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**MONOGRAPH**

# Molecular barcoding applied to the Mediterranean turtles biological matrices (Reptilia Cheloniidae)

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**ABSTRACT** *Chelonia mydas* (Linnaeus, 1758) together with *Caretta caretta* (Linnaeus, 1758) is the most representative Cheloniidae species in the Mediterranean basin. Currently, at the National Reference Centre in the "Istituto Zooprofilattico" of Sicily (Italy), damaged subjects are rehabilitated before they are released again. Clinical, physiological and molecular parameters were collected from each subject. We analysed 46 turtles which samples were collected. Species specific Cytochrome oxidase I sequences for the identification of marine turtle species were obtained. Barcoding is a new tool of classical taxonomy that allows the characterisation of living species and the differentiation of very morphologically similar species. It is a practical tool that can be used in cases of damaged samples and is also useful for taxonomical characterisation of specimen at immature development stages. In our region, in the centre of the Mediterranean area, we represent a reference centre for injured animals both stranded on the beach and captured in offshore.Turtles caught in fishing lines generally retain the fishing hooks in their throat or oesophagus, as visible by X-ray investigations. After the cure and samples collection, the animals are released into the sea. The polymorphisms could be related to the geographical distance of the turtles following different routes during their life. The large-scale sequencing of a single or few genes in taxonomic studies, denominated by species barcoding, aims at offering a practical method for species identification, as well as for providing insights into the evolutionary diversification of life.

**KEY WORDS** Turtles; Mediterranean Sea; molecular barcoding; speciation.

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## INTRODUCTION

Three species of sea turtles lives in Mediterranean Sea: the leatherback turtle, *Dermochelys coriacea* (Vandelli, 1761), the green turtle, *Chelonia mydas* (Linnaeus, 1758), and the loggerhead turtle, *Caretta caretta* (Linnaeus, 1758). Two other species of sea turtles have been occasionally reported in the Mediterranean Sea: the hawksbill sea turtle, *Eretmochelys imbricata* (Linnaeus, 1776), and the Kemp's ridley sea turtle, also called the Atlantic ridley sea turtle, *Lepidochelys kempii* (Garman, 1880) (Casale & Margaritoulis, 2010). They are considered endangered, at regional and global level, and therefore it is protected by international laws and by numerous conventions.

*Dermochelis coriacea* is a Vulnerable species following IUCN (Wallace et al., 2013), *Chelonia*

*mydas* is Endangered (Seminoff, 2004), and *Caretta caretta* is Vulnerable (Casale & Tucker, 2017). Only *Chelonia mydas* and *Caretta caretta* breed in the Mediterranean basin and the nesting areas are concentrated in the eastern half of the Mediterranean Sea. These sea turtles are found, also, with particular frequency in some rest and feeding areas (like northern Adriatic Sea, Ionian Sea, Tunisia and Libya and Spanish coasts).

*Chelonia mydas* frequents the eastern Mediterranean with the nesting sites that are principally in Turkey, Cyprus, and Syria. Foranging areas are also in Greece and Libya and green turtles are found occasionally in the Adriatic Sea, Tunisia and other areas of the western Mediterrannean Sea.

*Caretta caretta* known also as common turtle, is the most widespread species and most representative Cheloniidae family species in the Mediterranean Sea, followed by *Chelonia mydas* (Dutton, 1996; Carreras et al., 2007; Casale & Margaritoulis, 2010; Naro-Maciel et al., 2010). *Caretta caretta* is the only species of sea turtle nesting along the Italian coast. In the past, loggerhead sea turtles nesting was a regular phenomenon and relatively widespread along the coasts of the southern Italy, but over the last years, few cases of nesting have been recorded on the islands and coasts of Sicily, Sardinia, along the Ionian coast of Puglia and those of Basilicata and Calabria. However, nests are now considered sporadic or occasional, except for the Lampedusa and Linosa Islands. In the Italian coasts, their strandings are strongly influenced by the impact of massive fishing, the alteration of marine and coastal habitats and climate changes (Tamura et al., 2004). Currently, at the National Reference Centre for sea turtles located in the Istituto Zooprofilattico Sperimentale of Sicily (IZS Sicily, Italy), damaged subjects rescued along the Sicilian coasts are hospitalized, cured and freed again in the marine environment. Many exams are conducted in each subject including molecular parameters. Currently, there is a new tool to help track this highly migratory and endangered group of marine animals: DNA barcodes. DNA barcodes are short genetic sequences that efficiently distinguish species from each other, even if the samples from which the DNA is extracted are minute or degraded. DNA barcodes are relatively short segments of mitochondrial DNA. A region of the COI, or cox1 gene (cytochrome c oxidase subunit 1) has been agreed upon by researchers as appropriate for barcoding, given that it is both highly variable and very specific. This portion of the genome mutates quickly enough to distinguish many closely related species but also slowly enough so that individuals within a species may have similar barcodes. The aim of the present study was to obtain species-specific COI barcode tags that can be used for identifying individually the marine turtle species studied. Indeed, the large-scale sequencing of a single or few genes in taxonomic studies, denominated the Barcode initiative, aims at representing a practical method for species identification, as well as for providing insights into the evolutionary diversification of life (Honda et al., 2002; Hudson & Buhlmann, 2002; Hebert et al., 2003).

## MATERIAL AND METHODS

Samples were collected from muscle tissue or blood of both dead and live turtles rescued. DNA was extracted with EZNA Tissue DNA kit (WVR) and spectroscopically quantized. The primers used for Cyt-b targeted PCR were 5'CTCACCAGACATCTCCATAGC-3' as forward and 5' GGGTTGTTTGAGCCTGTTTCGTG-3' as reverse amplifying a 545 bp long fragment. PCR program was optimised as follow: 94°C for 8 minutes; 40 cycles of the repetitions 94°C for 50 seconds, 55°C for 50 seconds, 72°C for 1 minutes; finally 72°C for 7 minutes. PCR products were visualized on a 1%

| Number of Subjects | BLAST code | SPECIES |
|---|---|---|
| 52 | AY678314.1 | *Caretta caretta* |
| 2 | AF385671.1 | *Caretta caretta* |
| 51 | JX454984.1 | *Caretta caretta* |
| 26 | KP256531.1 | *Caretta caretta* |
| 8 | FR694649.1 | *Caretta caretta* |

Table1. BLAST code relative to the identified subjets by mtDNA sequencing.

agarose gel, purified and employed in sequencing analysis by Big Dye sequencing kit, (Applied Biosystems), according to the manufacturer instructions. After purification, the products were analyzed on Abi Prism 3130 Genetic Analyzer (Thermo). ClustalW2 software was employed for Gene Bank sequence data comparison and sequence multiple alignment for polymorphism detection. Data obtained in this work was used to create a barcode database for the Cyt-b gene sequence characterizing each turtle cured at the National Reference Centre for sea turtles (Istituto Zooprofilattico Sicilia - Sicily, Palermo).

## RESULTS

Out of 110 animals studied, all the sequences analyzed by Clustal W2 software ruled out a 99-100% sequence similarity to *Caretta caretta* species. Multiple alignment revealed a certain percentage of polymorphic SNPs, which could mark genetic variability through the various subject of the same species. The data here obtained allow to develop a database for the sequences barcoding. From the search for polymorphisms there is the possibility of identifying, on the one hand the species, on the other the small differences that may have a phylo-

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1               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 111
8               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 102
12              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 103
14              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 103
19              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 104
6               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 112
20              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 105
16              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 103
2               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 112
4               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 108
3               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 111
18              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 111
10              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 111
11              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTACCTATACAAAGAAAC 115
7               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 112
9               GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 115
15              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 114
23              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 113
22              GCATCTACCTCCACATCGGACGAGGAATCTACTACGGTTCCTATCTATACAAAGAAAC 111
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1               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 171
8               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 162
12              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 163
14              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 163
19              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 164
6               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 172
20              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 165
16              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 163
2               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 172
4               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 168
3               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 171
18              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 171
10              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 171
11              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 175
7               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 172
9               CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 175
15              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 174
23              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 163
22              CTGAAATACCGGAATCATCCTCTTACTACTAGTAATAGCCACCGCATTCGTAGGCTACGT 171
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Figure 1. Multiple alignment of sequences of some turtles examined. No significative polymorphic bases were found.

genetic meaning useful for understanding the mechanisms of evolution and species. Moreover the analysis of the incidence of these polymorphisms can give indication on the mutational effects, on possible linkage maps, on the frequency of SNPs in the various subjects examined. However, from the sequence analysis among all the examined animals, no SNPs were found.

## DISCUSSION AND CONCLUSION

As previously described in other animal species or in other geographical areas, the barcode is here proposed as a new tool of classical taxonomy which allows the characterization of living species as well as the differentiation of the individuals from the same origin and the others from a similar population within the same species. This practical and economical tool can also be used in cases of damaged samples and of immature state of development of the individual investigated including lost nests. The polymorphisms found in our results could be related to geographical distance of the turtles, which follow different routes during their, life and the Mediterranean Sea for the warm temperature during their reproductive activities. However, this preliminary data should be amplified in a larger population to better understand the migration faced by these animals into the Mediterranean basin as well as in other geographic areas of the world (Tamura et al., 2004). The potential for DNA barcoding applications is significant and often means that the species identity or geographic origin of a product is difficult to ascertain using conventional means. Barcoding items collected by wildlife management permit to track international trade in wildlife products. In addition, protected animals trapped can be identified through DNA barcoding. To assist in these efforts, barcode sequences from this study have been supplied to the Barcode of Life database and GenBank (Kocher et al., 1989), so that the data are freely available. DNA barcoding promises to be a powerful tool for species identification (Stoeckle, 2003; Stuart & Parham, 2004; Vargas et al., 2009), and other conservation genetic applications in marine turtles, which are unique on the evolutionary tree of turtles for occupying the marine realm, and widely known for their extensive migrations. Species identification, one of the main goals of the DNA barcoding initiative, was successfully carried out using their COI sequences (Naro-Maciel et al., 2010). Distance based analysis of COI sequences consistently grouped members of the same species, although a complete sample was necessary for correct assignment using phenetic methods. There was no convincing evidence of cryptic species revealed in this research, a result that is concordant with many other genetic studies of marine turtles. In addition, the barcodes provided insight into population structure and history. However, hybridization is an important source of error for analyses relying solely on a mitochondrial marker, including in this group that is known to hybridize despite ancient separations.

Cytochrome c oxidase subunit I barcodes were obtained for each marine turtles, using discrete characters, more consistent with classical taxonomy than distance based methods. Importantly, the character based approach was reliable, no species diagnoses could be made if the query sequences did not contain the relevant diagnostic characters.

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