The uncertainty of Late Pleistocene range expansions in the western Mediterranean: a case study of the colonization of south-eastern Spain by the spur-thighed tortoise, *Testudo graeca*

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ABSTRACT

Aim Recent biogeographical studies have postulated a North African, Late Pleistocene, origin for some species of the Iberian Peninsula. However, a robust assessment of such range expansions requires high-resolution molecular tools to resolve overlapping biogeographical and cultural processes. Here we aim to determine whether the spur-thighed tortoise, *Testudo graeca*, arrived in south-eastern Spain during historical or prehistoric times, and whether its dispersal to the Iberian Peninsula was human-mediated.

Location The western Mediterranean Basin (south-eastern Spain, northern Algeria and north-western Morocco).

Methods Using 428 samples from 19 sites in North Africa and 18 in south-eastern Spain, we obtained mitochondrial sequences from the cytochrome *b* gene and genotypes derived from seven microsatellite loci. These data were employed to obtain population genetics descriptors, haplotype networks, Bayesian cluster analyses and isolation-by-distance patterns. Moreover, we used a Bayesian demographic approach to delimit the dates involved in the range expansion.

Results We found lower levels of genetic variability and weak mitochondrial differentiation in the south-eastern Spanish tortoises compared with the North African ones. However, exclusive haplotypes occurred in the Iberian samples and microsatellite cluster analyses revealed moderate levels of admixture across both sides of the Mediterranean. A coastal area in the west of Algeria and the central-southern region in south-eastern Spain are suggested as the most probable founder and arrival places, respectively. Finally, we identified signatures of an ancient bottleneck event approximately 20,000–30,000 years ago.

Main conclusions The spur-thighed tortoise probably arrived in south-eastern Spain during Late Pleistocene sea-level low stands. The role that humans may have played as dispersers across the Mediterranean remains unclear. Our results are in accordance with other recent findings of trans-Mediterranean expansions during this period and highlight the importance of employing precise methodological approaches before a species can be considered as historically introduced.

Keywords

Cytochrome *b*, demographic history, human-mediated dispersal, Mediterranean Basin, microsatellites, North Africa, population structure, species conservation, Strait of Gibraltar, transmarine dispersal.

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INTRODUCTION

Mediterranean biodiversity has been influenced by humans for at least the last 10,000 years (Blondel, 2006). As a consequence, it is challenging to distinguish between a human-mediated or natural origin for some Iberian populations. This is particularly true for organisms that crossed the Strait of Gibraltar in the last 100,000 years or so, as any imprecision in dating or calibration could change the evidence favouring, or not, human-mediated transportation. Some Iberian species, including the common genet, *Genetta genetta*, the Mediterranean chameleon, *Chamaeleo chamaeleon*, and the Egyptian mongoose, *Herpestes ichneumon*, have traditionally been considered as having been introduced by humans (Morales, 1994; Pleguezuelos *et al.*, 2008; Riquelme-Cantal *et al.*, 2008). Their status as 'historically introduced species' was a consequence of their cultural linkage to historical civilizations, having their main ranges in Africa and the lack of any fossil record in Europe. However, although these species share the same genetic pattern of low mitochondrial differentiation across both sides of the Strait of Gibraltar and lower genetic variation in the Iberian populations, two alternative prehistoric scenarios could be responsible for the current pattern (Paulo *et al.*, 2002; Gaubert *et al.*, 2010).

1. Natural transmarine dispersals during periods of low sea level of the last glaciation (the Würm period, from 125,000 to 18,000 years ago), when the Mediterranean sea dropped by approximately 120–140 m relative to the present, reducing the sea strait between Iberia and North Africa to a width of only 3.5 km (Zazo, 1999; Flemming *et al.*, 2003).
2. Dispersal as travelling companions with the first human migrations across the Strait of Gibraltar; as could be the case

for species consumed by prehistoric cultures, including tortoises or hedgehogs (Fernández-Jalvo *et al.*, 1999; Blasco, 2008; Vamberger *et al.*, 2011), or those linked to human activities, such as has been proposed for the white-toothed shrew, *Crocidura russula* (Cosson *et al.*, 2005), and the Moorish gecko, *Tarentola mauritanica* (Harris *et al.*, 2004).

However, the assessment of when humans crossed the Strait of Gibraltar for the first time is a complex issue with many uncertainties. Although it is generally thought that human movements can be traced back to the mid- or Late Pleistocene, the first definite evidence for deep-sea fishing and navigation within the region dates to only the 12th millennium BC (Straus, 2001; Broodbank, 2006). Consequently, due to the complexity in clarifying which scenario is associated with each expansion event, there is a need to deepen our knowledge of the genetic spatial structure of populations across their Mediterranean range, by comparing their genetic signatures with both conservative and rapidly evolving markers.

Here we use this approach to unravel the case of a North African expansion: the origin of the endangered spur-thighed tortoise, *Testudo graeca* Linnaeus, 1758 (Testudinidae), in south-eastern Spain. The majority of the western Mediterranean distribution of this species lies in North Africa, with a few small and isolated western European populations confined to the Iberian Peninsula (Doñana and south-eastern Spain) and on a few islands (Mallorca, Sardinia, Sicily; Fig. 1). In general, western European populations are thought to be the result of historical introductions, which is supported by the absence of any fossil record in western Europe (Morales-Pérez & Sanchis-Serra, 2009) and low mitochondrial differentiation between the European and North African populations (Álvarez *et al.*, 2000; Harris *et al.*, 2003;

Figure 1 Approximate distribution of the five western Mediterranean mitochondrial lineages of *Testudo graeca* as described in Fritz *et al.* (2009). I corresponds to *T. g. soussensis*, II to *T. g. marokkensis*, III to *T. g. graeca*, IV to *T. g. nabeulensis* and V to *T. g. cyrenaica*. Grey areas represent the global range of the species, while black dots correspond to the western European populations of North African origin. The rectangle shows the study area.

Fritz *et al.*, 2009; Salinas *et al.*, 2011; Vamberger *et al.*, 2011). However, for the south-eastern Spanish tortoises an incipient evolutionary isolation has recently been suggested by the discovery of unique haplotypes and an explicit genetic pattern with spatial coherence (Fritz *et al.*, 2009; Graciá *et al.*, 2011; Salinas *et al.*, 2011), suggesting a more ancient origin for this expansion.

In the present study, we aim to determine whether the spur-thighed tortoise colonized south-eastern Spain in historical or prehistoric times. To do so, we estimate the time interval since the species' arrival using Bayesian demographic analyses. In addition, we characterize this range expansion by assessing differences in the spatial genetic patterns among the North African and south-eastern Spanish tortoises using a clustering approach to estimate the areas of origin and arrival of founder populations. Furthermore, we apply common exploratory methods of population genetics to assess the role of humans. The absence of a signature of recent bottlenecks, an isolation-by-distance pattern across the entire Iberian study area, a decrease of expected heterozygosity with increasing geographical distance from the putative centre of origin and genetic units established in a spatially coherent context would be evidence against recent human-assisted dispersal.

MATERIALS AND METHODS

Sampling and genetic analyses

This study broadly covers the range of the subspecies *Testudo graeca graeca*, being based on 428 samples of wild tortoises from the Moulouya Valley in Morocco (seven from a single location), the Tell and Saharan Atlas in Algeria (97 samples from 18 sites) and south-eastern Spain (325 samples from 18 sites). Additionally, we considered 37 previously published sequences from known localities (see Appendix S1 in Supporting Information).

The complete cytochrome *b* gene and 25 bp of the adjacent tRNA-Thr gene (1164 bp; cyt *b*) was amplified by polymerase chain reaction (PCR) and subsequently sequenced. Additionally, seven microsatellite loci previously used for these studied populations, namely GmuD16, GmuB08, GmuD51, Test 76, Test 21, Test 71 and Gp96 (Graciá *et al.*, 2011; Salinas *et al.*, 2011), were analysed. All loci were examined for genotyping errors, allelic dropout and null alleles using MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004). For further extended information regarding the marker selection and laboratory procedures see Appendix S2.

Mitochondrial DNA data analyses

Sequences were aligned with CLUSTAL W using default parameters as implemented in MEGA4 (Tamura *et al.*, 2007) and collapsed into haplotypes using TCS 1.2.1 (Clement *et al.*, 2000). We used the same software to calculate a parsimony network, which connects the different haplotypes via a minimal number of mutational steps. With this

approximation it is possible to have information about the relative age of each haplotype; those that have more connections and are located in the interior of the network are considered to be older than tip haplotypes (Posada & Crandall, 2001). Haplotype diversity (H_d), nucleotide diversity (π) and the average number of nucleotide differences (P_i) were obtained for the North African and south-eastern Spanish groups using DNASP 5.10 (Librado & Rozas, 2009).

Microsatellite data analyses

Genotypic linkage disequilibrium between each pair of microsatellite loci was tested using the Markov chain Monte Carlo (MCMC) method implemented in GENEPOP 4.0 (Rousset, 2007). To discard the linkage of the used loci is a premise for the use of the two Bayesian clustering methods we employed, STRUCTURE 2.3.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003; Hubisz *et al.*, 2009) and GENELAND 3.3.2 (Guillot *et al.*, 2005a,b). These clustering programs identify groups (K) that maximize the Hardy–Weinberg equilibrium (HWE) and the linkage equilibrium within them.

First, we ran STRUCTURE from $K = 1$ to $K = 10$, without prior population information, using the admixture model and the option 'correlated allele frequencies'. The burn-in was set to 10,000 and the number of iterations to 100,000. We repeated each analysis 10 times to evaluate the convergence and to estimate the optimal value of K following the method proposed by Evanno *et al.* (2005). Given that it is known that in south-eastern Spanish populations conservation actions including introductions and translocations have been carried out (Graciá *et al.*, 2011; Salinas *et al.*, 2011), we used the 'locprior' option, considering all the different sampling sites, and the optimal obtained value of K as priors, with the aim of detecting animals potentially introduced from North Africa (Hubisz *et al.*, 2009). In order to corroborate these results we compared multilocus information in an assignment/exclusion test using GENECLASS2 software (Piry *et al.*, 2004), selecting the Bayesian method for computation suggested by Rannala & Mountain (1997) and the algorithm by Paetkau *et al.* (2004) for 1000 simulated individuals ($\alpha = 0.05$). Those individuals detected as introduced in the south-eastern Spanish range were removed from further analyses. All these results were graphically displayed by DISTRICT (Rosenberg, 2004).

The second clustering method, GENELAND, has some similarities to STRUCTURE but allows the use of the geographical location of each sample as prior information. We ran the MCMC five times from $K = 1$ to $K = 10$, with the following parameters: 500,000 MCMC iterations, a thinning of 100, maximum rate of Poisson process fixed to 100, uncertainty attached to spatial coordinates fixed to 1 km, minimum K fixed to 1 and maximum fixed to 10, maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 300 and a Dirichlet model for allelic frequencies (given that it has been demonstrated that this performs better than the alternative model; Guillot *et al.*, 2005b). We inferred the optimal value

of K from its modal value in these first five runs and ran the software five additional times with K fixed to this obtained value. The posterior probability of population membership for each pixel in the spatial domain ($x = 50$; $y = 50$) was computed for these 10 runs after a burn-in of the first 10% of saved iterations. Once again the consistency of results was evaluated among the different runs.

GENALEX 6.0 (Peakall & Smouse, 2006) was employed to estimate diversity and differentiation indices among and within clusters inferred by STRUCTURE and GENELAND. Allele frequencies, the mean number of alleles and effective alleles, the number of private alleles and expected heterozygosities were obtained using this software. Moreover, we used this program to obtain pairwise F_{ST} values as a measure of genetic distance among clusters and between sampling sites within them. These genetic distances were employed using this software in two subsequent analyses: (1) in hierarchical analysis of molecular variance (AMOVA) to determine how genetic variation is distributed among regions, populations and individuals, when considering the groups suggested by the clustering analyses results; and (2) in two Mantel tests to detect the relationship between genetic isolation and geographical distance of North African and south-eastern Spanish populations (among sampled sites). In these last analyses, genetic distance was described by $F_{ST}/(1-F_{ST})$ and geographical distance by considering the natural logarithm of the Euclidean distance among sampled sites. Finally, we correlated the expected heterozygosities at sites with the geographical distances to the putative point of arrival of the species to south-eastern Spain, which was obtained as the centroid of those sites sharing at least a 5% of their assignment with the North African clusters in the GENELAND analyses. In this correlation, in AMOVA analyses and in Mantel tests, only those sites with more than seven individuals were considered to avoid problems due to small sample sizes.

Demographic history analyses: dating the expansion process

To infer the colonization process in south-eastern Spain, we explored the demographic history of the species there through different analyses of the microsatellite data. Tortoises from south-eastern Spain were treated as a single population, with the exception of one analysis, which was repeated for the location with the highest number of samples (see below).

First, we performed two different analyses using the software BOTTLENECK 1.2.02 (Piry *et al.*, 1999). We tested the temporary excess of heterozygosity that results from a reduction of the population size under the stepwise mutation model (SMM), and the two-phase mutation model (TPM) with 95% of SMM and 12% of variance (Cornuet & Luikart, 1996). To evaluate the significance of this analysis, we used the Wilcoxon signed-rank test based on 10,000 repetitions. Using this software we also carried out a mode-shift test,

which discriminates bottlenecked populations from stable populations, to detect a distortion of the expected L-shaped distribution (Luikart *et al.*, 1998).

Secondly, MSVAR 1.3 (Beaumont, 1999; Storz & Beaumont, 2002), based on a Bayesian coalescent method, was used to infer historical changes in the effective population size of the south-eastern Spanish populations. This method is based on the observed allele distributions and allele frequencies, assuming that a stable population with a determinate size (N_1) started to decrease (or increase) linearly or exponentially some time ago (T) towards the current population size (N_0). Based on this assumption and using the MCMC approach the method not only allows the estimation of N_0 and N_1 , but also T . Mutation rates ($\theta = 2N_0\mu$) were assumed under the SMM model and, like the rest of the parameters, were inferred separately across loci using priors assumed to be under a lognormal distribution. Because we were testing founder and bottleneck processes, we ran the program under the exponential growth model, using different wide uninformative priors. The generation time of *T. g. graeca* was set at 17.72, the mean value taken from those estimated by Díaz-Paniagua *et al.* (2001) from different years and scenarios. To evaluate the stability of the results we ran the program five times for one of the employed priors (with mean 100,000), with the lowest interval being 2×10^4 iterations. We discarded the first 10% of total iterations to avoid bias in parameter estimation due to starting conditions. The distribution of the remaining data was plotted against prior distributions to see the consistency of the results over the different runs and used to obtain the lower (10%), the median (50%) and upper (90%) quantiles of the posterior distributions. Given that genetic structure can generate false bottleneck signals when using this method (Chikhi *et al.*, 2010) or that multiple arrivals at different places could have given rise to south-eastern Spanish populations with different past population demographic histories, we repeated these analyses for the sampled site with the highest sample size, Galera ($n = 74$). We note that although it seems counterintuitive to use monomorphic loci in demographic analyses, it has been reported that their exclusion could lead to an overestimation of the decrease in population (Beaumont, 1999). Therefore, in order to estimate the possible bias of the obtained results, the analyses were run a further three times but excluding the two monomorphic loci.

To delimit further the dates and agents involved in this species' expansion, we used the data obtained from the MSVAR analyses including the two monomorphic loci. Specifically, we tested the subsequent hypotheses: (1) arrival before the last glacial period (more than 125,000 years ago); (2) arrival by transmarine dispersal during the last glacial period (18,000–125,000 years ago); (3) arrival by prehistoric introduction during the last glacial period (18,000–30,000 years ago, assuming that humans could have crossed during conditions of low sea level); (4) arrival by introduction after the development of prehistoric navigation (3100–14,000 years ago); (5) arrival by introduction by

Arabs or Phoenicians (520–3100 years ago); and finally (6), although not very likely, arrival by recent introduction as a consequence of the international pet trade during the last 100 years.

We calculated the density of iterations of T accumulated until the beginning and end of each hypothesis and, after this, the *odds* in favour of the time (T) periods that delimit each hypothesis (e.g. for the fifth hypothesis the inferior limit is $T \leq 3100$ and a superior limit is $T > 520$). The *odds* in favour of an event permit the comparison of two complementary alternatives, being expressed as the ratio between them $P/(1-P)$. It is usually considered that ratios greater than 3 represent positive evidence in favour of the considered alternative, and greater than 7 are considered significant (as in Sousa *et al.*, 2008). Consequently, values below 0.33 and 0.14, respectively, were considered as positive evidence and significantly in favour of its complementary alternative (in relation to the previous example, the complementary T periods would be $T > 3100$ and $T < 520$ for the lower and upper limits, respectively).

RESULTS

Mitochondrial genetic structure

Our dataset for the North African and south-eastern Spanish *T. g. graeca* included a total of 36 different haplotypes for cyt *b*. Nine of these haplotypes were previously reported by Fritz *et al.* (2009) and given that we amplified the same fragment, we followed the nomenclature of these authors (from B₁8 to B₁34; see Appendix S1 for GenBank accession numbers). The parsimony network was fully connected under the 95% criterion, with few missing node haplotypes. With two exceptions, all haplotypes belonged to the B₁ clade, corresponding to the subspecies *T. g. graeca* (Fig. 2a).

The remaining two haplotypes, A₆ and A₇, were found at El Kala, an Algerian site close to the Tunisian border, where another subspecies (*T. g. nabeulensis*) is present (Fritz *et al.*, 2009). Within the B₁ clade, the two haplotypes B₁2 and B₁3 were shared among Spanish and North African tortoises, while 28 haplotypes were only found in North Africa and four were exclusive to south-eastern Spain. For North Africa, an internal substructure was revealed in which the different haplotypes were mostly shared among neighbouring sites, with the exception of some common haplotypes with broad geographical distribution (such as B₁2, B₁3 or B₁29; Fig. 2b). The six haplotypes present in south-eastern Spain were distinct by one mutation step each (Fig. 2a). Additionally, a strong spatially coherent structure was revealed in which: (1) haplotype B₁4 was restricted to the central-southern part of the study area (Vera Basin and the Bédar and Cabrera mountains); (2) haplotypes B₁3 and B₁1 were common in the central and northern study area (from the Los Lobos area and the Almagro Mountains to the north of Lorca and the Almenara Mountains); (3) at Marinica, a site located in a central coastal place of the study area, the three unique haplotypes B₁2, B₁15

and B₁16 were found, the last two of which had not previously been reported for south-eastern Spain (Fig. 2b).

Haplotype diversity, nucleotide diversity and the average number of nucleotide differences were all higher in North African tortoises ($H_d = 0.854$; $\pi = 0.0029$; $P_i = 3.4$) than in south-eastern Spain ($H_d = 0.715$; $\pi = 0.0013$; $K = 1.5$). From these analyses North African samples belonging to the A clade were excluded, given that this lineage does not represent the *T. g. graeca* subspecies.

Genetic diversity and structure inferred from microsatellites

Two loci (Test 76 and Gp96) were monomorphic. For the other five, we detected a total of 83 different alleles, ranging from 6 (Test 71) to 28 (*GmuD51*) per locus (Table 1). We did not find evidence for genotyping errors or allelic drop-outs and no significant linkage disequilibrium was found for any of the 10 pairwise locus combinations. However, MICROCHECKER showed potential null alleles for Test 71 at Mouloya and Sotomayor sites and for *GmuB08* at Galera.

Population structure inferred from STRUCTURE analyses indicated that $K = 2$ was the most appropriate cluster solution using the ΔK statistic proposed by Evanno *et al.* (2005) (modal value of $\Delta K = 975.0$ when $K = 2$; $\Delta K = 49.68$ when $K = 3$). Although this method is not able to detect when $K = 1$ is more appropriate than $K = 2$, we considered this result reliable given that the different runs of $K = 2$ were consistent over repetitions and divided North African and south-eastern Spanish individuals in the two different clusters without any sampling prior information (99% and 92.3% of North African and south-eastern Spanish individuals, respectively, were assigned to their expected geographical group; Fig. 3a). After using the sampling locations as prior information, we detected in south-eastern Spain two potentially recently introduced tortoises, which were assigned to the North African cluster with membership probabilities > 0.90 . An assignment test in GENECLASS corroborated these results, and these two individuals were removed from further analyses.

North African tortoises are characterized by a higher mean level of allelic diversity, but a similar level of expected heterozygosities (number of alleles, $N_A = 12$; number of effective alleles, $N_E = 6.21$; expected heterozygosity, $H_E = 0.54$) compared with south-eastern Spanish tortoises ($N_A = 8.43$; $N_E = 3.30$; $H_E = 0.49$; Table 1). Additionally, 26 private alleles were found in North Africa, but only one in south-eastern Spain. After sequential Bonferroni correction, the two loci *GmuB08* and *GmuD51* did not conform to HWE in south-eastern Spanish tortoises, showing homozygote excess ($P < 0.003$). Pairwise F_{ST} values were in general significant (data not shown). The weakest differentiation (0.04) was found between a western coastal site in Algeria (Saf Saf) and one of the southernmost sites in south-eastern Spain (Teresa, located in the Cabrera Mountains), while the highest level of differentiation (0.24) was found between the National Park El Kala, in the east of Algeria, and Malacate, a site in the

Figure 2 Mitochondrial structure. (a) Parsimony network for mitochondrial DNA haplotypes of North African and south-eastern Spanish spur-thighed tortoises (*Testudo graeca*). Each line joining haplotypes indicates one nucleotide substitution, except when hatches across lines are present; then each hatch indicates one step. Colours indicate the haplotypes found in south-eastern Spain. The hatched area within each symbol represents the proportion of south-eastern Spanish haplotypes against the proportion of North African haplotypes and symbol size corresponds approximately to haplotype frequency. Thick lines connect the described haplotypes for south-eastern Spain, shaping a perfectly connected subnet. Only those haplotypes present in south-eastern Spain or mentioned in the text are highlighted. (b) Haplotype composition of each sampled site. Site abbreviations are defined in Appendix S1. Colours correspond to those in panel (a) and represent south-eastern Spanish haplotypes, while white symbols indicate haplotypes which occur only in North Africa. Symbol size corresponds approximately to the individual sample size for each place. The black line encloses the approximate distribution of the subspecies *T. g. graeca*.

Table 1 Statistical descriptors by locus for the south-eastern Spanish and the North African spur-thighed tortoises (*Testudo graeca*).

Locus	South-eastern Spanish tortoises (<i>n</i> = 323)							North African tortoises (<i>n</i> = 100)						
	Allele size range	<i>N_A</i>	<i>N_E</i>	<i>N_P</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>	Allele size range	<i>N_A</i>	<i>N_E</i>	<i>N_P</i>	<i>H_O</i>	<i>H_E</i>	<i>F_{IS}</i>
<i>GmuD16</i>	186–254	17	6.76	0	0.84	0.85	0.01	186–270	21	13.92	4	0.91	0.93	0.02
<i>GmuB08</i>	206–242	13	5.36	0	0.76	0.81	0.07*	200–257	19	12.61	6	0.89	0.92	0.03
<i>GmuD51</i>	143–239	21	5.06	0	0.76	0.8	0.06*	139–251	27	10.6	7	0.87	0.91	0.04
Test 76	114	Monomorphic						114	Monomorphic					
Test 21	200–214	3	1.87	0	0.46	0.47	0.02	190–214	9	2.59	6	0.58	0.61	0.05
Test 71	128–132	3	2.02	1	0.53	0.51	−0.05	128–138	6	1.74	3	0.33	0.42	0.22
Gp96	140	Monomorphic						140	Monomorphic					
Overall	–	8.43	3.30	–	0.48	0.49	0.02	–	12	6.21	–	0.51	0.54	0.07

*Significant deviation from Hardy–Weinberg equilibrium after Bonferroni sequential correction ($P < 0.005$).

N_A, number of alleles; *N_E*, number of effective alleles; *N_P*, number of privative alleles; *H_O*, observed heterozygosity; *H_E*, unbiased expected heterozygosity; *F_{IS}*, fixation index.

Figure 3 Genetic structure of North African and south-eastern Spanish spur-thighed tortoises (*Testudo graeca*) inferred from microsatellite loci. (a) Bar plots generated by STRUCTURE (Pritchard *et al.*, 2000). Vertical bars represent individuals, while the amount of each colour indicates the proportion of each inferred cluster. The initial model assumed admixture and correlated allele frequencies. The arrow indicates the two individuals sampled in the south-eastern Spanish group significantly identified as introduced from North African when using the 'locprior' option (see Results). (b) Proportion of each site attributed to each of the four GENELAND clusters (Guillot *et al.*, 2005a,b) using the geographical location of each sample as prior information. Site abbreviations are defined in Appendix S1. The black line encloses the approximate distribution of the *T. g. graeca* subspecies.

interior of the south-eastern Spanish study area, in the Almagro Mountains. The AMOVA analysis statistically supported the two clusters and indicated that 8.5% of the global genetic variation was distributed between them, 5.6% among sampled sites within these two groups, 5.2% among individuals within each site and 80.7% corresponded to individual variation ($F_{ST} = 0.14$; $F_{IS} = 0.06$; $F_{IT} = 0.19$; all P -values = 0.01). Mantel tests reported significant correlations between genetic and geographical distances both within North Africa ($r = 0.56$; $P = 0.03$) and within south-eastern Spain ($r = 0.37$; $P = 0.01$).

In the five GENELAND runs, $K = 4$ was the suggested number of different populations when the whole study area was considered. The next five posterior runs with the number of populations fixed to four showed good consistency: south-eastern Spanish individuals were gathered in a single cluster, while the three remaining clusters were located within North Africa, differentiating the individuals from the Moulouya Valley, from Ain Chorfa (a coastal sampling site in the west of Algeria) and from the remaining sampling sites in Algeria

(Fig. 3b). These clusters exhibited pairwise F_{ST} values which ranged from 0.03 (between Ain Chorfa and the remaining samples in Algeria) to 0.16 (between south-eastern Spain and the Moulouya Valley). The other two F_{ST} values with respect to the south-eastern Spanish group were 0.12 and 0.10 for Ain Chorfa and the rest of Algeria, respectively. The AMOVA test, using the proposed clusters as regions, revealed that 9.2% of genetic variation was distributed among regions, 5.2% among sampled sites within regions, 5.2% among individuals within sites, and 80.4% within individuals ($F_{ST} = 0.14$; $F_{IS} = 0.06$; $F_{IT} = 0.20$; all P -values = 0.01). It is noteworthy that within the south-eastern Spanish range, only the cluster which mainly comprised Ain Chorfa appeared to be represented, although in a low proportion and concentrated in the centre-south of the studied area (Vera Basin and the Bédar and Cabrera mountains; Fig. 3b). Therefore, the locations of Chinas, Centinares, Teresa, Judio and Cintas (with at least 5% of their genetic variation assigned to the North African cluster) were used for calculating a centroid between them, considered as the putative arrival of the

species to south-eastern Spain. We detected a significant decrease of the expected heterozygosities at Iberian sites with increasing distance from this point ($P = 0.08$; $r = -0.62$).

Past demographic history and the arrival of the species in south-eastern Spain

The results obtained with BOTTLENECK did not suggest any evidence of a recent bottleneck event for the south-eastern Spanish tortoises. However, using MSVAR 1.3 we found a clear signal of an ancient population collapse both for the Galera site and for all the south-eastern Spanish tortoises (Fig. 4, Appendix S3). For both datasets, the effective population N_1 decreased over 100 times from a large ancestral effective size, estimated at 39,810 individuals (10th–90th quantiles = 7943–125,892 and 5011–100,000, for Galera and south-eastern Spain, respectively), to the current effective size N_0 of a few hundred (199 individuals, 10th–90th quantiles = 25–631 for Galera; and 251 individuals, 10th–90th quantiles = 63–1000, for the whole Spanish dataset; Fig. 4a). The bottleneck was dated to approximately 32,000 years ago (31,622 years, 10th–90th quantiles = 3981–100,000) when the whole Spanish dataset was considered, and to approximately 20,000 years ago when only the data from Galera were used (19,952 years, 10th–90th quantiles = 2511–79,432; Fig. 4b). The obtained posterior densities of N_0 , N_1 and T were consistent over the different runs and the different priors used. The similarity in the estimated parameters both for the whole Spanish dataset or Galera alone is notable. Additionally, the obtained values were also similar when the two monomorphic loci were not considered. However, as was reported by Beaumont (1999), when these markers were excluded, the distributions were discretely shifted to the left, suggesting a slightly more recent process (Appendix S3).

To summarize these results, the hypothesis of an arrival before the last glaciation can be rejected. Likewise, the hypotheses of a very recent introduction as a consequence of the pet trade or the introduction of the species in historical times are not supported. The remaining three hypotheses (Table 2: trans-marine dispersal, prehistoric introduction during the last glacial period, or prehistoric introduction by prehistoric seafarers) are suggested as the most plausible ones. The relative temporal proximity of these three hypotheses (in part even overlapping) did not allow a decision regarding which is the most probable.

DISCUSSION

Dating the arrival of the species in south-eastern Spain

Different classes of molecular markers provide different insights into the temporal and geographical scales of the life history of a species. Whereas the study of genealogies can elucidate population processes, phylogeographical events or even speciation, allele frequencies can yield information on demographic trends (Sunnucks, 2000). The combination of these

Figure 4 Demographic inference for the arrival of *Testudo graeca* in south-eastern Spain. Results obtained from the analyses with MSVAR 1.3 software (Storz & Beaumont, 2002). Thin solid lines correspond to the posterior distribution of the parameters obtained in the five runs for the Galera sampled site. Thick lines correspond to the posterior distribution in the five runs for the whole south-eastern Spanish dataset. The wide uninformative priors employed in the analyses are represented by the dashed lines. (a) Posterior distribution of ancestral (N_1) and present (N_0) population effective sizes. (b) Posterior distribution of the time (in years ago) of the species' arrival in south-eastern Spain. The intervals of time between braces (from i to vi) correspond to the hypotheses for the species' arrival put forward in the last paragraph of Materials and Methods.

data types provides a more complete understanding of how an organism has responded to changes in the biogeographical landscape in a broad temporal spectrum, and the use of complementary analyses allows the study of different components of a species' evolutionary history (Garrick *et al.*, 2010). In this sense, the scale of resolution achieved by previous studies which employed mitochondrial DNA (mtDNA) only allowed rejection of the hypothesis that *T. graeca* arrived in the Iberian Peninsula before the Strait of Gibraltar opened (Álvarez *et al.*, 2000; Fritz *et al.*, 2009; Salinas *et al.*, 2011). By contrast, studies based on allele frequencies of microsatellites suggested that Spanish tortoises were not introduced very recently (Graciá *et al.*, 2011; Salinas *et al.*, 2011). Our results, based on the Bayesian analyses of the historical demography of the species, allowed us to delimit the interval of time for its arrival in south-eastern Spain to prehistoric times, to a period predating the maritime expansion of the Greeks and Phoenicians

Table 2 Inference of the plausible hypotheses for the arrival of the spur-thighed tortoise (*Testudo graeca*) to south-eastern Spain using Bayesian demographic data from MSVAR 1.3 and the odds analyses in favour of the different periods of time (T ; see Materials and Methods).

Hypotheses for the species' arrival	T period (years ago)	Inferior limit of the hypothesis		Superior limit of the hypothesis		
		Odds in favour of the T period		Odds in favour of the T period		
		South-eastern Spain	Galera	T period (years ago)	South-eastern Spain	Galera
(i) Transmarine dispersal before the LGP	–	–	–	$T > 125,000$	0.05§	0.04§
(ii) Transmarine dispersal during the LGP	$T \leq 125,000$	19.29†	24.14†	$T > 18,000$	1.44	0.82
(iii) Facilitated by humans during the LGP	$T \leq 30,000$	1.75	3.00*	$T > 18,000$	1.44	0.82
(iv) Introduced by prehistoric seafarers	$T \leq 14,000$	0.69	1.21	$T > 3100$	10.09†	5.58*
(v) Introduced in historical times	$T \leq 3100$	0.1§	0.18‡	$T > 520$	81.63†	250.00†
(vi) Introduced in the last century	$T \leq 100$	0.0001§	0.0003§	–	–	–

LGP, last glacial period.

*Ratios greater than 3, which suggest positive evidence in favour of the T period.

†Ratios greater than 7, considered significant in favour of the T period.

‡Ratios below 0.33, which represent positive evidence in favour of the complementary T period.

§Ratios below 0.14, considered significant in favour of the complementary T period.

in the western Mediterranean, and after the beginning of the last glaciation process (between approximately 3100 and 125,000 years ago). Our analyses for Spanish tortoises revealed a bottleneck approximately 20,000–30,000 years ago with a strong reduction of the ancestral effective population size, coinciding with one of the minimum Mediterranean sea level phases (Flemming *et al.*, 2003; Masseti, 2009). These results basically keep open two different possibilities for the species' arrival: natural transmarine dispersal, which has been reported for other species of tortoise (e.g. Caccone *et al.*, 1999; Gerlach *et al.*, 2006), or its introduction as a consequence of the first cultural expansions of humans from North Africa (late Palaeolithic or early Neolithic; Broodbank, 2006).

Unfortunately, the role that humans could have played in the species' dispersal across the Mediterranean remains unclear. Although a strong connection between tortoises and primitive Mediterranean cultures due to consumption habits is well known (e.g. Blasco, 2008; Morales-Pérez & Sanchis-Serra, 2009), it remains unclear exactly when humans crossed the Strait of Gibraltar for the first time (Flemming *et al.*, 2003; Broodbank, 2006). One of the main traditional arguments claiming the recent arrival of *T. g. graeca* to the Iberian Peninsula is the lack of fossils, in contrast with the abundant fossil remains of another Spanish tortoise species, *Testudo hermanni*. Nevertheless, in a recent work Morales-Pérez & Sanchis-Serra (2009) reported the absence of *Testudo* fossils in the Iberian Mediterranean region for the last 30,000 years, due to the species' rarity or even its local extinction. The rarity of *T. hermanni* as a consequence of climatic aridification occurring during this period, or even due to its possible overexploitation, could have led to a change in the consumption habits of humans (Morales-Pérez & Sanchis-Serra, 2009). Therefore, the absence of fossil remains of *T. graeca* could be explained as a consequence both of the low initial size of the population and of a change

in prey selection of modern humans, similar to the situation Stiner & Kuhn (2006) reported for several sites from Italy, in which 'fast prey' replaced 'slow prey'.

Disentangling an expansion process

We found an incipient differentiation process between North African and Spanish tortoises that is easily explained by the North African origin of the south-eastern Spanish populations (Álvarez *et al.*, 2000; Harris *et al.*, 2003; Fritz *et al.*, 2009; Graciá *et al.*, 2011; Salinas *et al.*, 2011). Microsatellite cluster analyses revealed moderate levels of admixture across the Mediterranean Basin and, when the spatial component was taken into account, allowed geographical location of the potential source and entry point of the species to south-eastern Spain. The most probable founder area in North Africa corresponds to some coastal sampled sites located in the west of Algeria, while the most plausible arrival point in south-eastern Spain is located in the central-southern part of the studied area. These results were additionally supported by the fact that the lowest pairwise F_{ST} values across both sides of the Mediterranean Basin corresponded to sampled sites located within these two areas.

Besides the differentiation among these populations, important different genetic patterns were found within them. The six haplotypes present in south-eastern Spain appeared clearly spatially distributed, showing a north–south differentiation pattern through the studied area. This spatial structure is in strong agreement with that reported by Graciá *et al.* (2011) using microsatellites. However, the North African mitochondrial genetic structure seems more diffuse across the region with some common haplotypes widely spread over the whole subspecies' range, some of them located at internal positions in the parsimony network (B_{12} and B_{13}). This North African pattern could be explained by ancient contrac-

tions and expansions of the species' range and population following climatic changes (Fritz *et al.*, 2009), and contrasts with the more structured mitochondrial pattern reported for the Spanish tortoises, which is probably a direct reflection of the colonization–expansion process. In any case, the finding of isolation-by-distance patterns within both ranges (identified from the microsatellite data) suggests that their present spatial structures are the result of a main differentiation–dispersal event across the space, which in the case of the south-eastern Spanish range suggests the natural expansion of the species within the newly colonized territory (as proposed by Graciá *et al.*, 2011). This hypothesis would be additionally corroborated by the detection of a spatial negative gradient in heterozygosities from the hypothetical arrival area in south-eastern Spain. However, the possibility of different origins for Iberian populations at different sites cannot be discarded without analysing in a landscape context the effect of geographical barriers on the genetic configuration of the populations. Such a methodology could clarify whether the exclusive haplotypes found in Marinica are the result of a particular colonization history or, alternatively, are the consequence of isolation and differentiation of the site.

Understanding recent Mediterranean range expansions

In this work we have used *T. g. graeca* as an example of a group of organisms from the Iberian Peninsula that show minimal mtDNA differentiation from North African populations, whose estimated dates of divergence are generally less than 100,000 years and which have complex and unresolved expansion processes. Recent genetic studies corroborated the historical introduction of some such species to the Iberian Peninsula, such as the Barbary macaque, *Macaca sylvanus* (Modolo *et al.*, 2005), or the common genet, *Genetta genetta* (Gaubert *et al.*, 2010); or contrarily suggested an ancient origin, as in the case of the white-toothed shrew, *Crocidura russula* (Cosson *et al.*, 2005), the Egyptian mongoose, *Herpestes ichneumon* (Gaubert *et al.*, 2010), or the snakes *Malpolon monspessulanus* and *Hemorrhois hippocrepsis* (Carranza *et al.*, 2006). For other species, including the Mediterranean chameleon, *Chamaeleo chamaeleon* (Paulo *et al.*, 2002), or the treefrog *Hyla meridionalis* (Recuero *et al.*, 2007), mtDNA genetic studies have failed to distinguish between historical and prehistoric origins of the Iberian populations. Our results highlight the importance of developing an accurate methodological approach to disentangle the particular expansion processes of each species. In fact, our assessment of *T. g. graeca* indicates that, even with extensive sampling and the use of microsatellites, it may be difficult or even impossible to ascertain with confidence whether such species naturally crossed during low sea level stands or were introduced by humans. In this sense, the range expansions of these species are at the limits of resolution of the tools available and constitute a current knowledge frontier.

It is clear, however, that the Late Pleistocene was an important period of faunal interchange between North Africa

and Europe and this framework should be taken into account from a species conservation point of view. Much caution should be taken when labelling a species as historically introduced because, as is shown in this work, disentangling the origin of some species can be a challenging task. The consideration of the species as an historical introduction may modify the social perception of the species (Pérez *et al.*, 2011), and could predispose to their conservation value being underestimated. Furthermore, given that conservation plans usually focus only on so-called native species, legal protection for the spur-thighed tortoise could be reduced (Colautti & MacIsaac, 2004). Assuming that sometimes it is impossible to distinguish using current methods between the native or human-mediated origin of some populations due to their complex biogeographical histories, the precautionary principle should therefore apply.

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REFERENCES

- Álvarez, Y., Mateo, J.A., Andreu, A.C., Díaz-Paniagua, C., Diez, A. & Bautista, J.M. (2000) Mitochondrial DNA haplotyping of *Testudo graeca* on both continental sides of the Straits of Gibraltar. *Journal of Heredity*, **91**, 39–41.
- Beaumont, M.A. (1999) Detecting population expansion and decline using microsatellites. *Genetics*, **153**, 2013–2029.
- Blasco, R. (2008) Human consumption of tortoises at level IV of Bolomor Cave (Valencia, Spain). *Journal of Archaeological Science*, **35**, 2839–2848.
- Blondel, J. (2006) The 'design' of Mediterranean landscapes: a millennial story of human and ecological systems during the historic period. *Human Ecology*, **34**, 713–729.
- Broodbank, C. (2006) The origins and early development of Mediterranean maritime activity. *Journal of Mediterranean Archaeology*, **19**, 199–230.
- Caccone, A., Gibbs, J.A., Ketmaier, V., Suatoni, E. & Powell, J.R. (1999) Origin and evolutionary relationships of giant Galápagos tortoises. *Proceedings of the National Academy of Sciences USA*, **96**, 13223–13228.

- Carranza, S., Arnold, E.N. & Pleguezuelos, J.M. (2006) Mitochondrial DNA indicates the Mediterranean snakes, *Malpolon monspessulanus* and *Hemorrhois hippocrepis* (Squamata, Colubridae), are recent colonizers of the Iberian Peninsula. *Molecular Phylogenetics and Evolution*, **40**, 532–546.
- Chikhi, L., Sousa, V., Luisi, P., Goossens, B. & Beaumont, M.A. (2010) The confounding effects of population structure, genetic diversity and the sampling scheme on the detection and quantification of population size changes. *Genetics*, **186**, 983–995.
- Clement, M., Posada, D. & Crandall, K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.
- Colautti, R.I. & MacIsaac, H.J. (2004) A neutral terminology to define invasive species. *Diversity and Distributions*, **10**, 135–141.
- Cornuet, J.M. & Luikart, G. (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001–2014.
- Cosson, J.F., Hutterer, R., Libois, R., Sarà, M., Taberlet, P. & Vogel, P. (2005) Phylogeographical footprints of the Strait of Gibraltar and Quaternary climatic fluctuations in the western Mediterranean: a case study with the greater white-toothed shrew, *Crocidura russula* (Mammalia: Soricidae). *Molecular Ecology*, **14**, 1151–1162.
- Díaz-Paniagua, C., Keller, C. & Andreu, A.C. (2001) Long term demographic fluctuations of the spur-thighed tortoise *Testudo graeca* in SW Spain. *Ecography*, **24**, 707–721.
- Evanno, G., Regnaut, M.S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.
- Falush, D., Stephens, M. & Pritchard, J.K. (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, **164**, 1567–1587.
- Fernández-Jalvo, Y., Andrews, P. & Denys, C. (1999) Cutmarks on small mammals at Olduvai Gorge Bed I. *Journal of Human Evolution*, **36**, 587–589.
- Flemming, N. C., Bailey, G. N., Courtillot, V., King, G., Lambeck, K., Ryerson, F. & Vita-Finzi, C. (2003) Coastal and marine palaeo-environments and human dispersal points across the Africa–Eurasia boundary. *The maritime heritage* (ed. by C.A. Brebbia and T. Gambin), pp. 61–74. Wessex Institute of Technology Press, Southampton, UK.
- Fritz, U., Harris, D.J., Fahd, S., Rouag, R., Graciá Martínez, E., Giménez Casaldueiro, A., Široký, P., Kalboussi, M., Jdeidi, T.B. & Hundsdoerfer, A.K. (2009) Mitochondrial phylogeography of *Testudo graeca* in the Western Mediterranean: old complex divergence in North Africa and recent arrival in Europe. *Amphibia-Reptilia*, **30**, 63–80.
- Garrick, R.C., Caccone, A. & Sunnucks, P. (2010) Inference of population history by coupling exploratory and model-driven phylogeographic analyses. *International Journal of Molecular Sciences*, **11**, 1190–1227.
- Gaubert, P., Machordom, A., Morales, A., López-Bao, J.V., Veron, G., Amin, M., Barros, T., Basuony, M., Djagoun, C.A.M.S., Do Linh San, E., Fonseca, C., Geffen, E., Ozkurt, S.O., Cruaud, C., Couloux, A. & Palomares, F. (2010) Comparative phylogeography of two African carnivores presumably introduced into Europe: disentangling natural versus human-mediated dispersal across the Strait of Gibraltar. *Journal of Biogeography*, **38**, 341–358.
- Gerlach, J., Muir, C. & Richmond, M.D. (2006) The first substantiated case of transoceanic tortoise dispersal. *Journal of Natural History*, **40**, 2403–2408.
- Graciá, E., Giménez, A., Anadón, J.D., Botella, F., García-Martínez, S. & Marín, M. (2011) Genetic patterns of a range expansion: the spur-thighed tortoise *Testudo graeca* in southeastern Spain. *Amphibia-Reptilia*, **32**, 49–61.
- Guillot, G., Estoup, A., Mortier, F. & Cosson, J.F. (2005a) A spatial statistical model for landscape genetics. *Genetics*, **170**, 1261–1280.
- Guillot, G., Mortier, F. & Estoup, A. (2005b) Geneland: a program for landscape genetics. *Molecular Ecology Notes*, **5**, 712–715.
- Harris, D.J., Znari, M., Macé, J.C. & Carretero, M.A. (2003) Genetic variation in *Testudo graeca* from Morocco estimated using 12S rRNA sequencing. *Revista Española de Herpetología*, **17**, 5–9.
- Harris, D.J., Batista, V., Lymberakis, P. & Carretero, M.A. (2004) Complex estimates of evolutionary relationships in *Tarentola mauritanica* (Reptilia: Gekkonidae) derived from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, **30**, 855–859.
- Hubisz, M., Falush, D., Stephens, M. & Pritchard, J. (2009) Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, **9**, 1322–1332.
- Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.
- Luikart, G., Allendorf, F.W., Cornuet, J.M. & Sherwin, W.B. (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity*, **89**, 238–247.
- Masetti, M. (2009) Mammals of the Mediterranean islands: homogenisation and the loss of biodiversity. *Mammalia*, **73**, 169–202.
- Modolo, L., Salzburger, W. & Martin, R.D. (2005) Phylogeography of Barbary macaques (*Macaca sylvanus*) and the origin of the Gibraltar colony. *Proceedings of the National Academy of Sciences USA*, **102**, 7392–7397.
- Morales, A. (1994) Earliest genets in Europe. *Nature*, **370**, 512–513.
- Morales-Pérez, J.V. & Sanchís-Serra, A. (2009) The Quaternary fossil record of the genus *Testudo* in the Iberian Peninsula. Archaeological implications and diachronic distribution in the western Mediterranean. *Journal of Archaeological Science*, **36**, 1152–1162.
- van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

- Paetkau, D., Slade, R., Burden, M. & Estoup, A. (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology*, **13**, 55–65.
- Paulo, O.S., Pinto, I., Bruford, M.W., Jordan, W.C. & Nichols, R.A. (2002) The double origin of Iberian peninsular chameleons. *Biological Journal of the Linnean Society*, **75**, 1–7.
- Peakall, R. & Smouse, P.E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.
- Pérez, I., Giménez, A. & Pedreño, A. (2011) A qualitative examination of the social practices and representations towards a species of endangered tortoise. *Wildlife Research*, **38**, 323–329.
- Piry, S., Alapetite, A., Cornuet, J.M., Paetkau, D., Baudouin, L. & Estoup, A. (2004) GENECLASS2: a software for genetic assignment and first-generation migrant detection. *Journal of Heredity*, **95**, 536–539.
- Piry, S.G., Luikart, G. & Cornuet, J.M. (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **90**, 502–503.
- Pleguezuelos, J.M., Fahd, S. & Carranza, S. (2008) El papel del Estrecho de Gibraltar en la conformación de la actual fauna de anfibios y reptiles en el Mediterráneo Occidental. *Boletín de la Asociación Herpetológica Española*, **19**, 1–17.
- Posada, D. & Crandall, K.A. (2001) Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, **16**, 37–45.
- Pritchard, J.K., Stephens, M. & Donnelly, P. (2000) Inference of population structure from multilocus genotypes data. *Genetics*, **155**, 945–949.
- Rannala, B. & Mountain, J.L. (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences USA*, **94**, 9197–9201.
- Recuero, E., Iraola, A., Rubio, X., Machordom, A. & Garcia-Paris, M. (2007) Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern. *Journal of Biogeography*, **34**, 1207–1219.
- Riquelme-Cantal, J.A., Simón-Vallejo, M.D., Palmqvist, P. & Cortés-Sánchez, M. (2008) The oldest mongoose of Europe. *Journal of Archaeological Science*, **35**, 2471–2473.
- Rosenberg, N.A. (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes*, **4**, 137–138.
- Rousset, F. (2007) *Genepop 4.0 for Windows and Linux*. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université Montpellier II, Montpellier, France.
- Salinas, M., Altet, L., Calvel, C., Almeda, R.M., Bayón, A., Burguete, I. & Sánchez, A. (2011) Genetic assessment, illegal trafficking and management of the Mediterranean spur-thighed tortoise in southern Spain and northern Africa. *Conservation Genetics*, **12**, 1–13.
- Sousa, V., Penha, F., Collares-Pereira, M.J., Chikhi, L. & Coelho, M.M. (2008) Genetic structure and signature of population decrease in the critically endangered freshwater cyprinid *Chondrostoma lusitanicum*. *Conservation Genetics*, **9**, 791–805.
- Stiner, M.C. & Kuhn, S.L. (2006) Changes in the ‘connectedness’ and resilience of Paleolithic societies in the Mediterranean ecosystems. *Human Ecology*, **34**, 693–712.
- Storz, J.F. & Beaumont, M.A. (2002) Testing for genetic evidence of population expansion and contraction: an empirical analysis of microsatellite DNA variation using a hierarchical Bayesian model. *Evolution*, **56**, 156–166.
- Straus, L.G. (2001) Africa and Iberia in the Pleistocene. *Quaternary International*, **75**, 91–102.
- Sunnucks, P. (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution*, **15**, 199–203.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596–1599.
- Vamberger, M., Corti, C., Stuckas, H. & Fritz, U. (2011) Is the imperilled spur-thighed tortoise (*Testudo graeca*) native in Sardinia? Implications from population genetics and for conservation. *Amphibia-Reptilia*, **31**, 9–25.
- Zazo, C. (1999) Interglacial sea levels. *Quaternary International*, **55**, 101–113.

SUPPORTING INFORMATION

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

Appendix S1 Geographical and genetic information by site.

Appendix S2 Laboratory procedures.

Appendix S3 Detailed demographic inference for the arrival of *Testudo graeca* in south-eastern Spain.

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Author contributions: E.G., A.G., J.D.A. and F.B. conceived the ideas and collected samples; E.G., J.H. and U.F. processed the samples; E.G. analysed the data; and E.G., F.B. and A.G. led the writing. All authors commented on and revised earlier manuscript versions.