Italian Journal of Zoology

ISSN: 1125-0003 (Print) 1748-5851 (Online) Journal homepage: https://www.tandfonline.com/loi/tizo20

Mitochondrial dna variation and divergence in three Hermann's tortoise *(testudo hermanni)* populations

Luca Mirimin , Cristiano Vernesi , Cristiano Bertolucci , Stefano Mazzotti & Giorgio Bertorelle

To cite this article: Luca Mirimin , Cristiano Vernesi , Cristiano Bertolucci , Stefano Mazzotti & Giorgio Bertorelle (2004) Mitochondrial dna variation and divergence in three Hermann's tortoise *(testudo hermanni)* populations, Italian Journal of Zoology, 71:S2, 199-201, DOI: 10.1080/11250000409356635

To link to this article: https://doi.org/10.1080/11250000409356635

Ital. J. Zool., *Suppl. 2*: 199-201 (2004)

Mitochondrial DNA variation and divergence in three Hermann's tortoise (*Testudo hermanni*) populations

LUCA MIRIMIN
CRISTIANO VERNESI
CRISTIANO BERTOLUCCI
Dipartimento di Biologia, Università di Ferrara,
Via Borsari 46, I-44100 Ferrara (Italy)

STEFANO MAZZOTTI
Museo Civico di Storia Naturale,
Via De Pisis 24, I-44100 Ferrara (Italy)
E-mail: *conszool@comune.fe.it*

GIORGIO BERTORELLE
Dipartimento di Biologia, Università di Ferrara,
Via Borsari 46, I-44100 Ferrara (Italy)
E-mail: *ggb@unife.it*

ABSTRACT

A 12SrRNA mitochondrial fragment was sequenced in 47 *Testudo hermanni* individuals from three different populations in northeastern Italy, Sicily, and Spain. All these locations fall within the distribution range usually recognized for the *T. h. hermanni* subspecies. Seven different sequences were identified, which clearly separate the highly variable northern Italian group from the homogenous individuals in Sicily and Spain. More interestingly, almost all the northern individuals had a DNA sequence identical or very similar to three sequences belonging to individuals from the Balkans and Greece, and morphologically classified as *T. h. boettgeri*.

KEY WORDS: *Testudo hermanni* - Genetic variation - mtDNA - Subspecies.

ACKNOWLEDGEMENTS
We thank Albert Bertolero (Parc Natural del Delta de l'Ebre, Spagna) for providing the Spanish samples, Antoinette van der Kuyl (Academic Medical Center, University of Amsterdam) for sharing her sequence data, and the rangers at 'Bosco della Mesola' reserve for their help and assistance.

(Received 29 November 2003 - Accepted 18 March 2004)

INTRODUCTION

Hermann's tortoise (*Testudo hermanni* Gmelin, 1789) is widespread in the Mediterranean regions from Catalonia to the Balkans, and to European Turkey (Bour, 1997). Two subspecies are recognised, *T. h. hermanni* and *T. h. boettgeri*, inhabiting the western (Spain, France, and Italy) and the eastern (all the other countries) part of the distribution range, respectively (Bour, 1997).

Recent human activities severely affected the distribution and the geographic structure of this species (Stubbs & Swingland, 1984). The urbanization of the coastline fragmented the habitat into small patches, and uncontrolled translocation of individuals probably resulted in hybridisation between genetically differentiated populations or subspecies. The real effects of these factors on the survival of this species are not known. However, several populations at least in Italy appear to display an increase in average age (Paglione & Carbone, 1990; Tomasetti G., 1997, Ph. D. Thesis, University of Catania; Mazzotti, 2004), and the potential risks of extinction were formally recognized about 20 years ago when the terrestrial tortoise was included in the list of strictly protected species by the Bern Convention.

In the present paper we present the first population genetics analysis of three groups of *T. h. hermanni* individuals from protected areas in Italy and in Spain. The main goal was to describe the levels of genetic variability and divergence within and between three isolated populations, thus providing an initial genetic framework useful for identifying conservation priorities and for developing management strategies.

MATERIALS AND METHODS

Blood samples were drawn from the femoral vein of 47 individuals living in three protected areas: the Mesola Wood Natural Reserve (close to Ferrara, northeastern Italy; $n = 20$), the Nebrodi Mountains Regional Park (close to Messina, Sicily; $n = 11$), and the Ebro Delta Natural Park (close to Tarragona, Spain; $n = 16$). Spanish samples are from individuals reintroduced in the Park from the surrounding areas (Bertolero *et al.*, 1995).

DNA was extracted from the blood samples using the phenol-chloroform procedure. A 395 base pair fragment of the 12S rRNA gene, located in the mitochondrial genome, was amplified via PCR using the following primers: 12S-L01091 (5'-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3') and 12S-H01478 (5'-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3') (Alvarez *et al.*, 2000). The purified PCR products were sequenced with the Big Dye Terminator Kit (Applied Biosystem), following the manufacturer's instructions; electrophoresis runs were accomplished by means of an ABI Prism 377 automated sequencer (Perkin Elmer Corporation, Norwalk, CT, USA).

Sequences were aligned by ClustalW 1.81 and re-checked by eye. The matrix of nucleotide differences between different alleles (also called haplotypes) was represented with a phylogenetic tree reconstructed using the Neighbor-Joining algorithm and the Phylip software. The homologous sequences of three subspecies of *T. graeca* available in GenBank (accession numbers: AF 175330, AF 175331, AF 175329) were used to root the tree. Simple measures of genetic variability, within and between populations, were computed using the Arlequin software (Schneider *et al.*, 2000).

RESULTS AND DISCUSSION

Seven polymorphic sites were identified, which define seven haplotypes (numbered from 1 to 7 in Table I). Compared to similar studies in Chelonians (Caccone *et al.*, 1999; Alvarez *et al.*, 2000; Palkovacs *et al.*, 2002), the values of gene diversity, 0.62, and nucleotide diversity, 0.47%, observed in the DNA coding region we analysed, suggest that this species retains a relatively high level of genetic variation, despite a probable reduction of census size in recent times. However, the demographic history of the three populations here considered was probably different: gene diversity and nucleotide diversity (whose expected values are independent of sample size (Tajima, 1983) in the Mesola Wood appear in fact higher than in the sample from Spain and in the monomorphic sample from Sicily (see Table II).

The phylogenetic tree in Fig. 1 identifies two clusters of haplotypes separated by three substitutions: cluster A, which includes the haplotypes from 1 to 4, and cluster B which includes the haplotypes from 5 to 7. Sequences belonging to cluster A are found only in the northeastern population of Mesola Wood, whereas all the individuals sampled in Spain and Sicily have haplotypes grouping together in cluster B. The genetic divergence of the Mesola Wood sample is also reflected in the pairwise F_{st} distances. In fact, F_{st} reaches the values of 0.81 and 0.83 (significantly higher than 0, $P < 0.05$) when this group is compared to the Sicilian and the Spanish samples, respectively, whereas F_{st} is not significant in the comparison between Sicily and Spain.

A possible explanation for the divergence between the Mesola Wood and the Sicily-Spain populations emerges when four additional sequences are considered (see Fig. 1). The sequences of three *T. h. boettgeri* individuals from Albania, Greece, and Macedonia (van der Kuyl *et al.*, 2002, kindly provided by the authors), fall well within the A cluster, with two of these sequences being actually identical to two sequences found in the Mesola Wood population. On the other hand, a fourth sequence available in GenBank from an Italian individual classified as *T. h. hermanni* (van der Kuyl *et al.*, 2002, kindly provided by the authors) clearly belongs to the B cluster. In other words, cluster A and cluster B seem to overlap with the *boettgeri* and the *hermanni* subspecies, respectively, with the northeastern Italian group genetically included in the *boettgeri* cluster.

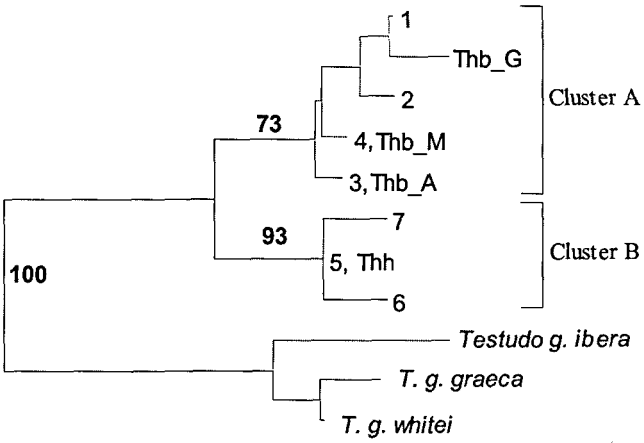

Fig. 1 - Neighbour-joining tree of the haplotypes (numbered as in Table I), rooted with the sequences of three subspecies of *T. graeca*. Four additional sequences (van der Kuyl *et al.*, 2002) from an Italian individual classified as *T. h. hermanni* (Thh) and three *T. h. boettgeri* individuals sampled in Greece (Thb_G), Albania (Thb_A), and Macedonia (Thb_M), are included in the tree. The bootstrap values of the most relevant nodes are reported.

TABLE I - *List of polymorphic sites identified in the different haplotypes, numbered starting from 1 in the sequences aligned with the* T. g. graeca *haplotype in GenBank (accession number AF175330). The number of individuals with each haplotype, and their geographic origin, is indicated in the last column.*

	1222333 6112335 8141087		
1	CTGCTCA	2	Mesola
2	CTGCCCA	1	Mesola
3	CTGCCCG	15	Mesola
4	CTGCTCG	1	Mesola
		13	Ebro
5	CTAACTG	11	Nebrodi
		1	Mesola
6	CCAACTG	2	Ebro
7	GTAACTG	1	Ebro

The genetic classification of almost all individuals (95%) from Mesola Wood within the *boettgeri* cluster, despite their morphological classification as *T. h. hermanni* and the location of this sample within the distribution range of the *hermanni* subspecies, call for an explanation. The Mesola Wood population is usually regarded as a native group, only partially affected by human activities (Mazzotti, 2004). Sporadic releases of animals of unknown origin are possible, but a massive translocation from the Balkans is not documented and the hypothesis that the present day population is almost exclusively constituted by released *boettgeri* animals or their descendents appears, therefore, very unlikely. Our data thus suggest that the geographic distribution of the different subspecies of *T. hermanni* should be reconsidered, possibly locating the suture zone within the Italian peninsula and more to the West than previously believed.

Finally, the high genetic divergence between two Italian populations suggests that the genetic structure in Italy might be substantial. If confirmed by the analyses of additional individuals, populations, and genetic markers, these results should be taken into account by any program of reintroduction and management of this species.

TABLE II - *Genetic variation within each sample and in total. Gene diversity corresponds to the expected heterozygosity (*sensu *Nei, 1987). Nucleotide diversity is the per nucleotide average percentage of differences between two sequences.*

	sample size	no. of haplotypes	no. of polymorphic sites	gene diversity	nuclotide diversity (%)
Mesola Wood	20	5	5	0.44	0.21
Nebrodi Mount.	11	1	0	0	0
Ebro Delta	16	3	2	0.34	0.09
Total	47	7	7	0.62	0.47

REFERENCES

Alvarez Y., Mateo J. A., Andreu A. C., Diaz-Paniagua C., Diez A., Bautista J. M., 2000 - Mitochondrial DNA. Haplotyping of *Testudo graeca* on both continental sides of the straits of Gibraltar. J. Hered., 91: 39-41.

Bertolero A., Carretero M. A., Llorente G. A., Martinez A., Montori A., 1995 - The importance of introductions in species conservation: the case of *Testudo hermanni* in the Ebro Delta Natural Park (NE Spain). Proc. Int. Congr. Chelonian Conserv., Gonfaron, France, pp. 187-191.

Bour R., 1997 - *Testudo hermanni* Gmelin, 1789. *In*: J. P. Gasc, A. Cabela, J. Crnobrnja-Isailovic, D. Dolmen, K. Grossenbacher, P. Haffner, J. Lescure, H. Martens, J. P. Martinez Rica, H. Maurin, M. E. Oliveira, T. S. Sofianidou, M. Veith, & A. Zuiderwijk (eds), Atlas of amphibians and reptiles in Europe. S.E.H. & Mus. Natl. Hist. Nat. (IEGB/SPN), Paris, pp. 178-179.

Caccone, A., Gibbs J. P., Ketmaier V., Suatoni E., Powell J. R., 1999 - Origin and evolutionary relationships of giant Galàpagos tortoises. Proc. Natl. Acad. Sci. USA, 96: 13223-13228.

Mazzotti S., 2004 - The Hermann's tortoise (*Testudo hermanni*): current distribution in Italy and ecological data on a population from the North Adriatic coast. Ital. J. Zool., 71: in press.

Nei M., 1987 - Molecular evolutionary genetics. Columbia University Press, New York.

Paglione G., Carbone M., 1990 - Biologia della popolazione di *Testudo hermanni* nel Parco della Maremma. *In*: G. Malacarne, L. Levi & A. Casale (eds), Atti VI Conv. Naz. Ass. 'A.Ghigi', Mus. Reg. Sci. Nat. Torino, pp. 197-199.

Palkovacs E. P., Gerlach J., Caccone A., 2002 - The evolutionary origin of Indian Ocean tortoises. Mol. Phylogen. Evol., 24: 216-227.

Schneider S., Roessli D., Excoffier. L., 2000 - Arlequin vers. 2.0, a software for population genetic data analysis. University of Geneva, Switzerland.

Stubbs D., Swingland I. R., 1984 - The ecology of the Mediterranean tortoise (*Testudo hermanni*): a declining population. Can. J. Zool., 63: 169-180.

Tajima F., 1983 - Evolutionary relationships of DNA sequences in finite populations. Genetics, 105: 437-460.

Van Del Kuyl A. C., Ballasina, D. L., Dekker, J. T., Maas J., Willemsen, R. E., Goudmit J., 2002 - Phylogenetic relationships among the species of the genus *Testudo* (Testudines: Testudinidae) inferred from mitochondrial 12S rRNA gene sequences. Mol. Phylogen. Evol., 22: 174-183.