Conserv Genet
DOI 10.1007/s10592-007-9457-1

RESEARCH ARTICLE

Patterns of nuclear and mitochondrial DNA variation in Iberian populations of *Emys orbicularis* (Emydidae): conservation implications

Guillermo Velo-Antón · Mario García-París · Adolfo Cordero Rivera

Received: 16 May 2007 / Accepted: 1 November 2007

Abstract The European pond turtle (*Emys orbicularis*) is threatened and in decline in several regions of its natural range, due to habitat loss combined with population fragmentation. In this work, we have focused our efforts on studying the genetic diversity and structure of Iberian populations with a fine-scale sampling (254 turtles in 10 populations) and a representation from North Africa and Balearic island populations. Using both nuclear and mitochondrial markers (seven microsatellites, ~1048 bp nDNA and ~1500 bp mtDNA) we have carried out phylogenetic and demographic analyses. Our results show low values of genetic diversity at the mitochondrial level although our microsatellite dataset revealed relatively high levels of genetic variability with a latitudinal genetic trend decreasing from southern to northern populations. A moderate degree of genetic differentiation was estimated for Iberian populations (genetic distances, F_{ST} values and clusters in the Bayesian analysis). The results in this study combining mtDNA and nDNA, provide the most comprehensive population genetic data for *E. orbicularis* in the Iberian Peninsula. Our results suggest that Iberian populations within the Iberian–Moroccan lineage should be considered as a single subspecies with five management units, and emphasize the importance of habitat management rather than population reinforcement (i.e. captive breeding and reintroduction) in this long-lived species.

G. Velo-Antón (✉) · A. Cordero Rivera
Grupo de Ecoloxía Evolutiva, Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, E.U.E.T. Forestal, Campus Universitario, 36005 Pontevedra, Spain
e-mail: guillermov@uvigo.es

M. García-París
Museo Nacional de Ciencias Naturales, C.S.I.C., c/ José Gutiérrez Abascal 2, 28006 Madrid, Spain

Keywords *Emys orbicularis* · Genetic diversity · mtDNA · nDNA · Population structure

Introduction

Turtle populations are declining in many areas of the world, due to a combination of overharvesting (Congdon et al. 1993), increasing demand from food markets (Altherr and Freyer 2000), pollution (Gasith and Sidis 1984; Bishop et al. 1998), habitat fragmentation and destruction (Joyal et al. 2001; Epperson and Heise 2003), pet trade (van Dijk et al. 2000; Moll and Moll 2004) and mortality on roads (Forman and Alexander 1998; Gibbs and Shriver 2002). Such conservation problems are particularly severe for freshwater animals, whose habitat is being lost at an accelerated rate.

The European pond turtle, *Emys orbicularis* has a widespread distribution from Eastern and Central Europe, to the Mediterranean countries and North Africa (Fritz 2001). *Emys orbicularis* was so abundant in prehistoric times as to become a frequent item in the European human diet (Schleich and Boehme 1994), but now the species is threatened and in decline in several regions of Europe. Several threats impinge on *E. orbicularis* populations, most of them a direct consequence of the disappearance of freshwater habitats and river contamination. The species has become rare in some regions of Europe (Fritz 2001). Within the Iberian Peninsula, *E. orbicularis* is rare in Portugal (Cabral et al. 1990; Rito de Araújo et al. 1997), while most of the northern populations in Spain are clearly in decline (Bertolero 2000; Mascort 1998; Gómez-Cantarino and Lizana 2000; Cordero Rivera and Ayres Fernández 2004).

Many different factors are contributing to the fragmentation of Iberian populations of *E. orbicularis*. The reduction in population size and the isolation between populations may potentially contribute to loss of genetic variability and local extinctions (Primack 1993; Frankham and Ralls 1998). The most recent distribution map for Iberian populations shows large gaps in the North (where summer temperatures might be too low for this species) and Southeast (where suitable freshwater habitats have almost disappeared) (Keller and Andreu 2002) (Fig. 1). Ayres Fernández and Cordero Rivera (2004) found that one Northwestern Iberian population, where *E. orbicularis* is considered the most endangered reptile (Galán 1999), shows surprisingly high levels of occurrence of accessory plates and malformations in their carapace and we found more recently a decreasing trend of anomalies from the northern to the southern regions of the Iberian Peninsula (Ayres Fernández et al. unpublished). This might be due, at least partially, to low genetic variability associated with small population size and inbreeding (Frankham et al. 2002). Iberian populations of *E. orbicularis* are currently assigned to four different subspecies, although their taxonomic status is still not solved. Two of them (*E. o. fritzjuergenobsti* and *E. o. hispanica*) are Iberian endemics, distributed over Eastern and Southwestern Iberia respectively (Fig. 1). Both subspecies were described based on biometrical measures and colour pattern of carapaces using samples from Valencia (*E. o. fritzjuergenobsti*) and Doñana (*E. o. hispanica*) populations (Fritz 1993; Fritz et al. 1996). Subsequent genetic studies, based on sequences of the mitochondrial *cytochrome b* gene, assigned the two Iberian endemic subspecies to one of the seven *E. orbicularis* lineages found across Europe (Lenk et al. 1999). This lineage seems to correspond to populations dispersed from the North Africa-Iberian refuge after the Pleistocene glacial ages (Lenk et al. 1999; Fritz et al. 2007). Representatives of two more subspecies (*E. o. orbicularis* and *E. o. galloitalica*) are present in Northeastern Iberia, likely as the result of postglacial expansions from eastern Europe populations (Fritz et al. 1996; Mascort 1998; Lenk et al. 1999). Specimens from *E. o. galloitalica* also occur in the Balearic islands due to intentional introductions by humans in the past (Fritz et al. 1998; Lenk et al. 1999) (Fig. 1).

In this paper we investigate the phylogeographic structure of *E. orbicularis* in the Iberian Peninsula using sequences of mitochondrial DNA (mtDNA), nuclear DNA (nDNA) and allelic variation at seven microsatellite loci, for 10 populations covering most of the range of the species in the area, plus one population from the Balearic Islands. Additionally, we also analyzed mtDNA and nDNA sequences from Moroccan samples (corresponding to the subspecies *E. o. occidentalis*) in order to study the relationships between Iberian and Northwestern African

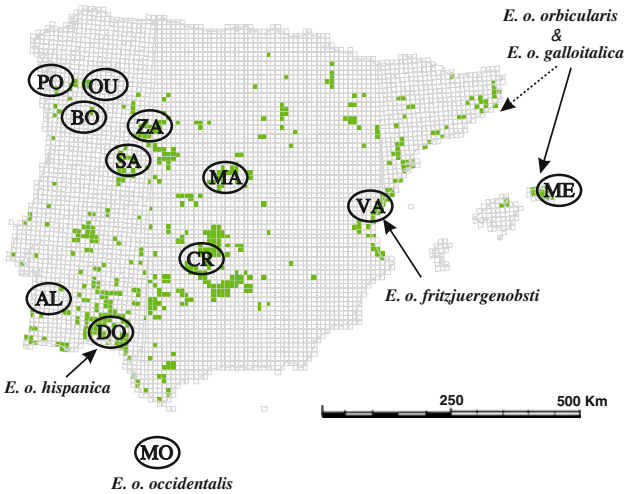

Fig. 1 Location of sampled populations (PO, Porriño; OU, Ourense; BO, Boticas; ZA, Zamora; SA, Salamanca, MA, Madrid; VA, Valencia; CR, Ciudad Real; AL, Alentejo; DO, Doñana; ME, Menorca and MO, Morocco) on a map of the distribution of *E. orbicularis* in the Iberian Peninsula (10 × 10 UTM squares). Distribution data for Spain are from Pleguezuelos et al. (2002), and for Portugal are provisional data from the "Projecto Atlas de Anfíbios e Répteis de Portugal" from the "Instituto da Conservação da Natureza". Iberian subspecies of *E. orbicularis* are shown in the map. Depicted arrows for *E. o. fritzjuergenobsti* and *E. o. hispanica* show the localities where these subespecies were described through morphologic characters. Arrows from *E. o. orbicularis* and *E. o. galloitalica* point at the contact zone with Iberian lineage in the Northeastern Iberian Peninsula and their occurrence in Balearic islands

(the southernmost localities) populations of *E. orbicularis*. The genetic structure and genetic diversity in the Iberian populations of *E. orbicularis* could be influenced by the glacial cycles that occurred during the Pleistocene. Many vertebrate, invertebrate and plant species in the Iberian Peninsula (e.g. *Chioglossa lusitanica*, *Lissotriton boscai*, *Salamandra salamandra*, *Lacerta schreiberi*, *Zootoca vivipara*, *Oryctolagus cuniculus*, *Chorthipus parallelus*, *Pynus silvestris;* see Gómez and Lunt (2007, for a review) show strong genetic subdivisions, containing at least two refuges for each species that prove not only the occurrence of different glacial refuges in the Iberian Peninsula, but also different glacial refuges for the same species. For *E. orbicularis* the occurrence of a glacial refuge has been suggested in North Africa (Fritz et al. 2007). This is the most comprehensive genetic dataset compiled for Iberian populations of *E. orbicularis* to date. With fine-scale sampling and using information from both nuclear and mitochondrial markers, this study allows us to estimate genetic diversity and structure of Iberian populations of this species. Our data also offer insights on patterns of gene flow between populations and have implications for the taxonomy of the species. These results will be useful for

the management and conservation of *E. orbicularis* in the Iberian Peninsula.

Methods

Sample collection

We collected samples of 254 turtles from 10 populations covering most of the species range in the Iberian Peninsula. In addition, 11 samples from Menorca (Balearic Islands) and three from Morocco were also included (Fig. 1, Table 1). Approximately 100 µl of blood were sampled from the vein of the tail or the occipital venous sinus (Martínez-Silvestre et al. 2002) of each specimen. Small portions of tail tissue was the source for DNA in individuals from Morocco. Genomic DNA was extracted using a lithium chloride protocol (Gemmell and Akiyama 1996). The resulting DNA pellets were air dried and resuspended in 50 µl TE, pH 8. The DNA was then diluted to a final concentration of 50 ng/µl for Polymerase Chain Reaction (PCR) amplifications.

Mitochondrial and nuclear DNA amplification and sequencing

We used material from 51 individuals from all sampling sites and sequenced a total of 2500 base pairs (bp) of mitochondrial and nuclear DNA for subsequent analyses (Fig. 1, Table 1). Previous studies indicate that cytochrome b (*cob*) is relatively invariant in *Actinemys marmorata* (Janzen et al. 1997) and it shows little variation among Iberian populations of *E. orbicularis* (Lenk et al. 1999). Thus, we assessed nucleotide variation in two alternative, relatively fast-evolving fragments of mtDNA: 660 bp from the control region (D-loop) and 829 bp from the nicotinamide adenine dinucleotide dehydrogenase subunit 4 (*ND4*) gene, which are the most variable regions of the mitochondrial genome in turtles (Starkey et al. 2003; Spinks et al. 2004). Moreover, in order to identify the subspecies occurring in our easternmost Iberian and Balearic populations, we have obtained *cob* sequences (~1000 bp length; mt-A and H-15909 were the primer pair used, see Lenk et al. 1999) for eight Menorcan samples and eight samples from Valencia (four individuals sequenced for the mt-nDNA dataset in this study and four more samples included in the microsatellite dataset) and then, compared with haplotypes deposited in GenBank. This was also done for eight individuals from Valencia (including the same four sequenced for D-loop and ND4), a population which is influenced by other two non endemic Iberian lineages (Fritz et al. 2007). For nDNA data, we

Table 1 Population identifiers (ID), population localities, sample size for microsatellites and sequences analyzed and genetic diversity measures for microsatellites (allelic richness, observed and expected heterozygosity (H_o and H_e) and number of private alleles) and mtDNA–nDNA haplotypes for all populations of *Emys orbicularis* analyzed

Number ID	Population	Localities	Subspecies	Sample size		Genetic diversity				MtDNA–nDNA Haplotypes
				Microsatelites	Sequences	Allelic richness	H_O	H_E	No. of private alleles	
1	Porriño	Gándaras de Budiño	[a]	30	5	4.37	0.70	0.67	0	I,II
2	Ourense	Baños de Molgas	[a]	32	4	4.80	0.68	0.70	1	I
3	Boticas	Vila Real	[a]	28	5	5.03	0.72	0.70	2	I
4	Zamora	Fornillos de Fermoselle	[a]	18	4	3.47	0.61	0.59	0	VII
5	Salamanca	Ciudad Rodrigo	[a]	[b]	4	[b]	[b]	[b]	[b]	III
6	Madrid	El Escorial	[a]	31	5	5.76	0.75	0.76	1	VII, VIII
7	Valencia	Sagunto, Burriana and Moro	*E. o. fritzjuergenobsti*	25	4	5.64	0.72	0.74	4	VI
8	Ciudad Real	Abenójar	[a]	23	4	5.74	0.77	0.74	2	V
9	Alentejo	Almograve	[a]	27	5	5.73	0.76	0.75	2	I
10	Doñana	Doñana Nacional Parq	*E. o. hispanica*	36	5	6.18	0.82	0.80	3	I,IV
11	Menorca	Ets Alocs and Tirant	*E. o. galloitalica*	11	4	6.01	0.68	0.67	12	X, XI
12	Morocco	Rif Mountains	*E. o. occidentalis*	[b]	3	[b]	[b]	[b]	[b]	IX

[a] Iberian populations which were not included in the description of Iberian subspecies using biometrical measures (Fritz 1993; Fritz et al. 1996)

[b] Microsatellites were not analyzed for these populations

included a 1048 bp fragment from the fingerprint protein 35 (R35) (Friedel et al. 2001; Fujita et al. 2004). The mtDNA and nDNA fragments have been previously used to assess phylogeographic patterns in *Actinemys marmorata* (Spinks et al. 2004). We collected both mtDNA and nDNA sequences from the same individuals. PCR conditions for mitochondrial genes were 2 min at 95°C followed by 37 cycles of 1 min denaturing at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. There was a final elongation cycle of 72°C for 10 min. These same conditions were used for R35 except that the annealing temperature was increased to 60°C. All PCR reactions were performed in a total volume of 25 µl, including 100 ng of DNA, 1 unit of Taq polymerase (Biotools, 5 U/ml), 0.3 µM of each primer, 0.30 mM dNTPs, 2 mM $MgCl_2$ and 1× PCR buffer (Biotools, Tris–HCl, pH = 8.3). Double-strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then re-suspended in 22 µl of ddH_2O. Sequencing reactions were performed for both strands on an ABI PRISM 3700 DNA sequencer following the manufacturer's instructions.

Microsatellite genotyping

Seven microsatellite loci (Cmu: B08, D16, D51, D87, D88, D93, D114) were screened in 261 turtles from ten populations (Fig. 1, Table 1). The primers used to amplify microsatellite loci were first described for *Glyptemys muhlenbergii* and tested for cross amplification in Emydidae by King and Julian (2004). One primer of each pair was synthesized with a fluorescent dye group—6 FAM, NED or HEX on the 5′ end to allow multiplex detection and sizing of fragments on an ABI Prism 3700 sequencer. The PCR reactions were performed using two different programs: program 1 was used for loci D88 and D114, and program 2 for the others. PCR reactions of 10 µl were performed using 75 ng of DNA, 0.2 µM of each primer, 0.25 mM dNTPs , 2 mM MgCl2, 1× PCR buffer (Biotools, Tris–HCl, pH = 8.3) and 1 unit of Taq polymerase (Biotools, 5 U/ml). PCR reactions for the loci in program 1 began with an initial denaturation of 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C and ended by a final extension step for 5 min at 72°C. For the loci in the program 2 group we performed a touchdown PCR (Don et al. 1991), starting 2 min at 94°C followed by 20 cycles (decreasing 0.5°C in each cycle) of 30 s at 92°C, 30 s at 60–50°C, 1 min at 72°C; this touchdown program was followed by 24 cycles with 30 s at 92°C, 30 s at 50°C, 1 min at 72°C and finished with 5 min at 72°C. Data collection, analyses and sizing were performed using ABI Prism Genescan software.

Mitochondrial and nuclear DNA sequence analysis

Sequences were read from both strands using ProSeq v.2.91 (Filatov 2002); since mitochondrial data displayed relatively low levels of variation, alignments were made by hand using the same program. Nucleotide diversity (Nei and Li 1979) was calculated using DnaSP (Rozas and Rozas 1997). We constructed a haplotype network on mtDNA–nDNA haplotype variation within the species using the software TCS 1.18 (Clement et al. 2000), which follows the statistical parsimony algorithm described in Templeton et al. (1992).

Microsatellite data analysis

We estimated the allelic richness, number of private alleles for each locus and population, and observed and expected heterozygosity over all loci using the software Genetix (Belkhir et al. 2004). Deviations from Hardy-Weinberg equilibrium (HWE) applying a Markov chain exact test (Guo and Thompson 1992) for each locus in each population and tests for linkage disequilibrium for each pair of loci in each population were performed using Genepop on the web (http://www.wbiomed.curtin.edu.au/genepop/). The HWE was also tested by the inbreeding coefficient F_{IS} values (Wright 1943) using Genetix (Belkhir et al. 2004). Sequential Bonferroni correction for multiple tests was used with HWE and linkage equilibrium (Rice 1989).

Evidence of recent population bottlenecks was assessed with Bottleneck (Cornuet and Luikart 1997). For the Wilcoxon's test, data were examined under all three mutational models (IAM, SMM and TPM). This program tests for recent reductions of effective population size by considering that alleles are generally lost faster than heterozygosity (Hedrick and Cockerham 1986), and recently bottlenecked populations will thus display an excess of heterozygosity based on the observed number of alleles. We followed the recommendations of the program for limited sample size and used the Wilcoxon test, and a qualitative descriptor of the allele frequency distribution (mode-shift indicator) to detect any evidence of population bottlenecks. Levels of population differentiation were calculated using pairwise F_{ST} values (Weir and Cockerham 1984) with Genetix (Belkhir et al. 2004) and pairwise R_{ST} values (Slatkin 1995) in Rstcalc (Goodman 1997). Significance of pairwise comparisons was tested through 1000 iterations. *P*-values were adjusted using the sequential Bonferroni correction (Rice 1989). However, Gaggiotti et al. (1999) recommend using F_{ST} values to calculate genetic differentiation among populations when few microsatellites ($\leq$10) are studied in small populations ($\leq$500) and for small sample size. Therefore, given than

less than 10 microsatellites were used, F_{ST} values are a conservative estimate more appropriate for our dataset. The PHYLYP-package (Felsenstein 1995) was used to calculate Cavalli-Sforza and Edwards's chord distance D_C (Cavallis and Edwards 1967) and to reconstruct phylogenetic relationships among sampling localities. According to Takezaki and Nei (1996), D_C performs better when recovering a topology compared to measures based on the stepwise mutation model. Bootstrap analyses were performed by first generating 1000 distance matrices using GENEDIST, which were then used to generate 1000 neighbour-joining trees with the program NEIGHBOUR in PHYLIP. These 1000 trees were summarized using the CONSENSE program in PHYLIP. To test for a possible scenario of isolation by distance, we analyzed the relationship between geographic distance and genetic differentiation between populations by means of Mantel tests (Sokal and Rohlf 1995) using GENETIX. In addition, and to test the hypothesis of a glacial refuge in the south, we tested for a correlation (Spearman's correlation coefficient) between genetic diversity (heterozygosity and allelic richness) and geographic distance for each population relative to the southernmost locality (Doñana). A model-based clustering method using multiloci genotype data to infer population structure and assign individuals to populations was done using a Bayesian approach performed in STRUCTURE (Pritchard et al. 2000). A non-admixture model with independent allele frequencies was used because studied populations are isolated from one another. No information about the population of origin for each individual was given. A series of three independent runs for K from 1 to 15 was performed. The results are based on 10^6 iterations, following a burn-in period of 1,000 iterations and five independent runs were performed. Individuals were assigned probabilistically to populations or jointly to two or more populations if their genotypes indicate that they are admixed using the multiloci genotype data.

Results

Mitochondrial and nuclear DNA sequence analysis

A total of 2537 bp were aligned for 51 individuals. Deletions or insertions were not detected. We found 22 polymorphic sites among all samples analyzed (18 transitions and four transversions; 21 parsimony-informative sites). The D-loop fragment was the most diverse marker with 10 variable sites, while the ND4 fragment showed eight variable sites. In contrast, the nuclear gene (R35) showed little divergence with four variable sites found when comparing sequences from the Iberian–Moroccan group with sequences from the population from Menorca. No variation was found in this gene across Iberian and Moroccan samples. Limited levels of variation across nuclear genes are usual at the intraspecific level in turtles (Caccone et al. 2004). Sequence divergences for the mtDNA–nDNA combined data set (uncorrected "p" distances) range from (i) 0.3% to 0.8% between Iberian-Morocco group and the Menorca population, (ii) 0.1–0.2% between Iberian populations and Morocco samples, and (iii) 0.0–0.1% among Iberian populations. A nucleotide diversity of 0.00186 (Pi, Nei 1987) and a haplotype diversity (Hd) of 0.809 were found for the total dataset while such measurements decrease reasonably when we calculate them for the Iberian dataset (Pi: 0.00041 and Hd: 0.740).

A total of 11 haplotypes were found in the combined mtDNA–nDNA dataset (Fig. 3). The haplotype network shows close relationships among Iberian populations. Six of the eight Iberian haplotypes (II, III, IV, V, VI, VII) differ by one base-pair substitution from haplotype I, while haplotype VIII differs by two substitutions. The most common haplotype (I) is widely distributed and it is found in Ourense, Boticas, Alentejo and Doñana populations. Moreover, Porriño and Doñana contain a second haplotype: II and IV respectively. The remaining Iberian populations have individual specific haplotypes except Madrid that shares haplotype VII with Zamora (Table 1). We found haplotype IX in the three samples analyzed from Morocco, differing by three substitutions from the most common Iberian haplotype. The introduced population in Menorca shows largely differentiated haplotypes, differing by 13 substitutions from the Moroccan haplotype and 14 from the closest Iberian haplotype. Furthermore, *cob* sequences showed relevant information about the subspecific status of the samples studied: (1) Menorcan samples correspond to *E. o. galloitalica*, the Italian lineage which reached the Northeastern Iberian coast after postglacial expansions and has also been introduced, in the past, on the Balearic islands; and (2) six of eight samples of Valencia (including the four samples of the mt-nDNA sequence dataset) correspond to the Iberian lineage while the other two correspond to *E. o. galloitalica.* All sequences were deposited in GeneBank under accession numbers EU277490–EU277645.

Microsatellite DNA analysis

None of 70 chi-square tests (for each locus in each population) showed significant deviations from Hardy–Weinberg equilibrium (HWE) at the 95% confidence interval after the sequential Bonferroni test. The inbreeding coefficient F_{IS} shows no departure from HWE in any population and the positive values found were not significant (data not shown; $P > 0.05$). Cases of linkage

disequilibria were found (after sequential Bonferroni test, $P < 0.05$) between loci D114-D88 (Porriño and Boticas) and D88-D51 (Doñana) although this probably does not mean physical linkage because it was not observed across all populations.

We analyzed polymorphism across seven loci in 261 individuals from 10 populations (Fig. 1, Table 1) identifying a total of 92 alleles. The number of alleles per locus ranged from 4 (CmuB08) to 21 (CmuD88) although 6 out of 7 microsatellites showed more than ten alleles with a mean of 15.4. Mean observed and expected heterozygosities and allelic richness ranged from the lowest values in the NW populations (Porriño, Ourense, Boticas and Zamora) to the highest values in the southernmost population (Doñana). Unique alleles were found in 8 out of 10 studied populations. Menorca had 44% unique alleles, while two Northwestern populations (Porriño and Zamora) had no private alleles. Valencia was the Iberian population where more private alleles were detected, although the southern group (Alentejo-Doñana) represents the area with the highest number of private alleles (Table 1). Genetic diversity (heterozygosity and allelic richness) tends to decrease from South (Doñana) to North (Porriño and Ourense) in the Iberian Península (Table 1).

Under none of the three mutation models was there any evidence for a genetic bottleneck for any of the 10 populations. The genetic relationship among the populations analyzed in our study was synthesized in an unrooted NJ tree based on Cavalli-Sforza and Edwards's chord distance D_C (Fig. 4). Overall, genetic distance estimates among populations ranged from 0.34 to 1.68 with low values of divergence if we compare only Iberian populations (up to 0.96, Table 2). Interestingly, the unrooted NJ tree (Fig. 4) group in a highly supported clade (100% bootstrap value) the westernmost population (Valencia) with the introduced Menorcan population. Because Menorca individuals have the same lineages of NE Iberia and the samples from Valencia group with them, this clade was called the "Northeastern clade". The Southwestern and Southern populations (Alentejo and Doñana respectively) are placed in different clades with moderate to low values of support. Central populations (Madrid and Ciudad Real) are grouped in the moderately supported "Central Clade" while NW Iberian populations (Porriño, Ourense, Boticas and Zamora) fall into the highly supported "Northwestern clade" (80% bootstrap value). Genetic similarity significantly decreases with geographic distance (Mantel test, $P = 0.020$). The linear measurement of geographical distances between each population and the southernmost locality (Doñana) resulted in significant and inverse correlation of heterozygosity with geographic distance (Spearman $r = -0.76$, $P = 0.021$) and allelic richness-geographic distance (Spearman $r = -0.81$, $P = 0.011$; Fig. 2).

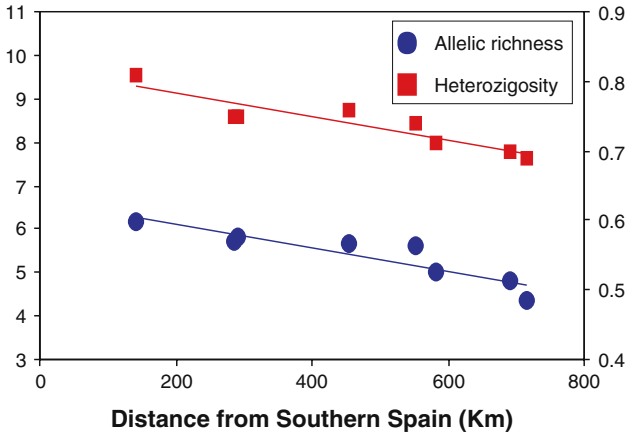

Fig. 2 Correlation between genetic diversity (allelic richness and observed heterozygosity) and distance from Southern Iberia (Doñana). Spearman correlation coefficient $r_s = -756$ ($P = 0.021$) for heterocygosity–geographical distance and $r_s = -812$ ($P = 0.011$) for allelic richness–geographical distance

Table 2 Pairwise F_{ST} (above the diagonal) and Cavalli-Sforza and Edward's chord distances (Dc) estimates (below the diagonal) between *Emys orbicularis* populations based on microsatellite data

Dc/F_{ST}	Porriño	Ourense	Boticas	Zamora	Madrid	Valencia	Ciudad real	Alentejo	Doñana	Menorca
Porriño	–	**0.10**	**0.09**	**0.12**	0.15	**0.19**	**0.13**	**0.14**	**0.10**	**0.25**
Ourense	0.36	–	**0.10**	**0.09**	**0.13**	**0.16**	**0.13**	**0.14**	0.10	**0.17**
Boticas	0.41	0.34	–	**0.12**	**0.11**	**0.16**	**0.12**	**0.11**	**0.09**	**0.18**
Zamora	0.41	0.42	0.40	–	**0.09**	**0.16**	**0.10**	**0.15**	**0.12**	**0.20**
Madrid	0.74	0.64	0.61	0.59	–	**0.13**	**0.08**	**0.14**	**0.10**	**0.15**
Valencia	0.92	0.80	0.90	0.90	0.76	–	**0.16**	**0.15**	**0.10**	**0.10**
Ciudad Real	0.52	0.60	0.60	0.59	0.45	0.94	–	**0.15**	**0.09**	**0.22**
Alentejo	0.50	0.61	0.51	0.54	0.86	0.96	0.85	–	**0.11**	**0.19**
Doñana	0.47	0.57	0.49	0.55	0.63	0.51	0.52	0.63	–	**0.12**
Menorca	1.51	0.85	1.11	1.30	0.95	0.47	1.68	1.20	0.67	–

F_{ST} values in bold were statistically significant after Bonferroni correction ($P < 0.01$)

Pairwise estimates of F_{ST} range from 0.08 (between Madrid and Ciudad Real) to 0.25 (between Porriño and Menorca). However, the higher F_{ST} value decreases to 0.19 (between Porriño and Valencia) when only Iberian populations are compared (see Table 2; $P < 0.01$). A moderate degree of genetic differentiation was estimated overall Iberian populations ($F_{ST} = 0.12$; $P < 0.01$) although higher differentiation was revealed with R_{ST} estimates ($R_{ST} = 0.19$; $P < 0.01$). However, most pairwise R_{ST} values (70%) were not significant after the sequential Bonferroni correction ($P > 0.05$).

The Bayesian method, implemented by STRUCTURE, revealed from the posterior probabilities L(K) and the second-order difference in ln(p) Δ(K), that the most likely number of clusters to explain our data is K = 11 (data not shown), indicating substructuring in our sample which almost agree with our a priori knowledge of ten independent populations. The Bayesian approach shows a high genetic subdivision revealing one cluster for each population and splitting Doñana and Ciudad Real into three clusters (with the maximum Ln probability). The analysis implemented for five clusters (taking into account the five groups resolved using genetic distances) joined NW populations in one cluster; Menorca and Valencia; Madrid and half of the samples of Ciudad Real; Doñana and the remainder from Ciudad Real and Alentejo with nine samples from Doñana (Figs. 4, 5). Assignment tests showed that 90% of individuals for 8 populations were assigned to their sampling location with a probability higher than 90% (not shown). However, Doñana and Ciudad Real genotypes were admixed, with their alleles grouped in three non-geographically cohesive clusters.

Discussion

Distribution of the genetic diversity in *E. orbicularis* in the Iberian Peninsula

Despite the use of rapidly evolving mitochondrial markers and a fairly good representation of *E. orbicularis* populations in the Iberian Peninsula, our results show low values of genetic diversity at the mitochondrial level. This is not unexpected, because evolutionary rates of mtDNA in turtles tend to be lower than other vertebrates (Avise et al. 1992). The low divergence found across populations of *E. orbicularis* throughout the Iberian Peninsula is thus concordant with low genetic differentiation found in previous genetic studies (Lenk et al. 1999; Fritz et al. 2007). The use in this study of more suitable mtDNA markers enabled us to reveal higher levels of genetic diversity than in recent phylogeographic studies of *E. orbicularis* (Fritz et al. 2007). However, our microsatellite dataset revealed relatively high levels of genetic variability within populations and some degree of population substructuring. Genetic variability in Iberian populations of *E. orbicularis* decreases from south to north, Doñana being the most diverse population (Table 1, Fig. 2). Habitat fragmentation can reduce genetic variability by decreasing local effective population size and by restricting gene flow (Harrison and Hastings 1996). Nevertheless, we did not find a dramatic diminution of genetic variability in Porriño and Ourense, two populations that are isolated and have low population size (Cordero Rivera and Ayres Fernández 2004). In contrast, we found similar genetic diversity levels among all NW Iberian sampled populations (Fig. 4) despite the larger population size and presumably the existence of high-quality habitat in Boticas and Zamora populations. The lowest genetic diversity was found in Zamora, the population with the smallest sample size.

Our results do not suggest significant effects of bottlenecks, as is usual in other studies of turtles, which show high genetic diversity even in small isolated populations (Cunningham et al. 2002; Kuo and Janzen 2004; Hauswaldt and Glenn 2005). Nevertheless, the absence of bottlenecks should be interpreted with caution, because it is difficult to detect such losses of genetic variability in animals like *Emys orbicularis* which can live up to 60 years. In fact, the Porriño population recently suffered a diminution when one of the three clay pits in which this species inhabits was destroyed (Cordero Rivera and Ayres Fernández 2004), but this is not reflected in its genetic structure.

Our study shows a clear correlation between genetic diversity levels (H_E, H_O, allelic richness) and geographic distance from each population to the most diverse population (Doñana; Fig. 2). There is no evidence to suggest that this trend may result from reduced population sizes or increased habitat fragmentation across northern and central populations. However, the latitudinal genetic trend found may be explained by a colonization effect from a south-Iberian glacial refuge (a similar genetic diversity pattern was found for *Chioglossa lusitanica*, Alexandrino et al. 2001) or from North Africa to the Iberian Península (as it has been found for fishes, Machordom and Doadrio 2001; reptiles, Fritz et al. 2006, Carranza et al. 2004, 2006, Pinho et al. 2007; and amphibians, Carranza and Arnold 2004, Recuero et al. 2007). Such colonization could be achieved by long-distance dispersers which fill the areas before others arrive. If this occurs many times over a long colonization route, it would reduce genetic variability in each migration event (Hewitt 1999, 2000).

Population relationships

The haplotype network (Fig. 3) shows a genetically homogeneous group of populations within the Iberian

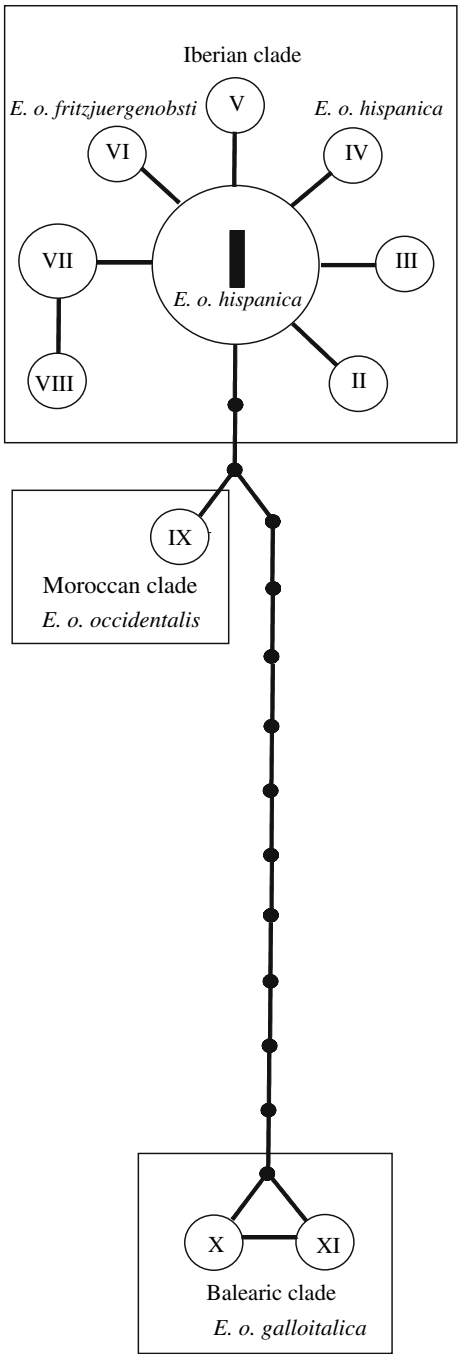

Fig. 3 Haplotype network based on mtDNA and nDNA sequences showing the three clades found (Iberian, Moroccan and Balearic). Black points represent mutational steps

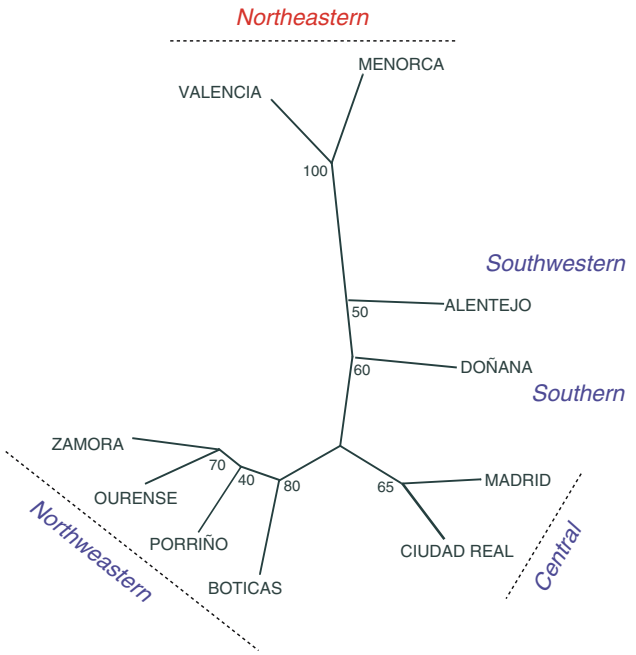

Fig. 4 Unrooted Neighbour-joining tree of *Emys orbicularis* populations based on the Cavalli-Sforza and Edwards's chord distance D_C from microsatellites. Over the branches are indicated the bootstrap support values based on 1000 replicates

Peninsula, with a common haplotype related to seven haplotypes that differ from it in only one or two substitutions. In our case this pattern is likely a consequence of the low mitochondrial mutation rate found in turtles rather than a consequence of limited sample size. The widespread haplotype is found in the southern and three NW Iberian populations (Fig. 5), whereas one NW population and the Central-Eastern populations present different haplotypes. There is little population subdivision in Iberian populations regarding mtDNA variation. With respect to microsatellites, a moderate overall F_{ST} value of 0.12 (Hart and Clark 1997) and significant pairwise differentiation levels indicate structuring in Iberian populations of the European pond turtle. However, estimates of F_{ST} were low compared to *Actinemys marmorata* (Spinks and Shaffer 2005), or other endemic Iberian taxa (e.g. *Chioglossa lusitanica*, Alexandrino et al. 2001; *Lacerta schreiberi* Godinho et al. 2003). The genetic differentiation found among Iberian populations of *Emys orbicularis* (genetic distances, F_{ST} values and clusters in the Bayesian analysis) may be a consequence of isolation by distance (Wright 1943). This is supported in this study by the significance of the Mantel test, where the genotypic differentiation increases with geographical distance across populations. This isolation by distance effect implies that populations are connected by gene flow or were connected in the recent past. Analysing all microsatellite data sets we detected 5 clades based on the Cavalli-Sforza and Edwards's chord distance D_C which are in agreement with F_{ST} values (Figs. 4, 5). The moderate genetic differentiation ($F_{ST} = 0.10$) found within the North-Western clade reflects recent gene flow on NW Iberia. Many river systems are found in this area, where mountain chains do not appear as important barriers for migration. The same pattern is found in the Central clade ($F_{ST} = 0.08$)

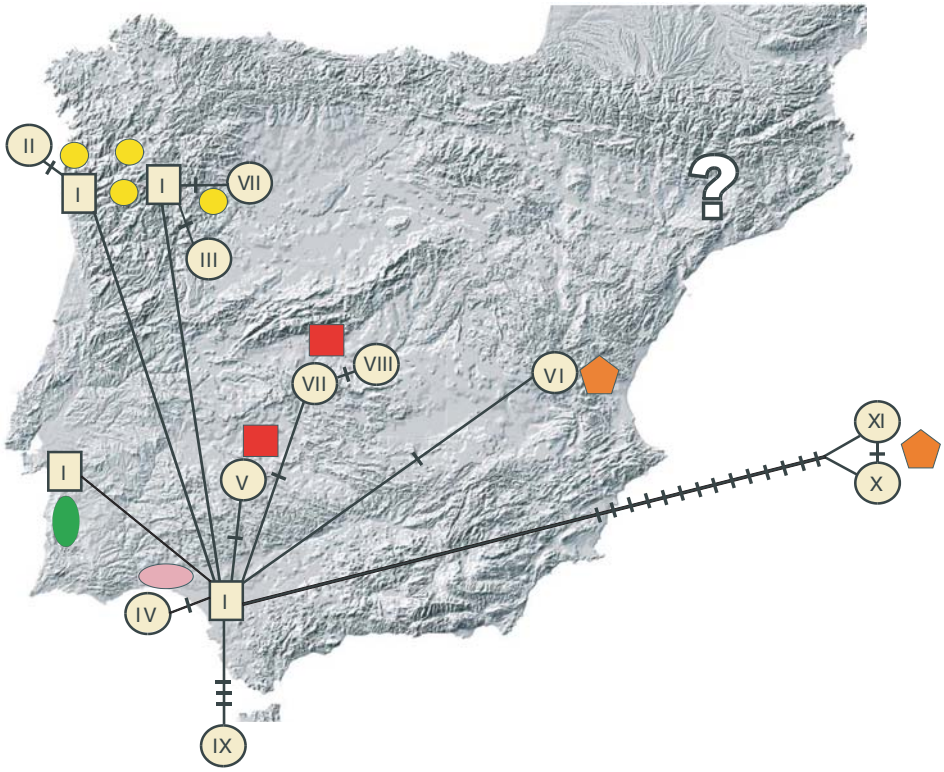

Fig. 5 Genetic relationships among studied populations of *E. orbicularis* depicted in an orographic map (downloaded from http://www.arqueotavira.com). Roman numerals show the different mtDNA and nDNA haplotypes in their correspondent geographic place. Each segment in the lines represents one mutation. Lines do not imply geographic migration routes. Symbols represent the five main groups assessed with the Cavalli-Sforza and Edwards's chord distance D_C and F_{ST} values using microsatellites

although, interestingly, the South-Western and Southern clades differ moderately ($F_{ST} = 0.11$) despite their closer geographic distance. Valencia and Menorca populations show moderate genetic differentiation and are grouped in a single clade which is not in agreement with results from our mtDNA analyses. The discordances between our mtDNA and microsatellite results (Figs. 4, 5) may be explained by recent and continuous migration of northern lineages (*E. o. orbicularis* and *E. o. galloitalica*) to the south, widening the contact zone (Fritz et al. 1996; Mascort 1998). Our limited mtDNA–nDNA sequence data set has failed to find evidence of such lineages in the population from Valencia, but *E. o. galloitalica* was found in Valencia and Menorca using cytochrome b in this study, as well as by Fritz et al. (2007), supporting the hypothesis of contact zone expansion in the Northeastern Iberian coast. Further analyses using microsatellites will be necessary in this region to delimitate the NE-Eastern Iberian contact zone.

Levels of heterozygosity and allelic diversity were moderately high among Iberian populations, but genetic differentiation was moderate to low. This genetic pattern is commonly found in microsatellite studies in turtles (Sites et al. 1999; Cunningham et al. 2002; Hauswaldt and Glenn 2005), although deeper genetic structure is also found in other tortoises (Beheregaray et al. 2003; Schwartz and Karl 2005).

Was Northern Morocco a glacial refuge for the Iberian populations?

Another freshwater turtle occurs in the Iberian Peninsula: the stride-necked terrapin, *Mauremys leprosa*, which is distributed in Iberia and Northwestern Africa. *Mauremys leprosa* and *Emys orbicularis* inhabit similar water bodies within Iberia, and we found both species in most sampled regions. A recent study on *M. leprosa* (Fritz et al. 2006) has revealed that the Atlas Mountains in Morocco acted as an important barrier that reduced gene flow between populations at both sides of the Atlas and split *M. leprosa* into two clades. One clade occurs in the Iberian Peninsula and Morocco North to the Atlas (*M. l. leprosa*), while the second clade is found South to the Atlas (*M. l. saharica*). Fritz et al. (2006) suggested that the little-diversified Iberian populations originated from northern Morocco, following a southwestern–northern colonisation path. Given habitat similarity between western *Mauremys* and *Emys* and the genetic diversity trend found in this study, a similar biogeographic scenario may also be possible for *E. orbicularis.* Unfortunately, we were unable to test among competing biogeographic scenarios for *Emys* due to a limited availability of samples from North Africa. The three samples analyzed from northern Africa did not share any haplotype with Iberian samples. However one Iberian haplotype is only moderately divergent from one of the

Moroccan haplotypes (0.1–0.2%). Further sampling will be helpful to establish a biogeographic scenario for *E. orbicularis* in the Western Mediterranean region.

Taxonomic and conservation implications

MtDNA and microsatellite results showed that sampled Iberian populations of *E. orbicularis* are genetically similar, and so they appear to form a single taxonomic unit. Our results do not support the separation between the two endemic subspecies of *E. orbicularis* in the Iberian Peninsula (*E. o. hispanica* and *E. o. fritzjuergenobsti*) since genetic differentiation between these subspecies is not larger than the differentiation shown between any other pair of populations. Therefore, given the genetic similarities among Iberian populations of *Emys orbicularis* we consider the sampled Iberian populations as a single subspecies: *E. o. fritzjuergenobsti*. However, further studies on the contact zone (NE–Eastern Iberia) using microsatellite markers and morphological data will be helpful to establish the distribution and taxonomic status of the different mitochondrial lineages and to delimit the contact areas among them.

As opposed to other genetic patterns in Iberian amphibians and reptiles which show deep genetic intraspecific structure (Alexandrino et al. 2001; Godinho et al. 2003; Martinez-Solano et al. 2006), Iberian populations of *E. orbicularis* show moderate genetic differentiation (microsatellite data, see results) and also low mtDNA genetic differentiation (0.0–0.1% uncorrected "p" distances), However, the management of this species should take into account the moderate genetic structuring (F_{ST} = 0.12; private alleles) of these highly isolated Iberian populations into five main geographic groups (Fig. 5) and therefore, *E. orbicularis* in the Iberian Peninsula should be handled taking into account five management units for future conservation programs. On the other hand, all Iberian populations share the same conservation problems, namely that conservation efforts centered on this species should be mainly done focusing on habitat preservation and restoration.

Our results show a latitudinal trend in genetic diversity along the Iberian Peninsula, however there is no evidence of dramatically low values of genetic variability in any of 10 sampled Iberian populations, having similar values of genetic diversity (Table 1), even when populations of different size are compared (i.e. Porriño and Boticas, data not shown). Small isolated populations are vulnerable to the loss of genetic diversity, which acts as a contributing factor to local extinction (Frankham 1995). Nevertheless, the small and isolated Porriño population does not show a significantly lower diversity, even when no more than 100 individuals survived recent catastrophic events due to human activity (Cordero Rivera and Ayres Fernández 2004). The same could be extrapolated to other isolated and small Iberian populations which are suffering similar threats. Thus, a loss of genetic diversity within the already small populations sampled is not appreciable and the differences in genetic diversity could be explained by a south to north migration trend. The fact that *E. orbicularis* populations continue to decline is due to a combination of several threats (i.e. destruction and habitat fragmentation, river contamination, pet trade), rendering Iberian populations rare, isolated and fragmented. However, a suitable genetic approach to study population genetic diversity (microsatellites) as used in this study was not able to confirm those menaces. Therefore, this study serves to emphasize the importance of habitat management more than population management (i.e. captive breeding and reintroduction programs) to preserve the fragmented populations in the Iberian Peninsula. In a fragmented landscape, metapopulations can persist for a long time thanks to migration between sources and sink areas (Hanski and Gilpin 1997). Adequate conservation planning of *E. orbicularis* habitat and conservation corridors among rivers and/or ponds within each population are necessary to ensure the persistence of small isolated populations and may help avoid or diminish population fragmentation in this species (Roe and Georges 2007).

Acknowledgements We thank all the people who helped us to collect samples: César Ayres from most populations; Pedro Segurado from Portugal; Gonzalo Alarcos from Salamanca and Zamora; Judith Hidalgo, Matati, Alex Portheault and Ana Andreu from the Doñana National Park; Vicente Sancho and Ignacio Lacomba from Valencia; Alberto Álvarez from Madrid, Carlos Gómez Lorenzo from Ourense. Alain Veysset provided samples from Menorca; David Donaire and Salvador Carranza supplied samples from Morocco. David Buckley, Iñigo Martínez-Solano and three anonymous reviewers made helpful comments and suggestions on the manuscript. Christopher Beatty improved English on the manuscript. GV-A is supported by a grant from Galician Government (Conselleria de Innovación, Industria e Comercio, Xunta de Galicia). Funding was provided by Research grants from Xunta de Galicia (PGIDT01MAM37101PR, PGIDIT05RFO37101PR) and Cátedra Filgueira Valverde-Universidade de Vigo to ACR.

References

Alexandrino J, Froufe E, Arntzen JW, Ferrand N (2001) Genetic subdivision, glacial refugia and postglacial recolonization in the golden-striped salamander, *Chioglossa lusitanica* (Amphibia: urodela). Mol Ecol 9:771–781

Altherr S, Freyer D (2000) Asian turtles are threatened by extinction. Turtle Tortoise Newsl 1:7–11

Avise JC, Bowen BW, Lamb T, Meylan AB, Bermingham E (1992) Mitochondrial-DNA a evolution at a turtles pace – evidence for low genetic variability and reduced microevolutionary rate in the testudines. Mol Biol Evol 9:457–473

Ayres Fernández C, Cordero Rivera A (2004) The incidence of asymmetries and accessory plates in *Emys orbicularis* from NW Spain. Biologia 14(59/Suppl):85–88

Beheregaray LB, Ciofi C, Caccone A, Gibbs JP, Powell JR (2003) Genetic divergence, phylogeography and conservation units of giant tortoises from Santa Cruz and Pinzon, Galapagos Islands. Conserv Genet 4:31–46

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, Montpellier

Bertolero A (2000) Suivi de la population de Cistude *Emys orbicularis* dans le delta de l'Ebre (NE Espagne). Chelonii 2: 63–68

Bishop CA, Ng P, Pettit KE, Kennedy SW, Stegeman JJ, Norstrom RJ, Brooks RJ (1998) Environmental contamination and developmental abnormalities in eggs and hatchlings of the common snapping turtle (*Chelydra serpentina serpentina*) from the Great Lakes-St. Lawrence River basin (1989–91). Environ Pollut 101:143–156

Cabral JM, Magalhães P, Oliveira E, Romão C (1990) Livro vermelho dos vertebrados de Portugal. Vol. I – Mamíferos, aves, répteis e anfibios. Serviço Nacional de Parques, Reservas e Conservaçao da Natureza, Lisboa

Caccone A, Gentile G, Burns CE, Sezzi E, Bergman W, Ruelle M, Saltonstall K, Powell JR (2004) Extreme difference in rate of mitochondrial and nuclear DNA evolution in a large ectotherm, Galapagos tortoises. Mol Phylogenet Evol 31:794–798

Carranza S, Arnold EN (2004) History of West Mediterranean newts, *Pleurodeles (*Amphibia: Salamandridae), inferred from old and recent DNA sequences. Syst Biodivers 1(3):327–337

Carranza S, Arnold EN, Wade E, Fahd S (2004) Phylogeography of the false smooth snakes, *Macroprotodon* (Serpentes, Colubridae): mitochondrial DNA sequences show European populations arrived recently from Northwest Africa. Mol Phylogenet Evol 33:523–532

Carranza S, Arnold EN, Pleguezuelos JM (2006) Phylogeny, biogeography, and evolution of two Mediterranean snakes, *Malpolon monspessulanus* and *Hemorrhois hippocrepis* (Squamata, Colubridae), using mtDNA sequences. Mol Phylogenet Evol 40: 532–546

Cavallis LL, Edwards AWF (1967) Phylogenetic analysis models and estimation procedures. American Journal of Human Genetics 19:233

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659

Congdon JD, Dunham AE, Sels RCV (1993) Delayed sexual maturity and demographics of Blandińgs turtles (*Emydoidea blandingii*) – implications for conservation and management of long-lived organisms. Conserv Biol 7:826–833

Cordero Rivera A, Ayres Fernández C (2004) A management plan for the European pond turtle (*Emys orbicularis*) populations of the river Louro basin (NW Spain). Biologia 14(59/Suppl):161–171

Cournet JM, Luikart G (1997) Description and power analysis of two tests for detecting recent population bottlenecks from allele frecuency data. Genetics 144:2001–2014

Cunningham J, Baard EHW, Harley EH, O'Ryan C (2002) Investigation of genetic diversity in fragmented geometric tortoise (*Psammobates geometricus*) populations. Conserv Genet 3:215–223

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Epperson DM, Heise CD (2003) Nesting and hatchling ecology of gopher tortoises (*Gopherus polyphemus*) in southern Mississippi. J Herpetol 37:315–324

Felsenstein J (1995) PHYLIP. [3.57c]. Department of Genetics, University of Washington, Seattle

Filatov DA (2002) PROSEQ: a software for preparation and evolutionary analysis of DNA sequence data sets. Mol Ecol Notes 2:621–624

Forman RTT, Alexander LE (1998) Roads and their major ecological effects. Annu Rev Ecol Syst 29:207–231

Frankham R (1995) Conservation genetics. Annu Rev Genet 29: 305–327

Frankham R, Ralls K (1998) Conservation biology: inbreeding leads to extinction. Nature 392:441–442

Frankham R, Ballou JD, Briscoe DA (2002) Introduction to conservation genetics. Cambridge University Press, Cambridge

Friedel RH, Stubbusch J, Barde YA, Schnurch H (2001) A novel 7-transmembrane receptor expressed in nerve growth factor-dependent sensory neurons. Mol Cell Neurosci 17:31–40

Fritz U (1993) On the intraspecific variation of *Emys orbicularis* (Linnaeus, 1758). 3. Two new subspecies from the Iberian Peninsula and North Africa, *Emys orbicularis fritzjuergenobsti*.nov. and *E.o.occidentalis* subsp.nov. (Reptilia, Testudines: Emydidae). Zool Abh Mus Tierdk Dresden 47:131–155

Fritz U (2001) *Emys orbicularis* (Linnaeus, 1758) – Europäische Sumpfschildkröte. In: Fritz U (ed) Handbuch der Reptilien und Amphibien Europas. Band 3/IIIA: Schildkröten I. Aula-Verlag, Wiebelsheim, pp 343–515

Fritz U, Keller C, Budde M (1996) A new subspecies of the European pond turtle from southwestern Spain, *Emys orbicularis hispanica* subsp.nov. Salamandra 32:129–152

Fritz U, Pieh A, Lenk P, Mayol J, Sättele B, Wink M (1998) Is *Emys orbicularis* introduced to Majorca? Mertensiella 10:122–133

Fritz U, Barata M, Busack SD, Fritzsch G, Castilho R (2006) Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). Zool Scr 35: 97–108

Fritz U, Guicking D, Kami H et al (2007). Mitochondrial phylogeography of European pond turtle (*Emys orbicularis, Emys trinacris*) – an update. Amphibia-Reptilia 28:418–426

Fujita MK, Engstrom TN, Starkey DE, Shaffer HB (2004) Turtle phylogeny: insights from a novel nuclear intron. Mol Phylogenet Evol 31:1031–1040

Gaggiotti OE, Lange O, Rassmann K, Gliddon C (1999) A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. Mol Ecol 8:1513–1520

Galán P (1999) Conservación de la herpetofauna gallega. Universidade da Coruña, A Coruña

Gasith A, Sidis I (1984) Polluted water bodies, the main habitat of the Caspian terrapin (*Mauremys caspica rivulata*) in Israel. Copeia 1984:216–219

Gemmell NJ, Akiyama S (1996) An efficient method for the extraction of DNA from vertebrate tissues. Trends Genet 12:338–339

Gibbs JP, Shriver WG (2002) Estimating the effects of road mortality on turtle populations. Conserv Biol 16:1647–1652

Godinho R, Paulo OS, Ferrand N, Luis C, Rosa HD, Crespo EG (2003) Major patterns of population differentiation in the Iberian Schreiber's green lizard (*Lacerta schreiberi*) inferred from protein polymorphism. Herpetol J 13:35–42

Gómez A, Lunt DH (2007) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Península. In: Weiss S, Ferrand N (eds) Phylogeography in southern European refugia: evolutionary perspectives on the origins and conservation of European biodiversity. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 155–188

Gómez-Cantarino A, Lizana M (2000) Distribución y uso del hábitat de los galápagos (*Mauremys leprosa y Emys orbicularis*) en la provincia de Salamanca. Bol Asoc Herpetol Esp 11:4–8

Goodman SJ (1997) RSTCALC: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. Mol Ecol 6:881–885

Guo SW, Thomson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hanski IA, Gilpin ME (1997) Metapopulation biology: ecology, genetics and evolution. Academic Press, San Diego

Harrison S, Hastings A (1996) Genetic and evolutionary consequences of metapopulation structure. Trends Ecol Evolut 11:180–183

Hart D, Clark A (1997) Principles of Population Genetics, 3rd edn. Sinauer Associates, Sunderland

Hauswaldt JS, Glenn TC (2005) Population genetics of the diamondback terrapin (*Malaclemys terrapin*). Mol Ecol 14:723–732

Hedrick PW, Cockerham CC (1986) Partial inbreeding: equilibrium heterozygosity and the heterozygosity paradox. Evolution 40:856–861

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc 68:87–112

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Janzen FJ, Hoover SL, Shaffer HB (1997) Molecular phylogeography of the western pond turtle (*Clemmys marmorata*): preliminary results. Chelelonian Conserv Biol 2:623–626

Joyal LA, McCollough M, Hunter ML (2001) Landscape ecology approaches to wetland species conservation: a case study of two turtle species in southern Maine. Conserv Biol 15:1755–1762

Keller C, Andreu AC (2002) *Emys orbicularis* (Linnaeus, 1758). Galápago europeo. In: Pleguezuelos JM, Márquez R, Lizana M (eds) Atlas y Libro Rojo de los anfibios y reptiles de España. Ministerio de Medio Ambiente, Madrid, pp 181–186

King TL, Julian SE (2004) Conservation of microsatellite DNA flanking sequence across 13 Emydid genera assayed with novel bog turtle (*Glyptemys muhlenbergii*) loci. Conserv Genet 5:719–725

Kuo C-H, Janzen FJ (2004) Genetic effects of a persistent bottleneck on a natural population of ornate box turtles (*Terrapene ornata*). Conserv Genet 5:425–437

Lenk P, Fritz U, Joger U, Winks M (1999) Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis (*Linnaeus 1758). Mol Ecol 8:1911–1922

Machordom A, Doadrio I (2001) Evidence of a cenozoic Betl-Kabilian connection based on freshwater fish phylogeography (Luciobarbus, Cyprinidae). Mol Phylogent Evol 18:252–263

Martínez-Silvestre A, Perpinan D, Marco I, Lavin S (2002) Venipuncture technique of the occipital venous sinus in freshwater aquatic turtles. J Herpetol Med Surg 12(4):31–32

Martinez-Solano I, Teixeira J, Buckley D, Garcia-Paris M (2006) Mt-DNA phylogeography of *Lissotriton boscai* (Caudata, Salamandridae): evidence for old, multiple refugia in an Iberian endemic. Mol Ecol 15:3375–3388

Mascort R (1998) Distribution and status of the European pond turtle, *Emys orbicularis*, in Catalonia. Mertensiella 10:177–186

Moll D, Moll EO (2004) The ecology, exploitation, and conservation of river turtles. Oxford University Press, New York

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Pinho C, Harris DJ, Ferrand N (2007) Contrasting patterns of population subdivision and historical demography in three western Mediterranean lizard species inferred from mitochondrial DNA variation. Mol Ecol 16:1191–1205

Pleguezuelos JM, Márquez R, Lizana M (2002) Atlas y Libro Rojo de los Anfibios y Reptiles de España. Dirección General de la Conservación de la Naturaleza-AHE, Madrid

Primack RB (1993) Essentials of conservation biology. Sinauer Associates, Sunderland

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Recuero E, Iraola A, Rubio X, Machordom A, García-París M (2007) Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern. J Biogeogr 34:1207–1219

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Rito de Araújo P, Segurado P, dos Santos NR (1997) Bases para a consevaçao das tartarugas de áuga doce (*Emys orbicularis e Mauremys leprosa*). Instituto da Conservaçao da Natureza, Lisboa

Roe JH, Georges A (2007) Heterogeneous wetland complexes, buffer zones, and travel corridors: Landscape management for freshwater reptiles. Biol Conserv 135:67–76

Rozas J, Rozas R (1997) DnaSP version 2.0: A novel software package for extensive molecular population genetics analysis. Comput Appl Biosci 13:307–311

Schleich HH, Boehme W (1994) Kupferzeitliche Schildkrötenreste aus der Grabung von Durankulak bei Tolbuchin in NO-Bulgarien (Reptilia: Testudines: *Testudo graeca*, *Emys orbicularis*). Mitteilungen der Bayerischen Staatssammlung fuer Palaeontologie und Historische Geologie 34:199–211

Schwartz T, Karl S (2005) Population and conservation genetics of the gopher tortoise (*Gopherus polyphemus*). Conserv Genet 6:917–928

Sites JW Jr, Fitzsimmons NN, da Silva NJ Jr, Cantarelli VH (1999) Conservation genetics of the Giant Amazon River Turtle (*Podocnemis expansa*): inferences from two classes of molecular markers. Chelonian Conserv Biol 3:454–463

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462

Sokal RR, Rohlf FJ (1995) Biometry, 3rd edn. W.H. Freeman, New York

Spinks PQ, Shaffer HB (2005) Range-wide molecular analysis of the western pond turtle (*Emys marmorata*): cryptic variation, isolation by distance, and their conservation implications. Mol Ecol 14:2047–2064

Spinks PQ, Shaffer HB, Iverson J, McCord W (2004) Phylogenetic hypotheses for the turtle family Geomydidae. Mol Phylogenet Evol 32:164–182

Starkey DE, Shaffer HB, Burke RL, Forstner MRJ, Iverson JB, Janzen FJ, Rhodin AGJ, Ultsch GR (2003) Molecular systematics, phylogeography, and the effects of Pleistocene glaciation in the painted turtle (*Chrysemys picta*) complex. Evolution 57:119–128

Takezaki N, Nei M (1996) Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144:389–399

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data III. Cladogram estimation. Genetics 132:619–633

van Dijk PP, Stuart BL, Rhodin AGJ (2000) Asian turtle trade: proceedings of a workshop on conservation and trade of freshwater turtles and tortoises in Asia. Chelonian Res Monogr 2:1–164

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370

Wright S (1943) Isolation by distance. Genetics 28:114–138