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DNA Barcoding and Newly Taxonomical Traits for the Shark Taxonomy: A New Approach

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ABSTRACT

This research aimed to validate new techniques in shark classification and perform a direct comparison to provide the best methodology to remove the boundaries against correct shark classification. The phenotype morphometrics of the dorsal and pectoral fins of two shark species (*Carcharhinus falciformis* and *Carcharhinus plumbeus*), inhabiting the Egyptian Red Sea water were investigated in comparison to the genetic identification through barcoding of COI from mitochondrial DNA. The results showed that phenotype morphometrics of the pectoral fins were better than dorsal fin morphometrics in differentiating between these shark species (*C. falciformis* and *C. plumbeus*). Condensed with previous results, DNA barcoding successfully features both species and clearly eliminates any confusion in their taxonomy. When the comparison level was raised across both species, pairwise distances significantly increased. COI gene sequencing produced an accurate method for unambiguous Egyptian Red Sea shark species differentiation. This might greatly help the active conservation and management efforts of those species in Egypt and throughout the world

INTRODUCTION

From an evolutionary standpoint, the cartilaginous fishes were the earliest group of extant jawed vertebrates. They are essential for understanding the evolution of bony vertebrates like teleost fish and humans **(Venkatesh *et al.*, 2007)**.

Sharks are the top predators within the marine food webs, providing regulatory control and maintaining the balance of marine ecosystem **(Cortés, 1999)**. Due to their traits of life-history, elasmobranch fishes are intrinsically more susceptible to human impacts. These features include sluggish development, delayed sexual maturation and poor reproductive capacity. In addition, there is often separation based on gender and age, as well as varied travel trends. It is commonly known that the extinction of apex predators due to human activities has severe effects on ecosystems **(Berger *et al.*, 2001)**.

In their morphological categorization, the exceptional morphological stability of elasmobranchs and the fact that essential diagnostic traits are only present in adults are recurring concerns **(Serena *et al.*, 2010)**. Several obstacles arise during the classical conventional taxonomical process, outlined here. Several challenges exist throughout the usual classical taxonomic procedure, which is discussed below. Persistent issues in the morphological taxonomy of elasmobranchs include their considerable morphological stasis and the reality that diagnostic characteristics are only present in adults **(Serena *et al.*, 2010).** Several challenges appear during the classical, traditional taxonomical method. For example,

some species under the genus *Carcharhinus*, including *C. altimus* and *C. plumbeus*, are barcoded and are known to be quite closely related (**Ward *et al.*, 2008; Moftah *et al.*, 2011)**.

DNA barcoding has become one of the most essential and influential scientific concepts in the last decade. As an innovative and useful method for species identification, DNA barcoding has gained global appeal. In 2003, Professor Paul Hebert and his colleagues from the University of Guelph, Canada introduced the revolutionary notion of DNA barcoding. The mitochondrial cytochrome C oxidase subunit 1 (COI) gene has been proposed as a distinct barcode area for mammals (**Hebert *et al.*, 2003)**. In animal forensics and conservation, DNA barcoding is an essential technique. It may identify endangered sea turtles by analyzing illegally trafficked flesh, corpses or eggs (**Vargas *et al.*, 2009)**.

Moreover, quantifying genetic diversity and metapopulation structure gives insights into the evolutionary history of a species and facilitates the development of suitable management measures (**Geraghty *et al.*, 2013)**. Traditional identification relies heavily on outward morphological diagnostic characteristics, resulting in conflicting findings in many instances (**Knebelsberger *et al.*, 2014**).

Not only is shark fishery poorly controlled, but it is also inadequately monitored, which permits improper identification and underestimation of targeted species, as well as pre-cladding estimates of the effect of fisheries on shark populations. The range of common names chosen to designate the samples evaluated in this investigation demonstrated this. Similarly, the high variety and unpredictability of common names used for sharks being caught make identification difficult for inspectors, especially if inspectors have poor taxonomic abilities for shark identification. In addition, fishermen use a variety of names to distinguish shark species. However, the popular term might change across landing places.

Therefore, the current work aimed to examine alternative shark taxonomy methodologies using the modern morphometric methodology and verify DNA barcoding as a potentially viable means of classifying sharks.

MATERIALS AND METHODS

Collection sites

The Red Sea is located to the East of Cairo. The major area for shark specimen gathering on the Red Sea shoreline was Attaka Harbor, located at 29.9, 32.47 (Fig. 1).

Samples collection

A total of 5 shark specimens representing the family Carcharhinidae were collected. The specimens were periodically gathered from the commercial catch at Attaka harbor during the 2022 season.

Freshly collected shark specimens were carefully measured for their total length to the closest millimetre and recorded. Shark specimens' tissue was stored in 100% Ethyl alcohol at -20°C for later laboratory study and delivered to the Marine Biology Laboratory, Zoology Department, Faculty of Science, Al-Azhar University (Cairo Branch), Egypt in furtherance DNA extraction. Specimens were classified as per **FAO (2005)**, and subsequent studies were carried out.

Fig. 1. Map showing the collection site

Morphometric measurements

Body morphometric measurements were calculated to the nearest millimetre to describe the obtained specimens' body measures (Fig. 2). These measurements included total length (TL), pre-pectoral fin length (PPL), pre-1st dorsal fin length (P1stDL), pre-second dorsal fin length (P2nd DL), pre-pelvic fin length (PPvL), pre-anal fin length (PAL) and pre-caudal fin length (PCL).

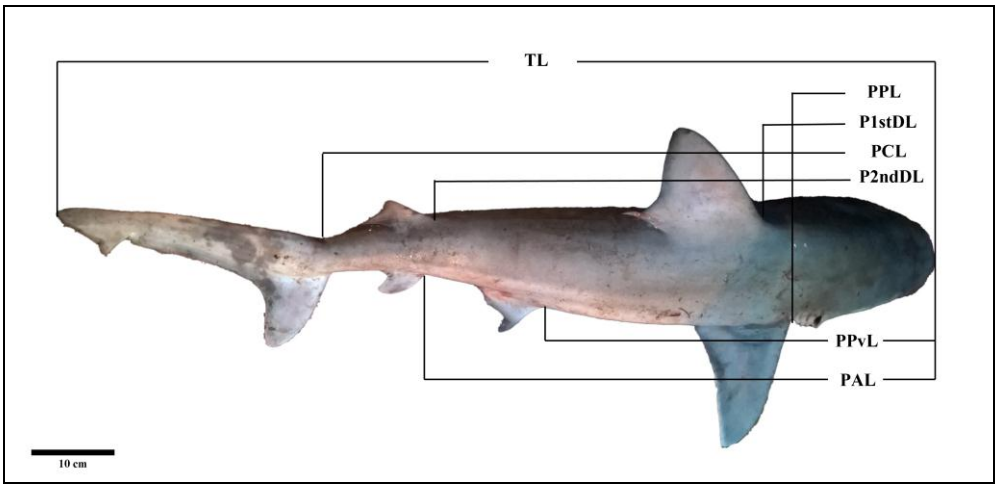

Fig. 2. Body morphometric measurements of shark

The new innovative methodology was used to accurately obtain dorsal and pectoral fin dimensional scales to validate the future potentiality of fin morphometrics in shark taxonomy. Photos of dorsal and pectoral fins were taken for all collected specimens with a scale bar. Then photos were applied to **IsharkFin V1.2** software to calculate the dimensional scales of each fin. Each dimensional measure was divided by the corresponding hypotenuse (Fig. 3).

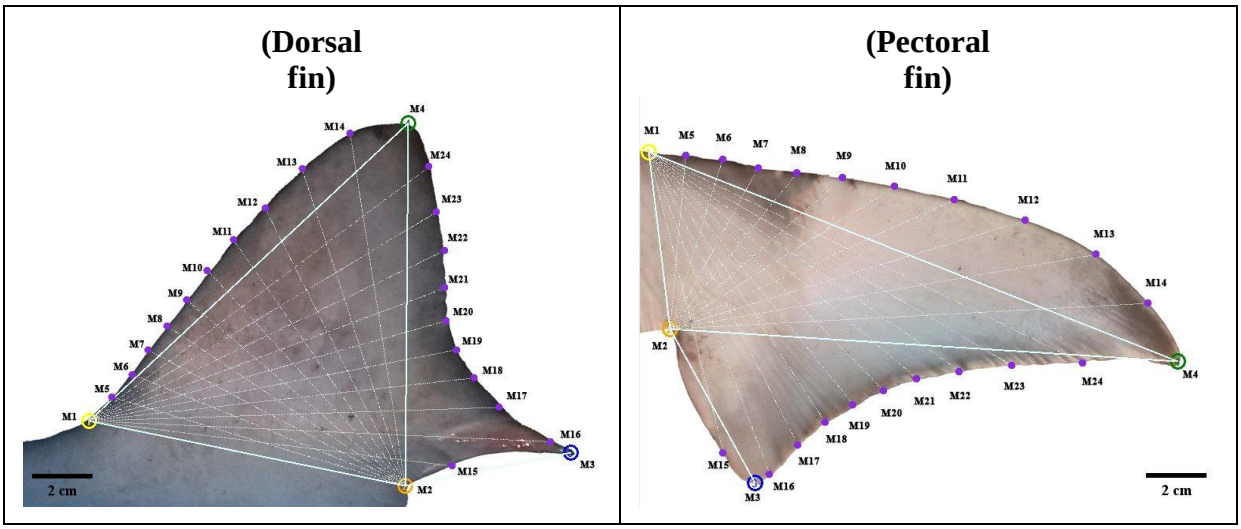

Fig. 3. Dimensional scales calculated using IsharkFin V1.2

Statistical data analysis

The SPSS V.22 statistical program was used to code and input the data. Quantitative variables were statistically represented, whereas categorical variables were defined by frequency. A probability value (*P*- value) of less than 0.05 was regarded as statistically significant. Data visualization becomes possible using R-studio V.4.1.3. Principal components analysis (PCA) was performed. While, the score of the ratios was determined using a distance-based biplot in the program Pc-Ord V5.0 software.

DNA extraction and analyses

Subsamples of tissue were excited from the gill slits of collected specimens, fixed in 100 percent ethyl alcohol and stored at -20 degrees Celsius. Al-Azhar University's Molecular Biology Laboratory, Department of Zoology, Faculty of Science (Cairo Branch) performed the molecular analysis for this research. According to the manufacturer's instructions, DNA was extracted from gill slits using the DNeasy Tissue Kit (QIAGEN). PCR was done for 679 bp of COI using defined primers (**Ward *et al.*, 2008**):

FishF2- 5'TCGACTAATCATAAAGATATCGGCAC3',
FishR2- 5'ACTTCAGGGTGACCGAAGAATCAGAA3'.

PCR reaction was done in 25µL per well containing 5.5µL of nuclease-free water, 12.5mL green master mix, 1µL forward primer, 1µM reverse primer, and 5pg extracted genomic DNA. Thermal cycler was used to accomplish the amplification. The thermal regime began with a 2-minute step at 95°C, followed by 35 cycles of 0.5 minutes at 94 °C, 0.5 minutes at 54 °C, and 1 minute at 72 °C, followed by 10 minutes at 72°C and then kept at 4°C. Amplicons were purified for sequencing after being quantified on 1.5 percent agarose gels. The pairs of PCR-primers were used for forward and reverse template strand sequencing at an Egyptian genetic facility (Color Laboratory Co.).

The obtained sequences were assembled using Chromas Pro 1.5 beta (Technelysium Pty., Tewantin, QLD, Australia). The newly COI sequences for shark species were aligned to those available in GenBank using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Muscle alignment was used to align the sequences using MEGA 11.0 software. Kimura's two-parameter calculates sequence divergences (K2P) (**Kimura, 1980**). To illustrate the patterns of species divergence, N.J. trees use the

Tamura 3-parameter method (**Tamura, 1992**). Bootstrapping was performed in MEGA 11.0 (**Kumar *et al*., 2004**), with 1000 replications. Visualization enhancement was done using ITOl software (**Letunic & Bork, 2021**). The minimum spanning network for haplotype divergence was evaluated using DnaSP v6.12.03 and PopArt v.3.0.

RESULTS

1. Body morphometric and description

Five specimens were obtained from Attaka Harbor. Two specimens belong to *Carcharhinus falciformis*, and 3 specimens are from *Carcharhinus plumbeus.* The total length of *C. falciformis* ranged from 85.32 to 187.55cm, while *C. plumbeus* ranged from 89.84 to 103.95 (Table 1).

Carcharhinus falciformis has a huge body, slender with a somewhat long, flat, and rounded head; large eyes; tiny jaws; oblique-cusped and serrated teeth. Dorsal fin origin is behind the free posterior tip of the pectoral fins.

At the same line, *C. plumbeus* has a triangular dorsal fin and a high status, exceptionally long pectoral fins. Typically, these individuals have bulky bodies and rounded snouts shorter than the ordinary shark's snout; their second dorsal fin and anal fin are around the same height, and their body color ranges from blue to brownish grey to bronze, with a white or light underbelly.

Table 1. Body morphometric measurements of both investigated shark species

Species	TL	PCL	P2ndDL	PAL	PPvL	P1stDL	PPL
C. falciformis	85.32-187.55	63.6-137.52	55.54-112.84	53.19-116.454	35.1-65.2	26.48-57.93	18.03-34.24
C. plumbeus	89.84-103.95	67.95-75.05	58.79-63.58	56.69-62.08	33.5-41.3	24.69-28.38	15.1-20.03

Measurement abbreviations according to that present in materials and methods

2. Dorsal and pectoral fins dimensional scaling

As shown in **Fig. (4),** the heatmap upper two cell lines represented *C. falciformis,* while the lower three cells represented *C. plumbeus* specimens. It showed that pectoral dimensional scaling is considered better than dorsal fin in differentiating between these two species.

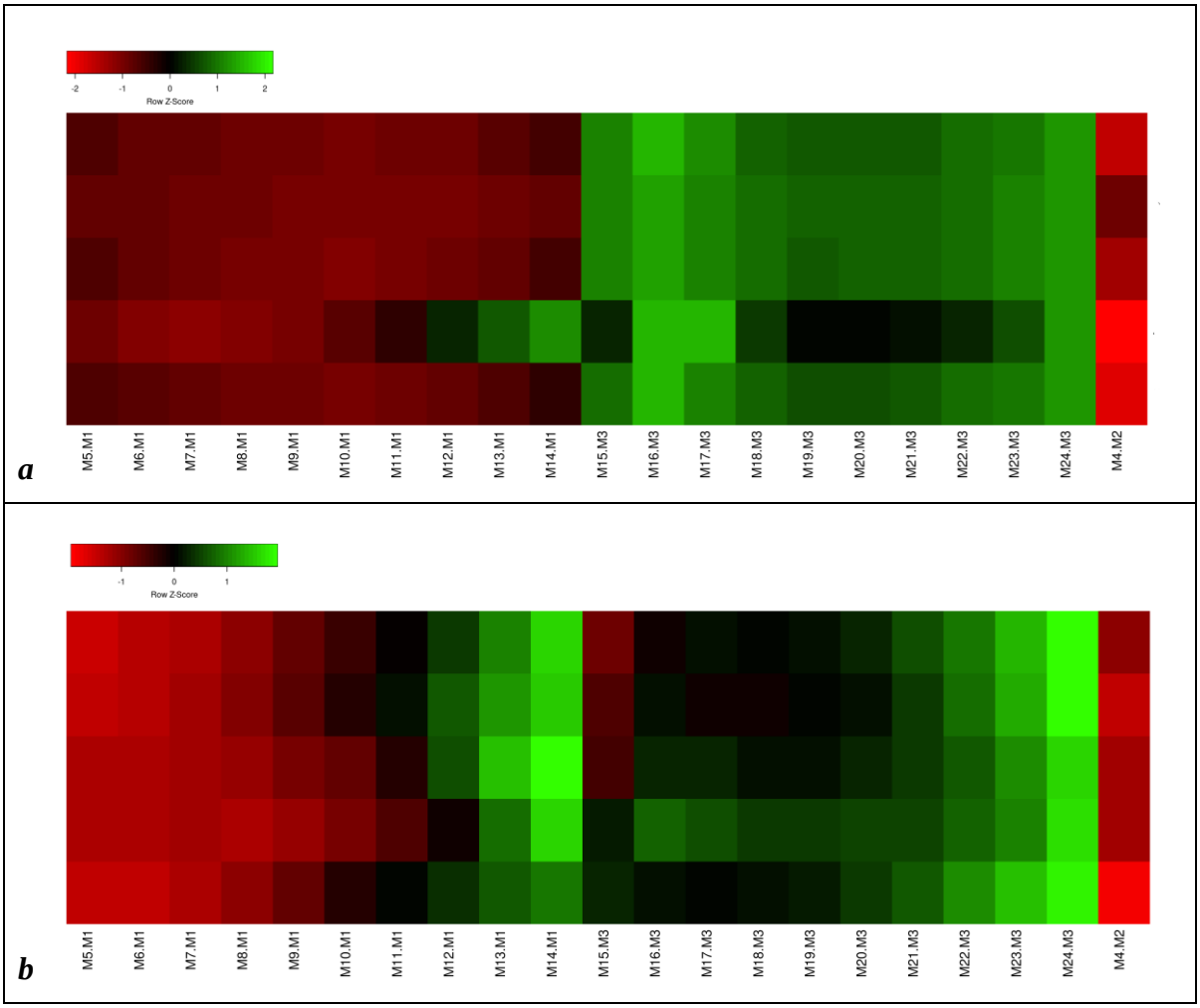

Fig. (4): Heat map showing the ratios of dimensional scaling for (*a*) dorsal fin and (*b*) pectoral fin in a head-to-head comparison with investigated species.

Principle Component Analysis (PCA) ordination **(Fig. 5)** represented the same result as shown in heatmap. Provide the potentiality of pectoral fin rather than dorsal fin with better distribution and accurate separation between *C plumbeus* and C *falciformis.*

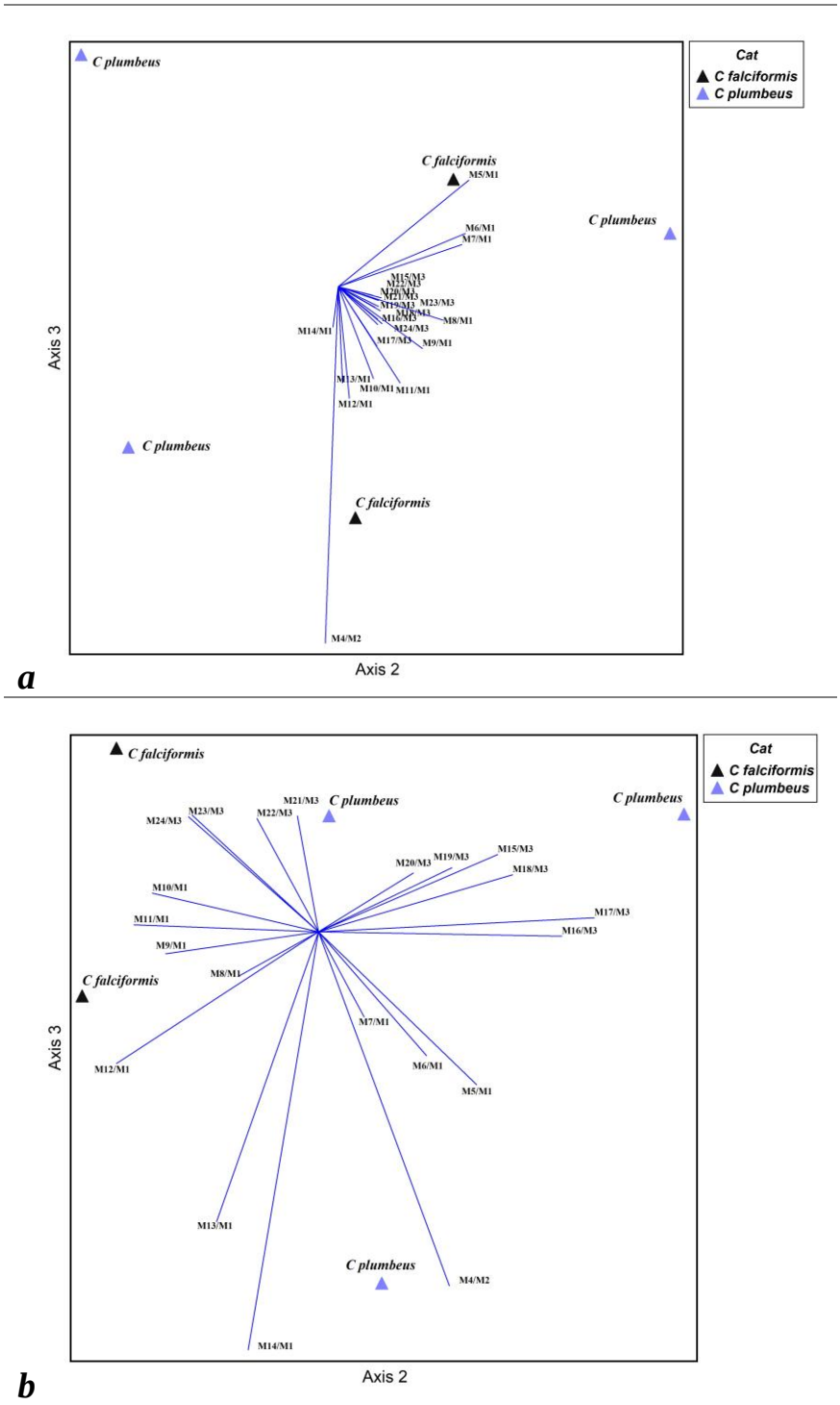

Fig. (5): PCA ordination for (a) dorsal fin (b) pectoral fin for investigated species using their feature scaling dimensions

3. DNA Barcoding

The PCR amplification of DNA specimens revealed positive amplification using primers that amplify the COI region. The amplicon size of both shark species was ~600 pb.

The Neighbor-Joining method inferred the evolutionary history **(Saitou & Nei, 1987).** The ideal tree is shown **in Fig. 6**. The proportion of duplicate trees in which the connected taxa grouped in the bootstrap test (1000 replicates) is shown by the colour-coded bootstrap value of the branches (**Felsenstein, 1985).** The phylogenetic tree is displayed to scale, with

branch lengths measured in the same units as the evolutionary distances used to build the tree. The evolutionary distances were calculated using the 3-parameter Tamura technique **(Tamura, 1992)** and are expressed as the number of base substitutions per location. This investigation included 28 nucleotide sequences (26 donor sequences from NCBI, two of which act as outgroups from higher taxa). Included codon, locations were 1st+2nd+3rd+Noncoding. All unclear locations were eliminated for each pair of sequences (pairwise deletion option). The final dataset had 613 locations in total. MEGA11 was used to do evolutionary studies **(Tamura *et al.*, 2021)**

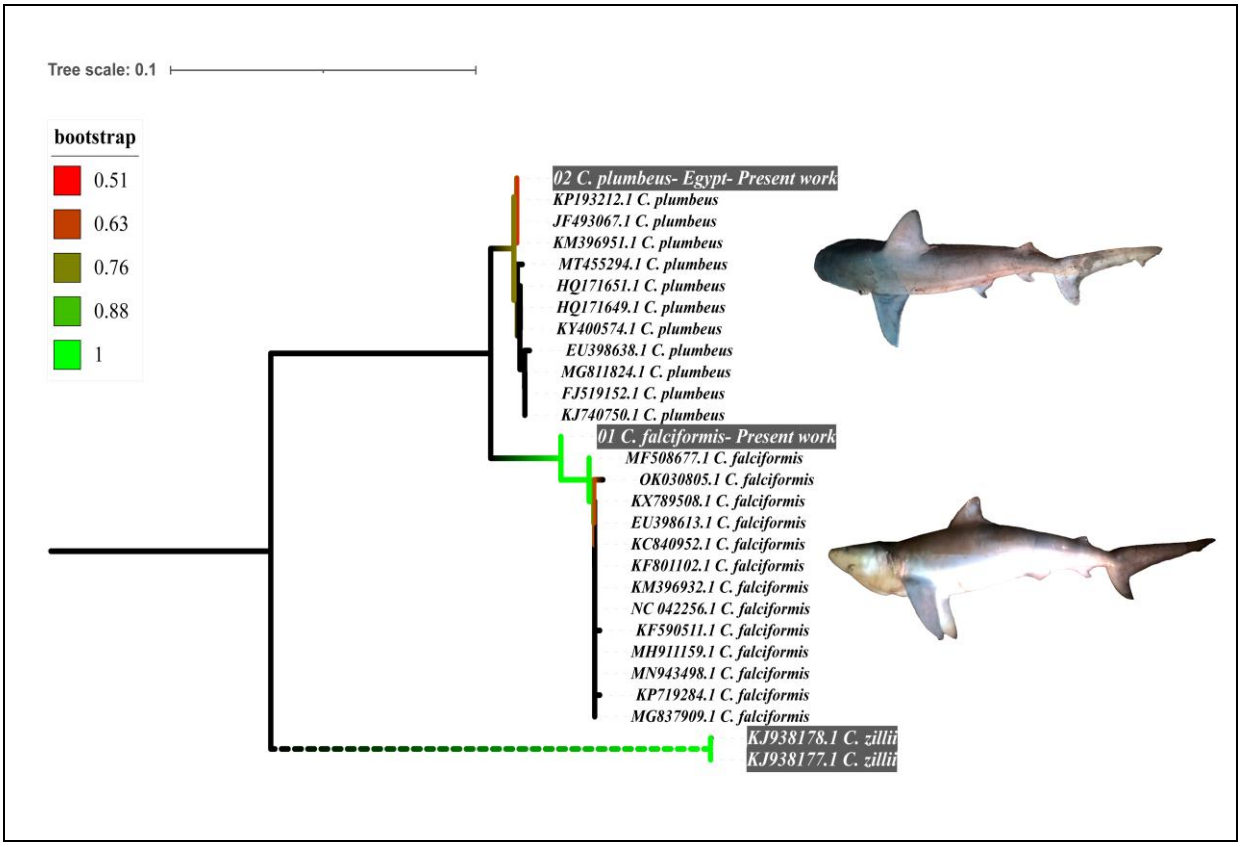

Fig. (6): Neighbor-Joining phylogenetic evolutionary tree

In addition, the minimum haplotype spanning network represented the haplotype and geographical location of the 28 nucleotide sequences evaluated in the present work. Revealing the haplotype diversity and change throughout geographical differences. Nucleotide diversity showed a pi-value of 0.128046; segregating sites were 162, while 159 parsimony-informative sites were observed. The Tajima's D statistic was calculated to be -1.95697 with P (D >= -1.95697) = 0.985844 **(Fig. 7).**

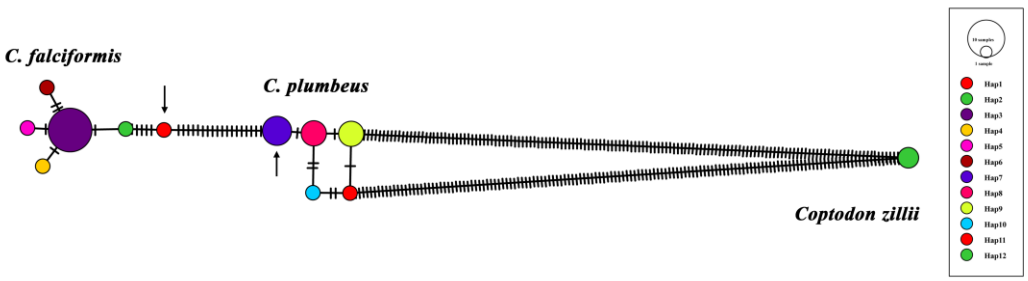

Fig. (7): minimum spanning network

DISCUSSION

Historically, fish morphology has been the major source of information for taxonomical species. Fish have distinctive forms, sizes, colour patterns, fin positions, and other exterior characteristics that help recognize, identify, and categorize **(Straüss & Bond, 1990)**. The shark's fins, especially dorsal and pectoral fins, are considered a key feature in the taxonomical identification of sharks in their natural habitat or after being captured by fishermen **(FAO, 2005)**.

In the current research, Carcharhinus plumbeus dorsal fin dimensions were larger than those documented for the same species in the southern Tyrrhenian Sea **(Consoli *et al*., 2004)** and the Middle Adriatic Sea **(Dragičević *et al*., 2010)**.

The current research demonstrated that the morphometric characteristics of shark pectoral fin have a greater potential for shark classification than dorsal fin **(FAO, 2005; Moftah *et al*., 2011; Akel & Karachle, 2017** and **IUCN, 2018)**. Although fish taxonomists widely employed these indices, they were exposed to several criticisms since they fluctuated according to individual characteristics like size and sex **(Osman, 2000)**.

DNA barcoding is a novel technique using short, standardized gene segments as internal species identifiers to provide quick, accurate, automated species identifications. In this work, standard morphological and biometric (morphometric) techniques were verified by molecular techniques.

The key finding of this work is that sequencing a 679 bp area of cox1 allows for 100% discrimination of two chondrichthyan species. A similar finding has been demonstrated by **Ward *et al.* (2008)** and **Moftah *et al.* (2011)**. The increase in the genetic distance at higher taxonomic levels is consistent with the significant increase in genetic divergence at species boundaries **(Hubert *et al*., 2008; Lakra *et al*., 2011** and **KeskİN & Atar, 2013).** Compared to one of the most comprehensive assessments of fish DNA barcoding, our results are among the most significant (**Ward *et al*., 2009); w**e observed that genetic distances were average.

The genetic connection between species was proven using a New Jersey (NJ) tree. Each species was associated with a distinct DNA barcode cluster, and their connection was emphasized. In terms of genetic divergence, closer species were grouped at the same nodes, and the distance between the terminal branches of the N.J. tree grew as the dissimilarity between them increased. Analyzing the N.J. tree reveals a clustering pattern that may be beneficial for establishing conspecific, congeneric and confamilial evolutionary relationships. **Ward *et al*. (2005)** It has been suggested that data obtained from a 655-bp fragment of a single mitochondrial gene may be used to arrange phylogenetic research. However, it is insufficient for achieving a high level of phylogenetic resolution.

The present phylogenetic study concurs with most of the connections provided by prior chondrichthyan research studies (**Compagno 1973; Shirai 1996; Carvalho & Freitas, 2013; Douady *et al*. 2003; Naylor *et al*., 2005** and **Moura *et al*., 2008**).

CONCLUSION

Pectoral fin morphometrics represents a high potential as a key taxonomical feature in differentiation between closely related shark species. In addition, modern technique as DNA barcoding is highly validating methodology in shark taxonomy.

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