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Assignment tests applied to relocate individuals of unknown origin in a threatened species, the European pond turtle (*Emys orbicularis*)

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Abstract. The pet trade is an important business around the world and one of the factors that might menace some wild populations. If wild animals are collected to maintain them as pets, this activity can produce several problems: i) an increase of population vulnerability, especially in the case of rare species; ii) the release of exotic pets in natural habitats, with the risk of competition with native species and the spreading of parasites and diseases, and iii) the maintenance of animals of unknown origin in Recovery Centres or zoos, which if too numerous are sacrificed or re-located to their supposed original regions. In this paper, we used seven microsatellite loci to analyze genetic diversity and genetic structure of the European pond turtle (*Emys obicularis*) covering the species range in the Iberian Peninsula. A Bayesian test revealed a genotypic differentiation between the regions sampled where most individuals (90%) were assigned to their sampling location with a probability higher than 95%. The likelihood values for individuals from Recovery Centres to came from one of our populations was higher than 90% in 22 out of 36 individuals. This work is a first step to relocate animals of unknown origin taking into account genetic similarities and contribute to reinforcement programs of endangered species.

Keywords: *Emys orbicularis*, population structure, genetic diversity, assignment tests, microsatellites.

Introduction

Some animals, especially vertebrates, are sometimes collected in the wild or reared to maintain them as pets. To give an idea of the importance of this market, more than 18.3 million live reptiles (over 600 different taxa), were imported to the United States from 1989 through 1997, including 5.7 million turtles and tortoises representing 142 taxa. At the same time, more than 57.8 million reptiles (570 taxa), were exported from the United States, including over 53.7 million turtles and tortoises representing 115 taxa (Telecky, 2001). Freshwater turtles are among the most popular pets in the world (Moll and Moll, 2004) and are therefore prominent in this trade in many parts of the world (van Dijk et al., 2000).

Removal of animals from wild populations might contribute to increase population vulnerability, especially in the case of rare species. When these animals become large, there is a tendency for people to release them in natural habitats, and the pet trade becomes a source of introduction of exotic species, some of which might behave as invasive species, or contribute to the spread of pathogens (Luiselli et al., 1997; Servan and Arvy, 1997; Cadi and Joly, 2000; Bringsøe, 2001; Cadi and Joly, 2004). Furthermore, some specimens are left in Recovery Centres, where they accumulate in large numbers. These animals are either maintained in zoos, sacrificed or re-located to their supposed original regions, in an attempt to reinforce natural populations.

A recurrent problem in the management of these species is how to determine the probable region of origin of a particular specimen. In some cases this can be unambiguously determined by phenotypic characters or because the species is restricted to a very small region

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in nature. But on most occasions, animals cannot be returned to their native region with confidence. The introduction of specimens from very different regions can cause genetic homogenization and hybridization. Outbreeding depression events can also occur when crossing of genetically differentiated populations (e.g. crossing species or subspecies) results in reduced reproductive fitness (Templeton, 1986). Furthermore, captive animals can be a source of new pathogens for wild populations.

The estimation of genetic variability between and within populations, a task that has been made affordable by recent developments in PCR-based technology, using harmless techniques, is therefore a solution to this problem, because it can produce assignment tests for animals from Recovery Centres, and contribute to reinforcement programs of endangered species.

In this paper we present the first data on intra- and inter-population genetic variability in Iberian populations of the European pond turtle (*Emys orbicularis*) using a set of seven microsatellites. *E. orbicularis* has a widespread distribution from East and Central Europe, to the Mediterranean countries and the North of Africa, but it is a species in clear regression in most of its area (Fritz, 2001). We have studied five populations that cover most of the range in the Iberian Peninsula, including two isolated Northern populations and the core populations of this species in Southern Spain, and samples from animals maintained in Recovery Centres of the same regions.

Although historically this species has been maintained as a pet, this practice is becoming less frequent because the species is now strictly protected. Nevertheless, turtles are sometimes relocated to areas distant from their home populations. For this reason, it is common to find animals of unknown origin in Recovery Centres. The main aim of our research was to test the ability of genetic algorithms like STRUCTURE (Pritchard et al., 2000) to identify possible source populations, and for this, we selected a suitable method for our purpose (Manel et al., 2005). This algorithm constructs genetic clusters from individual multiloci genotypes, estimating for each individual the fraction of its genome that belongs to each cluster. The assignment of animals of unknown origin to populations has often been done on the basis of phenotypes or sampling locations but a correct assignment of these animals must also account for genetic similarities. This is important because genetically similar individuals might be labeled differently due to distinct geographical origin or different phenotypes although this need not imply that the individuals are different. Also, phenotypically similar animals might have distinct genetic origin. Given that this species shows several mitochondrial lineages in the Iberian Peninsula (Lenk et al., 1999), it is crucial to release captive animals in their home areas. However, the low genetic divergence showed by mitochondrial DNA within *E. orbicularis* (Lenk et al., 1999) makes necessary the use of more polymorphic markers, such as microsatellites, to assess the source localities of individuals whose origin is unknown.

Material and methods

Samples

Approximately 100 μl of blood were sampled from the tail or head of 153 specimens of *Emys orbicularis* turtles from five populations (Porriño, Ourense, Madrid, Valencia and Doñana) covering the species range in the Iberian Peninsula and 36 animals of unknown origin from across four different Recovery Centres (Oleiros, GREFA, CRARC and Valencia) (fig. 1). Individuals from Recovery Centres were used to test the ability of our analyses to assign them a probable origin.

DNA extraction and microsatellite loci amplification

Genomic DNA was extracted using the lithium chloride method (Gemmell and Akiyama, 1996). The resulting DNA pellets were air dried and suspended 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. The primers used to amplify microsatellite loci were first described for *Clemmys muhlenbergii* and tested for cross amplification in Emydidae turtles (King and Julian, 2004). Eight sets of primers were chosen for amplification of which seven were polymorphic (D88, D114, D16, D93, D87, D51 and B08, King and Julian, 2004). Six of these markers

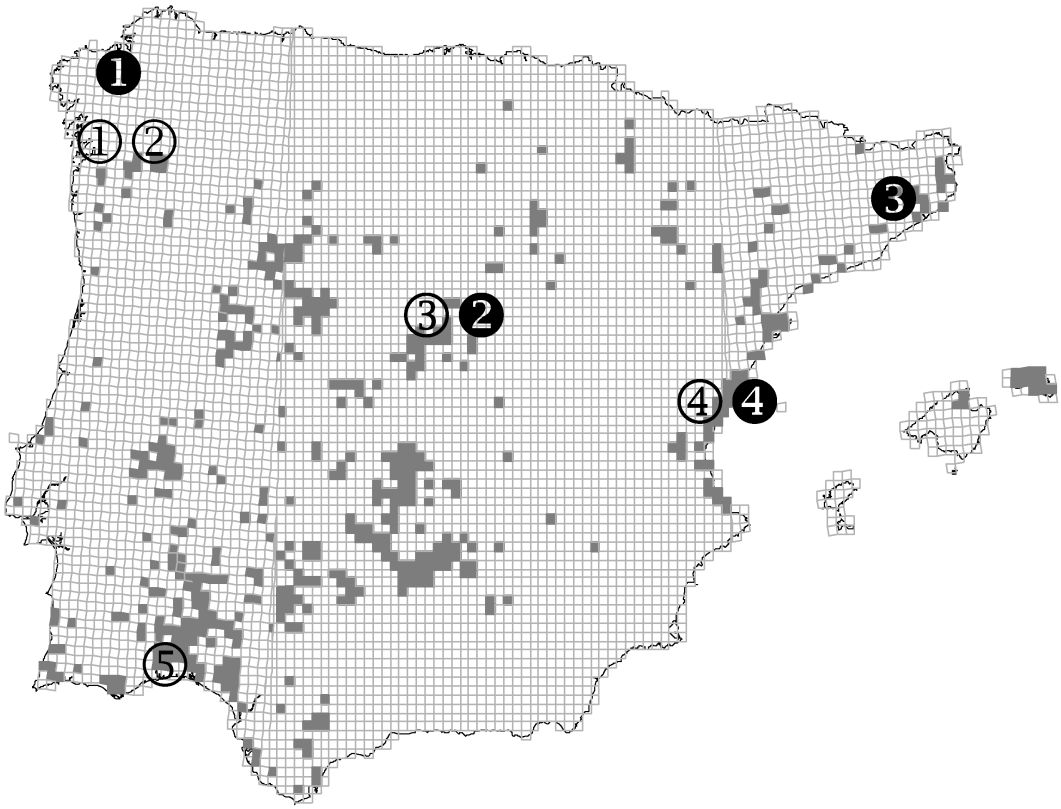

Figure 1. Location of sampled populations (open circles: 1, Porriño; 2, Ourense; 3, Madrid; 4, Valencia and 5, Doñana) and Recovery Centres (closed circles: 1, Oleiros; 2, GREFA; 3, CRARC and 4, Valencia) on a map of the distribution of *E. orbicularis* in the Iberian peninsula (10 × 10 UTM squares). 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".

were recently used for this species for paternity analysis in Doñana population (Roques et al., 2006).

The PCR reactions were performed using a Geneamp PCR System 2700 Thermocycler using two different programs with the following combination of loci: (A) D88, D114 and (B) D16, D93, D87, D51, B08. PCR reactions of 10 μl were performed using 75 ng of DNA, 0.25 mM dNTPs, 0.2 μM of each primer, 1 × PCR Buffer, 2mM $MgCl_2$ and 1 unit Ecotaq DNA Polymerase. One primer of each pair was labeled with a fluorescent dye.

PCR reactions for loci in the A group 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 loci in the B group we performed a touchdown PCR (Don et al., 1991), starting 2 min at 94°C followed by 20 cycles (going down 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 of 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.

After checking the size of the fragments in a 1.5% agarose gels and to minimize costs, 3 μl from the PCR reactions were mixed with the following combination of loci: (A) D114, B08 and D51; (B) D87 and D93; (C) D88 and D16. One microlitre of each multiplex mixture was added to 2 μl of a loading buffer containing 0.25 μl of GS350 Tamra standard (PE Applied Biosystems), 0.30 μl of dye, and 1.45 μl of formamide. Data collection, analysis and sizing were performed using ABI Prism Genescan software.

Data analysis

Genetic diversity within populations. The estimation of genetic diversity within populations helps to define the conservation status of local populations. The genetic variability was calculated using the Hardy-Weinberg expected heterozygosity (H_e), the observed heterozygosity (H_o) and the average number of alleles per locus (N_a). All these parameters were calculated for each locus for each population, and averaged over all loci using the GENETIX software (Belkhir et al., 2004). Deviations from Hardy-Weinberg equilibrium and tests for linkage disequilibrium were performed at all pairs of loci using GENEPOP on the web (http://wbiomed.curtin.edu.au/genepop/).

Population differentiation and assignment tests. The levels of population differentiation were calculated using pairwise F_{ST}. The value of F_{ST} (Weir and Cockerham, 1984) was calculated using GENETIX software (Belkhir et al., 2004) with significance of pairwise comparisons tested using 1000 iterations. The PHYLYP-package (Felsenstein, 1995) was used to calculate Nei's standard genetic distance (Ds) (Nei, 1972) and to construct Nei's distance tree (NJ) (Saitou and Nei, 1987). Bootstrap analysis was 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 then summarized using the CONSENSE program in PHYLIP. We calculated a Mantel test (Sokal and Rohlf, 1995) using GENETIX to test for correlation between genetic and geographic distances.

A model-based clustering method for using multiloci genotype data to infer population structure and assign individuals to populations was done using STRUCTURE (Pritchard et al., 2000). We have assumed a model in which there are K unknown populations, each of which is characterized by a set of allele frequencies at each locus. The most likely value of K is assessed by comparing the likelihood of the data for different values of K. A non-admixture model with independent allele frequencies was used because the studied populations are currently isolated among them. No information about the population of origin for each individual was given. A series of four independent runs for K from 1 to 10 was performed. The results are based on runs of 10^6 iterations, following a burn-in period of 10,000 iterations. 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.

To evaluate the probable origin of 36 individuals from Recovery Centres we used the same model and the same parameters with $K = 5$, and introduced the source population for all individuals excepting these ones.

Results

Genetic diversity measures

A total of seven microsatellite loci were analyzed in five *Emys orbicularis* populations representing almost the whole distribution area of the species in Iberian Peninsula.

Three out of 35 chi-square tests (for each locus in each population) showed significant deviations from Hardy-Weinberg equilibrium at the 95% confidence interval (one at 99%). Two of them occurred in the Madrid population for loci D114 and D93 and the third was observed in Doñana population for locus B08. Two linkage disequilibria, out of the 21 pair comparisons, were found between loci D114-D88 and D88-D51 although this probably does not mean physical linkage because it was not observed across all populations.

Table 1. Genetic diversity measures for seven loci screened for five populations of *Emys orbicularis*. Sample size (N), expected heterozygosity (H_e), observed heterozygosity (H_o), number of exclusive alleles, and the mean number of alleles per locus (N_a). Populations are ordered from North to South.

Population	N	H_e	H_o	No. of private alleles	N_a
Porriño	30	0.6729	0.7274	0	5.8571
Ourense	32	0.7059	0.6734	3	6.5714
Madrid	31	0.7752	0.7573	5	7.2857
Valencia	23	0.7224	0.7628	7	7.5714
Doñana	36	0.8005	0.8214	7	8.5714

Table 2. Pairwise F_{ST} values for the five studied populations of *Emys orbicularis*.

	Doñana	Madrid	Valencia	Ourense
Porriño	0.1027	0.15421	0.19207	0.10542
Doñana		0.09818	0.09487	0.11278
Madrid			0.13254	0.12797
Valencia				0.16341

The number of alleles per locus ranged from 4 (CmuB08) to 16 (CmuD16) with a mean of 11.86. A latitudinal trend in all genetic diversity measures studied was observed (heterozygosity, mean number of alleles and number of exclusive alleles), increasing from populations in the Northwest Iberia (Porriño and Ourense) to the Southernmost population (Doñana) (table 1).

Population differentiation. The analysis of population structure in *E. orbicularis* revealed a moderate degree of genetic differentiation with an overall population estimate of $F_{ST} = 0.12$. Not surprisingly, when pairwise comparisons of populations were calculated, the higher values of population differentiation were observed between the farthest populations (Porriño and Ourense with Valencia had $F_{ST} = 0.19$ and 0.16, respectively) (table 2). The genetic relationship between the five populations analysed in our study was synthesized in one unrooted NJ

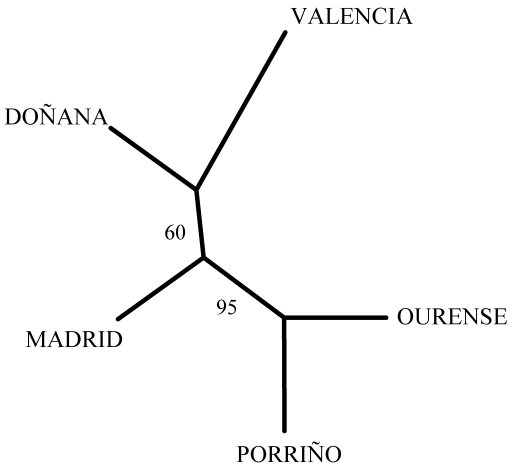

Figure 2. Unrooted Neighbour-joining tree of *Emys orbicularis* populations based on the Nei's genetic distances. In the branches are indicated the bootstrap support values are based on 1000 replicates.

tree made using Nei's genetic distances (fig. 2). Interestingly, the populations display in the tree by their geographical proximity being one extreme the Norwestern populations of Porriño and Ourense and the other extreme the Eastern population of Valencia. In the middle of the the cladogram is situated the central population of Madrid. Genetic similarity decreases with geographic distance, and a significant relationship (Mantel test, $p = 0.012$) was found amongst sampled locations.

When estimating by the Bayesian method the number of populations present in our data it was achieved the maximum Ln probability of the data with $K = 5$ (data not shown) (fig. 3) which fully agree with our a priori knowledge of five independent populations. Using this test we were able to allocate most individuals (90%) to their sampling location with a probability higher than 95%. This result gave us confidence in assessing the genetic differentiation of *Emys orbicularis* populations using the selected set of seven microsatellites to predict the sampling location of origin.

Assignment tests. Likelihood ratio values obtained from the assignment tests with probability higher than 95% showed that 87% of individuals in the Porriño population were correctly assigned, and the rest were joined to the second NW population. The Ourense population had 90% of individuals correctly assigned with just one individual assigned to Doñana population. Madrid, Valencia and Doñana had 97%, 92% and 86% of individuals correctly assigned, respectively. The reminders in the last three populations were all assigned to their own population with probabilities larger than 50% except for two individuals of Doñana which were assigned to Valencia population.

Interestingly, when the same Bayesian analysis was performed including individuals from the Recovery Centres, it was observed a high likelihood value for assignment of these individuals to one of the five populations studied. Out of 36 individuals, 22 were assigned to one of the five populations with a likelihood value higher than 90% (table 3). The GREFA Recovery Centre (Madrid) had five individuals assigned to Madrid population and three to Ourense population. The two samples from Oleiros Recovery Centre (NW Iberia) were assigned to Ourense. CRARC Recovery Centre (NE) had three samples assigned to Ourense, three to Valencia and two assigned to Madrid. Valencia Recovery Centre (East), which is the place which has more unknown individuals, had 6 samples assigned to NW populations (four to Ourense and two to Porriño), two individuals were assigned to Madrid and four to the SW population (Doñana), while six of them were assigned to the Valencia population.

Discussion

Our results indicate a moderate overall F_{ST} value of 0.12 and significant pairwise differentiation levels. Therefore the genetic structure of the Iberian *E. orbicularis* populations was enough to use microsatellite markers to assign animals to their original population with high confidence (91% of specimens). The Bayesian method revealed one cluster for each population (with the maximum Ln probability) but the analysis for four clusters joined NW popula-

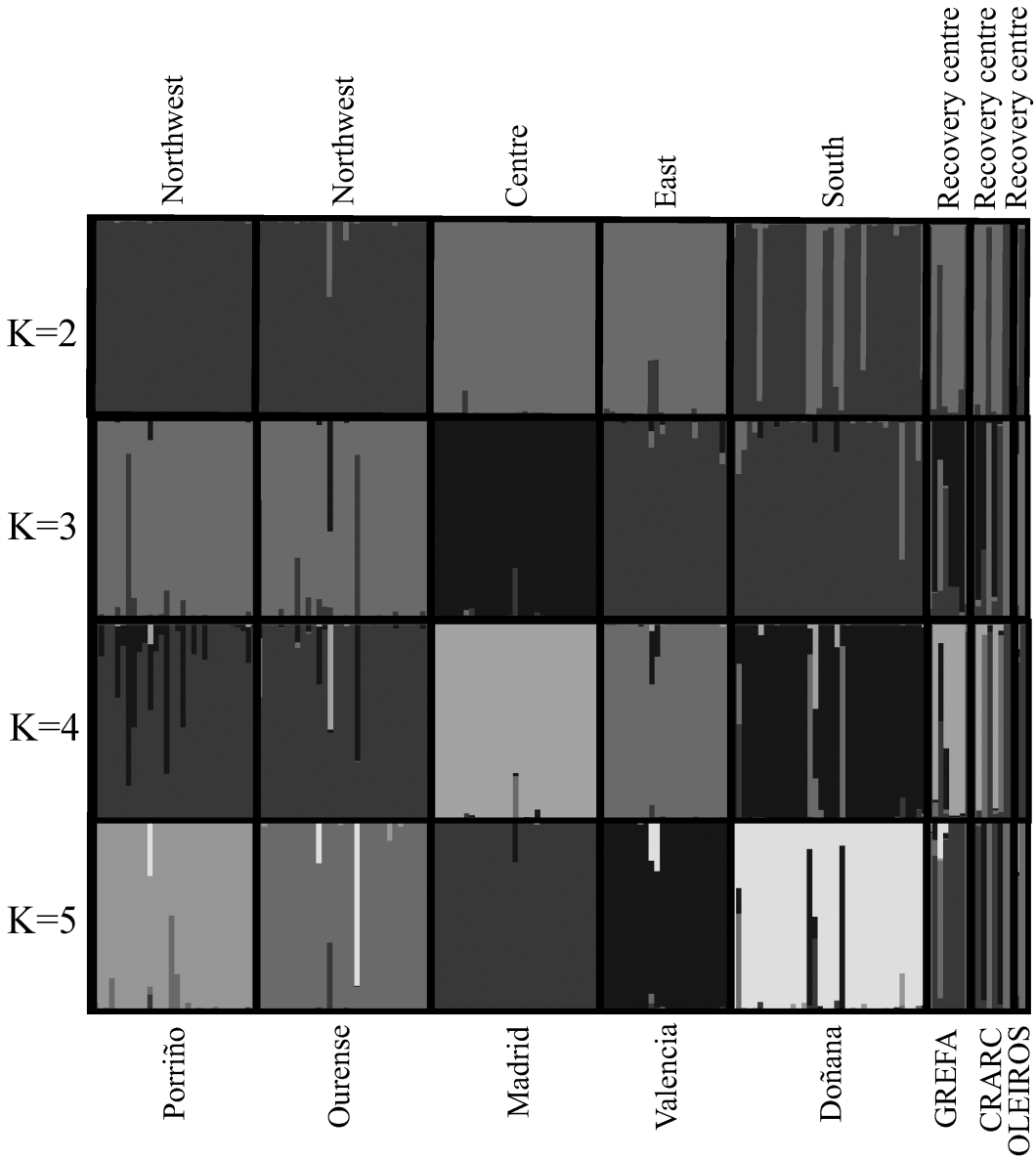

Figure 3. Estimated population structure (from K = 2 to K = 5). Black lines separate individuals of different populations which are labeled bellow the figure, with regional affiliations above it. Individuals from three Recovery Centres are represented to show the high diversity of their location origin. Each individual is represented by a thin vertical line, which is partitioned into K colored segments that represent the individual's estimated membership fractions in K clusters.

tions in one cluster an the reminders in the other, reflecting the similarities and past gene flow between Porriño and Ourense (fig. 3).

Freshwater turtles usually show population structure between adjacent river systems. In a giant Amazonian river turtle (*Podocnemis expansa*) a study combining mtDNA and microsatellites found that most of genetic variability (87%) was between two river systems whose centers were separated by 2400 km, suggesting very little migration between them (Sites, Jr. et al., 1999). However this differentiation can be due to isolation by distance (Wright, 1943). In fact, our data (Mantel test is significant) sug-

Table 3. Assignment test (%) of 36 *E. orbicularis* individuals from four Recovery Centres in Spain. In bold, it is highlighted the most likely population of origin for each individual. The proportion of missing data indicates cases where one or more loci failed to amplify.

SAMPLE	% Missing data	Ourense	Madrid	Porriño	Valencia	Doñana
GREFA-1	0	**85.90**	0.10	2.50	1.20	10.40
GREFA-2	14	5.90	**76.00**	0.00	12.20	5.60
GREFA-3	0	**78.30**	4.30	1.30	0.00	16.10
GREFA-4	0	2.00	**80.80**	0.50	8.20	8.40
GREFA-5	0	0.00	**98.10**	0.10	0.10	1.60
GREFA-6	0	0.00	**98.10**	0.10	0.10	1.60
GREFA-7	0	0.70	**97.50**	1.10	0.00	0.60
GREFA-8	0	**99.90**	0.00	0.00	0.10	0.00
CRARC-1	0	0.00	0.00	0.00	**100.00**	0.00
CRARC-2	0	11.00	**88.00**	0.00	0.80	0.20
CRARC-3	0	0.00	2.20	0.00	**97.80**	0.00
CRARC-4	0	**98.60**	0.00	1.20	0.00	0.20
CRARC-5	28	0.00	**98.60**	0.20	1.20	0.00
CRARC-6	0	0.20	1.40	0.30	**97.20**	0.90
CRARC-7	0	**100.00**	0.00	0.00	0.00	0.00
CRARC-8	0	**99.80**	0.00	0.20	0.00	0.00
OLEIROS-1	28	**63.10**	6.90	26.10	3.10	0.80
OLEIROS-2	0	**99.60**	0.00	0.40	0.00	0.00
VAL-1	7	**100.00**	0.00	0.00	0.00	0.00
VAL-2	7	2.30	0.00	0.00	**97.70**	0.00
VAL-3	0	0.00	15.70	0.00	33.10	**51.20**
VAL-4	0	0.00	0.00	0.00	**98.50**	1.50
VAL-5	0	**79.30**	0.00	0.00	20.70	0.00
VAL-6	14	0.00	0.00	0.00	**90.70**	9.30
VAL-7	0	1.60	0.10	0.00	**58.80**	39.50
VAL-8	0	0.50	0.00	**61.60**	0.20	37.70
VAL-9	0	**95.60**	2.00	0.20	1.70	0.50
VAL-10	14	16.20	0.00	0.00	**80.90**	2.90
VAL-11	0	**96.40**	0.00	0.40	0.90	2.30
VAL-12	0	0.00	0.00	0.20	0.00	**97.70**
VAL-13	0	0.00	0.00	**99.40**	0.00	0.60
VAL-14	0	8.60	0.00	0.00	**91.40**	0.00
VAL-15	0	0.00	0.00	0.00	0.00	**100.00**
VAL-16	14	24.80	**42.80**	0.00	29.80	2.60
VAL-17	14	26.90	**62.30**	0.10	1.00	9.70
VAL-18	0	0.00	0.00	0.00	0.00	**100.00**

gest that there is a correlation between genetic distance and geographical distance in Iberian populations of *E. orbicularis*. This isolation by distance effect implies that populations are connected by gene flow or were connected in the recent past.

The high level of differentiation between the two NW populations suggest an influence of genetic drift/or limited gene flow as indicated by assignment tests, particularly in Porriño and Ourense populations, where all samples were assigned to their original population although some of them had less than 95% likelihood. A low level of differentiation was detected in pairwise comparisons between all populations and Doñana which is the most diverse population.

Assignment tests as a conservation tool

Several molecular methods are being used to help to enforce wildlife conservation laws by identifying the species of origin from mitochondrial or nuclear DNA (Palumbi and Cipriano, 1998; Roman and Bowen, 2000) and by matching individuals to tissues through DNA finger-

printing (Taberlet and Luikart, 1999; Waits et al., 2000). It is also possible to determine the geographic origin of a sample using hypervariable molecular markers (e.g. microsatellites) and statistical approaches called assignment tests. Assignment tests are a way of using an individual's DNA to find out where that individual was born. This information can then be used to look at whether the individual was born in the same place where it was found or whether it has moved during its lifetime. This approach can also detect trade routes, and help in the management of captive-breeding programs by excluding non-target individuals (Olsen et al., 2000). When a turtle is assigned to a population it is assigned on the basis of how likely it is to have come from that population rather than any other. It is possible to assess the accuracy of an assignment of a turtle to a particular population using the likelihood that the turtle came from this population and comparing it to the likelihood that the turtle came from any of the other populations. In this study, most (90%) of the genotyped individuals had more than 95% likelihood of belonging to one of the sampled populations.

The individuals of unknown origin from Recovery Centres had in some cases lower assignation probability, clearly because we have not sampled all possible populations. The accuracy of this methodology could be improved by increasing the number of populations, and this work is currently under way. Of the animals of unknown origin, 33% of individuals were assigned to Ourense; 25% to Madrid and Valencia, 11% to Doñana and only 1% to Porriño. This suggests an important movement in the whole Peninsula. However of these individuals only four (from Valencia) were assigned to the Doñana National Park, which might indicate that this protected area impedes the capture of individuals, avoiding in this way the transfer of animals to other regions. Nevertheless, given the great distance from Doñana to the Recovery Centres included in this study, this could also explain why Southern animals do not appear in these Recovery Centres.

If we compare the movement in each region, the two samples from Oleiros (NW) come from one of the NW populations, however in Valencia 66% of the samples are not from the Valencia region and 38% of the samples in GREFA did not come from Madrid. We have not sampled in the NE so for individuals from GREFA 100% are from outside but considering Valencia as the closest population 38% of individuals are from Madrid and Ourense. It seems that central and NW Iberian populations have a tendency to lose individuals with this practice and East and NE regions to receive them.

As discussed above, our ability to assign animals to the correct population is obviously limited by the number of studied populations. However, given the strategic localization of the samplings, for most animals at least it is possible to indicate a region of origin. Therefore, these animals can now be released in the wild minimizing their genetic differences with the local animals, which is a matter of concern in many relocation studies. Moreover, the significant pairwise differentiation levels found among Iberian populations and the high likelihood of the assignment tests show that microsatellites are better markers than mtDNA to determine the geographic origin of a sample.

To conclude, this study provides the first description of the genetic structure of the European pond turtle in the Iberian Peninsula although further studies are needed comparing mtDNA and nDNA data with a finer sampling. Most (90%) individuals were correctly assigned with high accuracy when Structure was used on real data sets. Therefore, these results have important management consequences for the conservation and relocations of individuals of the European pond turtle.

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