, these results suggest allopatric fragmentation between central–south-east and south-west groups (clade 2–2) and between the north-east group and the rest of the populations (total cladogram) (Table 4).

DISCUSSION

All our data support a marked phylogeographical structure in the Iberian populations of *M. wagneri*. Phylogenetic analyses have revealed three main clades in the Iberian Peninsula, corresponding with populations located in north-east, central–south-east and south-west Iberia. The south-west clade was the most restricted geographically, being only presented in two close localities, whereas the central–south-east clade extended over 18 populations separated up to 276 km (Fig. 1). The north-east clade showed an intermediate range distribution, with four populations separated up to 76 km (Fig. 1). Geographical

Table 3. Population pairwise F_{ST} values

	UTX	SAL	CHI	GRA	OCA	HUE	VIL	CER	ALT	LON	LAR	TIR	PEN	LSA	YEG	SCO	ALC	MAN	AGR	MAR	TIN	CAS
UTX	–																					
SAL	**0.19**	–																				
CHI	0.21	–0.01	–																			
GRA	–0.16	**0.15**	**0.19**	–																		
OCA	**0.96**	**0.98**	**0.99**	**0.96**	–																	
HUE	**0.97**	**0.99**	**1.00**	**0.97**	0.00	–																
VIL	**0.97**	**0.99**	**1.00**	**0.97**	0.01	0.00	–															
CER	**0.95**	**0.97**	**0.98**	**0.95**	**0.17**	0.22	0.24	–														
ALT	**0.95**	**0.97**	**0.98**	**0.95**	0.07	0.11	**0.12**	–0.08	–													
LON	**0.97**	**0.99**	**1.00**	**0.97**	0.00	0.00	0.00	0.22	0.11	–												
LAR	**0.96**	**0.97**	**0.99**	**0.96**	0.00	0.01	0.02	0.00	–0.08	0.01	–											
TIR	**0.97**	**0.99**	**1.00**	**0.97**	–0.01	0.00	0.00	0.21	0.10	0.00	**0.00**	–										
PEN	**0.97**	**0.99**	**1.00**	**0.97**	0.00	0.00	0.00	0.22	0.11	0.00	0.01	0.00	–									
LSA	**0.95**	**0.97**	**0.98**	**0.96**	0.05	0.07	0.08	–0.05	–0.09	0.07	–0.09	0.06	0.07	–								
YEG	**0.95**	**0.97**	**0.98**	**0.95**	0.17	0.22	0.24	–0.11	–0.08	0.22	0.00	0.21	0.22	–0.04	–							
SCO	**0.96**	**0.98**	**1.00**	**0.96**	–0.08	0.00	0.00	**0.11**	0.01	0.00	–0.08	0.00	0.00	–0.02	**0.11**	–						
ALC	**0.97**	**0.99**	**1.00**	**0.97**	0.00	0.00	0.00	0.22	0.11	0.00	0.01	0.00	0.00	0.07	0.22	0.00	–					
MAN	**0.97**	**0.99**	**1.00**	**0.97**	0.00	0.00	0.00	0.22	0.11	0.00	0.01	0.00	0.00	0.07	0.22	0.00	0.00	–				
AGR	**0.96**	**0.99**	**1.00**	**0.96**	–0.08	0.00	0.00	**0.11**	0.01	0.00	–0.08	0.00	0.00	–0.02	**0.11**	0.00	0.00	0.00	–			
MAR	**0.97**	**0.99**	**1.00**	**0.97**	0.00	0.00	0.00	0.22	0.11	0.00	0.01	0.00	0.00	0.07	0.22	0.00	0.00	0.00	0.00	–		
TIN	**0.96**	**0.98**	**0.98**	**0.96**	**0.96**	**0.98**	**0.98**	**0.94**	**0.95**	**0.98**	**0.96**	**0.97**	**0.98**	**0.96**	**0.94**	**0.97**	**0.98**	**0.98**	**0.97**	**0.97**	–	
CAS	**0.96**	**0.98**	**0.98**	**0.96**	**0.97**	**0.98**	**0.98**	**0.95**	**0.96**	**0.98**	**0.96**	**0.98**	**0.98**	**0.96**	**0.95**	**0.97**	**0.98**	**0.98**	**0.97**	**0.97**	0.06	–

Only populations with five or more individuals are included. Values in bold are statistically significant ($P < 0.05$).

areas did not share any haplotype, suggesting that gene flow among them is absent, and nested clade analysis revealed that they have probably become differentiated through vicariance. *Mioscirtus wagneri* shows certain phylogeographic concordance with some zooplanktonic organism also inhabiting saline environments across the Iberian Peninsula (rotifers: Gómez *et al.*, 2000, 2007; shrimps: Muñoz *et al.*, 2008), which suggests some shared biogeographical histories. However, *M. wagneri* clearly shows a much more marked population genetic structure, probably because its low dispersal potential in comparison with zooplanktonic organisms frequently subjected to waterfowl-mediated gene flow (Gómez *et al.*, 2000; Figuerola, Green & Michot, 2005; Gómez *et al.*, 2007; Muñoz *et al.*, 2008).

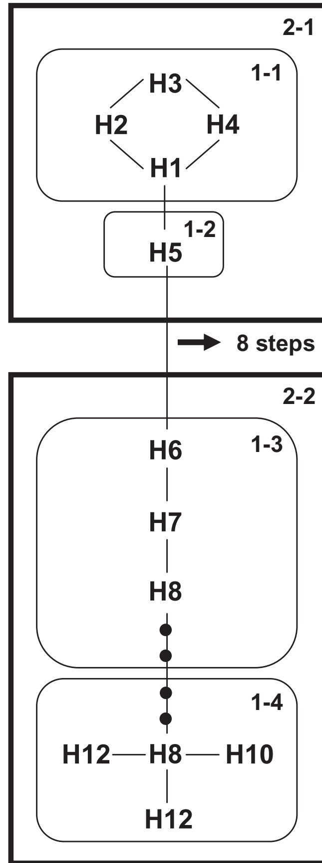

Figure 4. Haplotype network and associated nested design for Iberian populations of *Mioscirtus wagneri*. Haplotype numbers correspond to those displayed geographically in Figure 3B. Each line within the network represents a single mutational step which connects two haplotypes states (except where indicated). Black dots represent hypothetical unsampled/ancestral haplotypes.

We did not find any geographical trend of genetic diversity across the Iberian populations of *M. wagneri*, suggesting that the 'southern-richness and northern-purity' pattern characteristic of recently expanded European populations (also reported within refugial peninsulas; Gómez & Lunt, 2006; Canestrelli, Cimmaruta & Nascetti, 2008) does not fit for our study species. A latitudinal cline of genetic diversity is only expected in species able to disperse from southern refugia and track their emerging suitable habitats after the last ice age (Hewitt, 1996, 2000). However, this is not likely to be the case in *M. wagneri* or other specialized organisms with low dispersal potential associated with hypersaline environments as these habitats have probably become progressively more fragmented from the Early Pleistocene up to present days.

Based on sequence divergence rates described for other arthropods, we can estimate the split time between the lineages of north-east/south-west and central–south-east/north-east areas around the Early Pleistocene, whereas central–south-east and south-west clades have probably diverged more recently. This suggests the existence of an originally continuous population during the Messinean saline crisis, a period in which, according to the pollinic records, the habitat suitable for *M. wagneri* was likely to be much more widespread over the Iberian Peninsula (Blanco *et al.*, 1997). Data on sequence divergence suggest that the fragmentation of a suspected continuous

Table 4. Nested clade analysis of the geographical distribution of *Mioscirtus wagneri* haplotypes in the Iberian Peninsula

Clade		Populations groups involved	*P*-value	Inference key sequence	Biological inference
2–2	1–3	Central–southeast	< 0.001	1, 19, NO	Allopatric fragmentation
	1–4	Southwest			
Total	2.1	Northeast	< 0.001	1, 19, NO	Allopatric fragmentation
	2.2	Central–southeast/Southwest			

Only clades with significant GeoDis results are shown. The *P*-value of the χ^2-square test and the sequence followed in the inference key from GeoDis are indicated.

population could have started in the Early Pleistocene, although the distant populations from central–south-west Iberia may have remained connected until recent times. In any case, the relationship between sequence divergence and time for *M. wagneri* may not follow the same pattern as observed in other organisms with available molecular clocks (Ayala, 1997, 1999) and our estimates should be only considered an approximate time window rather than an attempt to establish a precise dating of the inferred processes (Hewitt, 1996).

All populations of *M. wagneri* exhibited very low levels of nucleotide diversity, with more than the half of the studied populations showing a complete lack of genetic variability at the studied mitochondrial DNA fragments. The low levels of genetic diversity could be a consequence of the small size of the studied populations which, in addition, are known to go through sharp demographic bottlenecks associated with periodical intense rainfall events which inundate the vegetation ring around hypersaline lagoons and low grounds used by this species to lay their eggs (P. J. Cordero, unpubl. data). Although these population crashes could have resulted from the inherently small-sized patches of these relict habitats, human-induced habitat alterations have also recently contributed to the progressive fragmentation and size reduction of these hypersaline environments.

Overall, this study shows that the Iberian populations of *M. wagneri* have a marked phylogeographical structure and highlights the importance of studying species with biogeographical histories which may greatly differ from that of the bulk of the Palaearctic organisms. Further, this study reveals that DNA divergences between the three main geographical areas are high enough to be considered as evolutionary-significant units which should be taken into account in future conservation strategies of Iberian hypersaline environments. Fortunately, several of these habitats have been protected or proposed for protection in recent years, offering a good network which could ensure their future conservation.

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