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Mitochondrial DNA (MtDNA) Fragment Sizing and Quantification of *Mobula tarapacana* and *Galeocerdo cuvier* spp. using Spectrophotometric Method

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Abstract

The morphological identification of certain species of shark and rays is very difficult. The mitogenome sequencing is noteworthy to solve the fish taxonomic uncertainties and phylogeny. The complete mitogenome of *Mobula tarapacana* (NCBI Accession No: MH669414) and *Galeocerdo cuvier* (NCBI Accession No: MH648005) were used to assess the phylogenetic and taxonomic status of these closely related species. Predominantly, the cytochrome oxidase I subunit (COI) was considered as a suitable marker gene for a species-level identification. The quality and quantity of the DNA were obtained by using spectrophotometricmethods. The average mean value of electropherogram peak displayed the library fragments in *M. tarapacana* and *G. cuvier* with size ranging between 531 bp (*M. tarapacana*) and 546 bp (*G. cuvier*) respectively. The phylogenetic relationships between the closed related species were evaluated using the Maximum likelihood method.

Keywords: PCR Amplification, DNA Quantification, Sequencing, Phylogeny, *M. tarapacana*, *G. cuvier*

Introduction

Shark and rays are the most important vertebrates in the food web of an aquatic ecosystem.[1,2] The population of these species has been drastically reduced as they are targeted worldwide in the recent days.[3-5] This population decrease is due to the outcome of significant human activities such as overfishing, habitat destruction, slow reproduction, long gestation periods and low fecundity.[6] Species identification is important for population studies and stock management. Morphological identification is quite difficult for closely related species and it may lead to misidentification and poor stock assessment.[7]Recently, many modern molecular approaches, sequencing techniques, protein electrophoresis were used for species identification.[8,9] The family *Mobulidae* consists of eleven species and they were

grouped into two classes: Manta rays (two species) and Mobula rays (nine species). The diversity of the *Mobulidae* families was well documented worldwide in all tropical, subtropical and temperate regions.[10-15] *M. tarapacana* species was mistakenly considered as *Mobula mobular* and which was reported by many authors. This species was distributed in various regions in the Indo-Pacific and Atlantic waters.[16]

On the other hand, the tiger sharks (*Galeocerdo cuvier*) were one of the largest species of *carcharhinids*, occupying tropical and subtropical waters worldwide.[17] This species population is reducing gradually due to illegal trade and targeted catch. This IUCN red-listed species is considered a "near-threatened" species. The microsatellite and mitochondrial markers are extensively used for population studies and genetic connectivity of tiger sharks.[18]

Molecular phylogenic implication provides more information about taxon sampling and revealed the evolutionary history of *Mobulidae*. Despite this, the mitochondrial genome is well known for solving taxonomic inference and the evolutionary relationship between the species.[19-21] In the present study, genomic DNA was extracted from sharks and rays tissue samples. The nucleic acid quantification was carried out using three different techniques: UV absorbance spectrophotometry,[22] NanoDrop (Thermo Scientific, US),[23] and dsDNA Qubit fluorimetry (Life Technologies, Grand Island, US).

DNA bar-coding is a useful tool for the identification of species. It uses universal primers for PCR based DNA amplification. Particularly, mitochondrial cytochrome c oxidase subunit I (COI) gene, in which about 650 bp was sequenced and compared to the reference genome that is available in the Barcode of Life Data System (BOLD) for species identification.[24,25] The accurate species identification leads to an effective population study and management of shark and rays across the globe, which is necessary to maintain a healthy aquatic ecosystem. The present study aims to quantify the DNA fragment size of *Mobula tarapacana* and *Galeocerdo cuvier*. Pure DNA was used for high throughput DNA sequencing and other applications.

Materials and Methods

Sample collection and isolation of mt DNA

The tissue samples from *Mobula tarapacana* and *Galeocerdo cuvier* were collected from Nagapattinam (10°45'04'' N / 79° 50'46'' E) fishing harbour Tamil Nadu, India. The samples were packaged with liquid nitrogen and transported to the laboratory. Five mg of the fish tissue was weighed and transferred to a microcentrifuge tube. Promptly, 180 µL of tissue lysis buffer (ATL) was added and incubated at room temperature. Subsequently, 20 µL of proteinase K was added to the mixture and agitated for 15 s. The microcentrifuge tube was placed in an orbital incubator at 56 °C, where the tissues were completely lysed. Further, 200 µL of 98 % ethanol was added and vortexed for 15 s and incubated for 5 m at room temperature. The entire lysate was centrifuged and transferred to the QIAamp MinElute column in a 2 mL collection tube and again centrifuged at 8000 rpm for 60 s. The collection tube was discarded and 0.5 mL of AW2 buffer was added in the QIAamp MinElute column and centrifuged at 8000 rpm for another 60 s. The QIAamp tube was centrifuged twice at 14000 rpm for 3 m, until the membrane is fully dry. A 20-100 µL of distilled water was added and incubated for 5 m at room temperature to increase the DNA yield. The DNA was collected after centrifugation at 14000 rpm for 1 m and used for further study.

Validation of DNA Purity and Quantification

The isolated DNA was evaluated for quantity and purity using an Eppendorf BioSpectrometer (Germany). The purity of the DNA was evaluated by measuring the absorbance at A_{260}/A_{280} and A_{260}/A_{230} using UV spectrometry. The absorbance value ranges from 1.8 to 2.0, which indicated that the DNA was pure without contaminations. The quality of the isolated DNA was determined using 0.8 % agarose gel electrophoresis and stained with ethidium bromide.

Amplification of DNA

The extracted DNA from *Mobula tarapacana* and *Galeocerdo cuvier* were PCR amplified using theprotocol described by Sun et al., 2006.[26] The primers were constructed based on the known genome references obtained from NCBI. The PCRs was performed with 50 µL reaction mixture containing 35 µL, 5 µL, 4 µL, 1.5 µL and 0.5 µL of nuclease free water, 10× Taq buffer (Mg^{2+}), dNTP (25mM), DNA, and forward/reverse primer respectively. The condition for performing PCR was as follows: 94 °C for 4 m followed by 35 cycles of 94 °C for 30 s, 45 - 58 °C for 40 s and 72 °C for 2 m; and the final extension process at 72 °C for 10 m.

Phylogenetic Analysis

The phylogeny of *Mobula tarapacana* and *Galeocerdo cuvier* were inferred using the Maximum Likelihood method and Kimura 2-parameter model.[27] The bootstrap consensus tree was inferred from 1000 replicates.[28] The tree with the highest log likelihood (-24165.12) and lowest log likelihood (-3100.12) are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The evolutionary analyses were conducted using MEGAX software.[29]

Results and Discussion

DNA Quantification: The quality and quantity of DNA molecule were determined by determining the absorbance spectra (220-270 nm) using NanoDrop and measuring the concentration of DNA. The absorbance was recorded at A_{260}/A_{280} and A_{260}/A_{230}.[30] The purity of DNA from *M. tarapacana* and *G. cuvier* were determined using Eppendorf BioSpectrometer (NanoDrop). The samples with an absorbanceratio of more than 1.8 affirmed that the extracted DNA was pure (Table 1). Similarly, the absorbance ratio at A_{260}/A_{230} was more than $A_{260}/A_{280,}$ which indicates that the extracted DNA was free from any other contamination. The impurities in extracted DNA and cross-contamination with any other proteins possibly reduce the ratio of A_{260} to A_{280} and an absorbance lower than 1.7.[31] An important entity to benoted is the contamination, which is a result of co-purification. A protocol that supports DNA/RNA quantification may have influential contamination of DNA with RNA molecules. NanoDrop UV analysis was not able to distinguish between molecules of DNA and RNA.[22,32,33] This trait can be tackled using fluorescence-based Qubit technology.[22,30-34] The combination of UV and Fluorescence methods yields the most accurate information on DNA, which makes it easier to quantify DNA. The DNA fragment size was analyzed using 0.8 % agarose gel electrophoresis ranges from 100 – 150 kb (Fig.1).

Validation of DNA fragment size: The DNA fragment size was evaluated by using 0.8 % agarose gel electrophoresis. The result revealed that the size ranges between 10 to 20 kb for *M. tarapacana* and *G. cuvier*. Capillary electrophoresis is an efficient molecular separation method that has been explored in the past decade. These complications have been overridden with the newly evolved Bioanalyzer 2100 by Agilent Technologies. This is a robust and reasonable technique to find out the size and quantity of the nucleic acid in a minute. It is easy to handle, which needs a gel-dye mixture, molecular ladder and experimental samples in appropriate wells of chips. The gel-dye mixture consists of a polymer and intercalating fluorescent dye. Molecular marker ladder mixture consists of both lower and upper size markers which act as a reference in DNA fragment sizing.[35] This Agilent 2100 Bioanalyzer (Biosizing software, Ver. A.01.05) was capable of generating stable fragment size and quantification factors. The mean of the library fragment size distributions was 531 bp and 546 bp for *M. tarapacana* and *G. cuvier* respectively (Fig 2 and Fig 3).

Phylogeny: Mitochondrial cytochrome oxidase subunit I (COI) is trustworthy for DNA barcoding and useful for species-level identification.[36,37] In the present study, the cytochrome oxidase subunit I (CoxI) genes from the mitogenome of *M. tarapacana* and *G. cuvier* were taken into account for species-level identification. The sequences of *M. tarapacana* are similar to other species of *M. tarapacana* (MH235672, KF899580) (Fig. 4). The *Mobula alfredi* and *Mobula birostris* formed a separate monophyletic clade in the *Mobulidae*. The genetic similarities of *Mobula mobular* are very similar to *Mobula japanica* and formed a single clade using strong bootstrap support. William et al., 2017 indicated that the species *Mobula Mobular* and *Mobula japanica* are closely related monophyletic group based on the assessment of mitochondrial genomes and nuclear exons.[36] The *Mobulidae* family consists of two genera, they are *Manta* and *Mobula*. The genus *Manta* comprised two species, *Manta alfredi* and *M. birostris*.[11] The genus *Mobula* consists of nine recognized species. The previous morphological andmolecular data evidenced that the family with monophyletic lineage comprises of three clades. The first clade contains large *mobulid* species, viz. *Manta* spp., *M. Tarapacana*, *M. mobular* and *M. japanica*. The second clade contains small species like *M. kuhlii*, *M. eregoodootenkee* and *M. thurstoni*. Third clade comprise of species such as *M. munkiana*, *M. rochebrunei* and *M. hypostoma*. [19-21, 39,40.]

Carcharhinidae is the largest family of sharks in the order of Carcharhiniformes with 60 species under 12 genera. These species group were found in marine and tropical freshwaters around the globe. The Sphyrnidae and the Carcharhinidae are the largest groups of sharks available in Carcharhiniformes that can grow up to 3 meters in length. *Galeocerdo cuvier* (tiger shark) is the most dangerous sharks in tropical waters around the world and that are only surviving species of genus *Galeocerdo*. Mitochondrial analysis of the shark species reveals interesting facts about the morphological and ecological data on a particular species. *Galeocerdo cuvier* exhibits a very distinct behavior from other Carcharhinus species. The cytochrome oxidase subunit I (Cox1) genes of *Galeocedro cuvier* CK01 (MH648005) was compared with other Carcharhinidae species. The results revealed that, *Galeocedro cuvier* exhibit genetic relationship with the other species of the same genus (NCBI Accession No: MT455748, MT455562) (Fig. 5).

Conclusion

A standardized DNA quantification protocol was necessary for performing high-throughput genome sequencing. The *Mobula tarapacana* and *Galeocerdo cuvier* mitochondrial DNA taxonomic and phylogenetic position was determined. The average mean value of electropherogram peak displayed the library fragments in *M. tarapacana* and *G. cuvier* with size ranging between 531 bp (*M. tarapacana*) and 546 bp (*G. cuvier*) respectively. Moreover, the species-level identification of *Mobula tarapacana* and *Galeocerdo cuvier* mtDNA was explored based on a suitable marker gene. The standardized DNA quantification protocol was required for high-throughput genome sequencing. The current study revealed a workflow for DNA quantification from sharks and rays that are suitable for advanced genomicstudies.

Conflicts of interest

The authors declared no conflict of interest.

Compliance With Ethics Requirements

This article does not contain any studies with human or animal subjects.

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Tables and Figures

Table 1: Quantification of mt DNA isolates of *Mobula tarapacana* and *Galeocerdo cuvier*.

S. No	Mt DNA Samples	$A_{260/280}$	$A_{260/230}$	NanoDrop Concentration (ng/µL)	Qubit Concentration (ng/µL)
1.	*Mobula tarapacana*	1.82	1.98	94.7	36.8
2.	*Galeocerdo cuvier*	1.86	2.02	102.9	43.4

Figures

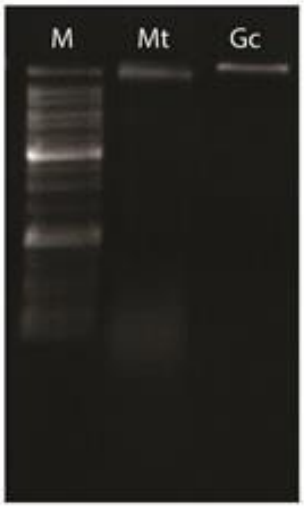
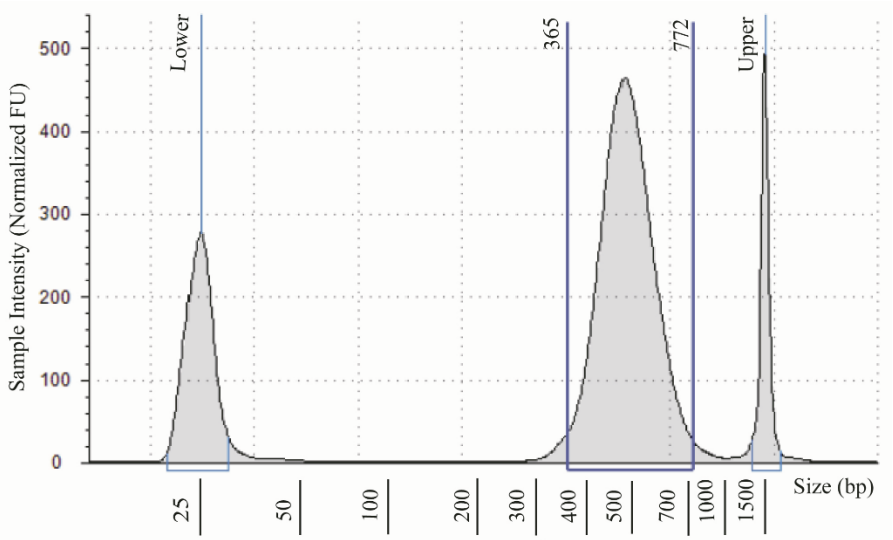
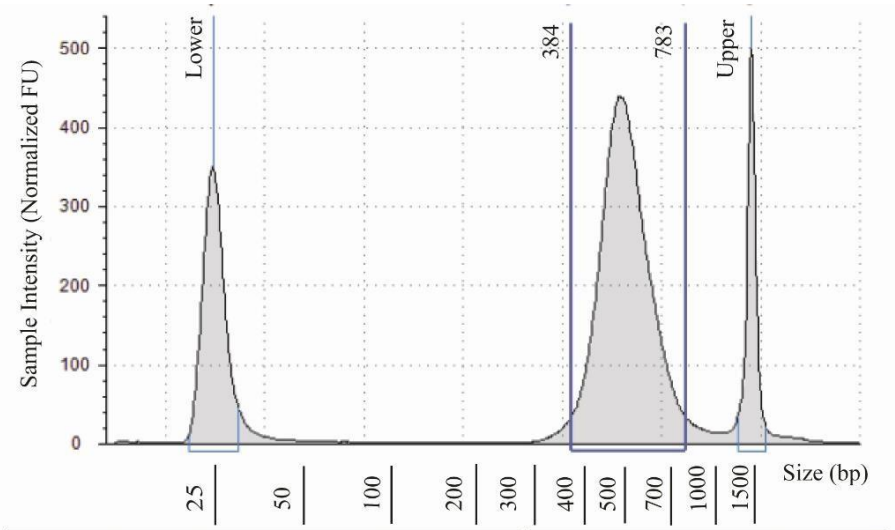


Figure. 1: Isolated mitochondrial DNA on 0.8 % Agarose gel electrophoresis (M: Marker; Mt: *M. tarapacana*;*Gc. Galeocerdo cuvier*)



From (bp)	To (bp)	Average Size (bp)	Conc. (pg/µl)	Region Molarity (pmol/l)	Total %
365	772	531	1470	4350	93.91

Figure. 2: Electropherogram report and library profile of *Mobula tarapacana* on Agilent Tape Station using High Sensitivity D1000 Screen Tape.



From (bp)	To (bp)	Average Size (bp)	Conc. (pg/µl)	Region Molarity (pmol/l)	Total %
384	783	546	1160	3350	90.16

Figure. 3: Electropherogram report and library profile of *Galeocerdo cuvier* on Agilent Tape Station using High Sensitivity D1000 Screen Tape.

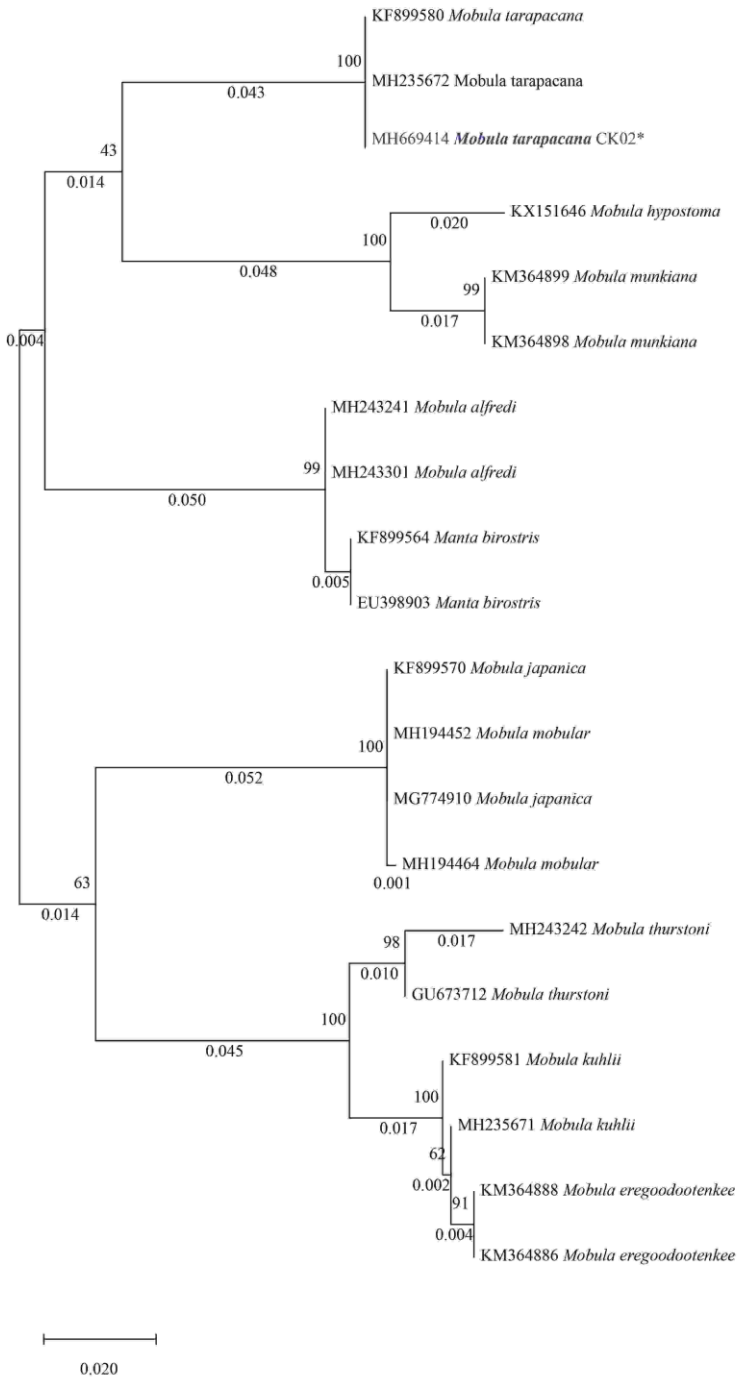


Figure 4: The phylogenetic tree of *Mobula tarapacana* based on COI gene sequences by using Maximum Likelihood method and Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates and the scale bar.

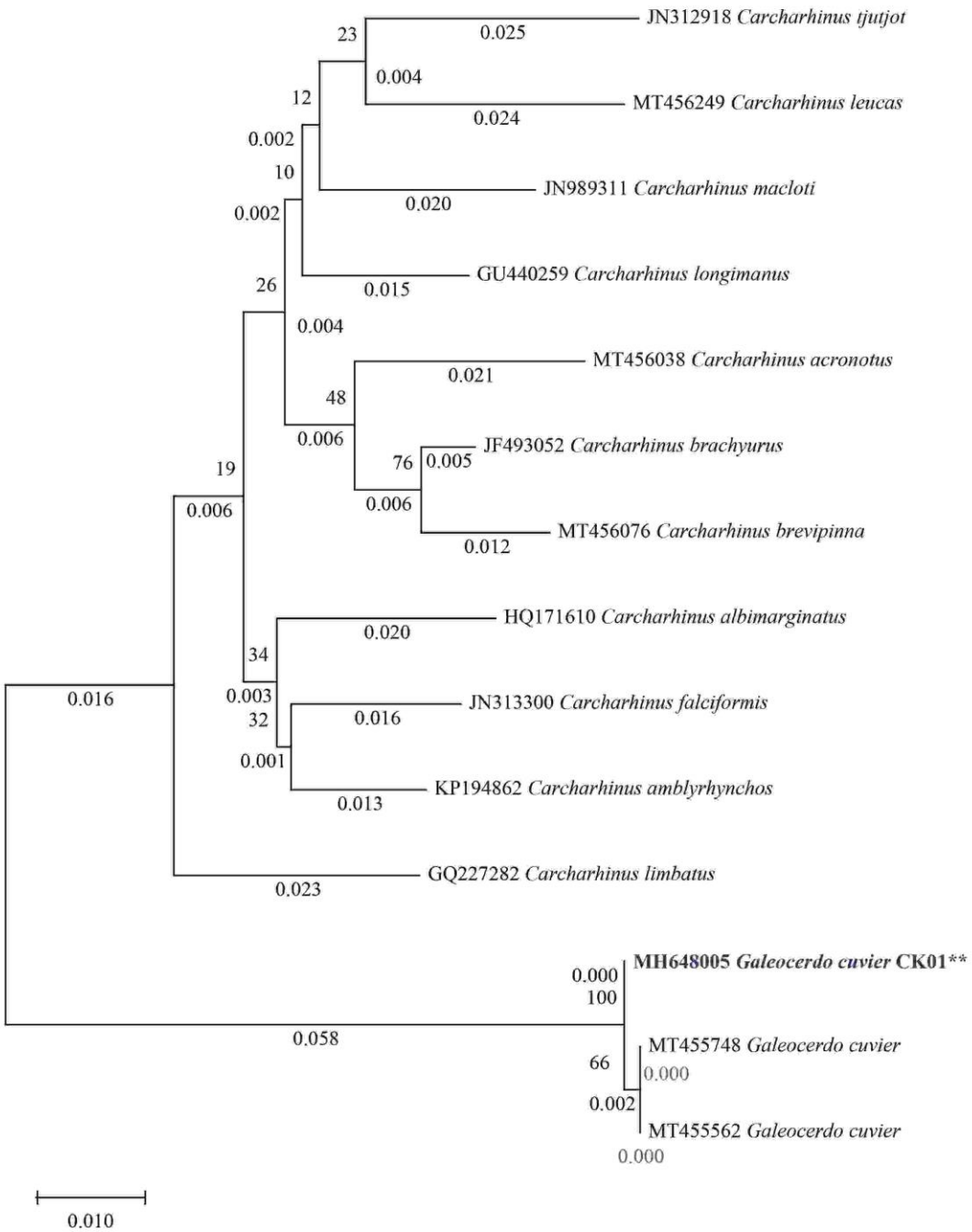


Figure 5: The phylogenetic tree of *Galeocerdo cuvier* based on COI gene sequences by using Maximum Likelihood method and Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates and the scale bar.