Amphibia-Reptilia 32 (2011): 49-61

Genetic patterns of a range expansion: The spur-thighed tortoise *Testudo graeca graeca* in southeastern Spain

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Abstract. In the present work we analyzed the genetic structure of the populations of the terrestrial tortoise *Testudo graeca graeca* in southeastern Spain, identified as a recent range expansion from North Africa. The study and interpretation of the species' genetic spatial pattern could provide clues to the processes related to the species' arrival and, because of its endangered status, is especially useful in implementing appropriate management measures. We used microsatellite markers to analyze 17 populations located in the coastal region of the species' range in southeastern Spain, and an external group of Algerian tortoises. Three genetic units with a high level of spatial coherence and moderate levels of admixture resulted from a cluster analysis, and an isolation-by-distance pattern covering the entire study area was detected. These results suggest that southeastern Spanish populations show a complex spatial genetic pattern resulting from their isolation from North African populations and their natural dispersal in this region. Finally, our work shows that conservation actions such as captive breeding, introductions or translocations, may have played a relevant role in the modification of the genetic structure of some populations in southeastern Spain. Therefore, these types of conservation measures should be carried out with more caution.

Keywords: conservation, endangered species, genetic patterns, range expansions, species management, *Testudo graeca*.

Introduction

The genetic structure of recent expansions of species ranges is especially revealing because it is possible to detect not only genetic patterns caused by the more typical dispersal, isolation and drift processes but also those patterns caused by the original founder events, which can still be genetically visible (Duglosch and Parker, 2008; Okada et al., 2009). The reconstruction of the origin of recent populations carries ecological, biogeographical and evolutionary interest (Hanski and Gilpin, 1997; Ingvarsson, 1997; Tremetsberger et al., 2003), and is relevant to conservation management of endangered species (López-Castro and Rocha-Olivares, 2005; Hedtke et al., 2007) and in understanding the dynamics of biological invasions (Kolbe et al., 2004; Wares et al., 2005).

In this work, we study the spatial genetic pattern of the spur-thighed tortoise (*Testudo graeca graeca*) populations in southeastern Spain (hereafter, SE). The species has a western Mediterranean distribution, with the majority of its range in North Africa with a few small and isolated European populations occurring in the Iberian Peninsula and on some islands (Mallorca, Sardinia and Sicily; fig. 1a).

The origin of SE populations has been suggested to be the consequence of human introductions or a natural range expansion from North Africa (Álvarez et al., 2000; Fritz et al., 2009). Salinas et al. (in press) also suggest that Spanish populations could originate from multiple introductions from North African populations as a result of the pet trade between Africa and Europe. However, the identification of exclusive haplotypes in SE suggests that the alternative hypothesis of an earlier natural colonization across the Mediterranean Sea cannot be rejected (Fritz et al., 2009; Salinas et al., in press).

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 DOI:10.1163/017353710X542985

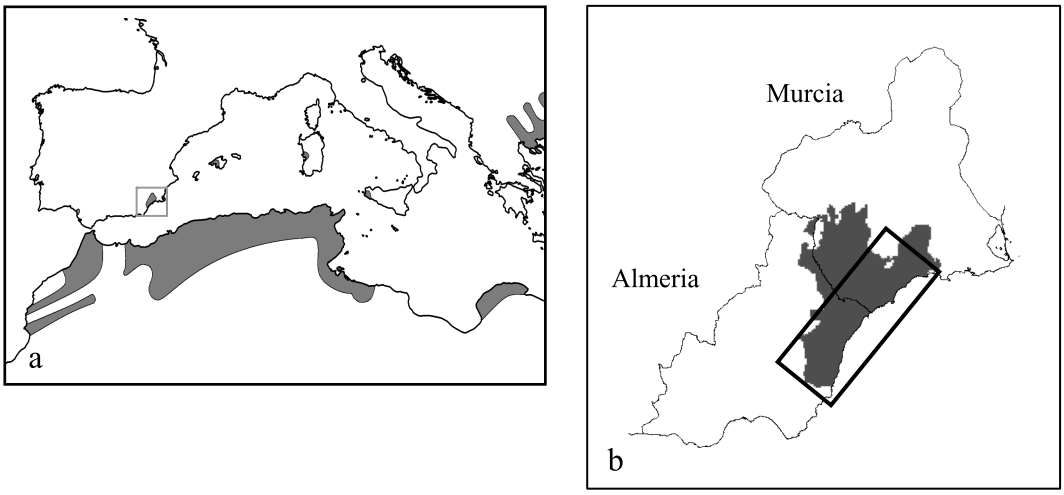

Figure 1. (a) Distribution of *Testudo graeca* in the Mediterranean region (modified from Fritz, 2009); SE Iberian populations are those included in a grey square. (b) Distribution in Southeastern Spain, provinces of Almería (Anadón et al., 2010) and Murcia (Anadón et al., 2007); sampled locations are inside the black rectangle.

Hence, the existing studies are unclear regarding the processes leading to the SE populations, with both a natural range expansion and a more recent human-mediated expansion being possible.

Until now, the spatial genetic structure of the population of *T. g. graeca* in SE has not been thoroughly investigated. Its study and interpretation may shed light on the processes that led to the present population in SE. Under the assumption of a recent range expansion (human-mediated or not) we can expect low levels of genetic diversity, no genetic differentiation among populations and the absence of spatial genetic structure (Hewitt, 1999). Nevertheless, it is known that multiple arrivals of genetically distinct individuals of different origins can lead to an opposite pattern with high genetic diversity levels and population differentiation (Beebee and Rowe, 2004; Marrs et al., 2008) but without a geographically coherent genetic structure. In this sense, more complex spatial patterns of genetic structure such as isolation by distance (IBD), caused by natural species dispersal, may require more time (Wright, 1943; Marrs et al., 2008).

In any case, the original genetic pattern could be altered by a more recent introduction of individuals (Pérez et al., 2004; Salinas et al., in press). The species is endangered in the SE region due to habitat loss and its collection as a pet (Giménez et al., 2004; Pérez et al., 2004). Its strong cultural dimension as a captive animal in the local communities and illegal trade from North Africa have led to the arrival of thousands of individuals of unknown origin at wildlife centers (Pérez et al., 2004). In past decades, public administration and NGOs have developed management schemes for these captive animals (i.e. captive breeding, introductions or translocations) with the aim of reinforcing wild populations (Pérez et al., 2004; Salinas et al., in press). However, the execution of these actions, without regard to whether or not the species presents a structured regional pattern of genetic diversity, threatens the evolutive potential of the species in this context through the loss of adaptive complexes (Álvarez et al., 2000). For this reason, the knowledge of the genetic spatial pattern of the species is especially relevant for its management.

In summary, we aim to: (i) describe the spatial patterns of genetic diversity of populations of *T. g. graeca* in southeastern Spain; (ii) discuss the possible processes involved in its origin and

configuration; and (iii) discuss conservation and management implications.

Materials and methods

Study area

SE populations occupy approximately 2600 km^2 of semi-arid coastal mountains on metamorphic substrates with sparse scrub with small patches of woodland and dry farming (Anadón et al., 2007, 2010). We sampled 243 tortoises from 17 wild populations located in the coastal region of the species' range in Murcia and Almeria Provinces (table 1), covering approximately 60% of its total range in SE Spain, (fig. 1b). Maximum and minimum distances among these sites were 63.7 and 2.8 km, respectively. Finally, 19 Algerian tortoises confiscated at Alicante's port were included in some analyses (see the last paragraph of data analysis).

Selection of molecular markers

In this work we selected three highly polymorphic microsatellite loci (CmuB08, CmuD16 and CmuD51; table 2), originally designed for the bog turtle, *Glyptemys muhlenbergii* (King and Julian, 2004), and previously used in *T. g. graeca* (Roques et al., 2004; Salinas et al., in press). Although a higher resolution in genetic structure could be achieved using more microsatellites (Evanno et al., 2005), equivalent results could be obtained in the precision of estimates of genetic distances between populations by examining a few loci with many alleles (Kalinowski, 2002) The three microsatellites employed in this work have been shown to yield a high number of alleles in *Testudo g. graeca*, with 53 alleles for the Doñana population (Roques et al., 2004), 54 for the SE population and 85 for the Northern Africa (Salinas et al., in press).

Sample collection and laboratory protocol

Blood samples were obtained by coccygeal vein puncture (Richter et al., 1977) and were preserved in 100% ethanol at 5°C. Genomic DNA was extracted using an alkaline digestion method adapted from Rudbeck and Dissing (1998). Polymerase chain reaction (PCR) was performed using the same conditions as Roques et al. (2004). PCR products were visualized on 9% polyacrylamide silver stained gels (Qu et al., 2005) by two different researchers. Allele sizes were estimated using a DNA ladder (*Roche, VIII:* 19-1114 bp) and known genotypes were used as standards across gels. In addition, with the aim to evaluate allele assignments, a subset of 15 samples was randomly selected and analyzed for a second time by repeating DNA extractions and genotyping of each sample.

Data analysis

As a first step in the evaluation of the reliability of the markers, all loci were examined for genotyping errors, allelic dropout and null alleles, using Monte Carlo simulations (bootstraps = 1000; confidence interval = 95% with Bonferroni correction) in MICRO-CHECKER 2.2.3 (Oosterhout et al., 2004). Genotypic linkage disequilibrium between each pair of microsatellite loci was tested using the Markov chain method (dememorization steps = 10 000; batches = 100; iterations per batch = 5000) in GENEPOP 4.0 (Rousset, 2007). To test the variability of the microsatellite markers we performed analysis of genotypic data for each locus to obtain estimates of the number of alleles per locus.

To evaluate variation in allelic diversity within sites, average allelic richness estimates (Rs) corrected for variable sample sizes and allelic frequencies to determine the number of private and rare alleles were calculated. These estimates were obtained in FSTAT 2.9.3.2 (Goudet, 2002). To avoid problems related to insufficient sampling, populations with less than eight individuals were excluded from the subsequent analysis. They were only included in some clustering analysis and in two posterior assignment tests (see below). Expected heterozygosities (H_S) for each locus across populations and for all loci across populations were calculated with POPGENE 1.32 (Yeh and Boyle, 1997, 1999). Significant deviations from Hardy-Weinberg equilibrium (HWE) and the heterozygote excess or deficit were tested in each population using the Markov chain approximation (dememorization steps = 10 000; batches = 200; iterations per batch = 5000) in GENEPOP 4.0 (Rousset, 2007). Sequential Bonferroni correction (Rice, 1989) was used for a significance level of 0.05. The inbreeding coefficients within populations (F_{IS}) were also obtained using this software.

The Algerian samples cannot be considered as a population due to the uncertainty of the exact geographic origin of each sample, so they were not consider in Rs, H_S, HWE and in F_{IS} analysis. In spite of this, these samples are valuable to estimate genetic differentiation between SE and Algerian tortoises using STRUCTURE 2.3.1 software (Pritchard et al., 2000). This program uses genotype data from unlinked markers and applies a Bayesian clustering approach to identify groups (K) that maximize HWE and linkage equilibrium within them. Two possible ancestry models are implemented in STRUCTURE: (i) the Admixture Model, in which individuals may have mixed ancestry (recent or current gene flow), and (ii) the No Admixture Model, where each individual comes purely from one of the K populations. Hubisz et al. (2009) extended these basic models to allow STRUCTURE to make use of information about sampling locations (LOCPRIOR) when the data indicate that this information would be helpful. In this sense, r parameter values near 1 or minors indicate that sampling locations are informative. The LOCPRIOR approach increases the STRUCTURE sensibility in clustering populations with low divergences or in analyzing datasets with few loci. Admixture and no Admixture models with and without sample information were run for all SE and Algerian tortoises, from $K = 1$ to $K = 5$ (MCMC repetitions = 100 000; burning period = 10 000; correlated allele frequencies). Calculations were repeated 4 times for each K and optimal K value was estimated following the method described by Evanno et al. (2005).

Table 1. Summary statistics for all loci across populations of *T. g. graeca* in Southeastern Spain.

Geographical location	Population	Pop. Code	n	Avg. R_S	Avg. H_S	Avg. F_{IS}	Rare alleles/rare and private alleles	Inferred cluster (prop. of membership)	Individuals excluded from the SE group
Almenara's Mountain	*North Bas*	NB	10	4.67	0.75	0.30	0/0	1 (0.85)	0
	South Bas	SB	17	5.62	0.84	0.14	1/1	1 (0.81)	2
	Galera	GA	31	5.81	0.86	0.04	2/1	1 (0.63)	2
	Villarreal	VI	14	4.91	0.81	0.03	1/0	1 (0.60)	1
Pinos' Mountain	*Aguilón**	AG	20	6.20	0.88	0.15	0/1	1 (0.88)	6
	Aljife	AJ	17	5.29	0.79	0.05	1/0	1 (0.89)	1
Almagro's Mountain	*Sotomayor*	SO	12	5.12	0.81	0.11	0/0	1 (0.40)	1
	Malacate	ML	6	3.61	–	–	0/0	–	0
Vera's Basin	*Palas*	PA	12	5.22	0.83	−0.04	0/0	2 (0.42)	0
	Marinica	MA	21	4.43	0.75	−0.02	1/0	2 (0.91)	2
	Sierrecica	SI	16	4.96	0.83	0.17	0/1	2 (0.74)	0
	Chinas	CH	21	5.27	0.84	0.11	0/0	3 (0.89)	2
Cabrera's Mountain	*Cintas*	CI	8	4.32	0.69	0.03	0/0	3 (0.73)	0
	Teresa	TE	8	5.55	0.84	0.11	0/0	3 (0.63)	0
	Peralicos	PE	5	5.33	–	–	0/0	–	3
Bédar's Mountain	*Centinares*	CE	19	5.20	0.82	0.06	0/0	3 (0.50)	1
	Piña	PI	6	4.85	–	–	0/0	–	0

(*) deviated from HWE after sequential Bonferroni with a significant deficit of heterozygotes. n = number of individuals genotyped. Avg. R_S = Average of allelic richness per population across loci. Avg. H_S = Average of Expected heterozygosities per population across all loci. Avg. F_{IS} = Average of F_{IS} per population across all loci. H_S and F_{IS} were not calculated for populations whose sample sizes were less than eight.

Table 2. Geographic distances among populations in km (lower diagonal) and pair-wise F_{ST} estimates (upper diagonal). All F_{ST} values except those in bold are significant ($p < 0.05$).

Population	Aguilón	Aljife	North Bas	South Bas	Centinares	Chinas	Cintas	Galera	Marinica	Palas	Sierrecica	Sotomayor	Teresa	Villarreal
Aguilón	–	0.04	0.05	**0.01**	0.03	0.05	0.12	0.02	0.09	0.03	0.04	0.05	0.03	0.03
Aljife	4.2	–	**0.03**	0.03	0.06	0.09	0.16	0.05	0.14	0.07	0.06	0.11	0.07	0.08
North Bas	24.4	28.6	–	**0.03**	0.07	0.10	0.21	0.05	0.16	0.09	0.09	0.08	0.11	0.11
South Bas	21.1	25.2	3.6	–	0.04	0.06	0.14	0.02	0.08	0.04	0.05	0.07	**0.04**	0.03
Centinares	31.1	26.9	55.4	52	–	0.03	0.14	0.04	0.10	0.08	0.05	0.08	**0.03**	0.05
Chinas	27	22.8	50.9	47.4	6	–	0.10	0.03	0.11	0.05	0.08	0.07	**0.02**	0.06
Cintas	40.5	36.4	63.8	60.2	13	13.9	–	0.09	0.15	0.06	0.13	0.11	**0.04**	0.15
Galera	15.8	19.7	15	13.6	45.6	42.2	56	–	0.10	0.04	0.06	0.03	**0.01**	0.04
Marinica	10.4	7	33.2	29.7	22.8	17.8	30.6	26.3	–	0.06	0.05	0.09	0.06	0.10
Palas	10.8	6.7	34.7	31.2	20.8	16.3	29.7	26.4	3.2	–	0.03	0.06	**0.03**	0.08
Sierrecica	13.6	9.4	37.5	34.1	18	13.5	26.9	29.1	5.1	2.8	–	0.07	0.05	0.08
Sotomayor	13.6	10	37.6	34.4	19.7	17.5	31.3	26.2	11.7	8.6	8.6	–	**0.03**	0.10
Teresa	38	33.8	61.6	58	10	11.2	3	53.3	28.3	27.2	24.4	28.5	–	**0.00**
Villarreal	18.4	22.4	13.3	12.5	48.4	44.9	58.7	2.8	28.8	29.1	31.8	29	56.1	–

SE population structure was inferred using the same parameters and procedures as for the rest in STRUCTURE, we ran a fifth and last Admixture Model with sampling information (using only SE populations whose $n \geqslant 8$). Pairwise F_{ST}, as a measure of genetic distances, were calculated among SE populations with ARLEQUIN 3.01 (Excoffier et al., 2005) and used in two subsequent analyses for the SE region. First, they were used in an analysis of molecular variance (AMOVAs), also in ARLEQUIN, to determine how genetic variation is distributed among the groups, populations or individuals, using the groups obtained in the last analysis of STRUCTURE. Secondly, we performed a Mantel test in IBDWS (Jensen et al., 2005), to detect the relationship between genetic isolation and geographic distance. Finally, we used GENECLASS 2.0 software (Piry et al., 2004) to test whether SE populations with small sample sizes ($n < 8$) were successfully assigned to nearby larger populations, showing coherence with the genetic pattern described (Assignment threshold of scores = 0.05; criteria for computation = Rannala and Mountain Bayesian method, 1997).

With the aim of screening for the presence of introduced individuals from Algeria, which could have been altering a previous genetic pattern of the species in this region, we compared multilocus information from Algerian and SE tortoises in an assignment/exclusion test for each individual in GENECLASS 2.0 (Criteria for computation = Rannala and Mountain Bayesian method, 1997; Algorithm = Paetkau et al., 2004; 1000 simulated individuals; alpha = 0.05). Algerian individuals were also included in the rest of analyses that take into account only the genotype of each individual (number of alleles per locus and the genotypic linkage disequilibrium), and not their population origin.

Results

GmuB08, GmuD16 and GmuD51 microsatellites in T. g. graeca

All three microsatellites were highly polymorphic. We detected a total of 45 distinct alleles (12, 17 and 16 alleles for GmuB08, GmuD16 and GmuD51, respectively; $n = 262$ individuals). We did not find evidence of genotyping errors, allelic dropout or null alleles. We could not reject the null hypothesis of no linkage disequilibrium between these markers (CmuB08/CmuD16, $p = 0.53$; CmuB08/CmuD51, $p = 0.54$; CmuD16/CmuD51, $p = 0.50$), therefore we considered their alleles as independently segregated. The 15 repeated samples used as a control were assigned to the same genotypes as their counterparts; hence, the genotype assignment process was considered reliable.

Genetic diversity and HWE in populations

Rs estimates for the 17 populations ranged between 3.61 for *Malacate* and 6.20 for *Aguilón* and we detected a total of 7 rare alleles in 13 individuals (allelic frequencies under 1%). They were located in seven populations in the north in the center of the study area and, in addition, four of them appeared in only a single population (private alleles).

Expected heterozygosities (H_S) ranged between 0.69 and 0.88 for *Cintas* and *Aguilón* populations, respectively. F_{IS} values were in general greater than 0, suggesting moderate levels of inbreeding within populations and only the *Aguilón* population deviated from HWE at several loci. A summary of these statistics is shown in table 1.

Genetic differentiation and spatial genetic structure

Moderate differentiation levels among SE and Algerian tortoises was suggested by STRUCTURE analysis with both Admixture and no Admixture models, being 3 the most supported value for K. Bar plots showed blurred evidences of genetic structure in SE area (figs 2a and 2b). In the two second analysis STRUCTURE increased the sensibility of its screening taking into account information about sampling locations, being able to difference Algerian and SE tortoises accurately (most supported value for $K = 2$). r values were 0.52 and 1.14 for the admixture and no admixture models respectively, so we can conclude that our sampling locations are informative for the analysis and SE and Algerian tortoises could be considerate into two different genetic units (figs 2c and 2d). Although the low admixture levels reported by these two cluster analysis some SE individuals were most probably assigned to the Algerian cluster. See the Appendix for variations in LnP and in its variance among the different K values for each STRUCTURE analysis.

In the fifth cluster analysis, three groups of populations were identified as the most sup-

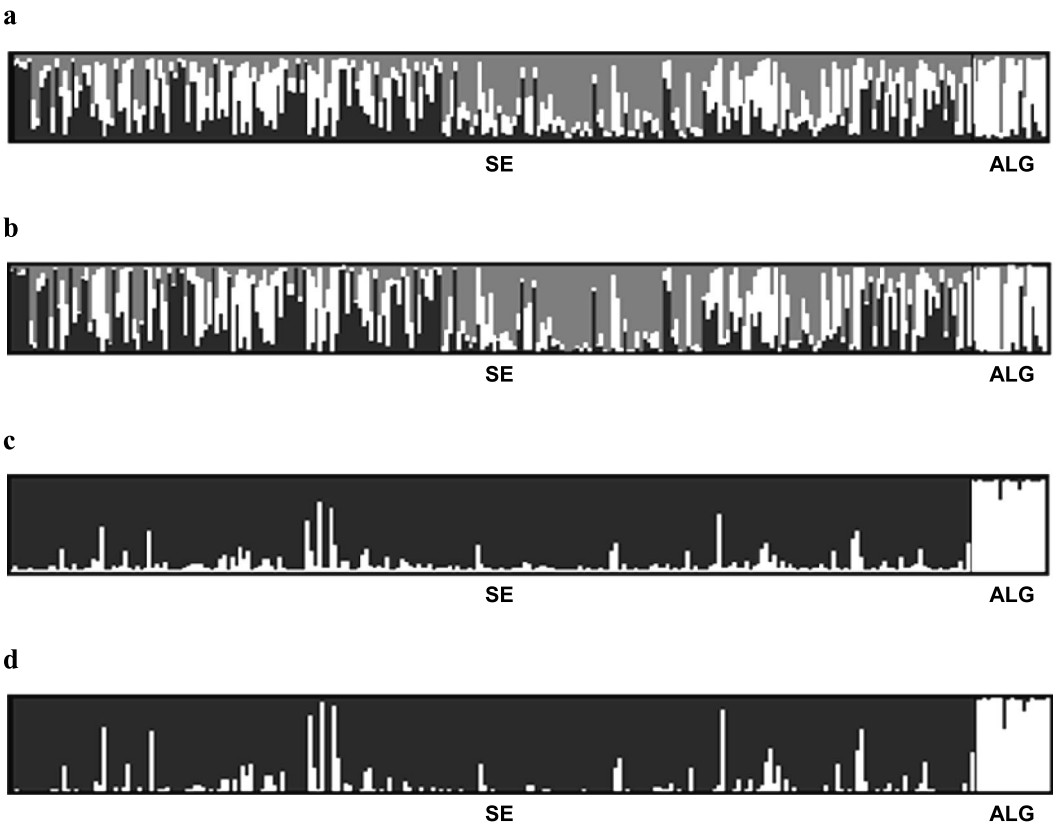

Figure 2. Bar plots generated by STRUCTURE (Pritchard et al., 2000) and graphically displayed by DISTRUCT (Rosenberg, 2004): (a) Admixture model; (b) No admixture model; (c) Admixture and LOCPRIOR model; (d) No admixture and LOCPRIOR model. SE = Southeastern Spanish tortoises; ALG = Algerian tortoises.

ported genetic substructure for SE populations, presenting high congruence with the geographical configuration of populations (table 1; fig. 3). Information about sampling populations was also informative in this case (r = 1.02). The six populations *Villarreal*, *Galera*, *North Bas*, *South Bas*, *Aguilón* and *Aljife*, mainly located in the north of the study area in *Almenara* and *Pinos* Mountains, were assigned to the first cluster. The *Sotomayor* population, located in the interior of the study area in Almagro's Mountain, was better assigned to this first cluster but with a low proportion of membership (40.2%) and high levels of admixture with the others. The *Palas*, *Marinica* and *Sierrecica* populations, located in the *Lobos Area*, were assigned to a second group and only *Palas* presented high levels of admixture. Finally, four populations located in the south of the study area, *Chinas*, *Centinares*, *Teresa* and *Cintas*, were brought together in a third cluster. These populations are located in the south of *Vera*'s Basin, and in *Bédar* and *Cabrera*'s Mountains. Although the assignment of all of these populations to the inferred clusters were geographically coherent, admixture levels in four of them (*Villarreal*, *Galera*, *Centinares* and *Teresa*) did not conform spatially.

F_{ST} distances between all possible pairs of populations were significant in most cases (80 of 91 pairs). Significant values ranged between 0.02 and 0.21. We found no significant estimates for 6 closely located pairs, as in the case of *North Bas-South Bas* (table 2). AMOVAs performed to assess the statistical support for the three main genetic units detected in the cluster

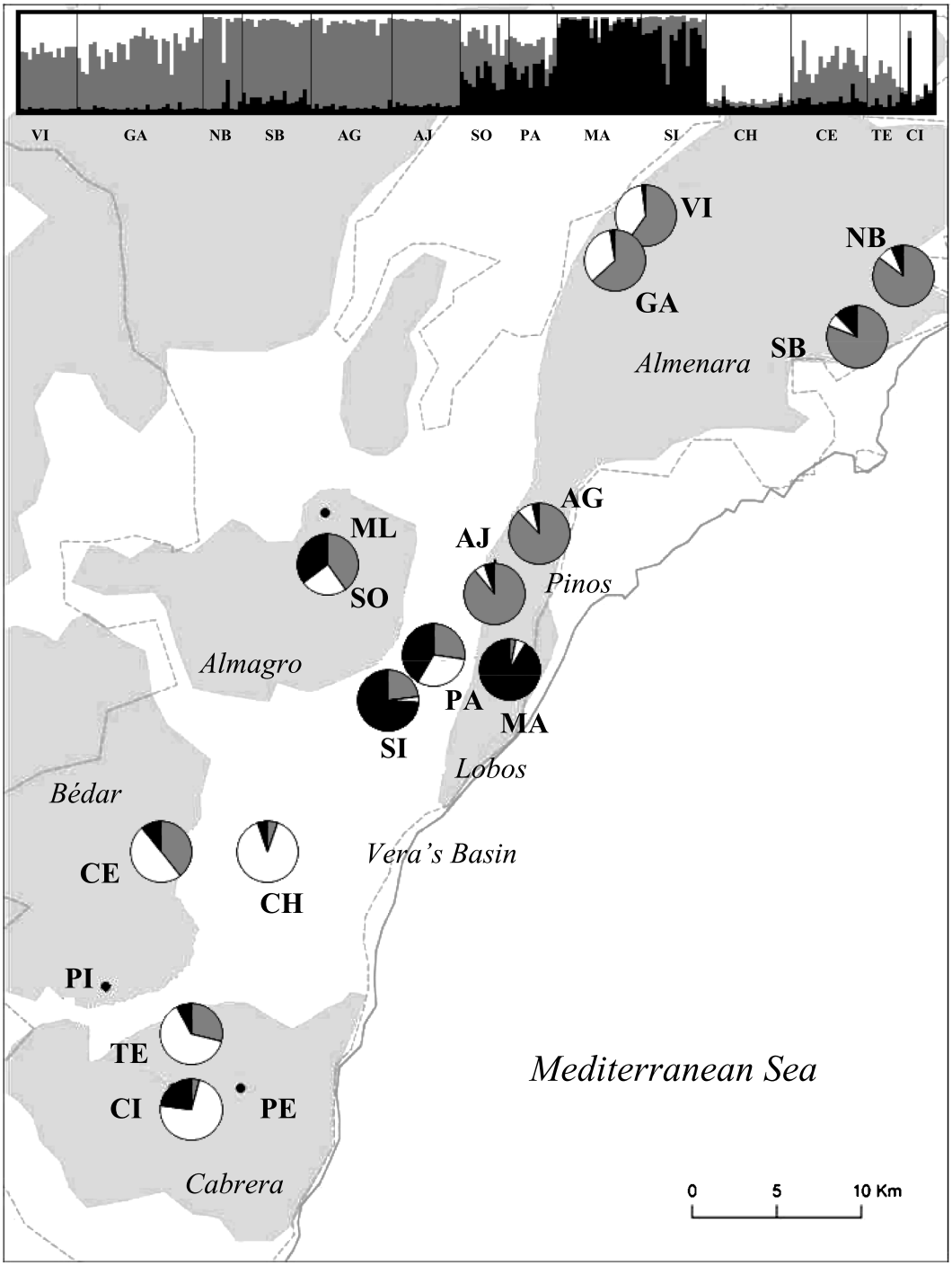

Figure 3. Results of STRUCTURE mapped onto the study area. K = 3 resulted in the most supported value for populations whose sample sizes were higher than 8 (ML, PE and PI were not included due to their small sample size). The bar plots were generated using the software DISTRUCT (Rosenberg, 2004). On the map, the grey dotted line indicates the distribution range of the species in SE and shaded areas indicate main mountain ranges. The complete names of the sampled populations are shown in table 1.

analysis were significant. Nevertheless, a relatively low percentage of variation was explained by groups and by populations within groups (among groups = 2.48%; among populations within groups = 4.63%; $F_{ST} = 0.07$; $F_{SC} = 0.047$; $F_{CT} = 0.024$; all p values < 0.0001). The Mantel test performed reported a significant correlation between genetic and geographic distances in the study area ($r = 0.25$; $p = 0.03$) providing evidence for spatial and genetic congruence.

For the assignment test of populations with small sample sizes to larger populations, two were assigned to their nearest sampled locations: *Malacate* was assigned to *Sotomayor* with a 99.99% assignment score and *Piñas* to *Teresa* with 97.87%. Geographical distances among these pairs of populations were 4 and 5 km, respectively. On the other hand *Peralicos* was assigned to *Aguilón* (assignment score = 80.15%). This last result did not show spatial coherence considering the distance between these two populations is about 38.5 km.

Finally, our three loci were enough to provide a relatively clear-cut separation between Iberian and Algerian tortoises in the assignment/exclusion test. We did not find any Algerian individual of the 19 analyzed whose genotype could be significantly excluded from its original group. On the other hand, we were able to detect 21 SE tortoises excluded from the SE sampled group (table 1), 20 of them being assigned to the Algerian group while one of them was also excluded from this group.

Discussion

As in previous works (Roques et al., 2004, Salinas et al., in press) the microsatellites employed here were highly polymorphic and reproducible, and appear adequate for population analysis. They provided a large number of independent alleles as is required for accurate estimates of genetic variation among populations (Kalinowski, 2002).

The populations studied showed high levels of microsatellite heterozygosity in contrast to findings obtained in other studies within the *Testudinidae* family (Schwartz et al., 2003; Forlani et al., 2005) but similar to those obtained with the same microsatellites in previous studies with the species (Roques et al., 2004; Salinas et al., in press). Departures from HWE (heterozygote deficit) were only detected in one population (*Aguilón*). In this sense, the Wahlund effect is not suggested because we sampled only closely situated individuals, according to the home-range described for the species (from 1 to 3 ha; Anadón et al., 2006). The presence of introduced Algerian individuals could have caused this effect in the *Aguilón* population (discussed further below).

Differentiation and structure of populations and origin of T. g. graeca *in SE*

The differentiation between Algerian and SE individuals provided by the cluster analysis as well as the assignment test agree with previous studies that describe SE and North African populations as genetically distinguished units (Fritz et al., 2009; Salinas et al., in press) and suggests historical isolation among these two populations. In any case, the low sample size of the Algerian populations could be masking the possibility that unsampled Algerian populations are harbouring the entire set of genetic variation found in Spain. In this sense, further studies with more extensive Algerian genetic sampling would be needed to completely elucidate this point.

The high genetic diversity in North African populations and the presence of shared haplotypes between the regions along with the lack of *T. g. graeca* fossils at SE, clearly indicate that SE populations were founded from North African individuals (Álvarez et al., 2000; Fritz et al., 2007, 2009; Salinas et al., in press). However, the role that humans may have played in the expansion of the species across the Gibraltar Strait is not clear. Historic introductions or even introductions from the pet trade have been sug-

gested (Álvarez et al., 2000; Fritz et al., 2007, 2009; Salinas et al., in press). Nevertheless, the existence of exclusive haplotypes found only in SE suggests that the alternative hypothesis of an earlier natural colonization or the existence of an ancestral polymorphism from which African and European populations derived cannot be rejected (Fritz et al., 2009; Salinas et al., in press), especially since recent range expansions from North Africa to the Iberian Peninsula have also been reported in other species of reptiles that are unlikely to be introduced by man (Carranza et al., 2004, 2006).

In this work we detected a spatially coherent genetic structure that is difficult to explain under the hypothesis of a very recent arrival. Firstly, pairwise F_{ST} were statistically significant in 87.9% of cases, indicating the existence of genetic divergence among populations. Secondly, three distinct genetic units were identified, grouping populations from the north, centre and south (fig. 3). Finally, the spatial coherence of this detected genetic substructure was also revealed by an IBD pattern covering the entire study area. The described pattern is typically due to species dispersal (Wright, 1943) and usually occurs over time (Marrs et al., 2008), reflecting a balance between genetic drift and gene flow, with the former increasing and the latter decreasing genetic divergence (Slatkin, 1993; Hutchison and Templeton, 1999). In this sense, spur-thighed tortoises are long-lived species with low to moderate dispersal abilities (Pérez et al., 2002; Anadón et al., 2006), so our results suggest a natural dispersal process inside SE Iberia and contradict the hypothesis of a very recent arrival.

Anthropogenic effects and species conservation

Clustering analysis as well as the assignment/exclusion test gave strong evidences of the presence of introduced North African tortoises in the SE area. In this last test among Algerian and SE individuals, twenty-one out of the 243 Spanish tortoises were better classified as being from Algeria. The distribution of these individuals among populations was skewed, particularly 6 out of 21 tortoises were sampled in *Aguilón* ($n = 20$) and 3 in *Peralicos* ($n = 5$). Both populations presented the highest percentages of individuals significantly excluded from SE (30% and 60%, respectively). The high proportion of likely introduced individuals could have led to the merge of these two populations in the assignment of populations with small sample sizes to larger populations, despite the large geographic distance between them. The *Peralicos* population is located in *Cabrera's Mountain*, where we know that introductions of captive animals have been carried out in recent decades as management schemes for species conservation (information obtained by the Regional Administration). It is especially remarkable that one tortoise sampled from this population was excluded from SE populations as well as from the Algerian group, making the possible origin of the animals introduced in SE even more unclear. On the other hand, *Aguilón* is the only sampled population showing a departure from HWE, possibly indicating the existence of genetically distinct groups (Selkoe and Toonen, 2006). *Aguilón* conformed to HWE when those 6 individuals were excluded from a second analysis, suggesting that these 6 individuals were indeed from a different genetic pool than the remaining 14 individuals. Moreover, it is also known that translocations of wild individuals have been carried out among populations along the SE range. Although these intraregional movements of animals alter the regional pattern of genetic diversity of the species in this context, its effects should be less perceptible than introductions from North Africa. Despite this, the high proportion of admixture levels among geographically distant clusters in *Galera*, *Villarreal*, *Teresa* and *Centinares* populations, contrasts with the general pattern detected in the SE region, which could be a consequence of the regional management of the species.

Our work thus suggests that conservation actions such as captive breeding, introductions

or translocations, may have played a relevant role in the modification of the genetic structure of some populations in SE. Despite this, we were able to detect a structured regional pattern of genetic diversity with individuals that were well differentiated from the Algerian outgroup. Therefore, it is possible that these manipulative management tools threaten the incipient evolutive potential of the species due to the loss of possible local adaptations. Under a principle of caution (Cooney, 2004), we recommend a better characterization of the genetic structure of *T. g. graeca* and the delimitation of genetic units at a regional scale, before carrying out these actions.

Acknowledgements. Financial support was granted by the Spanish Ministry of Education and Science (project: CGL1335-2004). EG was funded by a FPI grant from the Regional Government of the Community of Valencia and JDA by Fundacion Seneca. Foundation 2001-Global Nature allowed us to work in its reserve. Wildlife Centre of Santa Faz (Alicante) provided us Algerian samples. We also give our thanks to field technicians and volunteers who took part in the field work. We appreciate the interest of the Junta of Andalucía and the Regional Government of Murcia. Finally, we thanks to the referees for their comments and suggestions that have improved this work.

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Received: March 25, 2010. Accepted: September 20, 2010.

Appendix: Obtained likelihood values (ln P) and variances of the bootstrap samples (varLn P) using STRUCTURE software for $K = 1$ to $K = 5$: (a) Admixture model; (b) No admixture model; (c) Admixture and LOCPRIOR model; (d) No admixture and LOCPRIOR model.