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VSEBINA / *INDICE GENERALE* / CONTENTS

SREDOZEMSKJE HRUSTANČNICE
SQUALI E RAZZE MEDITERRANEE
MEDITERRANEAN SHARKS AND RAYS

Hakan KABASAKAL & Murat BİLECENOĞLU

A Review of Occurrences of Hammerhead Shark
(*Carcharhiniformes: Sphyrnidae*) on Turkish Seas
over the Past Five Decades

*Pregled pojavljanja kladvenic (Carcharhiniformes:
Sphyrnidae) v turških morjih v zadnjih
petih desetletjih*

Alen SOLDI & Rigers BAKIU

Additional Historical Records of the Great
White Shark, *Carcharodon carcharias*
(*Lamniformes: Lamnidae*) in the Eastern
Adriatic: Updating Regional Occurrence
of a Critically Endangered Shark

*Dodatni historični zapisi o pojavljanju belega
morskega volka, Carcharodon carcharias
(Lamniformes: Lamnidae) v vzhodnem
Jadranskem morju: aktualno regionalno
pojavljanje kritično ogrožene vrste*

**Farid HEMIDA, Christian REYNAUD &
Christian CAPAPÉ**

First Records of Sawback Angelsharks *Squatina*
Aculeata (*Squatinidae*) from the Algerian
Coast (Southwestern Mediterranean Sea)

*Prvi zapisi o pojavljanju trnastega sklata
Squatina aculeata (Squatinidae) iz alžirskih
voda (jugozahodno Sredozemsko morje)*

**Cemal TURAN, Mevlüt GÜRLEK, Servet Ahmet
DOĞDU, Deniz ERGÜDEN, Ali UYAN, Ayşegül
ERGENLER, Nuri BAŞUSTA & Alen SOLDI**

Phylogenetic Relationships and
Conservation Implications of Shark
Species from Turkish Waters

*Filogenetski odnosi in posledice ohranjanja
vrst morskih psov v turških vodah*

**Farid HEMIDA, Christian REYNAUD &
Christian CAPAPÉ**

On the Occurrence of Undulate Ray, *Raja*
undulata (*Rajidae*), from the Algerian Coast
(Southwestern Mediterranean Sea)

*O pojavljanju valovito progaste raže, Raja
undulata (Rajidae), iz alžirske obale
(jugozahodno Sredozemsko morje)*

**Sara A.A. ALMABRUK, Abdulghani ABDULGHANI
& Francesco TIRALONGO**

First Record of *Himantura* Müller & Henle, 1837
in Libyan Waters: a Comprehensive Discussion
of Misidentification Issues and Ecological
Implications in the Mediterranean Sea

*Prvi zapis o pojavljanju rodu Himantura Müller &
Henle, 1837 v libijskih vodah: celostna razprava
o problemu napačne identifikacije in ekoloških
posledicah v Sredozemskem morju*

**Hakan KABASAKAL, Ayşe ORUÇ, Ebrucan
KALECİK, Efe SEVİM, Nilüfer ARAÇ &
Cansu İLKİLİNÇ**

Recent Occurrences of *Rhinoptera marginata*
and *Mobula mobular* in Turkish Aegean and
Mediterranean Waters

*Recentno pojavljanje vrst Rhinoptera
marginata in Mobula mobular v turških
egejskih in sredozemskih vodah*

IHTIOFAUNA

ITTIOFAUNA

ICHTHYOFAUNA

**Deniz ERGUDEN, Servet AHMET DOĞDU &
Cemal TURAN**

On the Occurrence of the Greater Pipefish
Syngnathus acus Linnaeus, 1758 in the
South-Eastern Mediterranean, Turkey

*O pojavljanju velikega morskega šila Syngnathus
acus Linnaeus, 1758 v jugovzhodnem
sredozemskem morju, Turčija*

**Deniz ERGUDEN, Servet AHMET DOĞDU &
Cemal TURAN**

First Record of Roche's Snake Blenny *Ophidion*
rochei Müller, 1845 (*Osteichthyes: Ophidiiformes*)
in the North-Eastern Mediterranean

*Prvi zapis o pojavljanju huja vrste Ophidion
rochei Müller, 1845 (Osteichthyes: Ophidiiformes)
v severovzhodnem Sredozemskem morju*

Osama A. ELSALINI & Laith A. JAWAD

Fluctuating Asymmetry in *Chelon auratus*
from the Libyan Mediterranean Coast
and the Ain Ziana Lagoon

*Nihajoča asimetrija pri zlatem ciplju
iz libijske sredozemske obale in
lagune Ain Ziana*

Francesco TIRALONGO & Enrico RICCHITELLI

Salaria basilisca (Actinopterygii: Blenniidae) in Mediterranean Waters: New Biological and Ecological Data Emerging from the Collaboration between Citizen Scientists and Researchers 87

Salaria basilisca (Actinopterygii: Blenniidae) v sredozemskih vodah: novi biološki in ekološki podatki na podlagi sodelovanja med ljubiteljskimi raziskovalci in raziskovalci

BIOTSKA GLOBALIZACIJA
GLOBALIZZAZIONE BIOTICA
BIOTIC GLOBALIZATION

Jakov DULČIĆ, Robert GRGIČEVIĆ & Branko DRAGIČEVIĆ

Additional Record of *Pterois miles* (Scorpaenidae) in Croatian Waters (Eastern Adriatic Sea) 95

Dodatni zapis o pojavljanju navadne plamenke *Pterois miles* (Scorpaenidae) v hrvaških vodah (vzhodno Jadransko morje)

Okan AKYOL & Zafer TOSUNOĞLU

On the Occurrence of the Indo-Pacific Nakedband Gaper *Champsodon nudivittis* (Champsodontidae) in the Sea of Marmara, Turkey 101

O pojavljanju zobate krokodilke *Champsodon nudivittis* (Champsodontidae) v Marmarskem morju, Turčija

Deniz AYAS, Sibel ALAGOZ ERGUDEN & Deniz ERGUDEN

Range Expansion of *Priacanthus hamrur* (Fabricius, 1775) in the Northeastern Mediterranean (Mersin Bay, Turkey) 107

Širjenje areala lunastorepega veleokega ostriža *Priacanthus hamrur* (Fabricius, 1775) v severovzhodnem Sredozemskem morju (zaliv Mersin, Turčija)

Malek ALI, Aola FANDI, Amina ALNESSER & Christian CAPAPÉ

Confirmed Occurrence of *Jaydia smithi* (Apogonidae) and *Seriola fasciata* (Carangidae) on the Syrian Coast (Eastern Mediterranean Sea) 113

Potrjeno pojavljanje *smithovega morskega kraljička* *Jaydia smithi* (Apogonidae) in *malega gofa* *Seriola fasciata* (Carangidae) na sirski obali (vzhodno Sredozemsko morje)

Deniz ERGUDEN, Deniz AYAS & Sibel ALAGOZ ERGUDEN

Range Expansion of *Synodus randalli* Cressey, 1981 in the Northeastern Mediterranean 119

Širjenje areala *Randalljevega morskega kuščarja* *Synodus randalli* Cressey, 1981 v severovzhodno Sredozemsko morje

Abdel Fattah N. ABD RABOU, Jehad Y. SALAH, Mohammed A. ABUTAIR, Sara A.A. AL MABRUK, Bruno ZAVA & Maria CORSINI-FOKA

Occurrence of *Cheilinus lunulatus* (Labridae), *Triacanthus cf. biaculeatus* (Triacanthidae) and Other Four Non-Indigenous Fish Species New to the Gaza Strip Waters, Palestine 125

Prvo pojavljanje vrst *Cheilinus lunulatus* (Labridae), *Triacanthus cf. biaculeatus* (Triacanthidae) in še štirih tujerodnih vrst v vodah ob Gazi, Palestina

FAVNA
FAUNA
FAUNA

Nour BEN MOHAMED & Abdelkarim DERBALI

Status of the Exploited Clam *Ruditapes decussatus* in the Littoral Zone of Sfax, Tunisia 137

Stanje komercialno izkoriščene brazdaste vongole *Ruditapes decussatus* v litoralnem območju Sfax, Tunizija

Izdihar Ali AMMAR

A Preliminary Checklist of Marine Heterobranchs (Mollusca: Gastropoda: Heterobranchia) of Syria 145

Preliminarni seznam morskih polžev zaškrjarjev (Mollusca: Gastropoda: Heterobranchia) Sirije

FLORA
FLORA
FLORA

Martina ORLANDO-BONACA, Diego BONACA, Romina BONACA, Erik LIPEJ & Domen TRKOV

Five-Year Monitoring of the Ecological Status of the *Cymodocea nodosa* Meadow near the Port of Koper 159

Petletno spremljanje ekološkega stanja travnika kolenčaste *cimodoceje* (*Cymodocea nodosa*) v bližini koprškega pristanišča

IN MEMORIAM**Alenka MALEJ**

Thomas Charlton Malone (7. september 1943 – 24. februar 2024) 171

Kazalo k slikam na ovitku 173

Index to images on the cover 173

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PHYLOGENETIC RELATIONSHIPS AND CONSERVATION IMPLICATIONS OF SHARK SPECIES FROM TURKISH WATERS

Cemal TURAN, Mevlüt GÜRLEK, Servet Ahmet DOĞDU, Deniz ERGÜDEN, Ali UYAN & Ayşegül ERGENLER
Iskenderun Technical University, Faculty of Marine Sciences and Technology, Molecular Ecology and Fisheries Genetics Laboratory,
31220 Iskenderun, Hatay, Türkiye

Nuri BAŞUSTA
Firat University, Fisheries Faculty, Elazığ, Türkiye

Alen SOLDÓ
Department of Marine Studies, University of Split, Split 21000, Croatia
e-mail: soldo@unist.hr

ABSTRACT

Phylogenetic relationships of seven shark species (Squalus blainville, Carcharhinus plumbeus, Galeus melastomus, Scyliorhinus canicula, Isurus oxyrinchus, Mustelus mustelus and Oxynotus centrina) sampled from Turkish marine waters were examined using the mtDNA cytochrome b region. There were 293 variable and 74 conservative nucleotides, 279 of which were parsimony informative over 367 bp sequences of the mtDNA Cyt b region. Overall mean genetic diversity was 0.433, with the smallest genetic distance (0.2384) observed between M. mustelus and S. canicula, and the largest (1.3040) between G. melastomus and I. oxyrinchus. All species were clearly separated in NJ and MP trees, exhibiting high bootstrap values, and the observed genetic divergence clearly discriminated among all the shark species examined. Additionally, the study identified low genetic diversity and the presence of regional haplotypes, which suggests the need for immediate conservation strategies that are tailored specifically to these ecologically important predatory shark species.

Key words: shark species, mtDNA Cyt b, phylogeny, DNA barcoding, Turkey, Mediterranean Sea

RELAZIONI FILOGENETICHE E IMPLICAZIONI PER LA CONSERVAZIONE DELLE SPECIE DI SQUALI DELLE ACQUE TURCHE

SINTESI

Le relazioni filogenetiche di sette specie di squali (Squalus blainville, Carcharhinus plumbeus, Galeus melastomus, Scyliorhinus canicula, Isurus oxyrinchus, Mustelus mustelus e Oxynotus centrina) provenienti dalle acque marine turche sono state esaminate utilizzando la regione del citocromo b del mtDNA. C'erano 293 nucleotidi variabili e 74 conservativi, 279 dei quali erano informativi per parsimonia su 367 bp di sequenze della regione del citocromo b del mtDNA. La diversità genetica media complessiva è stata di 0,433, con la distanza genetica più piccola (0,2384) osservata tra M. mustelus e S. canicula, e la più grande (1,3040) tra G. melastomus e I. oxyrinchus. Tutte le specie sono state chiaramente separate negli alberi NJ e MP, mostrando alti valori di bootstrap, e la divergenza genetica osservata discriminava chiaramente tra tutte le specie di squali esaminate. Inoltre, lo studio ha identificato una bassa diversità genetica e la presenza di aplotipi regionali, il che suggerisce la necessità di strategie di conservazione immediate, specificamente adattate a queste specie di squali predatori ecologicamente importanti.

Parole chiave: specie di squalo, mtDNA Cyt b, filogenesi, DNA barcoding, Turchia, Mediterraneo

INTRODUCTION

Chondrichthyans, particularly the group of sharks, are an extraordinarily diverse group of large predatory animals inhabiting the world's seas. As primary predators of many species they play a significant role in oceanic ecosystems (Soldo, 2003; Vélez-Zuazo & Agnarsson, 2011; Kabasakal, 2021a). However, due to their life history characteristics, such as long generation times and low intrinsic population growth rates, these species are especially susceptible to over-exploitation. In many seas around the world, sharks are commercially captured for their meat, fins, gill plates, and liver oil, resulting in documented serious declines (Davidson *et al.*, 2016). The first global assessment by the IUCN (International Union for Conservation of Nature), which is widely recognized as the most comprehensive and scientifically-based source of information on the global status of plant and animal species, estimated that one quarter of sharks were in danger of extinction, making sharks the most threatened vertebrate lineage after amphibians (Dulvy *et al.*, 2014). For the Mediterranean region, the IUCN reported vulnerability of chondrichthyans and a general lack of data. Over half of the species assessed, specifically 39 out of 73, were found to be regionally threatened (critically endangered, endangered, or vulnerable), while 13 species were data deficient (Dulvy *et al.*, 2016). This makes the Mediterranean the world region with the highest level of threat to chondrichthyans. Furthermore, in some areas of the Mediterranean, the situation is even more serious; a recent assessment of the conservation status of chondrichthyan species in the Adriatic Sea, for example, has revealed that 70% are regionally threatened and three species are regionally extinct (Soldo & Lipej, 2022).

As members of chondrichthyans, sharks comprise over 500 documented species distributed among nine orders: Hexanchiformes, Heterodontiformes, Orectolobiformes, Lamniformes, Carcharhiniformes, Squaliformes, Echinorhiniformes, Pristiophoriformes, and Squatiniformes (Van der Laan *et al.*, 2024). Diversity patterns across these shark lineages are highly uneven. Over 75% of extant shark diversity is found within two lineages, the Carcharhiniformes and the Squaliformes. The Squatiniformes, Lamniformes, and Orectolobiformes exhibit moderate species diversity: ~4%, ~3%, and ~12%, respectively. The Echinorhiniformes, Heterodontiformes, and Pristiophoriformes are species poor, accounting for ~3% of the total described shark species diversity combined (Sorenson *et al.*, 2014).

Scientific data for chondrichthyans related to life parameters, particularly for the Mediterranean area, are available only for a few common species (Fowler *et al.*, 2005). In Turkey, limited research has been conducted on sharks, with a significant portion focusing

on distribution and bycatch data only (Yaglioglu *et al.*, 2015; Başusta, 2016; Kabasakal, 2021a; 2021b; Gül *et al.*, 2022; Kabasakal *et al.*, 2023). Overall, 37 species of sharks have been reported so far in Turkish marine waters (Kabasakal, 2021a).

Genetic markers are a valuable tool for describing species and monitoring genetic diversity levels in an exploited species (Turan, 2008). Molecular genetic studies utilizing mtDNA have proven advantageous in investigating the phylogeography and phylogeny of marine fish species (Turan *et al.*, 2009; Avise, 2012; Doğdu & Turan, 2021; Yağlıoğlu *et al.*, 2023; Uyan *et al.*, 2024). Sequence analysis of mtDNA regions is a common tool used for elucidating phylogenetic relationships of marine species (Avise *et al.*, 1994; Carvalho & Hauser, 1994; Turan *et al.*, 2017). The cytochrome b gene (Cyt b), whose phylogenetic performance is comparable to that of COI, has been widely used for fish species identification and enhancing our understanding of phylogenetic relationships (Farias *et al.*, 2001; Dettai & Lecomte, 2005; Sevilla *et al.*, 2007; Doğdu & Turan, 2016; Karan *et al.*, 2019).

In this study, we aimed to analyze the phylogenetic relationship of seven shark species (*Squalus blainville* (Risso, 1827), *Carcharhinus plumbeus* (Nardo, 1827), *Galeus melastomus* Rafinesque, 1810, *Scyliorhinus canicula* (Linnaeus, 1758), *Isurus oxyrinchus* Rafinesque, 1810, *Mustelus mustelus* (Linnaeus, 1758), and *Oxynotus centrina* (Linnaeus, 1758)) from Turkish waters by sequencing the Cyt b region.

MATERIAL AND METHODS

Seven shark species, comprising *Squalus blainville* (5 specimens), *Carcharhinus plumbeus* (2 specimens), *Scyliorhinus canicula* (5 specimens), *Galeus melastomus* (3 specimens), *Isurus oxyrinchus* (2 specimens), *Mustelus mustelus* (5 specimens), and *Oxynotus centrina* (3 specimens), were collected from Iskenderun Bay, in the northeastern Mediterranean. Tissue samples were collected in Eppendorf tubes from the specimens caught by fishermen and transported to the laboratory in bags with crushed ice. All tissue samples were then stored in 98% ethanol at –20 °C until analysis.

Total genomic DNA was extracted from muscle samples using a slightly modified phenol-chloroform procedure (Sambrook *et al.*, 1989). The total DNA was visualized by agarose gel electrophoresis (1.5%) and quantified by spectrophotometric assay. Polymerase chain reaction (PCR) amplification was performed with the following universal mtDNA Cyt b primers (Kocher *et al.*, 1989):

Cyt b- Forward 5'- AAA CTG CAG CCC CTC AGA
ATG ATA TTT GTC CTC -3'

Cyt b- Reserved 5'- CGA ACG TTG ATA TGA AAA
ACC ATC GTT- 3'

Tab. 1: The number of haplotype and its distribution among the species.**Tab. 1: Oznaka in prisotnost haplotipa in njegova razširjenost med vrstami.**

Haplotype	<i>S. blainville</i>	<i>C. plumbeus</i>	<i>S. canicula</i>	<i>I. oxyrinchus</i>	<i>M. mustelus</i>	<i>G. melastomus</i>	<i>O. centrina</i>
Hap_1	1	-	-	-	-	-	-
Hap_2	1	-	-	-	-	-	-
Hap_3	1	-	-	-	-	-	-
Hap_4	1	-	-	-	-	-	-
Hap_5	1	-	-	-	-	-	-
Hap_6	-	1	-	-	-	-	-
Hap_7	-	1	-	-	-	-	-
Hap_8	-	-	1	-	-	-	-
Hap_9	-	-	1	-	-	-	-
Hap_10	-	-	1	-	-	-	-
Hap_11	-	-	1	-	-	-	-
Hap_12	-	-	1	-	-	-	-
Hap_13	-	-	-	1	-	-	-
Hap_14	-	-	-	1	-	-	-
Hap_15	-	-	-	-	1	-	-
Hap_16	-	-	-	-	1	-	-
Hap_17	-	-	-	-	1	-	-
Hap_18	-	-	-	-	1	-	-
Hap_19	-	-	-	-	1	-	-
Hap_20	-	-	-	-	-	1	-
Hap_21	-	-	-	-	-	1	-
Hap_22	-	-	-	-	-	1	-
Hap_23	-	-	-	-	-	-	1
Hap_24	-	-	-	-	-	-	1
Hap_25	-	-	-	-	-	-	1
Total	5	2	5	2	5	3	3

The PCRs were conducted in a total volume of 50 µl with 0.4 µM of each primer, 0.2 mM of dNTP, and 1.25 U of Taq DNA polymerase in a PCR buffer containing 20 mM of Tris-HCl (pH 8.0), 1.5 mM of MgCl₂, 15 mM of KCl, and 1–2 µl of template DNA. The pre-denaturation step at 95°C for 1 min was followed by 5 cycles of denaturation at 94°C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. This was repeated for 30 cycles, with a final extension step at 72 °C for 7 min. The PCR products were visualized using electrophoresis on a 1.5% agarose gel. The DNA sequencing was per-

formed to determine the order of the nucleotides in the mtDNA Cyt b region. The chain termination method by Sanger *et al.* (1977) was applied with Bigdye Cycle Sequencing Kit V3.1 and ABI 3130 XL genetic analyzer. The initial alignments of partial Cyt b sequences were carried out using the Mega X program (Kumar *et al.*, 2018), and the final alignment was completed manually with BioEdit (Hall, 1999). Haplotype diversity, genetic diversity (Nei, 1987), and mean number of pairwise differences (Tajima, 1983) were calculated using the Arlequin program (Schneider *et al.*, 2000).

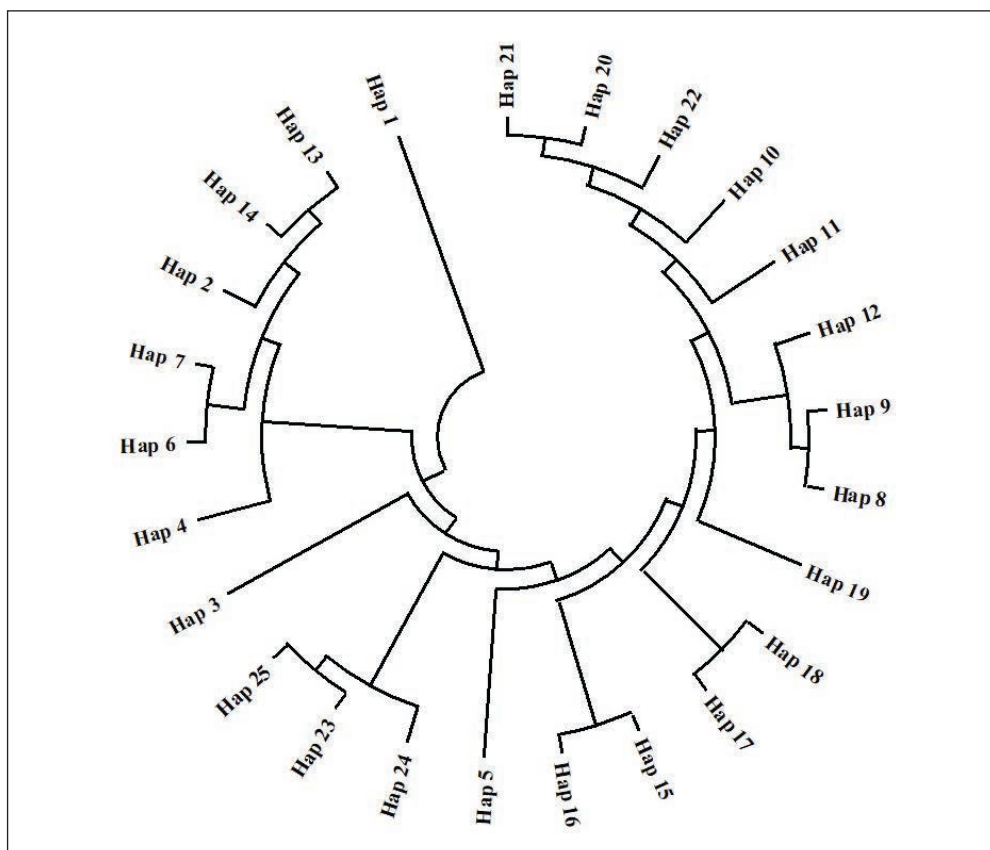


Fig. 1: A minimum spanning tree illustrating the relationships among the haplotypes.
Sl. 1: Minimalno vpeto drevo, ki kaže na odnose med haplotipi.

We also created a haplotype network for shark species. Haplotypes were obtained with the DnaSP 6 program (Rozas, 2017). The minimum spanning tree (Excoffier & Smouse, 1996) of haplotypes was produced with the FigTree (v1.4.3) program.

The molecular phylogenetic tree was constructed using Mega X (Kumar *et al.* 2018), employing neighbor joining (NJ) (Nei & Kumar, 2000) as the distance-based method and cladistics as the maximum parsimony (MP) criterion. The reliability of the inferred phylogenies was evaluated using the bootstrap method (Felsenstein, 1985) with 1000 replicates.

RESULTS

There were 293 variable and 74 conservative nucleotides, 279 of which were parsimony informative over 367 bp sequences of the mtDNA Cyt b gene region. The average nucleotide composition was 28.1% A, 31.3% T, 24.9% G, and 15.7% C. Twenty-five haplotypes were found, all of them unique, resulting in a haplotype diversity of 1.00 (Tab. 1). The minimum spanning tree revealed the relationships among the haplotypes (Fig. 1), with ancestral haplotypes 1, 2, and 4 only found in *S. blainville*.

The Tamura 3-parameter method was selected as the best method for analyzing intra- and interspecific variations (BIC score: 6923.7398). The mean genetic diversity across all species was found to be 0.43367. The matrix of pairwise genetic distances within species is presented in Table 2. The smallest genetic distance was observed between *M. mustelus* and *S. canicula* (0.2384), the largest between *G. melastomus* and *I. oxyrinchus* (1.3040). The lowest genetic diversity within species (0.024) was detected in *O. centrina*.

In the neighbor-joining phylogenetic tree, two main phylogenetic nodes were detected, with *I. oxyrinchus* separately branched as one main node. In the second main node, four sub-branches were identified, with each sub-branch comprising one species with a high bootstrap value (Fig. 2).

The maximum parsimony tree showed a different topology, with two main phylogenetic nodes detected. In the first node, *G. melastomus* was branched separately, whereas in the second node, four sub-branches were generated: *O. centrina* was closely grouped with *S. blainville*, while *C. plumbeus* was closely grouped with *I. oxyrinchus* (Fig. 3).

Tab. 2: Pairwise genetic distances between species and diversity within species (transversal diagonal) given in bold.
Tab. 2: Parne genetske razdalje med vrstami in genetska variabilnost med vrstami (transverzalna diagonalna) je podana v krepkem tisku.

Species	1	2	3	4	5	6	7
<i>Squalus blainville</i> (1)	0.0324						
<i>Carcharhinus plumbeus</i> (2)	0.3683	0.0278					
<i>Scyliorhinus canicula</i> (3)	0.3290	0.4343	0.0357				
<i>Isurus oxyrinchus</i> (4)	1.1958	1.2574	1.3715	0.0686			
<i>Mustelus mustelus</i> (5)	0.3003	0.3854	0.2384	1.1601	0.0555		
<i>Galeus melastomus</i> (6)	0.3255	0.4020	0.3077	1.3040	0.3294	0.0871	
<i>Oxynotus centrina</i> (7)	0.2569	0.4530	0.3530	1.2713	0.3269	0.3854	0.024

DISCUSSION

The study investigated the phylogenetic relationships of the seven shark species distributed in Turkish marine waters. All the species resulted separated in the NJ and MP trees with high bootstrap values. No shared haplotypes were detected between species, with the identified haplotypes distinct for each examined shark species.

The number of examined species was low due to the endangerment of shark species in the studied area. However, despite the small number, the results show a clear discrimination of each species. *O. centrina* had the lowest genetic diversity (0.024), which may call into question its conservation status (VU) as assessed by the IUCN. The genetic diversity of *G. melastomus* (0.0871) was the highest among the examined species. Castilho *et al.* (2007) studied the morphological and mitochondrial DNA divergence of *G. melastomus* and *Galeus atlanticus* (Vaillant, 1888) in the Iberian Peninsula, Portugal, using the mtDNA ND2 gene. Haplotype diversity and genetic diversity of *G. melastomus* were found to be 0.911 and 0.1, respectively, and seven haplotypes were observed. Their results for this species were similar to the findings presented herein. The genetic diversity of *M. mustelus* was also found to be low. Pereyra *et al.* (2010) studied the population genetic structure of *Mustelus Schmitt* Springer, 1939, in Río de la Plata, southwest Atlantic Ocean, using mitochondrial cytochrome b gene region. The genetic and haplotype diversity resulted to be 0.226 and 0.0015, respectively. Much lower genetic diversity was observed on the Atlantic coast, which may suggest a more conserved status of the species in the Mediterranean. Kousteni *et al.* (2015) studied the population genetic structure of the small-spotted catshark, *S. canicula*, in the Mediterranean using the mtDNA COI gene region. The investigation revealed high haplotype diversity (0.808) and low genetic diversity (0.0032). The

detected genetic diversity of *S. canicula* in the present study was found to be low (0.0357), but still much higher than that established by Kousteni *et al.* (2015). Coastal and demersal chondrichthyans, such as *S. canicula*, are expected to exhibit genetic differentiation in complex geomorphology like the Mediterranean Basin due to their limited dispersal ability (Kousteni *et al.*, 2015). Furthermore, Kousteni *et al.* (2015) suggested that strong genetic subdivision in both mitochondrial and nuclear microsatellite DNA data between the western and eastern Mediterranean is indicative of the geographic isolation of the two basins, with the deep sea acting as a significant barrier to dispersal. Kousteni *et al.* (2015) sampled from the Aegean and Egyptian coasts, while the present study is the first to sample from the northeastern Mediterranean for this species and has indicated that more conserved populations may exist in this area.

The genetic diversity of *C. plumbeus* in the present study was observed to be low (0.0278). Geraghty *et al.* (2014) investigated the genetic structure of the dusky shark, *Carcharhinus obscurus* (Lesueur, 1818), and the sandbar shark, *C. plumbeus*, in the Indo-Australian region using the mtDNA ND4 region. They found haplotype diversity and genetic diversity to be 0.2814 and 0.0009, respectively. The genetic diversity detected herein was relatively higher than that established by Geraghty *et al.* (2014), which may indicate a more conserved status for sandbar sharks in the Mediterranean Sea.

The level of genetic and haplotype diversity of other shark species from the Atlantic and Pacific Oceans was also low compared to that observed in the Mediterranean Sea. Murray *et al.* (2008) studied the relationships of sleeper sharks *Somniosus microcephalus* (Bloch & Schneider, 1801), *Somniosus pacificus* Bigelow & Schroeder, 1944, and *Somniosus antarcticus* Whitley,

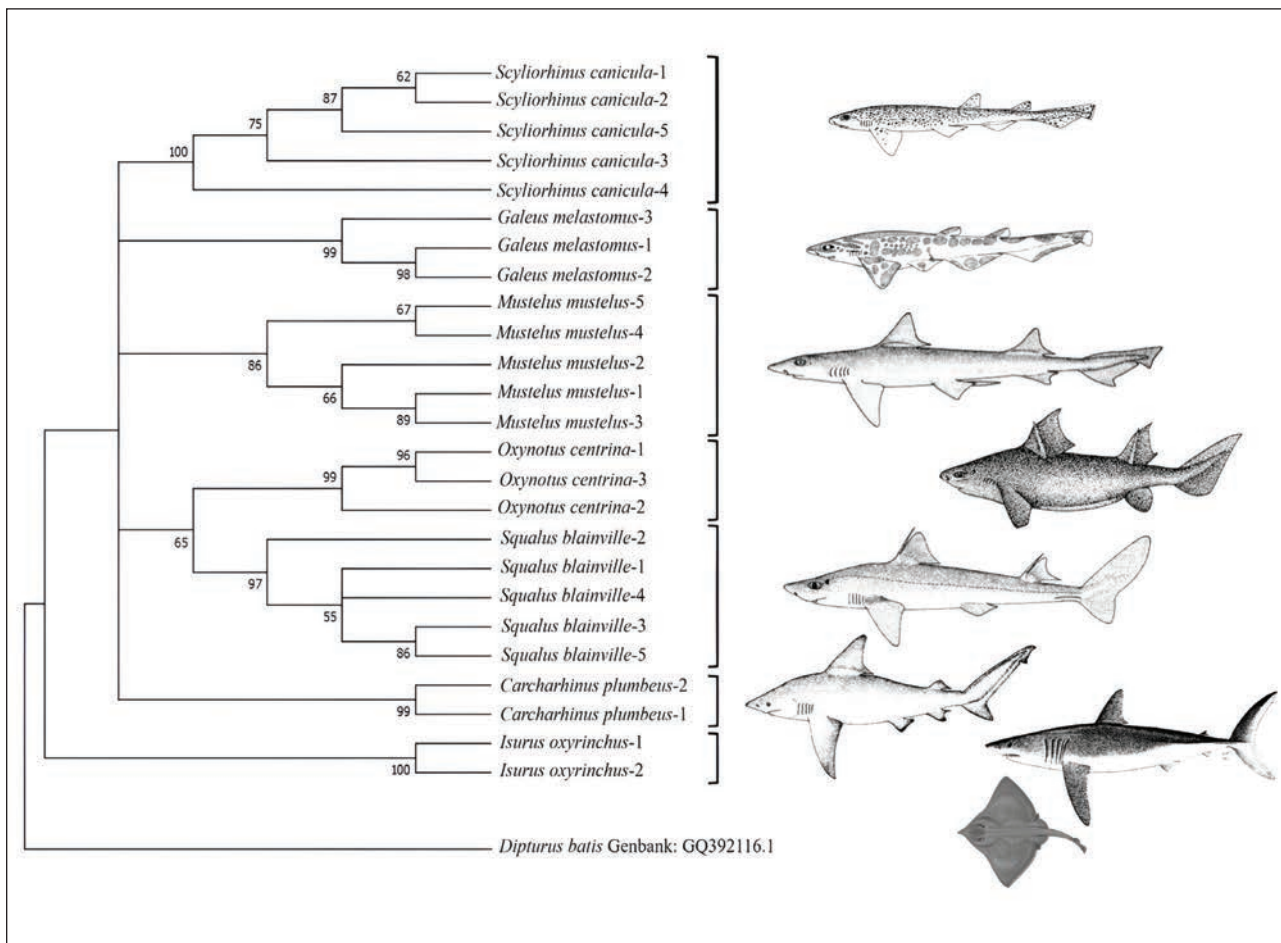


Fig. 2: A neighbor-joining phylogenetic tree based on Cyt b sequences. *Dipturus batis* (Linnaeus, 1758) was used as an outgroup. Bootstrap values are indicated on the nodes. Fish illustrations from Froese & Pauly (2023).

Sl. 2: Filogenetsko drevo združevanja sosedov, ki temelji na sekvencah Cyt b. Kot stranska skupina je bila uporabljena vrsta *Dipturus batis* (Linnaeus, 1758). Vrednosti bootstrap so navedene na vozliščih. Ilustracije rib so povzete po Froese in Pauly (2023).

1939, using the mtDNA Cyt b region. They observed a haplotype diversity of 0.7937 for *S. pacificus*, 0.6667 for *S. antarcticus*, and 0.7750 for *S. microcephalus*. Li *et al.* (2017) studied the genetic differentiation of *Prionace glauca* (Linnaeus, 1758) in the central Pacific Ocean, and found the haplotype diversity and genetic diversity to be 0.768 and 0.0011, respectively.

Although *S. blainville* had ancestral haplotypes, the highest genetic differentiation in this study was observed in *I. oxyrinchus*. This may be related to the species' low genetic diversity, which could be caused by anthropogenic factors such as fishing pressure and distraction of fishing grounds since limited spawning grounds of this species are known (Costa *et al.* 2002).

In phylogenetic analysis, all the species of the seven genera resulted aggregated into clusters in the NJ and MP trees, with high bootstrap values. The discrimination success of the present study can be associated

with the detected low levels of sequence differentiation within species and higher levels between species, as also reported by studies by Holmes *et al.* (2009) and Barbuto *et al.* (2010). The discrimination performance of the Cyt b gene is reasonable and applicable in future barcoding studies, especially in comparison to the performance of the COI gene, which has been widely used for identifying fish species and resolving fish phylogenies (Chen *et al.*, 2003; Dettai & Lecointre 2005; Sevilla *et al.*, 2007; Seyhan & Turan, 2016).

This study indicates that *O. centrina*, *C. plumbeus*, *S. blainville*, and *S. canicula*, which have low genetic diversity, are under threat. These results are only partially in accordance with the status of the mentioned species in the Mediterranean, as *O. centrina* is indeed assessed as critically endangered, and *C. plumbeus* as endangered. On the other hand, *S. canicula* is categorized as least concern and *S. blainville* as data deficient (Dulvy *et al.*,

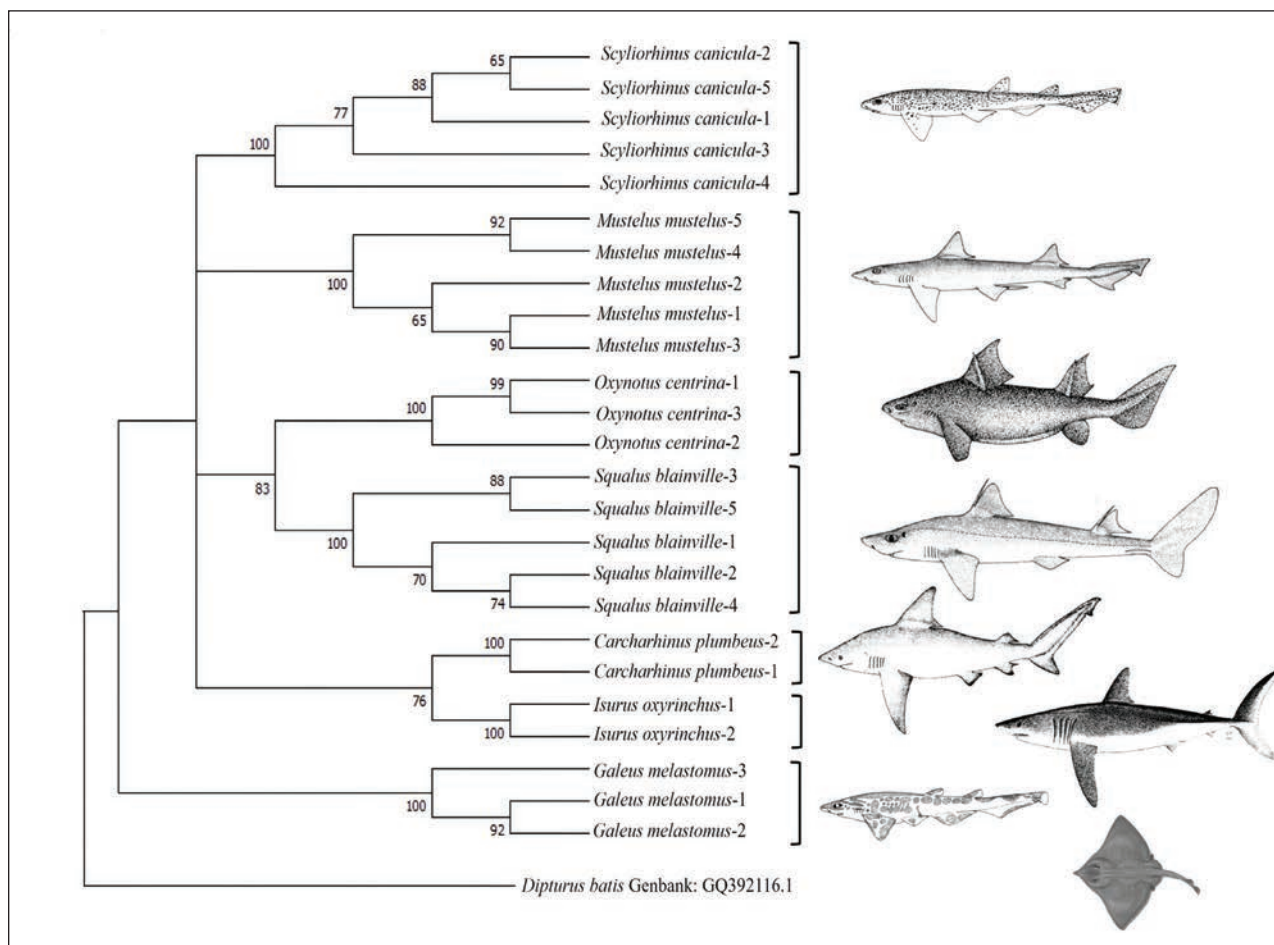


Fig. 3. A maximum parsimony phylogenetic tree based on *Cyt b* sequences. *Dipturus batis* was used as an outgroup. Bootstrap values are indicated on the nodes. Fish illustrations from Froese & Pauly (2023).

Sl. 3: Filogenetsko drevo z največjo parsimonijo (varčnost), ki temelji na sekvencah *Cyt b*. Za stransko skupino so uporabili vrsto *Dipturus batis*. Vrednosti bootstrap so navedene na vozliščih. Ilustracije rib so povzete po Froese in Pauly (2023).

2016). These two species belong to a group of demersal chondrichthyans that are highly impacted by various fishing gears, especially bottom trawls (Soldo & Lipej, 2022). Soldo & Lipej (2022) warned that even if these species are protected by various legislation, this does not prevent them from being caught. This is especially true for those inhabiting inshore areas where fishing effort is highest and a broad range of various unselective bottom fishing gear is used in both small-scale and large-scale fisheries. Soldo & Lipej (2022) therefore proposed that the ultimate conservation priority should be to identify and map the critical habitats of these species and, subsequently, implement in these relatively small areas such management measures that will only allow the use of highly selective bottom fishing gear. This approach is aimed at avoiding strong conflicts with the interests of fisheries, which can prevent any conservation measure.

An overview of IUCN extinction risk assessments shows that most of the Mediterranean populations of elasmobranchs are at a higher risk of extinction than their counterparts elsewhere (Dulvy *et al.*, 2016; Vella & Vella, 2023). Due to the infrequency of shark encounters in the Mediterranean Sea, conducting detailed studies on these species poses a challenge. Consequently, there are still knowledge gaps that need to be addressed in order to better understand and safeguard these species. There is an increasing need to improve the data on sharks to more accurately assess their status in the region. This study has identified low genetic diversity and the presence of regional haplotypes, highlighting the importance of developing immediate conservation strategies that are specifically tailored to these ecologically significant predatory species.

FILOGENETSKI ODNOSI IN POSLEDICE OHRANJANJA VRST MORSKIH PSOV
V TURŠKIH VODAH

Cemal TURAN, Mevlüt GÜRLEK, Servet Ahmet DOĞDU, Deniz ERGÜDEN, Ali UYAN & Ayşegül ERGENLER
Iskenderun Technical University, Faculty of Marine Sciences and Technology, Molecular Ecology and Fisheries Genetics Laboratory,
31220 Iskenderun, Hatay, Türkiye

Nuri BAŞUSTA

Firat University, Fisheries Faculty, Elazığ, Türkiye.

Alen SOLDÓ

Department of Marine Studies, University of Split, Split 21000, Croatia
e-mail: soldo@unist.hr

POVZETEK

Avtorji so raziskovali filogenetske odnose pri sedmih vrstah morskih psov (*Squalus blainville*, *Carcharhinus plumbeus*, *Galeus melastomus*, *Scyliorhinus canicula*, *Isurus oxyrinchus*, *Mustelus mustelus* in *Oxynotus centrina*) iz turških voda z uporabo mtDNA citohrom *b* regija. Bilo je 293 variabilnih in 74 konzervativnih nukleotidov, od katerih jih je bilo 279 varčnih informativnih v 367 bp sekvencah regije mtDNA *cyt b*. Skupna genetska diverziteta je bila 0,433, z najmanjšo ugotovljeno genetsko razdaljo (0,2384) med vrstama *M. mustelus* in *S. canicula*, in največjo (1,3040) med vrstama *G. melastomus* in *I. oxyrinchus*. Vse vrste so se dobro razlikovale na nivoju NJ in MP dreves, ki so pokazale visoke zagonske vrednosti, opažena genetska divergences pa je jasno razlikovala med vsemi raziskanimi vrstami morskih psov. Poleg tega je raziskava odkrila nizko genetsko variabilnost in prisotnost regionalnih haplotipov, kar kaže na potrebo po takojšnjih strategijah ohranjanja, ki so posebej prilagojene tem ekološko pomembnim plenilcem.

Ključne besede: vrste morskih psov, mtDNA *Cyt b*, filogenija, DNA barcoding, Turčija, Sredozemsko morje

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