# Sequencing of Cytochrome C Oxidase Subunit I Gene of Mitochondrial DNA from *Chelonia mydas* in Qatar.

Khaled Elmeer, Paul McCormick and Ameenah Almalki.

Biotechnology Centre, Ministry of Environment, Doha Qatar.
elmeer@gmail.com

**Abstract**: DNA of Qatari *C. mydas* samples were successfully sequenced using the Folmer forward and reverse primers. The identification with BOLD of approximately 688 base pairs sequence revealed maximum homology (99.84%) with *C. mydas* , which is a species of turtle has been declared "extinct in wild" by IUCN. The next closest species 93.79% , was *N. depressa* which has a restricted geographical distribution and was reported to be endemic to the Australian continental shelf. The finding of characteristic species-specific COI sequences offers the prospect of identifying marine turtle species by using DNA barcode methodology as an auxiliary tool for taxonomy. This can also be used during field work when identifying lost nests, animals stranded on beaches or those killed as part of catching in fishery nets. A further use is in forensic litigation when turtle eggs or meat are the only available material and for the development of Qatar gene bank information.
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**Key word:** DNA Barcoding, Green sea turtle, Cytochrome Oxidase, and COI sequencing.

## 1-Introduction

Sea or marine turtles superfamily *Chelonioidea* are marine reptiles. They were on earth for over 100 million years (Hirayama, 1998), and occupy a diverse ecosystems except the Arctic poles through their throughout highly migratory life cycles (Bjorndal and Jackson, 2003).

There are seven species of marine turtles in the world that include: the leatherback, *Dermochelys coriacea*; loggerhead, *Caretta caretta*; green, *Chelonia mydas*; flatback, *Natator depressa*; hawksbill, *Eretmochelys imbricata*; olive ridley, *Lepidochelys olivacea* and kemps ridley, *Lepidochelys kempi* (Storelli and Marcotrigiano, 2003). Of all the sea turtle species, *E. imbricate* along with *C. mydas* are the only two species found predominantly in the coral reefs of the gulf of Oman (Hossein *et al.*, 2011). Limpus, (1995) had alarmingly reported that the number of sea turtles present in all the world's oceans is declining. The rapid decline in number of sea turtles is due to many well documented factors, most of them are due to human interactions. For example the excessive use of egg and turtle protein as food consumption and alteration of nesting, foraging habitats, mortification in marine waste, vessel strikes and incidental capture in commercial and recreational fisheries, are some of the major factors driving sea turtles toward extinction (Storelli and Marcotrigiano, 2003).

To save the sea turtles from extinction, all of the clearly recognized species are listed and protected under Appendix 1 of the Convention on International Trade in Endangered Species of Fauna and Flora (CITES), so to ensure an international trade ban of the specimens of the Sea Turtles (Schouten, 1992). Furthermore, all species except *N.depressa*, are listed in Appendices I and II of the convention on the Conservation of Migratory Species (CMS) of wild animals (IUCN, 1995). The IUCN Red List considers *C. mydas*, as endangered in 1980 Bonn Convention (IUCN, 1996).

The 'Consortium Barcode of Life (COBOL), is an international collaborative organization whose purpose is to use "DNA barcoding" to generate a unique genetic standard barcode of every species of life on earth for taxonomic aims.

The DNA barcoding is a taxonomic method that uses a short genetic marker of an organism's DNA to identify it as belonging to a particular species (Stoeckle 2003).The higher the sequencing resolution of a particular gene, the better or more accurate results in taxonomic studies.

The Subunit 1 (COI or Cox1) gene is responsible for producing "Cytochrome Oxaidase C enzyme" which regulates and controls the respiratory process of the cell. Because of its unique structure and functionality from species to species, Subunit 1 (COI or Cox1) gene is therefore utilized as an accurate or optimum barcode identifier for a species. This was fully demonstrated by scientists when the Subunit (COI) gene was used to identify the North American bird species in 2004

(Hebert *et al.*, 2004). Since then many other vertebrate species' COI barcodes have been utilized for similar purposes (Chaves *et al*., 2008). This stipulates the imminent conclusion that COI provide the most optimum and rapid solution for species' classification or taxonomy (Moritz and Cicero, 2004). The initiative opens up and enhances our recorded knowledge and understanding of taxonomy in general, and makes it easy for researchers to look at the results, which could lead further improved ways of species conservations (DeSalle and Amato, 2004).

Even so, prior to year 2010, globally threatened marine turtles were poorly represented in the DNA barcoding initiative (Eugenia *et al.*, 2010). Most recently the DNA barcoding technique has been utilized to identify species classification of sea turtles. This is the first study of its kind in Qatar that has been carried out to tackle the DNA barcoding of sea turtles, which is leads to identify lost nests, animals stranded on beaches or those killed as part of the by catch in fishery nets and in forensic litigation when turtle eggs or meat are the only available material. In addition to enhance the conservation of genetic natural resources of Qatari gene bank.

**2-Materials and methods**

Blood samples were collected from *C. mydas* in Pearl Lake of Qatari coast on the Arabian Gulf.

DNA was purified from 200µl of EDTA-anti coagulated blood with a QIAamp Blood Mini kit (Qiagen, Basel, Switzerland). The isolated DNA were quantified and qualified by using NanoDrop® ND-1000 spectrophotometer, for further estimation of the DNA quantity 2 µl was loaded on 0.85% agarose gel at 100V for 30 min. The gels were stained in ethidium bromide and visualized under UV light.

PCR amplification of COI gene fragment using the Universal Animal Barcoding primer recommended by the Consortium for Barcoding of Life was used according to Folmer *et al.*, (1994), by the following sequence : BLCO1490F 5'- GGT CAA CAA ATC ATA AAG ATA TTG G-3' and BHCO2198R 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA-3'

Polymerase Chain Reaction was performed in a total reaction mixture of 20µl containing 1µl (5 ng) of DNA template, 10µl of AmpliTaq Gold® 360 Mastermix (Applied Biosystems), 0.25µl (10pmol/µl) of forward primer and reverse primer in addition to 8.5µl of nuclease free water. Amplification was carried out in a Veriti 96 Well Fast Thermal cycler (Applied Biosystems) according to Folmer *et al.*, (1994), which consisted of an initial denaturation at 95$^{o}$C for 10 minutes followed by 35 cycles of denaturation, annealing and chain extension at 95$^{o}$C (60s), 40$^{o}$C (60s), 72$^{o}$C (90s) respectively, The final chain extension step was for 7 minutes at 72$^{o}$C and a final hold at 4$^{o}$C.

The PCR amplifications were visualized on 2.0 % agarose gel using ethidium bromide staining then the PCR products were purified using ExoSap-IT.

DNA sequencing was carried out with forward as well as reverse primers of the universal primer according to standard protocol provided for Big Dye Terminator kit® V 3.1 (Applied Bio systems) using ABI 3130 genetic analyzer as 1 µl of cleaned PCR product was used for each 10 µl reaction.

The DNA sequence data was analyzed and edited with ABI Sequencing Analysis V 5.2 software. The species from the representative DNA sequence for the DNA samples was identified using the search engine of BOLD. In addition to this species identification was done from the DNA sequence using Basic locus alignment search tool (BLAST) of GenBank / NCBI.

**3-Results and discussion**

The mitochondrial COI gene of *C. mydas* was successfully sequenced to obtain good quality forward and reverse sequences of approximately 688 base pairs. The introduction of DNA barcoding has highlighted the expanding use of the COI as a genetic marker for species identification (Dawnay *et al.*, 2007).

DNA barcoding promises to be a powerful tool for species identification 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 is one of the main goals of the DNA barcoding initiative, was successfully carried out using their COI sequences (Eugenia *et al.*, 2010). The multiple sequence alignment from samples showed no intra-specific variations as revealed by the similarity matrix based on the pair-wise analysis obtained by a bootstrap procedure (1000 replicates), analyses were conducted in MEGA4, which shows that there are no single differences between the sequences of Qatari *C. mydas* as follows:

TTAAAATTCTTTTTTAGCTGTGCAGGATAG
TCGGCACAGCACTCAGTTTATTAATCCGCGCA
GAACTAAGCCAACCAGGAACTCTTCTAGGAGA
TGACCAAATCTATAATGTCATCGTTACAGCTCA
TGCCTTTATTATAATCTTCTTCATAGTTATACC
AATTATAATTGGTGGCTTCGGAAATTGACTTGT
TCCCCTAATAATTGGCGCACCAGACATAGCAT
TTCCACGTATAAATAATATAAGCTTTTGACTCC
TACCCCCTTCACTACTACTACTTCTAGCATCAT

CAGGAATTGAAGCAGGCGCAGGTACAGGTTGA
ACAGTATATCCCCCATTAGCCGGAAACCTGGC
TCACGCCGGTGCTTCCGTAGACCTAACTATCTT
CTCCCTCCACCTAGCCCGGCGTATCTTCAATCT
TAGGGTGCCATCAACTTCATTACCACAGCAAT
CAACATAAAATCCCCCGCCATATCACAAATAC
CAAACACCCTTATTTGTATGATCCGTACTAATC
ACAGGCCGTCCTATTTACTACTTTCACTGCCAG
TACCTCGCCGCAGGCATTACCATACTACTTACA
GACCGAAATCTAAATACAACCTTCTTCGACCTT
CAGGGGAGGAGACCAATCCTATACCAACACTA
TTCTGATTTTTGACCCCCCCACAAGTATAAATA
AAAAAA.

An ideal DNA barcode should allow fast, reliable, automatable, and cost-effective species identification by users with little or no taxonomic experience (Hebert and Gregory, 2005). Representative sequences were used for species identification through BOLD and GenBank / NCBI. The sequence identification with BOLD Identification Engine revealed that the sequence showed maximum homology (99.84%) with *C. mydas* (Table 1), which is a species of turtle has been declared "extinct in wild" by IUCN (2008). Identifications are usually made by comparing unknown sequences against known species DNA barcodes via distance-based tree construction (Hebert *et al.*, 2004), alignment searching e.g., BLAST; (Altschul *et al.*, 1990; Altschul *et al.*, 1997), or methods recently proposed such as the characteristic attribute organization system (CAOS) (Kelly *et al.*, 2007), decision theory (Abdo and Golding, 2007), and the back-propagation neural network BP based species identification (Zhang *et al.*, 2008).

The next closest species 93.79% (Table 1), was *N. depressa* which has a restricted geographical distribution and was reported to be endemic to the Australian continental shelf (Cogger and Linder, 1969; Limpus *et al.*, 1988).

The third closest species 92.52% (Table 1), was *E. imbricata* is a critically endangered sea turtle belonging to the family *Cheloniidae*, it is the only species in its genus. The species has a worldwide distribution, with Atlantic and Pacific subspecies. *E. imbricata* is the Atlantic subspecies, while *E. imbricata bissa* is found in the Indo-Pacific region. The fourth closest species 92.38% (Table 1), was *L. olivacea.* The match statistics for Qatari *C. mydas* samples as derived from BOLD are given as (figure 1).

Similarly, sequence identification with BLAST tool of NCBI revealed that there are sequences for COI gene available for the genus *Chelonia* on NCBI. Min and Hickey (2007) showed that the CO1 barcoding region provides a quick preview of mitochondrial genome composition.

The COI marker was more suitable for barcoding objectives than mitochondrial control region sequences. 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 (Seminoff *et al.*, 2003; Lara-Ruiz *et al.*, 2006). Based on the BOLD identification engine and BLAST analysis, COI sequences of most closely related to *C. mydas*, were obtained from the sequence data base of GenBank/NCBI. These sequences were aligned and compared with COI sequences generated for the Qatari *C. mydas* sample using MEGA. Phylogenetic and molecular evolutionary analyses were carried out with MEGA. The Distance matrix was calculated using Kimura 2-parameter and the Neighbour joining tree (NJ tree) was plotted using the Kimura 2-parameter (Figure 2).

Besides its usefulness in taxonomy, the barcode methodology is expected to be of great utility in conservation biology, for example, when performing biodiversity surveys. It could also be applied when traditional methods are inefficacious, as in the identification of eggs and larval forms, and in the analysis of stomach contents or excreta to determine food webs (Stoeckle, 2003). Furthermore, it can be potentially employed in forensic cases to identify the source of tissue samples obtained from the illegal commerce or use of eggs and meat.

**Table 1.** Match statistics (Top Four matches) for Qatari *C. mydas* sequence generated through BOLD.

| Phylum | Class | Order | Family | Genus | Species | Specimen Similarity (%) |
|---|---|---|---|---|---|---|
| Chordata | Reptilia | Testudines | Cheloniidae | Chelonia | mydas | 99.84 |
| Chordata | Reptilia | Testudines | Cheloniidae | Natator | depressa | 93.79 |
| Chordata | Reptilia | Testudines | Cheloniidae | Eretmochelys | imbricata | 92.52 |
| Chordata | Reptilia | Testudines | Cheloniidae | Lepidochelys | olivacea | 92.38 |

**Figure 1.** Identification tree generated through BOLD (Qatari *C. mydas* sample in blue).

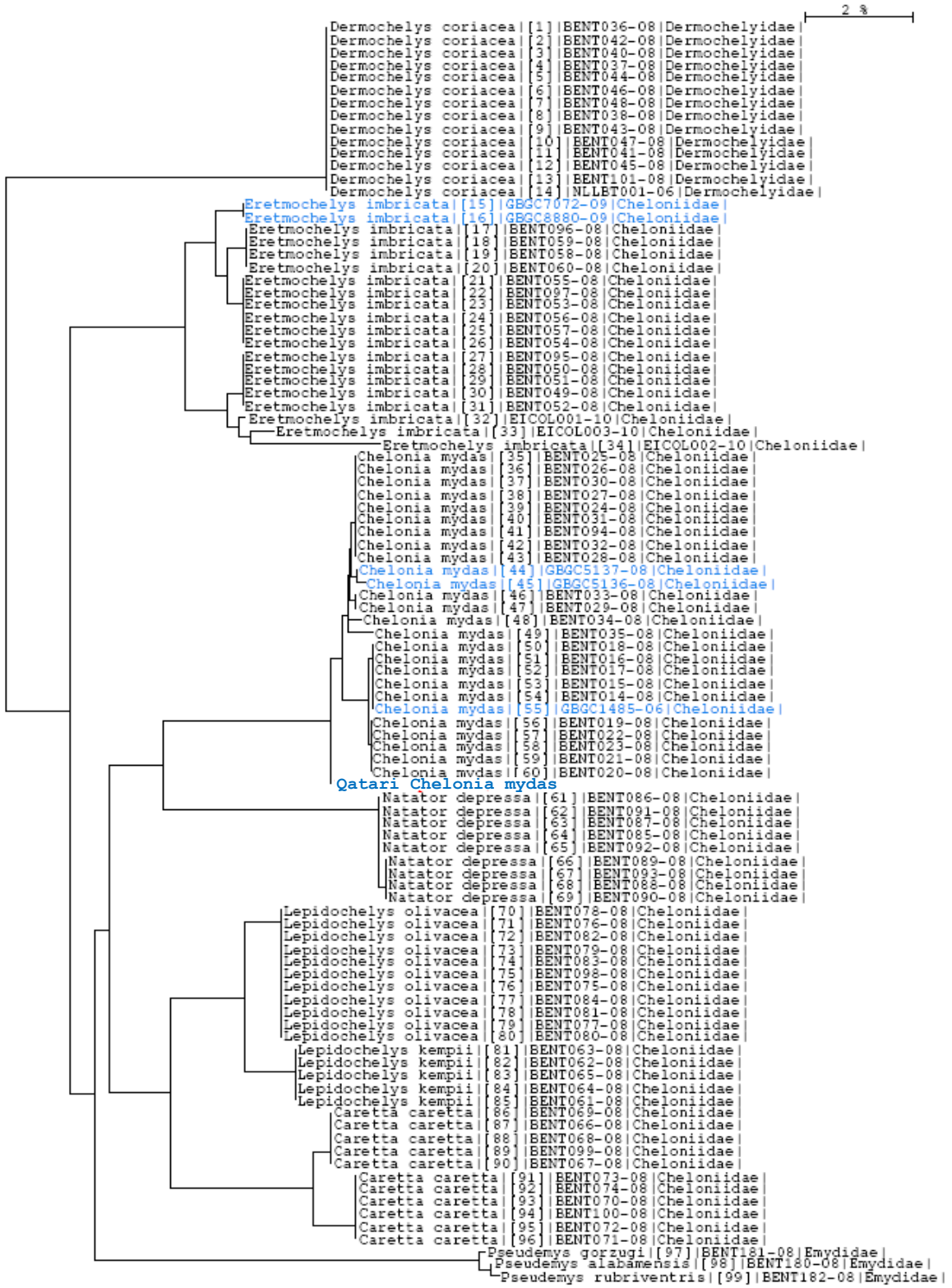
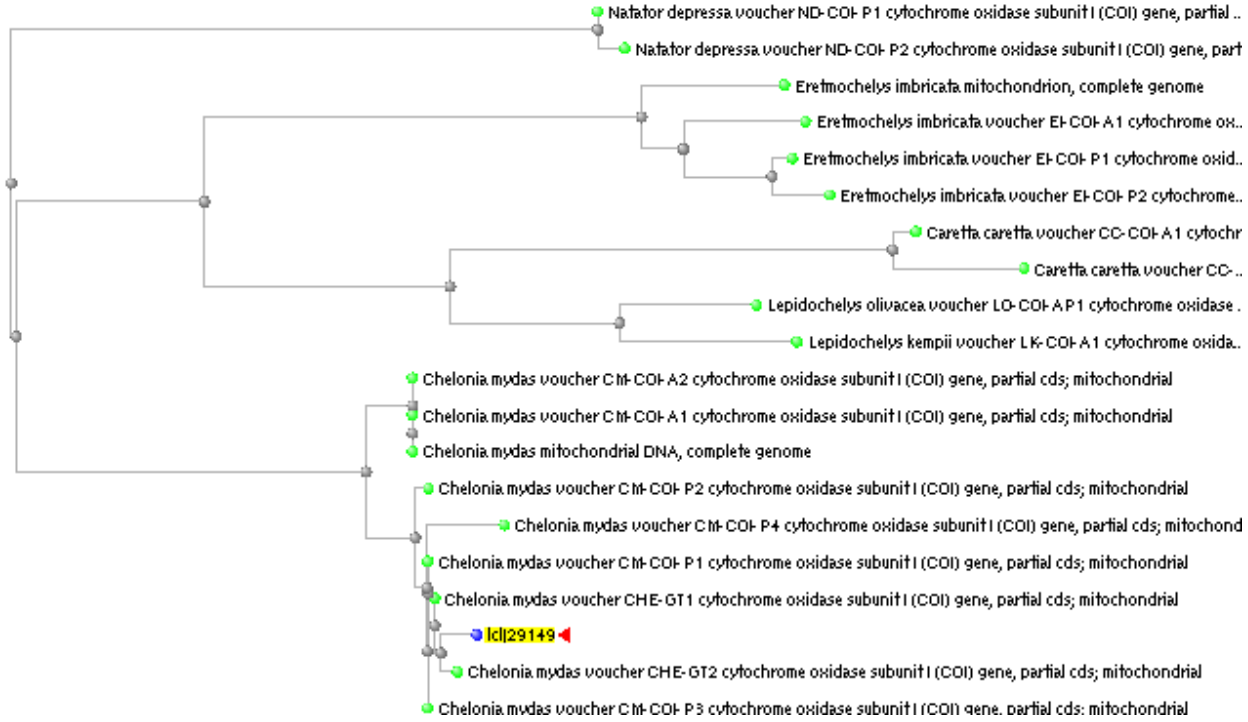


**Figure 2.** Tree was produced using BLAST pairwise alignments. Neighbour joining tree with Max Sequence Difference 0.1 (Qatari *C. mydas* sample lcl|29149)

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