The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2012, **105**, 591–606. With 4 figures

Effects of landscape features and demographic history on the genetic structure of *Testudo marginata* populations in the southern Peloponnese and Sardinia

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Received 26 May 2011; revised 19 September 2011; accepted for publication 20 September 2011

Testudo marginata, the largest European land tortoise, is suffering habitat degradation and destruction. Some populations, in markedly degraded habitats, are characterized by divergent morphotypes. However, the evolutionary significance of these morphotypes is of debate. Using 11 polymorphic microsatellites, we studied: (1) marginated tortoises from Sardinia that display a divergent morphotype – this population was potentially introduced from Greece; and (2) an area in the southern Peloponnese that includes a small and degraded zone in which marginated tortoises are dwarf. Genetic analyses run without any *a priori* assignment clearly acknowledge the specimens sampled in the territory of the dwarf form as a single group whilst Sardinian specimens are clustered with other specimens from the northern part of the area sampled in Greece. Demographic analyses suggest that Sardinian tortoises originated recently from some of the populations sampled in the northern part of the area sampled in Greece. Over locations sampled in Greece, a landscape-genetic analysis allowed us to detect potential landscape features that may reduce gene flow between the dwarf form territory and surrounding areas. Our results suggest that the territory of the dwarf form is particularly propitious for marginated tortoises and that conservation regulations in Greece should be reinforced to protect this area from increasing impact of human activities changing from traditional agriculture to mechanization and extensive use of chemicals. *Biological Journal of the Linnean Society*, 2012, **105**, 591–606.

ADDITIONAL KEYWORDS: bottleneck – clustering method – gene flow – Greece – isolation by distance – migration – morphology – phenotypic plasticity – *Testudo weissingeri*.

INTRODUCTION

The marginated tortoises (*Testudo marginata* Schoepff, 1793) are the largest European land tortoises. Their distribution extends throughout Greece (except the north-east), south-western Albania, and northern Sardinia where they have probably been introduced (Bringsøe, Buskirk & Willemsen, 2001). The species is suffering habitat degradation and destruction, mortality due to machines and chemicals, and pet collection (Bour, 1995).

Some geographically restricted populations (Sardinia, Greek Peloponese) are characterized by divergent morphotypes (Bour, 1995), although they

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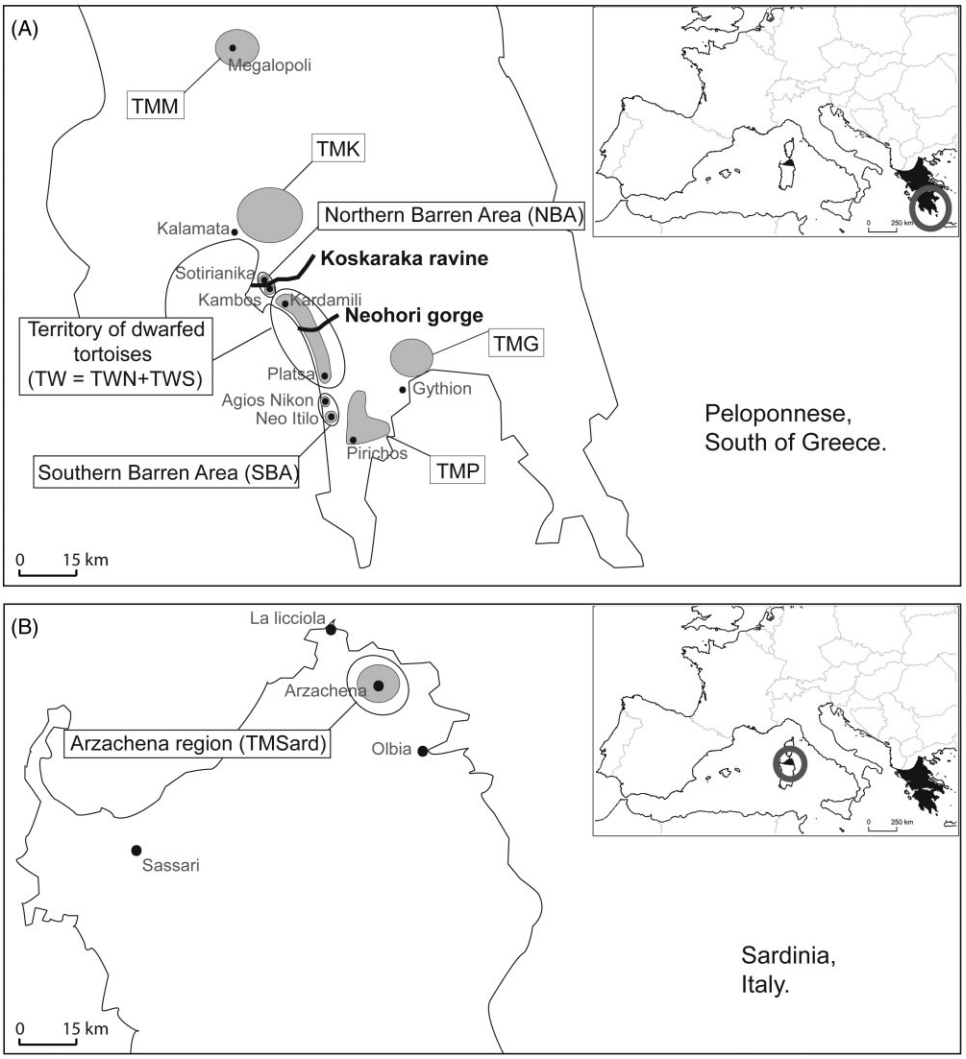

Figure 1. Sampled sites (in grey): A, in Greece, south of Peloponnese; B, in Italy, north-eastern Sardinia. Species range in Europe is in black.

are still acknowledged as belonging to *T. marginata* (e.g. Rhodin *et al*., 2010). In Sardinia, Mayer (1992) described *Testudo marginata sarda* based on morphological and coloration differences from Greek tortoises. This subspecies was never recognized as valid and Fritz *et al*. (2005) confirmed its taxonomic similarity with Greek tortoises. This thriving population was supposedly imported from Greece during Antiquity, by the Etruscans (Angelini, 1899; Tiedemann, 1978; Mayer, 1992). However, Bruno (1986) suggested that the introduction may have occurred at the beginning of the 19th century. Fritz *et al*. (1995) hypothesized that the morphologically distinctive features of the Sardinian marginated tortoises may reflect past demographic events. In the southern Peloponnese, Bour (1995) described *Testudo weissingeri*, a dwarf form of *T. marginata*, characterized by a smaller size, a moderate posterior border, and a less contrasted coloration. This form is restricted to a small area, planted with olive groves and some phrygana (Greek scrubland), of about 15×5 km between Kardamili and Platsa (Fig. 1). Mechanization of agriculture, extensive use of chemicals, and the expansion of suburban areas have strongly altered the environment (Bour, 1995).Van Der Kuyl *et al*. (2002) and Fritz *et al*. (2005), using mitochondrial DNA and inter-simple sequence repeats (ISSRs), did not detect molecular differentiation between the dwarf form and the other marginated tortoises. They relegated it to the synonymy of *T. marginata*. Besides this taxonomic debate, the poor variability of the genetic markers and the limited sampling gave no insight into the evolutionary significance of the dwarfism. Yet, since then, the morphological and ecological

differences from other populations of marginated tortoises have been confirmed by several authors (Artner, 1996; Bringsøe *et al*., 2001; Perälä, 2002).

Such distinctiveness may reflect ongoing demographic or ecological processes among populations and/or a phenotypic plasticity of morphological features reflecting differences in the habitats. Discerning the cause of population differentiation would help determine the evolutionary potential of the species and inform management schemes.

Several recently developed genetic analysis methods use highly polymorphic markers to identify genetic units free from *a priori* hypotheses (e.g. based on morphology, geography or any other criteria). We used 11 microsatellite loci to determine if some landscape features (e.g. mountains and gorges) could prevent gene flow, indicating that the morphotype restricted to the small and perturbed area in Greece could be considered as a management unit (MU; Moritz, 1999 and references therein), and if morphological distinctiveness of both Sardinian and Greek dwarf populations may be linked to past demographic events.

MATERIAL AND METHODS

Study area and sampling

Sampling encompassed the whole territory of the dwarf form, its surrounding areas, and the Sardinian population. In Greece, 191 individuals were sampled from Megalopoli to Pirichos (approx. 83×38 km; Fig. 1), where rocky mountains alternate with natural gorges (altitude 0–1161 m). The Koskaraka ravine is very deep with steep slopes. The Neohori gorge is less deep, less steep and it enlarges into a scrubland area when nearing the sea. The distribution of tortoises was not uniform over the sampling area. All detections were made by sight. The dwarf form was only found in both sides of the Neohori gorge (TW, Fig. 1). We sampled 61 dwarf tortoises on the northern side (TWN) and 69 individuals on the southern side (TWS). The territory of the dwarf form is bordered by two areas in which tortoises are rare: in the south (around Agios Nikon and Neo Itilo), only five tortoises were found in a very arid and rocky area of 30 ha with very scarce vegetation (Southern Barren Area, SBA); in the north, around Kambos and Sotiarinika, only three tortoises were found over 10–25 ha (Northern Barren Area, NBA). This very low density may be due to the urbanization of the landscape in the south of Kalamata. Beyond these barren areas, we sampled tortoises with the usual morphology of marginated tortoises: 14 tortoises around Pirichos (TMP), seven close to Gythion (TMG), 25 close to Kalamata (TMK) and seven around Magalopoli (TMM). In Sardinia, 18 marginated tortoises were sampled (TMSard) over about 225 ha at low elevation around Arzachena (Fig. 1).

Blood was collected from the caudal vein (0.3 mL) and geographical positions were recorded (Garmin GPS). Specimens were identified by scute notches and released in their capture site.

Molecular methods

Genomic DNA was extracted using an ABIPrism6100 Nucleic Acid PrepStation and the corresponding blood extraction protocol (Applera). We used eight microsatellite loci characterized in Perez *et al*. (2006: L61, MD51, Q113, S190, L221, R106, I61, and T113) and added three polymorphic microsatellite loci out of the six characterized by Forlani *et al*. (2005) on *T. hermanni* (Test10, Test21, Test56). Fragments amplified in the published PCR conditions were separated using an ABIPrism310 DNA sequencer and analysed with GeneScan software (Applera).

Genetic diversity

The number of analysed individuals (N), total number of alleles per locus (Al), observed (H_o) and expected (H_e) heterozygosities were computed for each locus and over all loci on the total sample using GENETIX-4.05.2 (Belkhir *et al*., 1996–2004). MICROCHECKER-2.2.3 (Van Oosterhout *et al*., 2004) was used to check for null alleles in the total dataset.

Genetic delimitation of population boundaries

We used genetic clustering methods to divide our sample into K homogeneous genetic groups without *a priori* hypotheses. Greek and Sardinian samples were analysed altogether using STRUCTURE-2.3.1 (Pritchard, Stephens & Donnelly, 2000). Analyses were run 15 times for each K-value (one to eight) under the model with admixture and independent allele frequencies. Other parameters were set to default values. After tests of convergence and consistency, we used Monte Carlo Markov Chain (MCMC) runs of 5×10^6 iterations (thinning = 100, burn-in of 10^5). We based our estimation of the most likely K on both ΔK statistic (Evanno, Regnaut & Goudet, 2005) and absolute posterior probability of the data.

GENELAND-3.0.0 (Guillot, Santos & Estoup, 2008) was used to define spatial genetic units and to infer K based on the spatial model with individual coordinates with or without the null allele option. We used the Dirichlet model for independent allelic frequencies. To infer the K-value and simultaneously check the consistency of results, we ran ten different MCMC with 10^8 iterations (thinning = 10^3, burn-in 50%,

maximum rate of Poisson process = 230, uncertainty attached to spatial coordinates of 0.1 km), K explored values ranging from one to eight with a starting value of six, and a maximum number of nuclei in the Poisson–Voronoi tessellation fixed at 500. The posterior probability of population membership on the spatial domain was computed for each of the ten runs and extra tests were performed by changing some parameter values to check for consistency. As Greek and Sardinian populations are separated by very large overseas distances, we used arbitrary coordinates for the Sardinian sample (i.e. closer to the continent than they really are) to avoid large discrepancies between the two distance classes that would group the Greek populations into a very small area, which would impair discrimination.

Analyses on the inferred populations

From the populations previously inferred by STRUCTURE and GENELAND, we computed linkage disequilibrium and deviation from Hardy–Weinberg equilibrium (HWE), observed (H_o) and expected (H_e) heterozygosity and number of alleles (N_A) using GENEPOP-4.0 (Rousset, 2008). A sequential Bonferroni correction (Rice, 1989) was used for all multiple tests performed on the same population. Allelic richness (A_R) was calculated using the rarefaction procedure in HP-RARE-1.0 software (Kalinowski, 2005) for each population and subpopulation. Wilcoxon tests were used to compare H_e, A_R, and N_A between populations. These parameters were also computed on TWN and TWS (Fig. 1).

Genetic differentiation, measured as F_{ST} values (Weir & Cockerham, 1984), was computed between all populations and for each population pair with GENEPOP. The F_{ST} values using the ENA correction for null alleles was estimated with FreeNA (Chapuis & Estoup, 2007), with confidence intervals computed using the bootstrap procedure. To estimate the variation attributable to the differences among populations and among sample sites within populations, hierarchical analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) based on F_{ST} was performed using ARLEQUIN-3.1 (Excoffier, Laval & Schneider, 2005).

Isolation by distance and migration rates

Isolation by distance (IBD) was assessed by regressing genetic distances between individuals (Rousset, 2000) over the logarithm of geographical distances and further tested using Mantel tests (3×10^4 permutations). The slope of the regression line is then an estimator of $D\sigma^2$, where D is the effective density of individuals on the sampled area, and σ^2 the second moment of parent–offspring dispersal distance (Rousset, 1997, 2000). IBD analyses were performed within and between the inferred Greek populations (Sardinia excluded as it is too remote), using the original dataset as well as the data corrected by FreeNA for null alleles. We first considered only pairs of individuals taken within a single population and discarded pairs of individuals taken from different populations (the 'within-population' analysis). We also conducted the opposite analysis which considered pairs of individuals taken from two different populations and discarded pairs of individuals taken from a single population (the 'between-populations' analysis). Those analyses were performed using GENEPOP-4.0 (Rousset, 2008) and R script (R Development Core Team, 2007), which modified the Mantel test to calculate rank correlation coefficients and to permute the pairwise distances within or between groups only. Such 'within- and between-populations' analyses are designed to analyse gene flow between different groups of individuals (e.g. different habitats, hosts or any categories) in the context of IBD (Rousset, 1999; see Martel *et al.*, 2003 for an example of such analysis). If there are very low levels of gene flow between different populations, the 'between-populations' comparisons will artificially increase the IBD pattern because of the large differentiation between individuals of different populations, most of them being separated by larger geographical distances than pairs of individuals from within populations.

To infer the migration–drift history of our samples, we used 2MOD (Ciofi *et al.*, 1999) to compare the likelihood of two models: (1) the pure drift model, in which an ancestral population splits into several independent units diverging purely by genetic drift; and (2) the constant gene flow model where populations are considered at drift–migration equilibrium under an island model of migration. The algorithm was run with 2×10^5 iterations and a burn-in of 40%.

BAYESASS-1.3 (Wilson & Rannala, 2003) was used to infer current migration rates (i.e. in the last 3–4 generations) between populations (m) based on individual assignation scores using an MCMC run with 3×10^6 iterations (burn-in of 10^6, thinning = 2×10^3, deltap = 0.15, deltam = 0.15, deltaF = 0.15, idum starting value = 10). We followed the recommendations of Faubert, Waples & Gaggiotti (2007) to analyse the results.

Demographic history of sardinian population

To detect population expansions or bottlenecks on the Sardinian sample, we used the heterozygosity test and the mode shift indicator implemented in BOTTLENECK-1.2.02 (Piry, Luikart & Cornuet, 1999), assuming infinite allele (IAM), stepwise

mutation (SMM), and two-phase mutation models (TPM) with various (70–90%) single-step mutations and variance among multiple steps of 12 and 30 (Piry *et al*., 1999).

We also used the *M*-ratio (ratio of the number of alleles over the range of allele sizes) to test the signature of population bottlenecks as implemented in M_p_val (Garza & Williamson, 2001). According to simulations, any dataset with at least seven microsatellite loci showing *M*-values smaller than 0.68 can be assumed to have gone through a recent reduction in population size. However, as this value of 0.68 may not be adequate in all situations (Leblois, Estoup & Streiff, 2006), we simulated an equilibrium distribution of *M* using the method described in Garza & Williamson (2001) which showed that there is a significant reduction in population size if less than 5% of the replicates (here 10^4) are below the observed value. The initial parameters for the calculations of the *M*-ratio were θ (4 x Ne x mutation rate) ranging from 0.1 to 10, *PS* (the proportion of one-step mutations) ranging from 0.7 to 0.9, and Δg (the mean size of multiple-step mutations) ranging from 1.5 to 3.5.

Evidence for demographic change was checked using MSVAR-1.3 (Storz & Beaumont, 2002), a method that infers posterior probability distributions of population parameters using MCMC simulations based on the observed distribution of microsatellite alleles. We ran analyses with 132×10^3 thinned updates and a thinning interval of 5×10^4 steps, leading to a total number of 6.6×10^9 iterations. The first 50% of updates were discarded as burn-in and the remaining data were used to obtain the posterior marginal distributions of the parameters. Three independent simulations were run on the Sardinian sample using a model with exponential variation in population size and different prior distributions (e.g. flat, default and peaked priors). Generation time was set to 1, so that time was expressed in generations rather than years.

The Sardinian sample was also analysed using the IM software (Hey & Nielsen, 2004) to infer divergence time and migration rates between this population and the northern Greece sample that was the suspected population of origin. The analysis was run using six independent Markov chains (3×10^7 iterations, burn-in = 10^6, thinning = 10).

RESULTS

Microsatellite amplification success over the 209 samples reached 90% (Table 1). The total number of alleles (N_A) detected per locus varied from two to 24 (mean = 10). Locus I61 exhibited the highest number of alleles (24) and appeared to be the most difficult to amplify. The average expected heterozygosity (H_e) varied from 0.37 (locus MD51) to 0.79 (R106 and Test10) on the whole sample size (mean = 0.61, Table 1). MICRO-CHECKER showed that four loci (L61, I61, T113, Test10) had potential null alleles (overall significant excess of homozygotes, evenly distributed across the homozygote classes).

Genetic delimitation of population boundaries

Results from STRUCTURE showed that lnP(D) increased sharply with *K* from one to three, more slowly with $K = 4$, and then decreased for $K \geq 5$. Evanno's highest value ΔK was obtained for $K = 2$, and the clusters correspond to: (1) tortoises from TWN and TWS, and (2) all the other tortoises from Greece and Sardinia (Fig. 2A). Considering $K = 3$ (Fig. 2B), the three clusters corresponded strictly to: (1) northern sites TMM and TMK plus Sardinian tortoises, TMSard; (2) southern sites TMP and TMG; and (3) TWN and TWS. The results obtained for $K = 4$ (Fig. 2C) showed a split between Sardinian tortoises and the northern Greek sites. The individuals from NBA and SBA are scattered in the inferred populations.

For $K = 3$, GENELAND detected a marked maximum posterior probability of the model greater than 60%, using the null alleles option or not. The three clusters (Fig. 3) strictly matched those detected by STRUCTURE at $K = 3$ and corresponded to (1) Tw = TWN + TWS; (2) TmS = TMP + TMG + SBA; and (3) TmN = TMM + TMK + NBA and TMSard. Note that individuals from Sotirianica and Neo Itilo (NBA and SBA, respectively) were assigned consistently over all runs to a single population. Yet, their probability of membership was always lower than 0.9. The only tortoise from Kambos (NBA) and the only individual from Agios Nikon (SBA) could not be consistently assigned to any of the three clusters ($P < 0.5$). GENELAND detected potential null alleles in our dataset, but the results given by GENELAND remained unchanged when these alleles were taken into account. G. Guillot and A. Estoup (pers. comm.) note that GENELAND still gives robust results even with a null allele frequency up to 30%.

In the following analyses, we thus considered the three Greek clusters defined by GENELAND for $K = 3$ (TmN, TmS, Tw), excluding non-assigned individuals from barren areas of Kambos (NBA) and Agios Nikon (SBA), composed respectively of 34, 25, and 130 individuals. The 18 individuals sampled in Sardinia are considered separately to analyse their relationships with the Greek populations and will be then called 'TmSard'.

Genetic analyses on the inferred populations

No linkage disequilibrium occurred for any pair of loci in any population ($P > 0.05$ after Bonferroni

Table 1. Genetic diversity within the total sample and within each population

					Greece																	
	Total sample ($Nt = 207$)				TmN ($N = 34$)			TmS ($N = 25$)			Tw ($N = 130$)			TWN ($N = 61$)			TWS ($N = 69$)			Sardinia TmSard ($N = 18$)		
Locus	N	Al	H_o	H_e	Ap	H_o	H_e	Ap	H_o	H_e	Ap	H_o	H_e	Ap	H_o	H_e	Ap	H_o	H_e	Ap	H_o	H_e
L61	186	13	0.52	0.73	8	0.56*	0.80	6	0.63	0.71	12	0.60*	0.76	9	0.61	0.77	10	0.58*	0.75	3	0.29	0.66
MD51	200	2	0.35	0.37	2	0.35	0.37	2	0.27	0.41	2	0.24	0.30	2	0.27	0.37	2	0.22	0.24	2	0.53	0.40
Q113	203	9	0.61	0.57	8	0.76	0.69	5	0.57	0.52	5	0.72	0.66	5	0.70	0.67	4	0.74	0.66	2	0.40	0.40
S190	204	3	0.47	0.46	2	0.50	0.51	2	0.28	0.39	3	0.52	0.50	2	0.63	0.50	3	0.41	0.50	2	0.60	0.43
L221	201	12	0.62	0.69	7	0.47	0.65	5	0.58	0.60	8	0.75*	0.80	7	0.73*	0.82	8	0.78	0.78	6	0.67	0.69
R106	199	22	0.80	0.79	15	0.85	0.83	15	0.90	0.88	17	0.68	0.75	10	0.68	0.70	16	0.67	0.79	7	0.73	0.69
I61	150	24	0.63	0.77	11	0.61	0.82	10	0.79	0.92	21	0.58*	0.75	15	0.45	0.56	20	0.72*	0.86	4	0.54	0.59
T113	188	5	0.47	0.66	4	0.47*	0.70	5	0.45*	0.73	5	0.57	0.70	5	0.61	0.72	4	0.54	0.66	3	0.40	0.50
Test56	200	2	0.44	0.45	2	0.53	0.51	2	0.37	0.46	2	0.40	0.40	2	0.32	0.31	2	0.48	0.45	2	0.44	0.41
Test21	201	4	0.29	0.47	2	0.26	0.41	2	0.38	0.37	3	0.39	0.44	3	0.40	0.43	3	0.38	0.44	4	0.11*	0.65
Test10	193	14	0.57	0.79	12	0.65*	0.87	7	0.60	0.85	11	0.65	0.75	7	0.57	0.70	9	0.73	0.79	5	0.39*	0.70
$H_e \pm$ SE			0.61 ± 0.15			0.65 ± 0.18			0.62 ± 0.21			0.62 ± 0.17			0.59 ± 0.17			0.63 ± 0.19			0.56 ± 0.13	
N_A			10			6.64			5.55			8.09			6.09			7.36			3.64	
A_R						4.25			4.20			3.91			3.63			4.06			3.11	

Nt, total number of specimens analysed. The number of specimens per inferred population using STRUCTURE is given in parentheses. For each locus: total number of analysed individuals (N), number of alleles (Al), expected heterozygosity (H_e) and observed heterozygosity (H_o) are given. For each population and subpopulation: number of alleles (Ap), expected heterozygosity (H_e) and observed heterozygosity (H_o) are given. Allelic richness (A_R) was calculated for all the loci for each sample, per population and subpopulation. Loci showing significant deviation from HWE after Bonferroni correction ($P < 0.05$) are indicated with an asterisk.

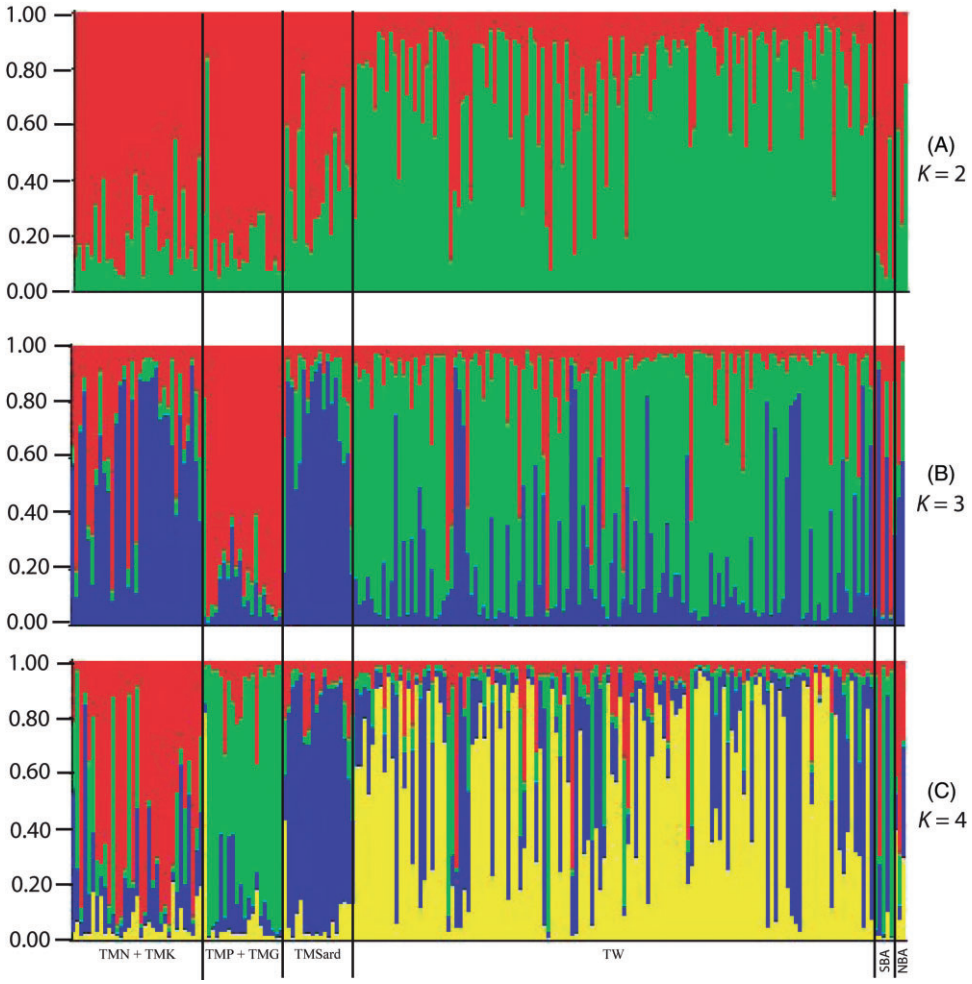

Figure 2. Barplot of the proportional membership of individual accessions for each of the 2–4 inferred clusters. Each accession is represented as a vertical bar comprising different coloured scale on the *x*-axis. Each group is represented with a different colour proportion.

correction). The average number of alleles per population (N_A) varied from 3.64 (TmSard) to 8.09 (Tw) with the number of alleles at loci L61 and I61 in Tw being twice as important as in the Tm populations (Table 1; all N_A values are significantly different among all population pairs except for Tw/TmN according to Wilcoxon tests; $P < 0.05$). Allelic richness (A_R) was similar among all Greek populations (Table 1), but the allelic richness of TmSard appeared significantly lower than the one of TmN and Tw ($P < 0.05$, Wilcoxon test). Mean expected heterozygosity (H_e) ranged from 0.56 (TmSard) to 0.65 (TmN) and Wilcoxon tests revealed a significant difference between TmN and TmSard ($P < 0.05$).

Tests performed using GENEPOP on these four populations revealed, after Bonferroni corrections, that nine loci/population combinations out of 44 did not conform to HWE (Table 1). Three combinations belonged to Tw. All involved heterozygote deficits. When Tw was divided to take the Neohori gorge into account, departure from HWE was observed only for one locus in TWN and two loci in TWS, suggesting a Wahlund effect. Such departure could result from the presence of null alleles in the dataset. This hypothesis was supported by the analysis of the dataset using MICRO-CHECKER.

Differentiation between populations

The F_{ST} estimated over all populations was 0.075 and all pairwise population F_{ST} values were significant ($P < 0.01$; Table 2). FreeNA confirmed the presence of null alleles but the ENA correction for null alleles did not change the significance of the pairwise F_{ST} tests. Confidence intervals (using FreeNA) never included zero value. The highest F_{ST} value was observed between TmSard and TmS ($F_{ST} = 0.159$) and the lowest between TmN and Tw ($F_{ST} = 0.048$). The F_{ST}

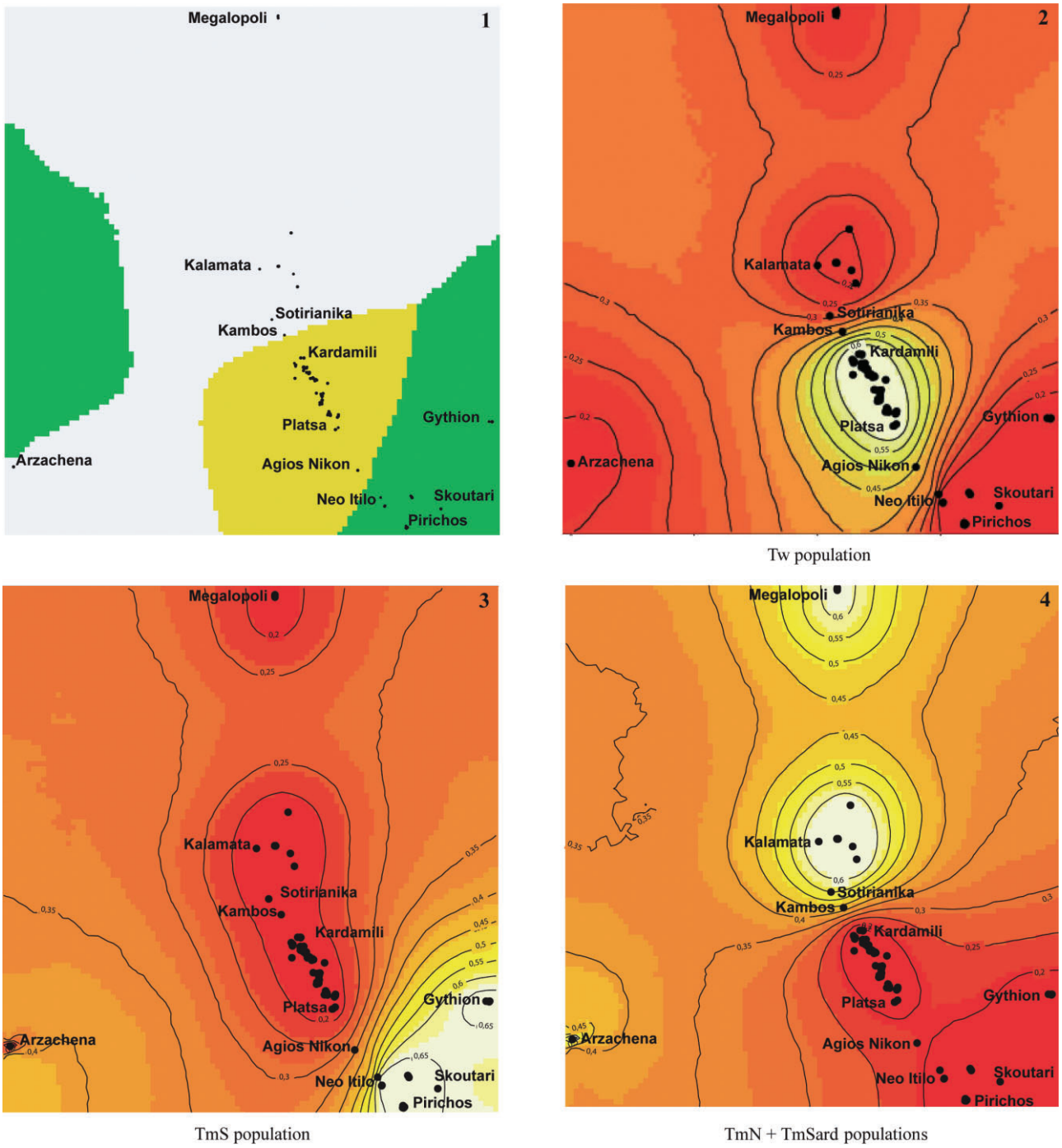

Figure 3. Mapping population membership (posterior probabilities) using GENELAND. Black dots represent the geographical position of individuals; lighter colour reflects probabilities of belonging to one of the three populations. 3.1, synthetic map; 3.2, Tw; 3.3, TmS; 3.4, cluster TmN + TmSard.

value between TmN and TmS (0.060) was significant ($P < 0.05$) and higher than that of Tw/TmN (0.048) but lower than that of Tw/TmS (0.096). The F_{ST} value between the two subpopulations of Tw on each side of the Neohori gorge was 0.016 (results not shown), three- to ten-fold lower than any other F_{ST} values between populations. Using AMOVA based on F_{ST}, the variation among inferred Greek populations explained about 6% of the total variation (significant, $P = 0.000$), while the variation among sample sites

Table 2. Pairwise genetic differentiation between populations calculated as F_{ST} values computed using GENEPOP (above diagonal) and FreeNA (below diagonal)

	TmN	TmS	TmSard	Tw
TmN	–	0.059† [0.017; 0.109]	0.066† [0.040; 0.093]	0.053† [0.027; 0.073]
TmS	0.060* [0.020; 0.110]	–	0.154† [0.083; 0.233]	0.099† [0.046; 0.151]
TmSard	0.063* [0.038; 0.090]	0.159* [0.084; 0.240]	–	0.084† [0.035; 0.137]
Tw	0.048* [0.026; 0.069]	0.096* [0.047; 0.150]	0.083* [0.034; 0.135]	–

*Significant positive F_{ST} values computed with FreeNA ($P < 0.05$).
†Significant genotypic differentiation test computed with GENEPOP ($P < 0.01$).
[;] = 95% confidence intervals computed with FreeNA.

Table 3. Isolation by distance in continental populations, indicating the slope of the linear regression between estimates of $F_{ST}/(1 - F_{ST})$ and geographical distance, as well as Mantel test significance level and 95% ABC bootstrap confidence interval details

	Populations (sample sizes)	Slope	Probability	Confidence intervals
Within-population analyses	TmN (34)	0.013	0.011	[–0.014; 0.037]
	TmS (25)	0.013	0.173	[–0.004; 0.031]
	Tw (130)	0.008	0.010	[0.002; 0.016]
	TmN + TmS (59)	0.013	8e-04	NA
	TmN + Tw + TmS (189)	0.010	0.027	NA
Within Tw subpopulations	TWN (69)	0.006	0.306	[–0.005; 0.015]
	TWS (61)	0.007	0.216	[–0.008; 0.027]
Between-population analyses	TmN + TmS (59)	0.021	0.000	[0.004; 0.043]
	TmN + Tw + TmS (189)	0.048	0.000	[0.031; 0.095]
	TmN + Tw (162)	0.026	0.000	[0.015; 0.039]
	Tw + TmS (155)	0.032	0.000	[0.019; 0.051]

within populations explained 3.4% of the variation ($P = 0.000$). Thus, we detect a low but significant genetic structure between Greek populations. The vast majority of detected variation (90.6%, $P = 0.000$) was due to variation among individuals within sample sites, consistent with the microsatellite variability.

Fine-scale population genetics of the inferred continental populations

A strong IBD pattern was found for the global 'within-population' treatment: the regression between a_r and log (geographical distance) had a slope of 0.010 and a Mantel test was significant ($\alpha = 0.05$, $P = 0.027$; Table 3). Similar values of the regression slope and significant Mantel test ($P < 0.05$) were found within each continental population, with a slightly higher slope for TmN and TmS (around 0.013) than for the Tw (around 0.008). To test for an effect of Neohori gorge, we examined IBD pattern within each TWN and TWS population. This IBD was comparable with the results obtained on the whole Tw population, but was no longer significant ($P > 0.05$).

IBD analyses were also performed using all pairwise comparisons (within and between populations). Regression slopes were higher 'between populations' than 'within populations', suggesting the presence of barriers to gene flow between the continental populations.

Slopes 'between populations' ranged from 0.021 for the TmN/TmS set to 0.032 for the TmS/Tw set and were significant ($P = 0.000$). In agreement with F_{ST} analyses, this suggested that Tw and TmN are less differentiated than Tw and TmS. Using the dataset corrected by FreeNA to check the influence of null alleles on IBD analyses did not alter the results; neither did the use of a smaller data set excluding loci with potentially null alleles. The software 2MOD indicated with great confidence (infinite likelihood ratio) that populations have evolved under a constant model of low gene flow rather than under pure drift. The overall F_{ST} was higher than 0.05, and thus we could use BAYESASS to infer present migrations with

Table 4. Migration rates between inferred populations using BAYESASS

	Rates from:			
	TmN	TmS	Tw	TmSard
To				
TmN	0.983	0.008	0.006	0.004
	(0.942; 0.999)	(0; 0.038)	(0; 0.030)	(0; 0.021)
TmS	0.015	0.957	0.020	0.007
	(0; 0.076)	(0.877; 0.999)	(0; 0.086)	(0; 0.036)
Tw	0.004	0.003	0.991	0.001
	(0; 0.020)	(0; 0.013)	(0.965; 1)	(0; 0.007)
TmSard	**0.276**	0.013	0.025	0.685
	(0.188; 0.322)	(0; 0.055)	(0; 0.100)	(0.667; 0.729)

Values are means of the posterior distributions of the migration rate into each population (m), and their respective 95% confidence intervals in parentheses. Values along the diagonal (underlined) are the proportion of individuals derived from the source population for each generation. Migration rates greater than 0.100 are in bold type.

confidence. This method only detected extremely low current migrations in the last 3–4 generations between Greek populations (Table 4). All inferred migration rates were similar (around 1%, with all overlapping credibility intervals containing zero).

Demographic history of the Sardinian population

BAYESASS (Table 4) showed an important migration rate from TmN to TmSard (27.6%), ten times higher than the rate between the other Greek populations and Sardinia. Although less than 0.33, these estimates should be taken with caution because estimated rates are higher than 0.1 and introduction may have been diverse (origin, timing) over the few last generations. The mode-shift indicator of BOTTLENECK did not provide significant results, and Wilcoxon tests showed a significant heterozygosity excess only when using the IAM mutation model ($P < 0.05$). The M-ratio showed a significant bottleneck signal in TmSard ($M = 0.596$). Less than 1% of the 10^4 simulated equilibrium samples had M-values smaller than 0.596 independently of the parameters. Using MSVAR with various prior distributions on parameters and various MCMC run lengths, all runs converged after 2×10^4 thinned iterations. As they all roughly gave the same posterior distributions, we present only the results for the peaked priors, with the longest runs and with a burn-in of 50% (Fig. 4). Posterior distributions clearly differed from prior distributions and all showed a marked peak on the parameter space explored despite very large credibility intervals for all parameters (also observed in Beaumont, 1999 and Storz & Beaumont, 2002; Girod *et al.*, 2011). The major signal detected by MSVAR was a founder event by a small number of individuals [infinite Bayes factor; mode and 95% CI = 25 (1.6×10^{-7}; 1.3×10^9) individuals], which occurred approximately 200 generations ago [224 (1.9×10^{-6}; 1.4×10^{10})]. MSVAR did not detect any signal of expansion, or increase in size. The size of the ancestral population providing these founders was estimated to be very large [2.0×10^5 (1.2×10^{-3}; 1.0×10^{13}) individuals] and the mutation rate estimate was 1.6×10^{-4} (2.4×10^{-12}; 1.9×10^4). Finally, despite many tests using IM software to infer divergence time and migration rates between the Sardinian sample and the northern Greece sample, we could not find any MCMC run showing good convergence, and different long runs were always contradictory.

DISCUSSION

Marginated tortoise populations sampled in this study displayed high levels of heterozygosity (H_e) and high allelic diversity (N_A). These data are comparable with those obtained for the rarest land tortoises in Africa, *Psammobates geometricus* (Cunningham *et al.*, 2002). According to Howeth, McGaugh & Hendrickson (2008), the long generation time of turtles/tortoises relative to the period of habitat fragmentation/reduction may buffer the loss of genetic diversity. The global genetic differentiation ($F_{ST} = 0.075$) between populations is within the usual range of a fair number of other Testudinidae species analysed with microsatellite loci (e.g. Rioux Paquette *et al.*, 2007; Fujii & Forstner, 2010; Hagerty & Tracy, 2010; Graciá *et al.*, 2011). Overall, our results indicate that the disturbed area where marginated tortoises display dwarfism is genetically distinct from surrounding areas and that genetic boundaries are linked to landscape features.

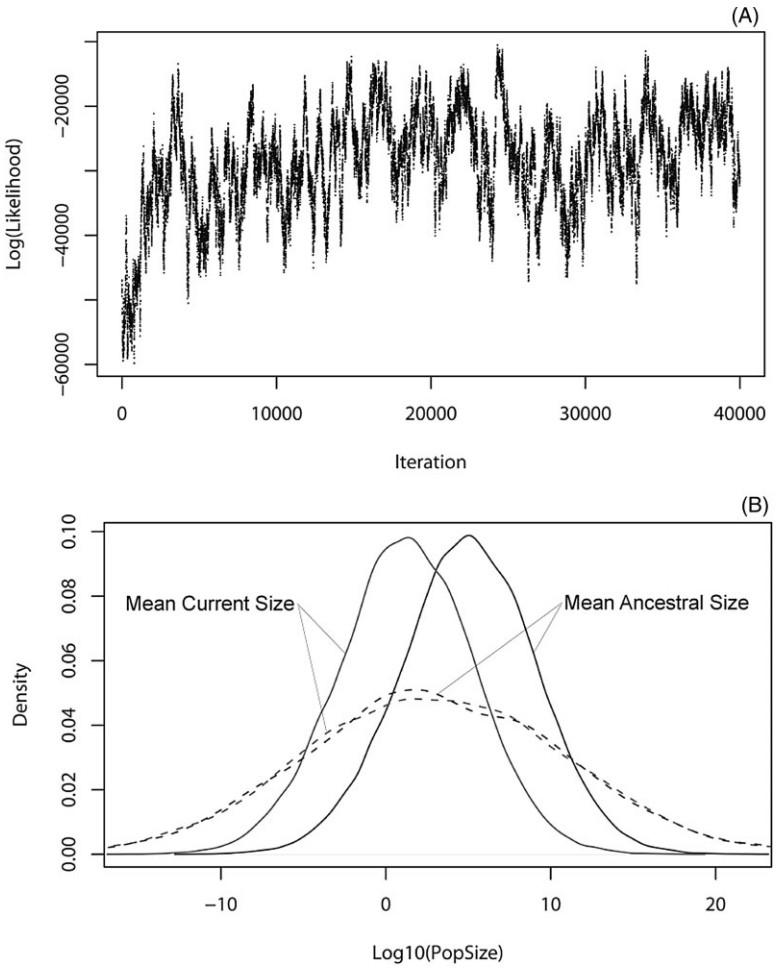

Figure 4. Inference of the demographic history of the Sardinian population using the software MSVAR. A, likelihood trace with a burn-in of 50%. B, this figure was obtained with normal hyperpriors on mutation rates with mean 10^{-4} and variance of 8. Dotted lines represent the prior probability distribution of the different parameters and solid lines show the posterior distribution of the same parameters using the genetic information of the sample. Population size is expressed as number of individuals.

Conversely, the Sardinian population of marginated tortoises, introduced more or less recently by humans, is not differentiated from some of the surveyed continental populations where specimens display the usual morphology.

Sardinian population

Clustering methods implemented in STRUCTURE (for $K = 3$) and in GENELAND reveal a genetic proximity between the Arzachena tortoises and those from TmN. Accordingly, genetic differentiation (F_{ST}) between TmSard and TmN is lower and migration rate from TmN to TmSard inferred with BAYESASS is ten times higher than from any other population. These results suggest that some individuals have been introduced to Sardinia from a Greek population with a similar genetic profile to that of TmN. Moreover, the low genetic diversity level in the Sardinian sample (low richness and expected heterozygosity) and the detection of a bottleneck using the *M*-ratio of Garza & Williamson (2001) suggest both a recent introduction and a low number of founders. MSVAR estimated that about 25 tortoises were introduced fewer than 200 generations ago. Considering that sexual maturity occurs between 13 and 16 years (Perez, 2007) and a lifespan of 60–80 years is typical (Bringsøe *et al*., 2001), 200 generations equates to around the beginning of Antiquity (4400–6700 years ago, 3500 BC to year 476). MSVAR may not have detected any population expansion because the signal of the founder effect was stronger than the signal of expansion. As repeatedly mentioned in the literature, we observed discrepancies between the methods. The

M-ratio method of Garza & Williamson (2001) supported a bottleneck event contrary to the heterozygosity excess test implemented in BOTTLENECK. BOTTLENECK lacks power when using small sample sizes or a small number of loci (Leblois *et al*., 2006). *M*-ratio detects events older than the heterozygosity excess test (Abdelkrim, Pascal & Samadi, 2005). Thus, the high recent migration rates from continental populations to Sardinia indicated by BAYESASS may have erased the signal of older founder effects that are detected by the *M*-ratio. Small sample sizes are likely to contribute to the very low resolution of the MSVAR estimates, even if classical sample sizes of 60 genes per population also lead to very large credibility intervals on natural parameters when uninformative priors are used (Girod *et al*., 2011). Moreover, as shown in Chikhi *et al*. (2010), multiple events of immigration from different sources into the Sardinian population may have accentuated the bottleneck signal detected by MSVAR.

Bruno (1986) proposed that individuals of the *marginata* form were introduced many times into Sardinia by Franciscan monks between the end of the 18th century and the beginning of the 19th century. Mayer (1992) also suggested transport by German soldiers during the Second World War and transport during Antiquity to the trading harbour of Olbia, in north-eastern Sardinia. Tortoises were often moved by humans and used as food or for religious reasons. Monks sometimes ate tortoise meat in place of fish (Mayer, 1992). Angelini (1899) and Tiedemann (1978) reported tortoise transportation during Antiquity whereas Ballasina (1995) mentioned the discovery of tortoise shells in antique Etruscan tombs (Tuscany), close to Greek artefacts. A hypothesis of multiple introductions associated with small sample sizes would also explain the contradictory results obtained by the different methods used and particularly with IM software.

Genetic structure in the dwarf form territory and surrounding areas

Both the clustering methods (e.g. STRUCTURE and GENELAND) and the IBD method ('between-populations' analyses) indicate a low but significant differentiation between the dwarf individuals and all other tortoises. Very low rates of recent migration were detected using BAYESASS (no recent migrant in the last 3–4 generations) and 2MOD analysis suggested that these populations have evolved under a constant model of low gene flow rather than complete isolation. Tortoises sampled around Sotirianica (NBA) and Neo Itilo (SBA) are consistently attributed, using GENELAND, to clusters of tortoises sampled outside the dwarf form territory. However, the presence of non-assigned tortoises from Kambos (NBA) and Agios Nikon (SBA) does not exclude some level of introgression between inferred populations. This result needs confirmation as only one individual from Kambos and one from Agios Nikon were sampled. These low exchanges may be limited to a very few individuals sparsely distributed in the 'barren areas' that fragment the suitable habitat for marginated tortoise (Bour, 1995; R. Bour & M. Perez, pers. observ.).

The IBD regression slopes for 'within-analysis' were lower than slopes for 'between-populations' analyses. This supports the hypothesis that barriers to gene flow exist between those areas. The geographical boundaries between inferred genetic units coincide with features of the landscape that may potentially act as barriers to dispersal for such phylopatric animals. For example, the arid and rocky area from Platsa to Neo Itilo (SBA) and the Koskaraka ravine (NBA) between Sotirianica and Kardamili coincide with the genetic limits. It must be difficult for tortoises to cross the SBA (12 km) if appropriate shelters are not available for thermoregulation and the impressive Koskaraka ravine has steep slopes and a rock face that is sheer, moist, and continuous between the sea and the mountains. Yet, the genetic differentiation and IBD 'between populations' is lower between the dwarf form territory (Tw) and the northern area (TmN) than between Tw and the southern area (TmS). This result suggests that the Koskaraka ravine may be easier to cross than the SBA. The presence of road bridges over the ravine or human-mediated translocation may explain how tortoises cross such an inhospitable area. Another explanation would be that, contrary to other marginated tortoises, the dwarf form may not be able to disperse through the mountains. Indeed, we never observed any dwarf individual above 554 m, while tortoises with the usual morphology of *T. marginata* have been reported up to 1100 m (Perez, 2007). Overall, our study suggests that the distribution of the dwarf form is limited in the north by the Koskaraka ravine and not at 5 km south of Kalamata as suggested by Bour (1995).

The impact of such geographical features on gene exchanges is also supported by the results obtained within the dwarf form territory. The genetic differentiation within this territory is weak (about three to ten times lower than those obtained for the other population pairs) and may result from an analytical artefact (i.e. resulting from the presence of null alleles, small sample size, etc.). Moreover, neither IBD (no significant weak slope and comparable results when considering Tw or TWN and TWS separately) nor clustering results detected a split within Tw. The Neohori gorge seems to have at most only a weak effect on the genetic structure of the dwarf form

population. This result matches field observations. Indeed, compared with the Koskaraka ravine, the Neohori gorge has a much more open topography with a lot of vegetation for shelters and food. Moreover, tortoises were observed in the gorge, indicating that this geographical feature is not insurmountable for the tortoises and that exchanges between individuals living on each side of the gorge are possible.

The global distribution of the Greek populations is also supported by a more detailed analysis of IBD. As most 'within-population' treatments were significant, this suggests that IBD occurs in these populations. The slope values (about 0.013 for TmS and TmN; about 0.008 for Tw) can be translated to neighbourhood size values (i.e. $4\pi D\sigma^2$, where D is the density of adult individuals and σ^2 is the second moment of the dispersal distribution) of 77 and 125 individuals, respectively (Rousset, 1997, 2004). Such values suggest low densities and/or very limited dispersal. Tortoises are well known to be philopatric (Geffen & Mendelssohn, 1988; Nougarède, 1998; Lagarde *et al*., 2003). The IBD pattern is extremely consistent for all 'within-population' analyses in terms of slope, suggesting that all populations considered in this study have roughly similar demographic behaviours (i.e. small densities and very limited dispersal for all populations). However, some differences between TmS/TmN and Tw could be due to greater dispersal abilities, or more probably, to greater adult densities of Tw. Field observations support this last hypothesis as, in similar sampling conditions (surface and time), the abundance of Tw was about three times higher than that of TmS/TmN (up to 15 tortoises from Tw per hour for two observers).

If we now examine the surroundings of the dwarf form territory, several tests (STRUCTURE and Evanno's method at $K = 2$, GENELAND and STRUCTURE for $K > 2$, a high F_{ST} value of 0.060 and IBD analyses with slope 'between TmS + TmN' > slope 'within TmS + TmN') support that they should be divided into two distinct populations (TmN and TmS). Yet, although a barrier to gene flow between TmN and TmS is detected by our analyses, 2MOD suggests that they have exchanged migrants recently (partial isolation). Several hypotheses could explain this result. First, on the oriental side of the Taygetos mountain tortoises are rare and/or difficult to reach (e.g. deep dens under limestone layers). If prospecting was insufficient, the sampling gap may have induced a discrete change in allelic frequencies between the two distant patches and would very likely be interpreted as a barrier by clustering algorithms (G. Guillot & A. Estoup, pers. comm.). Secondly, TmS and TmN may be fragmented because of intense agricultural activities (orange orchards with bare soils due to intensive use of herbicides). Human-mediated translocation or a genetic cline may explain the low inferred gene flow. TmN and TmS could be the northern and the southern ends of a more or less continuous population, the cline resulting from the low dispersal/density indicated by the estimated neighbourhood values.

Insights into the evolutionary significance of the dwarfism

Tortoise populations are known to display decreasing body sizes when disturbed (e.g. Stiner *et al*., 1999 in a heavily exploited tortoise population in prehistoric times). In other tortoise species, smaller sized or even dwarf populations are observed in suboptimal habitats (Fritz *et al*., 2010). The low genetic differentiation between the dwarf form and the other marginated tortoises compared with the morphological and biometric differences detected by Bour (1995), Perälä (2002), and Perez (2007) led Bringsøe *et al*. (2001) and Fritz *et al*. (2005) to suggest that the morphological differentiation could be due to phenotypic plasticity. Following this hypothesis, the dwarfism would not reflect an ongoing process of differentiation within the species. However, our genetic analysis suggests that landscape features surrounding the territory of the dwarf form limit gene exchanges with tortoises from surrounding areas. This population could undergo either intense genetic drift and/or selection within the very distribution area of *T. marginata*. Life-history traits such as phylopatry or poor ability to disperse coupled with geographical features that impair movements, such as barren areas, high mountains, gorges, and ravines, may have incidentally favoured an accelerated genetic drift and/or natural selection in this small territory. Moreover, the features of this territory may trigger some specific selection. Rocky terraces on which olive trees are cultivated offer many but small shelters for thermoregulation. The narrowness of the terraces and of the shelters might be a protection against predators, pet collection, agricultural machines, and chemicals present on the site, but would also favour dwarf individuals. As this environment was largely shaped by human activities, such a shift of the ecological niche might result from historical anthropogenic pressures.

Both morphological (size and colour) and genetic characters allow us to distinguish this dwarf form from the neighbouring *T. marginata* populations. *Testudo marginata* is well protected by international laws with respect to trade (CITES) and its conservation within its range (EEC laws). It is globally ranked 'Least Concern' in the IUCN Red List (IUCN, 2010). If this dwarf population was considered as a subspecies (*T. marginata weissingeri* Bour, 1995), it could benefit from a specific listing in the IUCN Red List as well as subsequent increased protection at the national and

local levels (e.g. action plan). However, taxonomic distinction may unintentionally encourage illegal trade and over-collection (Stuart *et al*., 2006). Our results suggest that conservation regulations in Greece should be reinforced.

ACKNOWLEDGEMENTS

We are grateful to Dr G. Handrinos and the Greek Ministry of Agriculture (Parks and Wildlife Management), A. Manca, and G. Vacca (Instituto regionale fauna selvatica, Cagliari) for collecting permits. We thank the SSM (MNHN), C. Bonillo, F. Noël, and B. Martinez-Cruz for the molecular work, S. Aroua, A. Doxa, F. Bour, M-N. Uhl, C. Azzara, and S. Soubzmaigne for collecting samples, A. Ohler, P. Chesselet, and V. Bouetel for comments and Computational Biology Service Unit from the MNHN (CNRS-UMS-2700). This work was financed by the MNHN and private associations (SOPTOM, A Cupulatta, Chelonian Research Foundation, Société des Amis du Muséum). We are grateful to Sarah Dalrymple (Bangor University) for proofreading and Eva Gracià and two other referees for their helpful comments on this manuscript.

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