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Genetic Variation and Population Structure in the Endangered Hermann's Tortoise: The Roles of Geography and Human-Mediated Processes

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Abstract

The Hermann's tortoise (*Testudo hermanni*) is an endangered land tortoise distributed in disjoint populations across Mediterranean Europe. We investigated its genetic variation by typing 1 mitochondrial locus and 9 nuclear microsatellites in approximately 300 individuals from 22 localities. Our goal was to understand the relative impact of natural and human-mediated processes in shaping the genetic structure and to identify the genetic priorities for the conservation of this species. We found that 1) all geographic areas are highly differentiated, mainly as a function of their distance but with a clear genetic discontinuity (F_{st} values larger than 0.4) between the Eastern and the Western subspecies; 2) the contact zone between subspecies is located farthest to the west than previously believed, and it probably coincides with the delta of the largest Italian river; 3) extinction events due to climatic conditions in the Upper Palaeolithic and subsequent human-mediated translocations in the Neolithic possibly explain the unexpected similarity among Spain, Sicily, and Corsica. For conservation purposes, the large majority of genetic pools appears native although hybridization among subspecies, related to extensive 20th century trade of tortoises across Europe, is observed in Spain and some Italian samples. Most populations do not seem at immediate risk of low genetic variation, except the French population, which has very low nuclear genetic diversity (heterozygosity = 0.25) and where 50 out of 51 sampled animals shared the same mitochondrial sequence. In general, restocking and reintroduction plans should carefully consider the genetic background of the individuals.

Key words: *conservation, microsatellites, mtDNA, phylogeography,* Testudo hermanni, *translocations*

The Hermann's tortoise (*Testudo hermanni* Gmelin, 1789) is distributed from Spain to the Balkans and in various Mediterranean islands (Cheylan 2001, see Figure 1). Pleistocene climatic fluctuations probably fragmented the once continuous distribution along the Mediterranean coasts (Fritz et al. 2006), and coastline urbanization clearly enhanced this process in more recent times. Habitat reduction, together with intensive agricultural practices and forest fires, is

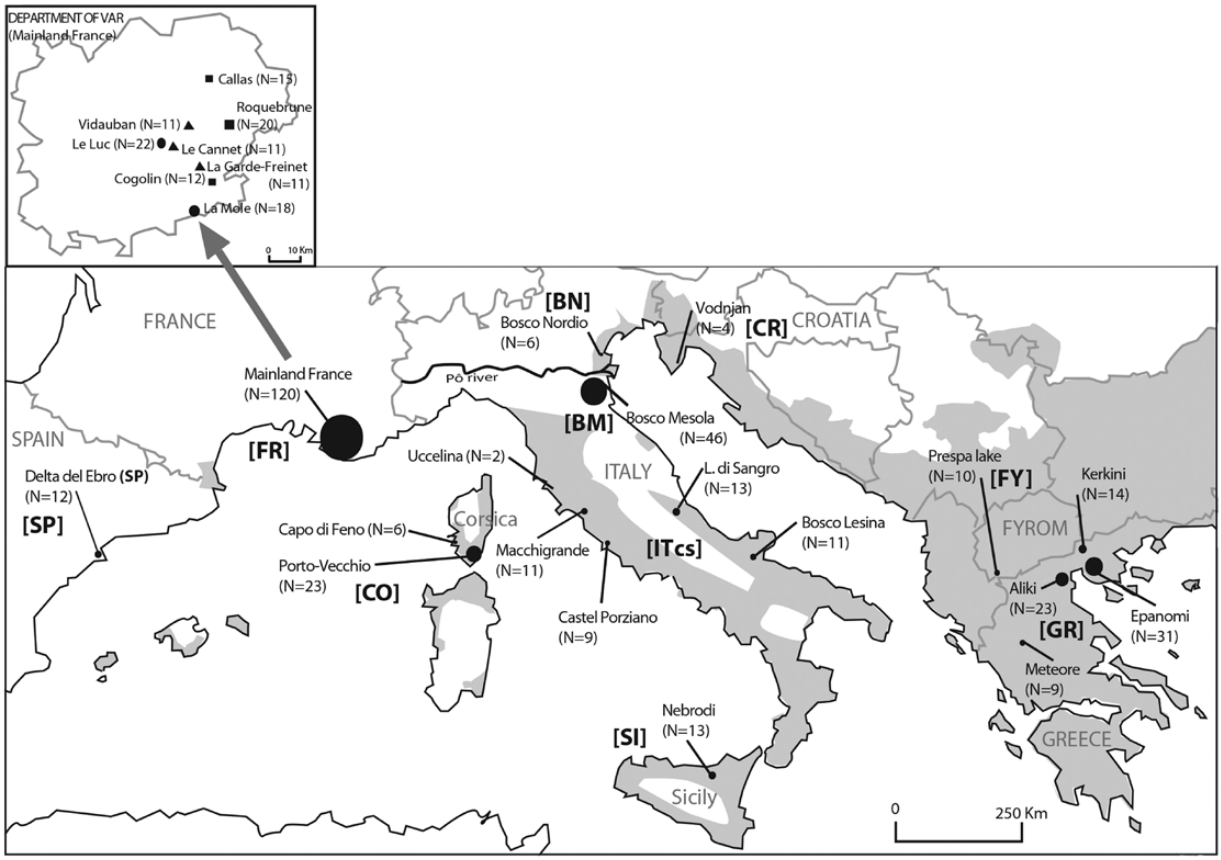

Figure 1. Current distribution of the species (in grey) and sampling localities. The size of the black circles is proportional to the sample size. Codes in square brackets correspond to populations or groups of population as used in the main text and the tables.

major cause of the reduction in population sizes in many Mediterranean areas (Stubbs and Swingland 1985). Intensive harvesting for the pet trade, especially before the 1980s when it was banned (Groombridge 1982; Ljubisavljevic et al. 2011), and occasional releases of nonnative individuals into local populations represent additional threats for this species (Bertolero et al. 2011).

Two subspecies are generally recognized, *Testudo hermanni hermanni* (Gmelin 1798) and *Testudo hermanni boettgeri* (Mojsisovics 1889), differing by 4 shell features (Bour 1986, 2004b; Wermuth 1952): the carapace length (larger in *T. h. boettgeri*), the color of the plastron (2 dark bands in *T. h. hermanni* vs. dark spots in *T. h. boettgeri*), the ratio between the pectoral and femoral midline seams (<1 in *T. h. hermanni,* but see Perälä 2002), and the pattern of scales on the forearm. The 2 subspecies also inhabit different geographical areas (Cheylan 2001; Bour 2004a): *Testudo h. hermanni* is described in isolated patches in Spain, France, and Italy and on the islands of Sicily, Sardinia, Corsica, Majorca, and Minorca. *T. h. boettgeri* inhabits the coastal areas of the Balkan Peninsula, from Croatia to Greece, including many islands in the Adriatic and Ionian seas, the European part of Turkey, and further inland in the former Yugoslavia, Romania, and Bulgaria.

Many characters (e.g., length or color) are affected by local environmental conditions, and the ratio between plastral seams varies with age. As a consequence, the evolutionary divergence between the subspecies, the taxonomic value of morphological traits, and the possible substructuring in genetically divergent groups are controversial (Cheylan 2001). Analysis of mitochondrial DNA (mtDNA) showed 2 major clusters of sequences at the 12S ribosomal RNA gene (van der Kuyl et al. 2002; Mirimin et al. 2004), approximately matching the morphological classification. Three major haplogroups, geographically located in different areas, were identified at the Cytochrome b (Cyt*b*) gene in *T. h. boettgeri* (Fritz et al. 2006). However, the phylogenetic signal at this marker was considered robust enough only to support the traditional 2 subspecies model, with incipient taxonomic differentiation possibly occurring in the eastern subspecies (Fritz et al. 2006).

In Italy, the existence of both subspecies was reported more than 50 years ago by Wermuth (1952), who considered the Apennine chain as the natural border between them. The status of the Italian populations has remained controversial since then (Bruno 1970; Bruno and Guacci 1993; see review in Cheylan 2001). Bruno (1970) described *T. h. boettgeri* along the Adriatic side of the Italian peninsula, but Cheylan (2001)

considered the Po River (Figure 1) in north-eastern Italy as a natural barrier to gene flow and as the boundary separating the 2 subspecies. The controversial status of populations in the north-eastern areas is also due to uncertainty about their true origin. For example, Bruno et al. (1973) considered that tortoises morphologically classified as *T. h. boettgeri* from that region have been recently introduced. Ballasina (1995b), on the contrary, suggested that a few populations are native to that area, but not the population from Bosco Mesola (Figure 1), which is regarded indigenous by other authors (Mazzotti 2004). Today, the general tendency is to limit the distribution of the eastern subspecies to the Balkans (Fritz et al. 2006).

Similar controversies affect the Spanish populations. Llorente and Montori (1995) questioned the natural origin of some animals from the Ebro Delta, but the source for most of the reintroduced individuals was probably along the Spanish coast or the close Balearic Islands (Bertolero et al. 1995).

In continental France, although the eastern *T. h. boettgeri* form has never been described as indigenous, morphological data suggested the presence of hybrids between the 2 subspecies in the wild (Guyot and Pritchard 1999), likely related to pet trade and unsupervised releases. In Corsica, morphological traits are much more variable than those from mainland France, with a mixture of features from each subspecies observed at the individual level (Cheylan 2001). Tortoises from Corsica also differ from their counterparts on the continent by several reproductive features (Bertolero et al. 2007a,b). Fritz et al. (2006) described Corsican tortoises as part of the *Testudo h. hermanni* subspecies based on Cyt*b* but also found some level of genetic divergence between Corsica and mainland France. Giacalone et al. (2009) using the same marker highlighted a link between Corsican, Sardinian, and Sicilian tortoises due to the sharing of a specific mtDNA haplotype.

In this article, we aim to quantify the level of genetic variation in this species and to clarify the geographic distribution of genetic diversity, between and within the 2 commonly recognized subspecies. We are then interested in understanding the relative impact of natural and human-mediated processes in generating the observed genetic structure. Our study includes the analyses of the 12S ribosomal RNA gene, a phylogeographically informative mtDNA marker typed in previous studies in limited number of individuals from few areas (van der Kuyl et al. 2002; Mirimin et al. 2004). For the first time in this species, we also analyze several nuclear markers, which allowed us to increase the power of the statistical inference especially in the study of the geographic structure within subspecies, to reach conclusions not limited to maternally inherited DNA fragments, and to study the admixture process at the individual level. The samples we collected allow a rather complete analysis of the western populations and include 2 important populations in the contact area between the 2 subspecies. Admittedly, however, the genetic structure within the eastern subspecies cannot be dissected in detail due to the fact that some areas are not covered as well as in other studies based only on single mtDNA markers (Fritz et al. 2006). The results we obtain, based on mtDNA and 9 nuclear markers, will have also practical implications for the management and the conservation of this species.

Materials and Methods

Sampling

Between 2001 and 2010, blood samples were gathered from 25 localities: Greece (4 sites), former Yugoslav Republic of Macedonia—FYROM (1), Croatia (1), mainland Italy (7), Sicily (1), mainland France (8), Corsica (2), and Spain (1) (see Figure 1 for sample sizes). Mitochondrial analysis was conducted on 299 individuals from 22 sampling locations. Microsatellite analyses were run on 330 samples from 22 sampling locations. Mainland Italy samples come from different protected areas (see Figure 1 and Supplementary Table S1). In Sicily, samples were collected in the Nebrodi area, the only native population in the island according to Ballasina (1995a). FYROM samples come from the island of Prespa Lake, and Croatia samples come from Vodnjan. Spanish samples were collected in the Ebro Delta, an area where individuals from the close locality of Montsia (where the species is now extinct), from the Balearic population, or with unknown origin, were introduced between 1987 and 1998 (Bertolero 1991; Bertolero et al. 1995; Cheylan 2001; Fritz et al. 2006). Before the reintroduction, all these individuals were carefully checked to have the morphological traits typical of the western subspecies (Bertolero et al. 2007c).

Molecular Analyses

Blood samples were preserved in heparin buffer or desiccated on sterile blotting paper and stored at −20 °C before being processed. Tissues were stored in 95% ethanol at −20 °C. Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer instructions.

We amplified approximately 400 nucleotides of the 12S rRNA mitochondrial gene using primers L01091 and H01478 (Kocher et al. 1989). Nucleotide sequences were deposited in Genbank, under accession numbers KF591452–KF591478.

Nine microsatellite markers were typed. Five of them (Test10, Test56, Test71, Test76, and Test88) were isolated from *T. h. hermanni* (Forlani et al. 2005), whereas other 4 markers (Gal136, Gal75, Gal73, and Gal263) were developed for *Geochelone* spp. (Ciofi et al. 2002).

The microsatellite data were checked for genotyping errors using the software MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004).

Additional details and laboratory protocols are provided online (Supplementary Protocols). In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

Statistical Analyses

mDNA

12S rRNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). Arlequin 3.5 (Excoffier and Lischer

2010) was used to calculate population average haplotype diversity (*h*) and mean number of pairwise differences (*k*). Partitioning of genetic variances within and among sampling sites was assessed by analysis of molecular variance (AMOVA; Excoffier et al. 1992), using as molecular distance between pairs of alleles the simple number of nucleotide differences. An unrooted network of haplotypes was obtained using the method of statistical parsimony implemented in the software TCS (Templeton et al. 1992). The robustness of the longest branch in the network was assessed by bootstrapping the aligned sites and reconstructing each time a maximum parsimony and a Neighbor-Joining tree using MEGA 3.1 (Kumar et al. 2004).

Nuclear Microsatellites

To identify the different genetic groups present in our dataset, 2 Bayesian clustering methods, implemented in STRUCTURE 2.3.3 (Hubisz et al. 2009; Pritchard et al. 2000) and GENELAND-3.1.5 (Guillot et al. 2008), were used. With STRUCTURE, 30 runs were performed for each value of *K* from 1 to 15 (burn-in period: 30 000, 10^7 iterations) under the admixture model and the assumption of correlated allele frequencies among populations. The most likely number of clusters (*K*) was inferred by looking at the variation of the likelihood of the data and following the approach based on likelihood ratios (Evanno et al. 2005). GENELAND was used to perform a spatial analysis of the genetic structure by explicitly integrating the geographic locations of the samples. We used the Dirichlet model for independent allelic frequencies. To infer the most probable *K* value, 10 Markov chain Monte Carlo (MCMC) with 10^6 iterations (thinning = 10^3, burn-in 50%, maximum rate of Poisson process = 330; spatial uncertainty = 0.1 km) were performed for each *K* values ranging from 1 to 15. Null alleles are accommodated by this method. The posterior probability of population membership on the spatial domain was computed for each of the 10 runs. STRUCTURE and GENELAND were primarily used to group sampling localities in homogenous genetic clusters. We also used STRUCTURE to study hybridization among subspecies (see below).

Descriptive statistics of nuclear genetic diversity were estimated within the homogenous inferred groups. The number of individuals analyzed for each locus (*N*), the mean number of alleles per locus (N_a), F_{IS}, and observed (H_O) and expected (H_E) heterozygosities were computed using Genalex 6.4 (Peakall and Smouse 2006) as well as deviation from Hardy–Weinberg equilibrium. Tests for linkage disequilibrium between pairs of markers were performed using Genepop-4.0 (Rousset 2008) using default parameter values. Allelic richness (A_R) was calculated using the rarefaction procedure in the Fstat 2.9.3.2 software (Goudet 2001) for each group.

Genetic differentiation measured as F_{ST} values (Weir and Cockerham 1984) was estimated for each pair of inferred genetic group with MSA 4.05 (Dieringer and Schlotterer 2003). Significance of the F_{ST} values was tested using 10 000 permutations, and *P* values were multiplied by the total number of comparison following the conservative Bonferroni approach for multiple testing.

Isolation by distance (IBD) was tested comparing genetic and geographic distances between each pair of sampling locations. $F_{st}/(1 - F_{st})$ were used as genetic distances, whereas geographic distances were computed as natural logarithms of the on-land shortest distance between 2 continental localities (see Supplementary Figure S1). In comparisons involving islands, Sicily was assumed to be an extension of Italy and Corsica as linearly connected to either the Italian or the French coast (whatever is the shortest distance in the comparison). Five different analyses were performed: all localities (IBD1), only continental localities (IBD2), only *T. h. boettgeri* (IBD3), only *T. h. hermanni* (IBD4), and only continental *T. h. hermanni* (IBD5). All *T. h. boettgeri* sampling sites are continental. The *P* values of the correlation coefficients were computed using the Mantel test with a permutation approach (10 000 permutations) and multiplied by the number of tests following the conservative Bonferroni correction for multiple testing.

The presence within each subspecies of migrants and F1 or F2 descendants of migrants was tested with the software STRUCTURE using the USEPOPINFO and GENSBACK options. Because we focused on recent mixing events between the 2 subspecies, we assumed $K = 2$. The model in STRUCTURE assumes that most individuals classified in each subspecies have pure ancestry from that subspecies but that a small proportion of individuals may have a proportion of ancestry from the alternative subspecies. Under this prior assumption, individuals with less than 50% posterior probability of having pure ancestry can be confidently assigned to the migrant or hybrid class (Falush et al. 2007).

Results

mDNA

The 12SrRNA gene was successfully sequenced in 93% of individuals. The alignment (296 bp) shows 27 different haplotypes, defined by 25 polymorphic sites and 26 substitutions (13 transitions and 13 transversions). Haplotype diversity is high in the total sample (0.812) but equal to 0 in 4 French mainland and a Corsican sites, and it reaches its maximum value (0.933) in Bosco Nordio (north-eastern Italy). Results for each population are reported in Supplementary Table S1. The AMOVA analysis suggests that populations are, on the average, highly divergent. In fact, the large majority of molecular genetic variation (82.55%) is due to differences between populations.

The statistical parsimony network splits the mtDNA sequences in 2 major groups of sequences, or haplogroups, separated by at least 3 substitutions and associated to the western and the eastern populations (Figure 2). We call these haplogroups THH and THB, respectively, indicating their relationship with the 2 subspecies. The bootstrap support for the branch separating the haplogroups is high in maximum parsimony (84%) and neighbor-joining (72%) trees.

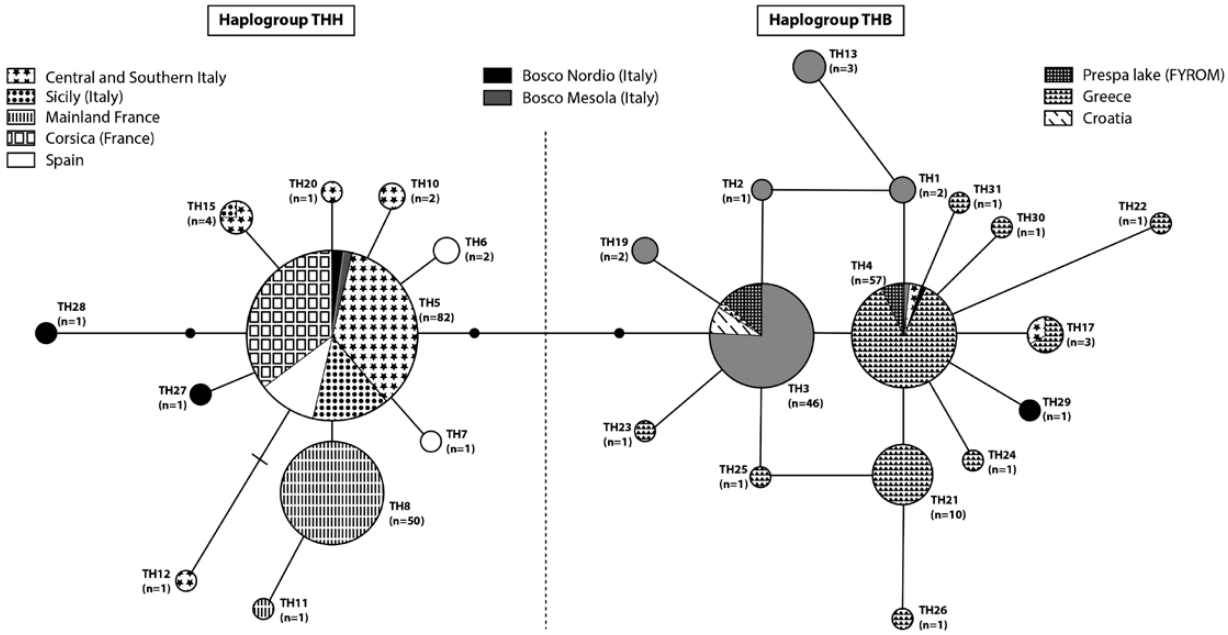

Figure 2. Parsimony network of *Testudo hermanni* 12S mtDNA haplotypes. *n* = haplotype frequency. The length of the lines connecting haplotypes is proportional to the number of mutations.

Haplogroup THH encompasses 11 haplotypes and is characterized by a star-like shape stemming from most frequent haplotype, TH5. It encompasses the majority of samples (96.5%) from central and southern Italy, Sicily, mainland France, Corsica, and Spain, that is, the typical distribution range of the *T. h. hermanni*. The mainland French samples all have the same haplotype, TH8, a sequence different but strictly related (1 mutation apart) from the most common *T. h. hermanni* haplotype TH5, except 1 individual showing the TH11 haplotype (1 mutation apart from TH8). Haplogroup THB includes 16 haplotypes found almost only in eastern populations (Greece, Croatia, and FYROM) and in north-eastern Italy (Bosco Mesola and Bosco Nordio). Most haplotypes sampled in Greece (66 out of 69) cluster together in a topology centered on haplotype TH4. TH3 is separated from TH4 by a single substitution and is observed in the 4 samples from Croatia and in 60% of the samples from FYROM (the remaining 40% were of TH4 type). In Italy, all individuals from Bosco Mesola have TH3 (78%) or a related haplotype, except 1 individual showing the haplotype TH5 from haplogroup THH. The only population where large fractions of both THH or the THB haplogroups are observed is Bosco Nordio, where however only 6 individuals are available. In this north-eastern Italian area, 5 different haplotypes are found, 3 of which are not observed elsewhere. Four and two individuals belong to the THH and THB haplogroups, respectively.

Microsatellite Markers

Genotyping Errors

MICRO-CHECKER was applied separately to the 9 genetic and geographic groups jointly defined by different statistical methods (see below): Spain, France, Sicily + Corsica, central and southern peninsular Italy, Bosco Mesola, Bosco Nordio, Croatia, Greece, and FYROM. No systematic signal of allelic dropout or stuttering anomalies was detected for any locus. Occasional excess of homozygotes was detected in Bosco Mesola, France, Greece, and central and southern peninsular Italy. This signal may be due to natural or artificial substructuring in these groups (see below). Allele frequencies adjustments were minor, and given also that the statistical analyses based on these groups (e.g., F_{st}) produced very similar results with or without frequency adjustments, we decided to keep the original genotypes.

Identification of Major Genetics Groups

A graphical analysis of STRUCTURE results indicates that the log probability of the data increases sharply from $K = 1$ to $K = 2$ and then more slowly from $K = 3$ to $K = 5$ (see Supplementary Figure S2a), where it reaches a plateau. The method of Evanno et al. (2005) provides similar evidence regarding the most likely number of groups (see Supplementary Figure S2b).

When K is fixed to 2, the results clearly support the mtDNA analysis (Figure 3a). The 2 nuclear genetic components correspond to the eastern and the western subspecies, with each population having a large fraction of its genetic pool that can be assigned to one of them: Spain, all mainland French populations, Corsica, and all the populations from central and southern Italy are predominantly *T. h. hermanni* (red component in Figure 3a); Bosco Mesola, Croatia, FYROM, and all the Greek populations are predominantly *T. h. boettgeri* (green component in Figure 3a); Bosco Nordio

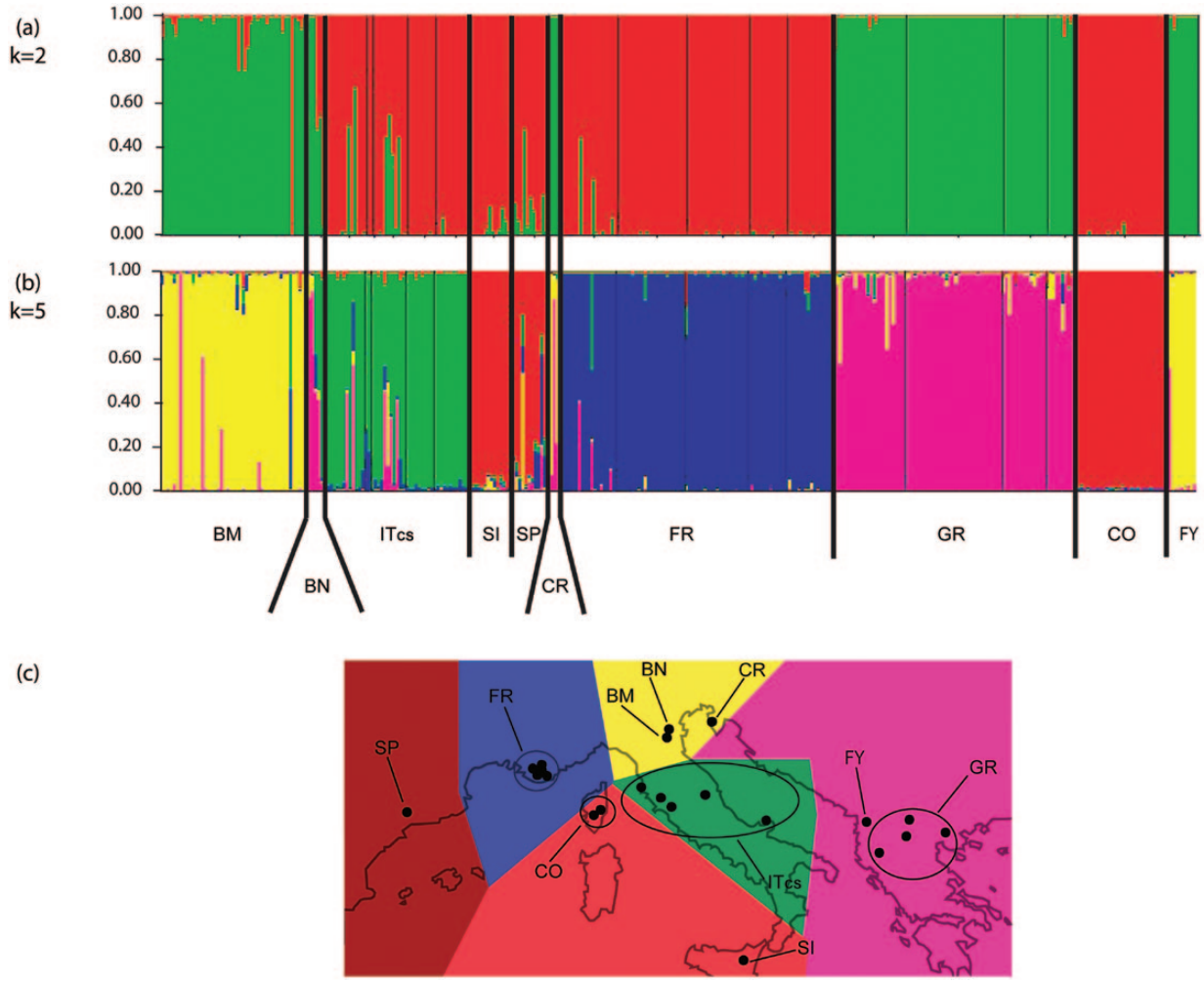

Figure 3. Bar plot representing the genetic composition of single individuals (thin vertical columns) as inferred from STRUCTURE with $K = 2$ (**a**) and $K = 5$ (**b**). The results provided by GENELAND are reported in panel (**c**), with colors matching as much as possible the groups inferred in STRUCTURE. Codes as in Figure 1.

cannot be easily assigned to 1 component or the other. Some individuals, especially in Italy and Spain, showed a mixed composition (see the specific analysis reported below).

For $K = 5$ (Figure 3b), most of the STRUCTURE runs (90%) suggested a subdivision of *T. h. hermanni* and *T. h. boettgeri* in 3 and 2 groups, respectively. The *T. h. hermanni* groups are the following: all the populations from central and southern peninsular Italy (ITcs), mainland France (FR), and Spain joined with the islands of Sicily and Corsica (SP + SI + CO). The *T. h. boettgeri* groups are Bosco Mesola and FYROM (BM + FY) and all the Greek populations (GR). Croatia (CR) is partially affiliated to GR and partially to BM + FY, and Bosco Nordio (BN) has both GR and ITcs components. When K was fixed to 3 (data not shown), most of the STRUCTURE runs (70%) suggest a subdivision of the *T. h. hermanni* component in 2 parts corresponding to ITcs + FR and SI + SP + CO. The results with $K = 4$ are very unstable across different runs of the analysis.

The results obtained with GENELAND (Figure 3c) suggest a subdivision in 6 groups. This subdivision is largely consistent with the major partitions provided by STRUCTURE, but some differences are observed: 1) the STRUCTURE group SI + SP + CO is further divided into SP and SI + CO; 2) FYROM is affiliated to GR and not to BM; 3) CR and BN are forced into a group with BM. These differences can easily be explained looking at Figure 3b (STRUCTURE results) and considering the approach of the method implemented in GENELAND. In fact, 1) Spain, Sicily, and Corsica share a large fraction of their genetic pools, but Spain includes also several mixed individuals; 2) the assignment procedure implemented in GENELAND is constrained by the geographic coordinates, and therefore, the low divergence between Greece, Bosco Mesola, and FYROM is forced in 2 geographically consistent groups; 3) Bosco Mesola, Bosco Nordio, and Croatia clearly share a certain fraction of genetic variation and are geographically close.

Genetic Diversity within and between Groups

The level of genetic variation within the 2 subspecies (defined geographically assigning Greek, FYROM, Croatian, and north-eastern Italian populations to the eastern subspecies, and all the other populations to the western subspecies) is similar (see Supplementary Table S2). *T. h. hermanni* shows a larger number of alleles, but the allelic richness values (i.e., the number of alleles standardized for the sample size) are similar. Heterozygosities are similar as well, and in both subspecies, the internal substructure generates large and positive (and again similar) F_{is} values.

Further analyses of genetic variation within and between groups were based on the clusters identified by STRUCTURE and GENELAND, but (conservatively) considering as separate the populations for which the 2 analyses disagree or unequivocal support for pooling was not reached. Within the 4 and 5 major populations or groups of *T. h. hermanni* and *T. h. boettgeri*, respectively, and considering that estimates in samples with very small size are imprecise, we note that 1) the level of variation is very low in France, 2) the other western groups show large variation values, together with the Italian *T. h. boettgeri* sample (BM), and 3) the Balkans samples (FY and GR) have intermediate levels of variation (see Supplementary Table S2). The admixed composition of Bosco Nordio (BN) observed at the mtDNA sequences is confirmed by its large values of allelic richness and heterozygosity at nuclear microsatellites. The fraction of loci deviating from Hardy–Weinberg equilibrium is larger than expected by chance, due to a deficit of observed heterozygosity. On the average, however, this deficit is quite limited (much lower than observed when localities within subspecies were pooled), and it is likely due to some level of additional internal substructuring in groups obtained pooling localities (e.g., ITcs, FR, and GR) or to the presence of few allochthonous animals (e.g., BM). We note, for example, that excluding from the analysis of BM only 2 homozygous individuals (out of 46 in the sample) carrying an allele at the locus GAL 156 which is very rare in this population (but common in some other populations), almost halves the number of loci showing significant deviation from Hardy–Weinberg equilibrium.

The genetic differentiation (F_{st}) is almost always high and significant between any pair of groups (Table 1). On the average, groups within subspecies show similar levels of pairwise divergence (0.24 and 0.27, respectively). As expected, when *T. h. hermanni* and *T. h. boettgeri* groups are compared, the average F_{st} increases substantially, reaching 0.42. The largest values are observed when the internally homogenous French group is compared with most of the other groups.

IBD

The IBD pattern is significant after Bonferroni correction in 4 out of 5 Mantel analyses ($P < 0.05$) and not significant only in the test with the smallest sample size(see Supplementary Table S3). The fit between genetic and geographic distances is similar in the 2 subspecies ($r = 0.40$ in IBD3 and IBD4), but it is almost twice as large in THH when islands (Sicily and Corsica) are excluded ($r = 0.73$ in IBD5).

Admixture between Subspecies

Eighteen individuals (6% of the sampled animals, see Table 2) can be confidently considered as migrants from one subspecies to the other or as hybrid individuals descendants from local animals mated with individuals that migrated either 1 or 2 generations ago. Given the low dispersal ability and the patchy distribution in this species, the inferred recent migration events are clearly related to human-mediated translocations.

Two *T. h. hermanni* migrants were identified in the *T. h. boettgeri* populations in north-eastern Italy, and both show as expected a mtDNA haplotype belonging to the THH haplogroup. In the same populations, 4 additional animals had either parents or grandparents from the *T. h. hermanni* group. Half of the individuals analyzed in Bosco Nordio (sample size = 6) clearly showed a *T. h. hermanni* contribution. Twelve individuals sampled in the *T. h. hermanni* distribution range showed mixed ancestry. The average frequency of individuals classified as hybrids in *T. h. hermanni* localities (migrants are absent) is around 9%, with higher values in Spain (25%) and central Italy (27% and 15% in Macchia Grande and Lecceta di Sangro, respectively). Among

Table 1. Pairwise differentiation (F_{ST}) between inferred groups

		THH				THB				
		SP	FR	SI + CO	ITcs	BM	BN	CR	GR	FY
THH	SP	—								
	FR	0.345	—							
	SI + CO	0.180	0.359	—						
	ITcs	0.156	0.202	0.176	—					
THB	BM	0.352	0.497	0.360	0.302	—				
	BN	0.212	0.468	0.288	0.177	0.159	—			
	CR	0.494	0.645	0.482	0.405	0.169	0.217	—		
	GR	0.414	0.579	0.465	0.416	0.313	0.159	0.338	—	
	FY	0.374	0.595	0.420	0.375	0.280	0.245	0.423	0.364	—

All values are statistical significant ($P < 0.05$ after Bonferroni correction), except for the comparisons CR × BN and CR × FY. See Figure 1 for population coding.

Table 2. Analysis of introgression among subspecies

	Individual ID	Sampling location	No hybrid ancestry	Prob. migrant	Prob. has migrant parents	Prob. has migrant grandparent	mtDNA haplotype (Haplogroup)
THB	Mesola291	BM	0.304	0.009	0.000	0.688	TH3 (THB)
	Mesola851	BM	0.487	0.000	0.000	0.513	TH2 (THB)
	MesolaNM3	BM	0.000	1.000	0.000	0.000	TH5 (THH)
	Nor4	BN	0.000	0.101	0.001	0.899	TH5 (THH)
	Nor5	BN	0.000	0.000	0.739	0.261	TH28 (THH)
	Nor6	BN	0.000	1.000	0.000	0.000	TH27 (THH)
THH	AbruzzoA8	ITcs	0.000	0.000	0.451	0.549	TH4 (THB)
	AbruzzoA10	ITcs	0.000	0.145	0.834	0.02	TH4 (THB)
	Macchia5	ITcs	0.000	0.000	0.259	0.741	TH17 (THB)
	Macchia6	ITcs	0.000	0.000	0.961	0.039	TH5 (THH)
	Macchia7	ITcs	0.000	0.000	0.115	0.885	TH15 (THH)
	Macchia9	ITcs	0.000	0.000	0.677	0.323	Nd
	Neb7	SI	0.302	0.02	0.001	0.677	TH5 (THH)
	Ebro2321	SP	0.331	0.022	0.002	0.645	TH5 (THH)
	Ebro1442	SP	0.21	0.000	0.000	0.79	TH6 (THH)
	Ebro4211	SP	0.358	0.000	0.000	0.642	TH5 (THH)
	Fr1H607	FR	0.000	0.000	0.008	0.992	TH8 (THH)
	Fr1H643	FR	0.055	0.000	0.002	0.944	TH8 (THH)

Individuals with a probability of being nonhybrid < 0.5 are shown. The probability of being a first-generation migrant or a F1 or F2 hybrid are indicated. The corresponding mtDNA haplotype and haplogroups of these individuals are also shown.

the 16 individuals classified as F1 or F2 hybrids in either the *T. h. boettgeri* or *T. h. hermanni* groups, 5 had a mtDNA haplotype corresponding to the immigrant subspecies. This suggests that both males and females were introduced.

Discussion

Results from mtDNA and 9 microsatellites markers support the existence of 2 distinct genetic pools in *Testudo hermanni*, corresponding to the 2 recognized subspecies *T. h. hermanni* Gmelin, 1789 and *T. h. boettgeri* Mojsisovics, 1889. The geographic distribution of the clades is consistent, with some differences in the position of the contact zone, with that described by Wermuth (1952) using morphologies or identified previously from mtDNA data (van der Kuyl et al. 2002; Mirimin et al. 2004; Fritz et al. 2006). Our new microsatellites data allowed a fine dissection of the genetic structure within subspecies, including the identification of admixed individuals and populations. We believe that these results are relevant for their scientific sake but also to help the development of future conservation and management plans.

Overall, the eastern subspecies shows a larger number of mtDNA haplotypes (with a slightly smaller number of sampled individuals) than the western subspecies. Higher variation at mtDNA in the eastern subspecies was observed also in a previous study based on Cyt*b* sequences (Fritz et al. 2006). The levels of nuclear genetic variation, on the contrary, are almost identical. The demographic dynamics within the 2 distribution ranges were probably different, with fragmentation and extinctions affecting more extensively the western areas (see below), and this difference might explain the genetic pattern at the marker more sensitive to genetic drift (mtDNA). Additional sampling in the eastern areas is however required to better interpret this result. More generally, genetic variation should be considered high enough to exclude reduced evolutionary potential at least at the species and the subspecies level. When compared with other species typed at microsatellite markers, in general and also specifically in Testudines, the expected heterozygosity observed in the 2 *T. hermanni* subspecies (0.53 and 0.56) is close to the lower limit in nonendangered species but much higher than values observed in endangered species (Frankham et al. 2010; Fritz et al. 2012; Güçlü et al. 2011; Hamilton 2011; Pedall et al. 2011; Vargas-Ramírez et al. 2012). Also single populations or geographic areas within subspecies do not show low level of genetic variation, except France.

Unsurprisingly for a sedentary animal, the geographic structure in this species is strong. We can summarize it in 3 features: 1) a major discontinuity between eastern and western areas (corresponding to the 2 subspecies), with a contact zone located in north-eastern Italy; 2) an overall pattern of correlation between genetic and geographic distances, modified by specific processes occurring only in some areas; 3) a series of recent secondary contacts among subspecies due to human translocations.

We discuss these points focusing on geographic areas and/or single populations that have been identified as genetically distinct groups using several statistical analyses. We note that even if the major discontinuity is observed between eastern and western areas corresponding to the 2 subspecies, genetic substructuring is evident also within these 2 groups. We discuss in turn 3 groups of populations: the eastern *T. h. boettgeri* group (Greece, FYROM, and Croatia), the western *T. h. hermanni* group (Spain, mainland France, Corsica, mainland Italy, and Sicily), and a group of 2 populations in the north-eastern Italy (Bosco Mesola

and Bosco Nordio), which are located in a region that likely corresponds to the contact area of the subspecies.

Eastern *T. h. boettgeri* Group

Our sampling of the eastern distribution range is discontinuous and limited to Greece, FYROM, and Croatia, and only few individuals are sampled from the latter 2 countries. It is therefore difficult to suggest a geographic pattern of genetic variation across this large area and also difficult to use the nuclear markers we typed to confirm or reject the hypothesis suggested by Fritz et al. (2006) that taxonomic divergence is occurring in the eastern subspecies. We therefore limit our conclusions to 2 inferences. First, in southern regions, populations are rather homogeneous. There is 1 typical mtDNA group of haplotypes in Greece centered on the most frequent sequence TH4, with FYROM having a combination of TH4 and the closely related TH3 haplotypes (typical of north-eastern *T. h. boettgeri* areas, i.e., Croatia and north-eastern Italy). Microsatellite markers cluster together all the GR, with FYROM having some intermediate features but still clearly related to the south-eastern populations when their geographic proximity is also considered (GENELAND analysis). Second, the 4 individuals sampled in Croatia have genetic relationships with both the Balkans and the north-eastern *T. h. boettgeri* Italian populations.

The absence of genetic structure in Greece contradicts the conclusion of Willemsen (1995) who suggested the existence of several taxa based on morphological traits. A genetic structure within GR was also suggested by Fritz et al. (2006) based on Cyt*b*, but this structure was mainly based on the genetic differentiation of western tortoises (Corfu and Epirus). Our dataset does not include these western populations and suggests that some morphological divergence observed by other authors may reflect environmental factors and not a deep genealogical divergence.

Western *T. h. hermanni* Group

When the nuclear markers were used to identify the genetic partition in this species beyond the subspecies level (i.e., the STRUCTURE analysis with $K = 3$), 2 groups were identified within the western subspecies: Spain + Sicily + Corsica and mainland France + mainland Italy. The former group is maintained also for larger K values using STRUCTURE but split in Spain and Sicily + Corsica using a different method (GENELAND). The second group can be further subdivided in the 2 mainland areas in Italy and France. Genetic data support therefore the existence of 4 major groups of populations: Spain, Sicily + Corsica, mainland France, and mainland Italy. These results, which are compatible with our analysis of mtDNA sequences as well as with previous works (Fritz et al. 2006; Giacalone et al. 2009), require at least a tentative explanation.

Spain, Sicily, and Corsica

Llorente and Montori (1995) described the Spanish Ebro population as having a complex history of multiple reintroductions. The origin of many introduced individuals, however, are the Montsia region (surrounding the Ebro Delta) and the Balearic Islands (facing the Ebro Delta), and all the introduced animals were carefully classified (morphologically) as belonging to the western subspecies (Cheylan 2001; Bertolero 2009). The Sicilian population we analyzed is considered native to the island (Cheylan 2001). Tortoises from Corsica have been described as morphologically different from those in mainland France (Cheylan 2001), and Giacalone et al. (2009) vaguely suggest some introduction events from Sicily to Corsica (and Sardinia) "either by natural oversea dispersal or translocation by man," without however specifying any time frame for this process.

Recent translocations, as suggested by the results of our admixture analysis, have certainly played a role, but massive translocations of individuals in the last centuries among these 3 areas (Spain, Sicily, and Corsica) is not documented. Instead, we believe that the geographic distribution of fossil records analyzed by Morales Pérez and Serra (2009), together with the hypothesis that many turtles and tortoises acted as "living cans" for seafarers during prehistoric and historic times (Vamberger et al. 2011), may help us to understand this pattern.

The archeological and paleontological record from western Europe suggests that *T. hermanni* disappeared from the end of the Middle Palaeolithic (around 30 000 years ago) in almost all the Iberian peninsula and in France and reappeared in these areas 3–4 thousand years ago (Cheylan 2001; Morales Pérez and Serra 2009). Consistent with that, some authors suggest that *T. hermanni* was introduced in the Balearic Islands around 3000 years ago (Mayol 1985; Fritz et al. 2006). This temporal gap of several thousand years, possibly related to the extinction or strong reduction of this species related to climatic Heinrich event that transformed the Iberian Mediterranean ecosystems in a semidesert landscape, is not observed in the Italian (including Sicily) fossil record (Morales Pérez and Serra 2009). Our genetic results suggest that *T. hermanni* from Sicily could have contributed to the repopulating of the Mediterranean Islands and the Spanish coast, likely through human-mediated translocations during prehistoric times. This hypothesis explains the clear genetic similarity we observe between Sicily and Corsica, and our study also indirectly supports the idea based only on mtDNA data that Sardinian and Sicilian *T. hermanni* are genetically similar because of human transportation (Vamberger et al. 2011). If Sicily was a major source of individuals found today in other islands, the fact that several individuals in the Ebro population may have had a very recent origin probably in the Balearic Islands (Cheylan 2001; Bertolero 2009) would not exclude the possibility that the Spanish population we analyzed derives indirectly from the diffusion of Sicilian tortoises.

Mainland Italy

The distribution of tortoises in mainland Italy is very fragmented along the coast, and illegal releases have been described in many areas (Mazzotti et al. 2007). Several individuals with

mixed genetic contribution, including hybrids with the eastern subspecies, are found. Nevertheless, the large majority of individuals (excluding the 2 north-eastern samples described in the next section) are typical *T. h. hermanni*. The 5 areas considered in this group (Lecceta di Sangro, Bosco di Lesina, Castel Porziano, Macchiagrande, and Uccellina, see Figure 1) all have a large contribution of a distinctive genetic component (see the Structure analysis with $K = 5$), not found in other regions where the species is distributed. Genetic variation is relatively high compared to the other *T. h. hermanni* populations. These results appear compatible with the hypothesis that the Italian peninsula acted as a refugium for the western subspecies during the last glaciation (Morales Pérez and Serra 2009). Also, they support the idea that the Apennines, with lowest altitudes around 500 meters, are not preventing some occasional migration among the 2 coastal regions (Fritz et al. 2006). We cannot however exclude the possibility that recent translocations across Italy partially favored the homogenization of the *T. h. hermanni* genetic pool in Italy.

Mainland France

The mainland French population shows only 2 mtDNA haplotypes, one of which is present in 50 out of 51 individuals, and none are observed in other regions. Genetic variation is also very low for microsatellite markers (heterozygosity is lower than any other *T. h. hermanni* population we analyzed). These markers support the distinctiveness of this population but also a certain level of affinity with the mainland *T. h. hermanni* Italian population (structure analysis with $K = 3$). This latter result is in agreement with the Cyt*b* data analyzed by Fritz et al. (2006). All in all, considering that no fossil records are documented in France between approximately 30 000 and 4 000 years ago (Morales Pérez and Serra 2009), we suggest that the genetic pattern we observe in France today is compatible with the hypothesis that this area was colonized in the late Holocene by few individuals of Italian origin, either naturally or through human transportation. Little gene flow and high genetic drift occurred thereafter, and mutation, especially at the slowly evolving 12S mtDNA (around 1% per millions years in *Testudo*, van der Kuyl et al. 2002), did not restore genetic variation because of the short time and/or the small population size.

North-Eastern Italy "Contact Zone"

The 2 north-eastern populations of Bosco Mesola (Po delta) and Bosco Nordio (around 25 km north of the previous location) are characterized by a *T. h. boettgeri* profile. BN and BM showed respectively a strong presence (50%) or weak presence (9%) of hybrids and migrants from *T. h. hermanni* populations. Yet the 2 Bosco do not seem to share a common history. Concerning Bosco Nordio, Mazzotti (personal communication) suggested that some tortoises from Castel Porziano and Rome (eastern coast) have been translocated since the 1960s, which could explain the presence of *T. h. hermanni* individuals together with *T. h. boettgeri*. Our results support this hypothesis. This suggests that at least some introduced animals have survived after the releases, but it remains unsure whether hybrids have been produced by a mixture of the 2 subspecies after their release, or if they have been produced in captivity and survived to their translocation. Concerning Bosco Mesola, the clear genetic affiliation with the eastern subspecies, together with the large genetic variation at both markers and the relevant divergence with other Balkan samples (F_{st} around 30% with the Greek and the FYROM samples), supports the view that this isolated population represents the most western area occupied by *T. h. boettgeri*. Some level of natural or more likely artificial gene flow with the neighboring western subspecies likely occurred, but previous conclusions that most individuals in Bosco Mesola are not native (Ballasina 1995b) should be dismissed. The paucity of natural populations in this area prevents a clear geographic identification of the contact zone among subspecies and an explanation for its origin. We suspect, however, that the Po River and its complex delta, together with the relevant changes of the coast line that occurred in that area during the last glaciation when the limit of the Po plain was approximately 300 km south of the modern shoreline (Simeoni and Carbau, 2009), played an important role in isolating eastern and western populations. Additional information on the contact zone among subspecies could be obtained by studying 2 close remnant populations occurring in the areas of Venice and Trieste.

Migrants and Admixed Individuals

The overall number of introduction or hybridization events concerning different subspecies is limited, but our Spanish sample and some Italian sites indeed showed relatively high frequencies of subspecies introgression. Interestingly, only in Italy introduced individuals or first-generation descendants of introduced individuals were observed. Mixed individuals found today in Spain and in France are probably related to introduction events that occurred 2 or more generations ago. This result is compatible with the presence of the subspecies contact zone in Italy because it is reasonable to suspect that translocation of individuals from 1 subspecies into the distribution area of the other subspecies is more frequent in case of geographic proximity. But it is also compatible with the temporal pattern of introduction in Western Europe of tortoises of eastern origin. For example, according to Ljubisavljevic et al. (2011), large numbers of individuals were exported in the last century from the former Yugoslavia to almost all European countries. The phenomenon had a peak in the 1970s and sharply declined in the 1980s after the introduction of trade restriction imposed by the European Union. In Italy, however, illegal introductions from the Balkans across the Adriatic Sea are probably common even today, as suggested by frequent confiscation of tortoises by custom authorities in southern port cities. Admixed individuals do not show preferentially the mtDNA corresponding to 1 subspecies or the other, suggesting that introductions were not sex biased.

Conservation Concerns

Overall, levels and patterns of genetic variation within species and subspecies do not appear to be cause of concern.

When single populations and areas are considered, 2 major warning for future management strategies emerge from our analysis: 1) the French population should be carefully monitored and protected, and the possible negative consequences of low genetic variation in this population should be investigated; 2) the strong geographic structure, together with the presence of hybrid and migrant individuals in some areas, which can seriously threaten the genetic identity of the 2 subspecies, suggests that future plans of restocking small populations or repopulating areas where the species is now extinct should preliminarily evaluate the genetic background of wild and captive-bred reintroduction candidates.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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