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Demographic structure and genetic variability of a population of *Testudo hermanni hermanni* (Reptilia: Testudines: Testudinidae) from Southern Tuscany (Central Italy): a case of "happy-ending" uncontrolled reintroduction

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Abstract
Testudo hermanni hermanni is becoming seriously endangered throughout its range. It has a scattered distribution, with a small number of residual populations found in Spain, France and Italy. In this study we sampled a population of *T. h. hermanni* from Southern Tuscany (Massa Marittima, Grosseto), composed of native and introduced individuals (recognizable due to residual signs of previous marking on the carapace). Overall, 95% of the captured individuals were adults and the sex ratio was slightly, but not significantly, biased in favour of females. Population density was relatively high in comparison with other Italian populations, although it was doubled by previous reinforcement. Genetic analysis performed on six polymorphic microsatellite loci revealed a high level of genetic variability and heterozygosity, with no evidence of current inbreeding processes. Moreover, introduced individuals presented genotypes similar to those of the native individuals, thus suggesting that the reinforcement intervention did not cause a significant change in the original genetic pool. Nevertheless, long-term monitoring of the population is necessary to ensure its stability and vitality. Furthermore, to preserve the genetic identity of the local population in the future, uncontrolled translocation events should be avoided.

Keywords: Testudo hermanni, *ecology, population structure, microsatellites, Italy*

Introduction

The Hermann's tortoise (*Testudo hermanni* Gmelin, 1789), is endemic to Southern Europe, with two recognized subspecies; *T. h. hermanni* inhabits the Western part of its distribution range (Spain, France and Central and Southern Italy) and *T. h. boettgeri* (Mojsisovics 1889) inhabits the Eastern part of its range (North Eastern Italy, Balkan regions, Greece and Turkey).

Past events, such as Pleistocene climatic fluctuations, forced *T. h. hermanni* populations to retreat to a small number of southern refuges (Fritz et al. 2006) and, due to this fragmentation, the distributional range of *T. h. hermanni* has been severely reduced. Presently, this subspecies is strictly confined to areas with a Mediterranean climate (Stubbs & Swingland 1985; Vetter 2006), and therefore has a scattered distribution, with some residual populations found in Catalonia, Southern France (Massif des Maures) and Central and Southern Italian coastal areas. Moreover, for several decades, human activities, such as urbanization and agriculture, and the collection of specimens for local and international pet trade have severely affected the geographical distribution of this taxon, contributing to the decline in population densities. Finally, many individuals belonging to non-autochthonous populations/subspecies/species have been probably introduced in some parts of the geographic range of *T. h. hermanni* (Fritz et al. 2006; Vetter 2006). These translocations may have led to genetic pollution events among diverged populations and to hybridization events between subspecies/species, hindering the genetic identity of local endemisms.

For all of these reasons, *T. h. hermanni* is now endangered throughout its range and is listed

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as "Near Threatened" in the 2012 International Union for Conservation of Nature Red List (IUCN 2012). Furthermore, the Washington Convention (1975) regulates international trade (App. II) of this species to avoid over-exploitation of natural populations. Thus, the planning of conservation strategies to protect existing populations and restock declining populations is of paramount importance to prevent the extinction of *T. h. hermanni* in the wild.

Hermann's tortoises occupy a relatively wide range of habitats, specifically termophilous forests along coastlines, scrubs, meadows and pastures (Ernst & Barbour 1989; Bertolero et al. 2011). The duration of the activity window and the reproductive season of these animals may vary at a local scale according to geographic parameters such as latitude, distance from the sea and average temperature (Vetter 2006); however, in the Mediterranean region the reproductive season generally ranges from spring to the beginning of autumn (Mazzotti 2006).

Since the 1980s, *T. h. hermanni* has been the subject of many studies, which have predominantly focused on ecology and population structure (Cheylan 1981; Stubbs & Swingland 1985; Bertolero et al. 1995; Carretero et al. 1995; Henry et al. 1998; Mazzotti et al. 2002; Corti & Zuffi 2003; Bertolero et al. 2007a, 2007b; Loy et al. 2007), and home-range and habitat use (Chelazzi & Francisci 1979; Chelazzi & Carlà 1986; Paglione & Carbone 1990; Bossuto et al. 2000). Studies of Italian populations have provided useful information concerning the vital space and habitat use of Tuscan populations (for example Chelazzi & Francisci 1979; Chelazzi & Carlà 1986; Paglione & Carbone 1990; Bossuto et al. 2000), while other authors have investigated the population structure and ecology of *T. h. hermanni* in Latium (Filippi et al. 2010), Po River Delta (Mazzotti & Vallini 1996; Mazzotti et al. 2002), Molise (Loy et al. 2007), Sicily (Tomasetti & Bossuto 2000), and Sardinia (Corti & Zuffi 2003).

Genetic studies were also performed, which mainly focused on systematic and phylogeographic questions through the use of mitochondrial markers (van der Kuyl et al. 2002; Fritz et al. 2006; Parham et al. 2006). A clear genetic separation between the two *T. hermanni* subspecies emerged (van der Kuyl et al. 2002), and a total absence of genetic differentiation among the European western populations was also revealed (Fritz et al. 2006).

However, little information concerning *T. h. hermanni* genetic diversity and divergence within and among Italian populations is available (Mantovani 2004; Mirimin et al. 2004; Bertorelle et al. 2006). Such information would be fundamental for the development of conservation strategies and translocation/reintroduction programs. Thus, we aimed to contribute to the sparse existing knowledge of *T. h. hermanni*, through the study of one of the few remaining natural populations in Southern Tuscany. Specifically, we aimed to (1) describe the population structure and estimate the population density, and (2) evaluate the level of genetic variation and its genetic pureness by means of genotyping six microsatellite loci.

Materials and methods

Study area

The study area was within the National Park of the Colline Metallifere Grossetane, near Massa Marittima (Grosseto, Tuscany). The surveyed area is approximately 12 ha and the altitude ranges from 174 to 214 m above sea level (a.s.l.). The climate is Mediterranean-temperate, with cold winters, rainy springs and autumns, and dry and hot summers. The vegetation is characterized by a mosaic of habitat types, primarily mixed forest dominated by *Quercus ilex* and *Q. cerris*, Mediterranean maquis and plantations including olive (*Olea europea*) and fruit trees. This area holds a known wild population of *T. h. hermanni* and is close to the ex-CARAPAX, the European Centre for Tortoise Conservation (which was closed in 2009).

Tortoise capture and measurement

Field sampling was carried out from April to June 2011. In order to capture study individuals, all habitat types within the entire study area were searched for a total of 92 person-hours. Once captured, animals were individually marked with a numeric code on the carapace with non-toxic paint. For each animal the following data were collected: sex, straight-line carapace length (SCL; Stubbs & Swingland 1985), carapace width and weight, together with a photo of the carapace and the plastron. In order to estimate the age of captured individuals, we counted the number of scute annuli, assuming that one growth annulus was added each year (Stubbs et al. 1984). This method is quite reliable in *Testudo* species and, despite its precision being criticized by some authors (see Bertolero et al. 2005), it is highly accurate in the aging of juveniles and subadults (Castanet & Cheylan 1979). Captured tortoises were then released back into their natural environment, after the end of our census, to avoid recapturing them.

Unexpectedly, during our surveys we captured some individuals with notches in their marginal scutes, a common marking method for tortoises

(Stubbs et al. 1984), that was used on all of the animals housed by the ex-CARAPAX (Rita Capecchi, pers. comm.). These individuals may have been intentionally introduced into the area to restock the existing population, or they may have escaped from the nearby ex-CARAPAX. To overcome this potential source of variation in the data, all analyses were performed taking into account the total population (TOT), and the two subsets of individuals, hereafter referred to as "wild" (W) and "introduced" (IN).

Demographic and biometric data (sex ratio M:F, population density, age distribution and percentage of juveniles, mean SCL and weight of males and females) were analyzed using the program Microsoft Excel. All measures reported in results are provided with ± standard error (SE). Statistical analyses were carried out to test whether these measures differed significantly between the two groups of individuals (IN and W). For these analyses, Microsoft Excel and SPSS (ver. 11.0.1) were used. All tests were two-tailed and alpha was set at 5%.

DNA sampling and genetic analysis

From each captured individual, a tissue sample was collected by means of a buccal swab; a non-invasive procedure that can also be used easily in small-sized individuals (such as hatchlings), avoiding the dangers associated with blood sampling (Wingfield 1999; Poschadel & Moller 2004; Broquet et al. 2007). Total genomic DNA was extracted from epithelial cells by combining alkaline and temperature lysis. All individuals were screened at six polymorphic microsatellite loci previously tested on *T. h. hermanni* by Cutuli et al. (2012): Leo10, Leo56, Leo76, Leo71, GmuB08 and GmuD51 [polymerase chain reaction (PCR) conditions are reported in Cutuli et al. 2012]. These loci, based on their allele sizes, also allow the identification of each *Testudo* species/subspecies and hybrid individuals (Cutuli et al. 2012). For detection of polymorphisms, the forward primer for each locus was 5′-labelled and then labelled amplicons from the six loci were divided into two sets (Leo10-NED + GmuBo8-HEX + Leo56-FAM and Leo76-NED + GmuD51-HEX + Leo71-FAM). For each set, 1–5 μL of each PCR product was combined with water in a final volume of 10 μL for successive dimensional analysis. Sizing was performed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with reference to the internal size standard ROX400, using GENOTYPER ver. 3.7 and GENESCAN ver. 3.7 (Applied Biosystems).

Linkage disequilibrium among loci was assessed using GENEPOP ver. 3.4 (Raymond & Rousset 1995), calculating *P*-values using a Markov chain with 1000 batches and 1000 iterations per batch, and applying the Bonferroni correction for multiple comparisons (significant corrected *P*-value = 0.003). GENEPOP was also used to calculate observed (*Ho*) and expected (*He*) heterozygosity; deviations from Hardy-Weinberg Equilibrium (HWE) for each locus in TOT, W and IN were assessed using a Markov chain with 1000 batches and 1000 iterations per batch. *F-statistics* were calculated using GENEPOP; inbreeding coefficient *Fis* was calculated according to Weir and Cockerhan (1984), using Markov chain with 1000 batches and 1000 iteration per batch. Allele frequencies were computed for TOT, W and IN using the software GENETIX ver. 4.05.2 (Belkhir et al. 1996), while FSTAT ver. 2.9.3 (Goudet 1995) was used to calculate allelic richness for each locus. Differences in allelic frequency between W and IN subsets of individuals were calculated with CHIFISH software (Ryman 2006). Finally, ML-Relate (Kalinowski & Taper 2006) was used to evaluate the extent of relatedness (which is correlated with inbreeding risk) in TOT, W and IN.

Results

Demographic structure

We captured a total of 62 tortoises, 34 of which were adult females, 25 adult males and three juveniles (i.e. not clearly sexually dimorphic). Taking into account the presence/absence of scute notches, the total population (TOT) was divided into two subsets; 27 individuals were placed in the W subset (9 males, 15 females and 3 juveniles) and 35 individuals were placed in the IN subset (16 males and 19 females). The sex ratios in TOT, W and IN were 0.73, 0.6 and 0.84, respectively. We did not observe significant differences in male/female ratio in the TOT group (Yates corrected CHI square observed vs. expected $x^2 = 0.442$, df = 1, $p = 0.51$) or in the IN ($x^2 = 0.01$, df = 1, $p = 0.9$) and W subsets ($x^2 = 0.34$, df = 1, $p = 056$). No significant differences were recorded when comparing the sex ratios of the two subsets of IN and W individuals ($x^2 = 0.13$, df = 1, $p = 0.72$).

The full sample of 62 individuals corresponded to a density of approximately 5.16 ind/ha within the study area, whereas in the W subset (27 individuals) population density was 2.25 ind/ha. We should mention that although precise population density estimates could not be calculated since the population was open and sampling was uneven, we found very few new individuals towards the end of the census. Thus, it is reasonable to assume that most of the

tortoises present in the area were detected during our survey and that the total sample captured was a close approximate of the actual population (as suggested in Stubbs & Swingland 1985).

Most individuals (56.45% of TOT) were aged between 11–15 years (i.e. presented 11–15 scute annuli); furthermore, the population appeared to lack juveniles and very old individuals (only 4.84% of TOT individuals were less than 5 years old, and only 3.23% were more than 20 years old). No significant difference was found in age estimates between females and males, 13.32 (± 0.58) and 13.96 (± 0.74) years old respectively ($t = 0.69$, df = 57, $p = 0.49$). Moreover, W individuals were slightly older than IN individuals (13.67 ± 1.02 and 12.69 ± 0.47 years old respectively), but this difference was not significant ($t = -0.95$, df = 60, $p = 0.3$).

The ratio of juveniles to adult females, which was proposed as an index of population stability in *T. hermanni* (Hailey et al. 1988), was very low when considering both TOT females (0.08) and only W females (0.2).

Within the TOT group, females were significantly larger than males, with an SCL of 143.5 mm (± 2.4 mm) and 120.2 mm (± 1.9 mm), respectively ($t = -7.45$, df = 57, $p < 0.001$). Correspondingly, females were significantly heavier than males, weighing 694.6 g (± 26.4 g) and 401.8 g (± 18 g), respectively. However, when we analyzed the W and IN subsets separately, we found no significant differences in average CL and weight between the two groups (data not shown).

Genetic data

(All loci tested were at HWE in TOT, W and IN (Table I). No linkage associations were evident from pairwise comparisons of the six loci analyzed (results are not shown).

Based on allele sizes of the analyzed microsatellites in different *T. hermanni* subspecies (Mantovani 2004; Cutuli et al. 2012), we excluded the presence of *T. h. boettgeri* and hybrid specimens within our population; only *T. h. hermanni* individuals were present in both the W and IN groups.

Data regarding genetic variability in TOT, W and IN are reported in Table II.

A comparison of the two subsets of individuals revealed that private alleles were present in W at loci Leo56, GmuB08 and GmuD51, and in IN at loci Leo10 and GmuB08. Considering all tested loci, CHIFISH analysis did not reveal significant genetic differentiation between the two groups of individuals, either by the Fisher's method ($x^2 = 13.75$, df = 12, $p = 0.31$) or by the Pearson's method ($x^2 = 17.3$, df = 14, $p = 0.24$). *Fis* values obtained using GENEPOP were not significantly different from 0 in any of the three groups (TOT: Fis = –0.012; W: Fis = –0.045; IN: Fis = 0.009).

Table I. Expected (He) and observed (Ho) heterozygosity for each locus in the total population (TOT), native individuals (W) and introduced individuals (IN). P = Hardy-Weinberg probability test (*P < 0.05).

Locus	TOT (n = 59)			W (n = 24)			IN (n = 35)		
	Ho	He	P	Ho	He	P	Ho	He	P
Leo10	0.5593	0.6044	0.78	0.6250	0.5842	0.52	0.5143	0.6232	0.08
Leo56	0.6250	0.5738	0.83	0.6364	0.6110	0.89	0.6176	0.5492	0.62
Leo71	0.1552	0.1444	1.00	0.1667	0.1560	1.00	0.1471	0.1383	1.00
Leo76	0.5254	0.5037	0.80	0.4583	0.5098	0.69	0.5714	0.5068	1.51
GmuB08	0.4407	0.4510	0.87	0.4167	0.3573	1.00	0.4571	0.5106	0.65
GmuD51	0.3729	0.3690	1.00	0.500	0.4672	1.00	0.2857	0.2882	1.00

Table II. Polymorphism analysis for each locus in the total population (TOT, n = 59), native individuals (W, n = 24) and introduced individuals (IN, n = 35). Na = numbers of alleles, A = allelic richness, Np = number of private alleles.

Locus	TOT				W				IN			
	Na	A	Np	Size range	Na	A	Np	Size range	Na	A	Np	Size range
Leo10	4	3.373	/	178–194	3	3	0	178–194	4	3.629	1	178–194
Leo56	4	3.377	/	201–209	4	4	1	201–209	3	2.96	0	201–205
Leo71	2	1.989	/	125–127	2	2	0	125–127	2	1.996	0	125–127
Leo76	2	2	/	116–118	2	2	0	116–118	2	2	0	116–118
GmuB08	5	3.979	/	209–221	4	3.911	1	209–221	4	3.865	1	209–218
GmuD51	3	2.373	/	129–163	3	2.917	1	129–163	2	2	0	129–145
Mean	3.33	2.85	/	/	3	2.97	0.5	/	2.83	2.74	0.33	/

Table III. Estimates of relatedness in the total population (TOT), native individuals (W) and introduced individuals (IN). PO = parent-offspring, FS = full siblings, HS = half siblings, UN = unrelated.

	PO	FS	HS	UN
TOT	13.9%	8.7%	11.4%	66%
W	12.3%	10.5%	7.6%	69.6%
IN	11.4%	9.8%	11.1%	67.7%

Percentages of relatedness obtained with ML-relate for TOT and for the IN and W subsets are reported in Table III. Unrelated individuals were estimated to comprise 66% of TOT, 69.6% of W and 67.7% of IN.

Discussion

Since the 1980s, Italian populations of *Testudo hermanni hermanni* have progressively declined, even within natural reserves and parks. For example, Zuffi and Foschi (2007, unpublished report) reported that the population of Pineta Granducale within the Parco Regionale dell'Uccellina (South Tuscany) hosted 0.6 individuals/ha, which is 10-fold less than the population density recorded 20–30 years earlier (see Paglione & Carbone 1990). Reinforcement programs and demographic and genetic studies of natural populations are therefore necessary. Our study takes place within this framework, and it focuses on one of the few natural extant populations of *T. h. hermanni* in Tuscany.

The presence of introduced, intentionally or accidentally, individuals from the ex-CARAPAX in the survey area could have influenced the demography and genetic structure of TOT population. In particular, uncontrolled translocation events could severely compromise the genetic identity of the local population. For this reason, our analyses primarily aimed to verify if the past reinforcement had an effect on the genetic variability of the original population and if hybridization with non-autochthonous individuals occurred.

Firstly, the population sampled during this study was characterized by a density of approximately 5 ind/ha, a value rather high with respect to other Tuscan populations (Zuffi & Foschi 2007, unpublished report), and doubled by the population reinforcement (density calculated for W individuals only was approximately 2 ind/ha). We should note that this past introduction of individuals, if intentional, taking into account the local genetic identity of the population, could have increased genetic variability and reduced the deleterious effects of inbreeding, which can severely affect isolated and small populations (Frankham 1995).

In this frame, genetic analyses of six microsatellite loci revealed that the TOT population was in HWE; additionally, the two groups IN and W also presented good levels of heterozygosity. *Fis* values and results obtained with ML-relate also confirmed that inbreeding processes appear to be absent in the population at the present time.

CHIFISH analysis did not reveal any significant genetic differentiation between the two groups of individuals. Moreover, private alleles found in IN (192 at Leo10 and 218 at GmuB08) were included in the size range of W. Unfortunately, no microsatellite data are available for Tuscan populations; however, our comparisons between the two groups indicate that the introduction of individuals into the native population did not cause significant changes in its genetic pool. These results also indicate that introduced individuals may have a similar geographical origin to the native individuals. This is supported by fact that we found no significant differences in body measurements, specifically average SCL and weight, and age between native and introduced individuals. Italian populations display a relatively high variability in their morphometrics, sex ratio, average age of individuals and density (Table IV).

In the Massa Marittima population, both the IN and W subsets and the TOT group had sex ratios not significantly different from 1:1, as commonly found in this species (Table IV).

Most of the sampled individuals were adults aged 11 to 15 years, and both the W and IN subsets presented a similar age distribution, with an average age of approximately 13 years. We found very few young individuals and, accordingly, the index of population stability (ratio of juveniles to adult females; Hailey et al. 1988) was very low; this result accords with other studies (Table IV). The percentage of juveniles recorded in different studies and areas is, however, highly variable, ranging from less than 0.01% (Loy et al. 2007) to more than 70% (Hailey 2000). This is probably due to the difficulties associated with detecting juveniles compared to adults, particularly in areas with thick vegetation cover. However, these low percentages of juveniles could indicate that the study site is disturbed by human activities, or by the large presence of predators such as wild boars, which are known to consume eggs and hatchlings. For our study site the latter hypothesis is more likely; although we do not have any data on egg predation rates, tracks of wild boars were observed frequently.

As observed in other populations (see Table IV; Cheylan 1981; Stubbs & Swingland 1985), males

Table IV. Comparison of available morphometric, density and demographical data between some Italian populations of *Testudo hermanni hermanni*. Data from the present study refer to all individuals sampled (TOT).

Study area	Massa Marittima, Tuscany	Maremma Natural Park, Tuscany	Monti Nebrodi, Sicily	Asinara Island, Sardinia	Bosco della Mesola, Emilia Romagna	Molise	Tolfa Mountains, Latium
References	Present study	Paglione and Carbone (1990)	Tomasetti and Bossuto (2000)	Corti and Zuffi (2003)	Mazzotti et al. (2007), Mazzotti (2004)	Loy et al. (2007)	Filippi et al. (2010)
Population density (specimens/ha)	5.16	3.44–5.1	11.8	4.88	0.94	21.2	1.2
Sex ratio (M:F)	0.73:1	1:1	1:2.37	1:1	1:1	1:1	0.6:1
Average age	F 13.32 ± 0.58	28.5% of individuals > 20 years	47.6% of individuals > 20 years	90% of individuals > 20 years	90% of individuals	F 16.93 ± 2.46	F 22.5 ± 5.39
	M 13.96 ± 0.74				> 20 years	M 16.88 ± 2.44	M 20.56 ± 4.62
% juveniles	4.84%	22%	6.80%	0.04%	2.07%	0.01%	50%
Straight carapace length, SCL (mm)	F 143.5 ± 0.24	F 146.6	F 158.6	F 168.26 ± 8.33	F 184.4 ± 12.1	F 142.11	F 148.9 ± 14.51
	M 120.2 ± 0.19	M 128.8	M 130.1	M 145.89 ± 7.7	M 162.2 ± 9.3	M 120.09	M 132.1 ± 8.57

were consistently significantly smaller than females in all of the morphometric measurements, which is a consequence of sexual size dimorphism in this species. Finally, average straight carapace lengths of sampled individuals accord with those measured in other Italian populations, with the exceptions of populations in Sardinia (Corti & Zuffi 2003) and Bosco della Mesola (whose body sizes are the largest recorded in Italy; Mazzotti 2004; Mazzotti et al. 2007). It should be mentioned that the presence of many *T. h. boettgeri* individuals has been revealed in Bosco della Mesola (Mantovani 2004), and hybridization with *T. h. hermanni* could not be excluded (Mazzotti et al. 2007). Indeed, this may have led to an overestimate in the average dimensions of individuals captured in that area, as *T. h. boettgeri* is characterized by remarkably larger body size measurements than *T. h. hermanni* (Bour 2004). In conclusion, based on the results of our study, the study population of *T. h. hermanni* presently has a good level of genetic variability, which will act to protect this population from the long-term deleterious effects of inbreeding processes. Thus, the past reinforcement (intentional or accidental) does not seem to have compromised the genetic identity of the population, but, conversely, likely played a role in increasing the genetic diversity of the population.

Notably, the number of juveniles detected in this area was remarkably low, which could indicate the presence of disturbing phenomena and could represent a long-term risk for population stability. Strategies for species conservation that incorporate periodic monitoring of the population status and dynamics should be carried out. Some intervention strategies to increase hatchling success (e.g. localize and protect the sites where females lay eggs with nets in order to prevent predation) could be useful to increase population vitality.

The combination of morphometric, demographic and genetic data is fundamental in the accurate assessment of population status, planning of long-term conservation strategies and development of guidelines to monitor *T. h. hermanni*. In this respect, our study provides an important contribution to the knowledge of *T. h. hermanni* status in Italy.

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