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# Genetic characterization of over hundred years old *Caretta caretta* specimens from Italian and Maltese museums

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**Abstract.** Museum collections have proven to be a useful source of samples for the reconstruction of evolutionary history and phylogeography of many taxa. This study was aimed at assessing the success rate in a genetic analysis of historical material, in order to explore the feasibility and eventually begin the diachronic description of *Caretta caretta* stocks in Italian and Maltese coastal waters. The endangered status of the species and the difficulty to study it in the wild make its common occurrence in Italian museum collections a valuable resource. We used minimally invasive methods to collect biological material from specimens dating from the end of the 19th century to 2003, belonging to four museums. As a control for amplification success and absence of cross-contamination, four dinucleotide microsatellite loci of different average length (Cc7, Cc141, Cm72 and Cm84) were typed. All individuals with two or more successfully amplified microsatellites (36%) displayed distinct genotypes, thus excluding contamination as a major flaw in the data. We then targeted 380 bp of the mtDNA control region, which allows comparisons with many living populations worldwide and represents the optimal marker for the philopatric behaviour of this species. All individuals but 2 were successfully sequenced. Haplotype CC-A2 was found in 68 individuals, whereas CC-A1 and CC-A3 were found only in one Tyrrhenian and one S-Adriatic specimens, respectively. This study demonstrates that genetic analysis of marine turtles from museum specimens is feasible. Data generated from cohorts of several generations ago are potentially useful for research and dissemination purposes.

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## INTRODUCTION

Museum collections have proven a useful source of samples for the reconstruction of the evolutionary history and phylogeography of many taxa (Wandeler et al., 2007). So far genetic analysis of museum specimens of turtles has been scanty (e.g. Parham et al., 2006; Feldman & Parham, 2004; Parham et al., 2004; Austin et al., 2003), yet revealing relevant hidden quotas of diversity (Poulakakis et al., 2008). Studies on marine turtle specimens from museum collections in Italy have never been conducted before, despite the availability of many chelonian collections (e.g. Mizzan, 1994; Mazzotti, 2010).

Genotyping of specimens from museums and other natural history collections is exposed to low success rate and a high risk of contamination, mainly due to low non-degraded DNA yields (Taberlet and Luikart, 1999; Wandeler et al., 2007). Time elapsed since preservation (specimen age) is not the only important factor affecting DNA yield and quality. Different preservation techniques may negatively influence the possibility to extract, amplify and sequence DNA. In addition, nucleotide misincorporation during amplification of damaged DNA or allelic dropout in microsatellite analysis can lead to the over- or under-estimation of genetic diversity in past populations, respectively (Wandeler et al., 2007). However, providing that these factors are kept under control, the sampling of museum-preserved material has the advantages of being promptly available and of reducing the needs for tissues from living individuals. This may be particularly valuable for species that are both endangered and difficult to study in the field.

We address here the most common marine turtle in the Mediterranean, *Caretta caretta,* which is widely represented in Italian museum collections. This species is suffering a dramatic reduction in the size of its foraging and reproductive stocks (current IUCN classification: Endangered). Thus, genetic analysis of museum samples may be important to catch changes in the pattern of diversity across generations, associated with population fluctuations and/or environmental shifts. We then aimed at assessing the success rate in a genetic analysis of historical *C. caretta* material through minimally invasive sampling methods, and eventually commence the description of the diachronic composition of *C. caretta* stocks in Italian and Maltese coastal waters.

## MATERIALS AND METHODS

The samples contributed by different museums, partitioned according to their geographic origin and preservation period, are summarized in Table 1.

The preserved starting material for DNA preparation consisted of dried bones (skulls, vertebrae, carapaces and nails), specimens in ethanol and ethnographic artifacts (painted carapaces). Each specimen was labeled with the museum's catalogue number and/or CITES ID. Only one source

of material was used for each specimen. Sample collection and subsequent molecular analyses were carried out in controlled conditions.

For dried material (including ethnographic artifacts), powder was obtained by means of minimally invasive drilling (drill bit diameter = 6 mm) on the ventral side of carapace or from hidden sites of bones and skulls, after discarding the superficial part; holes were later sealed with bee wax. For internal parts, remains of meninges and spinal cord were collected by gentle grasping the inner side of neural arches of neck and trunk vertebrae of preserved skeletons, carapaces and skulls. For liquid-preserved material a few $mm^3$ tissue biopsy was obtained from the front flipper with a sterile blazer, after discarding the superficial part (epidermis) (Table 2).

While the majority of specimens could be measured and assigned to three major age classes, information on sex was available only if recorded at the time of collection or for large, stuffed and mounted whole animals (Table 3).

No more than 10 samples were extracted simultaneously (NucleoSpin Tissue kit, Macherey Nagel GmbH, Duren, Germany), to decrease the risk of cross-contamination. As a control for amplification success and absence of cross-contamination, typing of DNAs at four dinucleotide microsatellite loci of different average length (Cc7, Cc141, Cm72 and Cm84, in order of increasing molecular weight) (Carreras et al., 2007, and conditions reported therein) was performed. This choice was dictated by the high polymorphic content of the loci and the possibility of comparing our results with literature data.

A fragment of 380 bp of the mitochondrial (mtDNA) control region, obtained using the primers TCR5 and TCR6 (Norman et al. 1994) was amplified (annealing Temp. 52°C; 32-36 cycles) and sequenced. A mtDNA fragment 815 bp long was sequenced from a single sample carrying haplotype CC-A1, in the conditions described by Garofalo et al. (2009).

In all cases sequencing was carried out with standard protocols for an ABI3100 Avant automated sequencer on both strands, with the same primers used for PCR. Electropherograms were visually inspected and sequences aligned with the BioEdit software (Hall, 1999). Mitochondrial DNA haplotype nomenclature follows that reported by the Archie Carr Center for Sea Turtle Research (http://accstr.ufl.edu/ccmtdna.html). In each lot of samples subjected to PCR, negative controls were performed. PCR and sequencing of the same sample was performed twice (Cooper and Poinar, 2000), by the same researcher.

## RESULTS

### *Sample composition*

The sample series reported here represent the totality of individuals available in the four museum collections analyzed (listed in Table 1) up to 2009. Most of the samples had been collected within the last 50 years (Table 1), whereas 12.5% (9/72) are older than 1900. The majority of specimens consisted of dried-preserved parts (Table 2), i.e. entire skeletons or isolated carapaces and crania. The large osteological collections of Rome and Venice contributed the majority of samples, leading likely to an overrepresentation of individuals from the Tyrrhenian and N-Adriatic seas in the overall series (Table 1).

The proportion of juveniles and adults is close to 50:50 in all geographic samples. Subadults were found only among Tyrrhenian specimens. In addition, while an even proportion of sexes was found among the N-Adriatic specimens, the Tyrrhenian adults of known sex were all females (Table 3).

**Table 1.** Sampling coverage and results according to the date of collection. Each entry reports the number of individuals successfully amplified at least at two microsatellite loci out of the total sampled from each institution. Source institution codes are: MCZRM (Museo Civico di Zoologia, Rome), MSNVE (Museo di Storia Naturale di Venezia, Venice), MZUBA (Museo di Zoologia, Università di Bari), NMNHM (National Museum of Natural History, Mdina, Malta), n.a. = not assigned.

| Source | Geographic origin | Before 1900 | 1900 -1950 | 1950 - 2003 | n. a. | Total |
|---|---|---|---|---|---|---|
| MCZRM | Tyrrhenian | 2 / 8 | - | 10 / 29 | 2 / 4 | 14 / 41 |
| MSNVE | N-Adriatic | - | 1 / 3 | 4 / 8 | 2 / 10 | 7 / 21 |
| MZUBA | S-Adriatic | 0 / 1 | - | 3 / 4 | - | 3 / 5 |
| NMNHM | Malta | - | - | 2 / 5 | - | 2 / 5 |

**Table 2.** Amplification success relative to preservation method of the original samples, shown as the number of individuals successfully amplified (at least at two microsatellite loci) out of all available individuals for each method.

| Groups | Dried bones[a] | Fluid specimens | Internal parts |
|---|---|---|---|
| Tyrrhenian | 14 / 41 | - | - |
| N-Adriatic | 5 / 19 | 2 / 2 | - |
| S-Adriatic | 2 / 3 | - | 1 / 2 |
| Malta | 2 / 4 | - | 0 / 1 |

[a]: includes ethnographic artifacts.

**Table 3.** Number of samples analyzed for each location and life stage (n.a. = not assigned). Individuals were considered juveniles if CCL < 50 cm, subadults if between 50 and 60 cm, and adults if > 60 cm. Only adults can be externally sexed.

| Sampling location | N° of samples | Life stage | | | | Sex | |
|---|---|---|---|---|---|---|---|
| | | Juveniles | Subadults | Adults | n. a. | F | M |
| Tyrrhenian | 41 | 15 | 11 | 15 | - | 7 | - |
| N-Adriatic | 21 | 9 | - | 8 | 4 | 2 | 2 |
| S-Adriatic | 5 | 2 | - | 1 | 2 | - | - |
| Malta | 5 | 2 | - | 3 | - | - | - |

*Control amplifications.* Loci Cc7, Cc141, Cm72 and Cm84 were amplifiable in 35, 29, 23 and 16 individuals, respectively (Table 4). As expected for fragmented DNA, longer loci displayed a reduced amplification rate [called “molecular behavior” in Cooper and Poinar, 2000]. Overall, 26 out of 72 samples could be amplified for at least two microsatellites, with replicable results. Tables 1 and 2 show the success rate in amplifying microsatellite loci according to different variables. Recent samples (1950-2003) produced good results in about 50% of instances, i.e. slightly better than average. Within this category six samples were older than 10 years. Moreover, amplification of DNA from material dating back to before 1950 was also possible. As to preservation methods, fluid-preserved speci-

**Table 4.** Allelic range at each microsatellite locus by location. Each entry reports the minimum and maximum allele size in bp (above) and the number of individuals successfully amplified (below).

| Groups | Cc7 | Cc141 | Cm72 | Cm84 |
|---|---|---|---|---|
| Tyrrhenian | 167-203<br>n = 17 | 187-207<br>n = 14 | 223-249<br>n = 12 | 313-323<br>n = 10 |
| N-Adriatic | 163-197<br>n = 10 | 197-203<br>n = 7 | 223-247<br>n = 7 | 313-323<br>n = 3 |
| S-Adriatic | 171-193<br>n = 4 | 183-203<br>n = 4 | 223-231<br>n = 2 | 315-323<br>n = 1 |
| Malta | 171-191<br>n = 4 | 187-207<br>n = 4 | 223-241<br>n = 2 | 313-323<br>n = 2 |

mens too yielded an adequate DNA quantity and quality for genetic analysis. However, bone is usually the preferred material for this kind of analysis, due to the high degree of conservation and the lower risk of external contamination.

All individuals with two or more successfully amplified microsatellites displayed distinct genotypes. This excluded contamination as a major flaw in the data.

Allele ranges at all the four STR loci (Table 4) fitted those of Mediterranean nesting populations (Carreras et al., 2007) and do not support a relevant presence of Atlantic alleles (reported in Moore and Ball, 2002). As to a more precise discrimination among different sources in the Mediterranean, this is currently not possible due to the lack of geographically-confined allele sizes (Carreras et al., 2007).

*Genetics. mtDNA.* PCR amplification of the mtDNA control region was attempted in all samples, in view of the larger representation of this target in cellular material (Mulligan, 2005). In no instances negative controls produced amplified material at the level of sensitivity of agarose gels. Overall 70 individuals, i.e. all but two (one from Venice [1988] and one from Bari [Muzac collection, 1742]), were successfully sequenced for the 380 bp in mtDNA in a single PCR reaction. Sequencing of both strands produced traces overlapping for 350 positions. In all cases a perfect overlapping was obtained, showing that electropherogram interpretation was unambiguous. In all cases the results of two independent DNA extracts of the same specimen or PCR reactions of the same extract produced identical results.

Haplotype CC-A2 was found in 68 individuals, whereas CC-A1 and CC-A3 were found only in one Tyrrhenian (juvenile, 2002) and one S-Adriatic (juvenile, 1980's) specimens, respectively. Haplotype CC-A3 differs from CC-A2 by a single nucleotide substitution out of the 380 bp segment scored, while haplotype CC-A1 is at 35 mutational steps from CC-A2. In order to further confirm the reliability of this latter result, the CC-A1 sample was amplified and sequenced also for a 815 bp fragment, flanking the previous one on both sides. The resulting sequence is identical to the CC-A1 subtype CC-A1.1. The fact that variation was observed only at positions already known to vary in Mediterranean and Atlantic haplotypes shows that PCR and sequencing did not introduce artifacts in our DNA series as far as mtDNA is concerned.

## DISCUSSION

Almost all individuals from our comprehensive series could be sequenced at the mtDNA control region, and the success rate of microsatellites genotyping was inversely related to the length of the targeted fragment. These results demonstrate that, adopting good laboratory practices and proper techniques, DNA contamination of old samples can be largely avoided, producing reliable genotyping results in roughly 1/3 of samples up to 50 years old. The higher rate of mtDNA genotyping, though somewhat expected, awaits further confirmation by resampling of the same specimens and testing in an independent laboratory, to fulfill the recommended guidelines (Willerslev and Cooper, 2005). We nevertheless show that genetic analysis of marine turtle museum specimens is feasible and can potentially provide data on cohorts of several generations ago.

One consequence of the complex migratory pattern of marine turtles is that many different variables may affect the genetic composition of a population stock and samples drawn from it. By controlling for the original provenance of each museum specimen (e.g. strandings, bycatch), our series can be compared with modern samples of similar origin.

In interpreting our results the strong philopatric behaviour of females must be taken into account. Under these circumstances mtDNA becomes, by and large, a marker for the geographic origin of the maternal 50% of an individual's genome. Conversely, the origin of the paternal component is uncertain, due to the loose philopatry of males to breeding grounds.

Our overall data indicate that most individuals collected along Italian coasts can be traced to a generic maternal "Mediterranean" source, given that the most common mtDNA haplotype in our series is CC-A2, also shared by all Mediterranean nesting populations. The finding of a single carrier of haplotype CC-A1, observed only in Atlantic nesting colonies, testifies the presence of sporadic juveniles from this ocean in central and western Mediterranean feeding aggregates. The individual carrying haplotype CC-A3 probably came from Turkish nesting sites, where this haplotype is common. Similar frequencies for both haplotypes have been reported by Maffucci et al. (2006).

All individuals from Malta showed haplotype CC-A2. Given that these specimens dated back to the 1970-1980's, they can potentially represent the remnants of the last generation born in the Island (a recent report of breeding in Malta, sometime in the 1960s, was reported by Deidun and Schembri, 2005). In this case, this nesting colony as well would have hosted the haplotype shared by all Mediterranean rookeries.

Several authors alerted on the limitations of molecular analyses of material with low DNA content (Cooper and Poinar, 2000; Pääbo et al., 2004; Taberlet and Luikart, 1999; Willerslev and Cooper, 2005). However, they concluded that DNA from historical specimens adds an important temporal dimension to the genetic study of endangered species. Studies of this kind remain pivotal in conservation genetics, for their power in revealing changes in population size and structure, phylogenetic relationships and to define Evolutionary Significant (and Management) Units (Wandeler et al., 2007).

If extended to additional series (for a comprehensive description of Italian Collections see Mazzotti, 2010) the combined use of genetic data and information on place and date of collection, sex, life stage and cause of death, can produce valuable knowledge on a species that is currently endangered, and is also difficult to study in the wild.

The present work is potentially relevant for the knowledge of the biology of *C. caretta*. In fact, the phylogeographic information here obtained can be easily incorporated into an "Identity Card" of the preserved specimens, that will augment their value in case of a re-examination for research purposes. Moreover, the display of the same card in exhibits will also serve dissemination purposes, by rising public interest towards genetics and conservation issues, and promoting museum collections as irreplaceable banks of the world's biodiversity.

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