



DNA barcoding and mitochondrial diversity of threatened sharks and rays from the northern Arabian Sea

Mohsan Raza^{1,2,3} · Sumaira Aziz² · Arif Muhammad Khan² · Azhar Abbas Khan⁴ · Chen Hong Li^{1,3} · Muhammad Ashfaq⁵

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Abstract

Elasmobranchii, including sharks, rays, and skates, face increasing threats from overfishing and habitat degradation, particularly in regions with limited monitoring and management. The present study used DNA barcoding to investigate the mitochondrial haplotype diversity and phylogenetic relationships of elasmobranch species from daily fish landings in the coastal area of Pakistan. Our findings provide baseline DNA barcode data and preliminary insights into mitochondrial variation of *Chiloscyllium burmense*, *Iago omanensis*, *Carcharhinus sorrah*, *Rhizoprionodon oligolinx*, *Rhizoprionodon acutus*, *Scoliodon laticaudus*, and the Critically Endangered species, *Rhina ancylostoma* and *Glaucostegus typus*. A clear barcode gap (5.91% COI divergence) distinguished the morphologically similar *R. acutus* and *R. oligolinx*. Haplotype analysis revealed shared COI haplotypes between Pakistani and geographically distant specimens of *R. ancylostoma* and *G. typus*, though sample sizes limit definitive inferences. Phylogenetic analysis based on COI sequences supported established taxonomic classifications and identified close relationships between morphologically similar species. The accumulation curve analysis reflects the sampling completeness within the study. Given that these species were collected from unmonitored daily fish landing facilities, our findings emphasize the urgent need for improved catch reporting, live release protocols for threatened species, and the establishment of marine protected areas in the region.

Keywords Sharks and rays · Species identification · Fisheries monitoring · Biodiversity assessment · Arabian Sea · IUCN Red List

Introduction

Sharks, rays, and skates (subclass Elasmobranchii) are cartilaginous fishes characterized by a skeleton composed of cartilage rather than bone, five to seven gill slits on the sides of the head, and pectoral fins that are not fused to the head (Compagno 1999; Ebert et al. 2021). They possess a suite of life-history traits that make them particularly vulnerable to anthropogenic stressors, including slow growth, late sexual maturation, low fecundity, long gestation periods, and extended reproductive cycles (Baum et al. 2003; Moazzam and Osmany 2021). These K-selected life-history strategies limit their capacity to recover from population declines caused by overfishing or habitat degradation (Stevens 2000; Hutchings et al. 2012). Ecologically, sharks occupy diverse trophic roles; many species are apex predators that exert top-down control on marine food webs, regulating lower trophic levels and maintaining ecosystem balance (Myers et al. 2007; Ferretti et al. 2010).

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✉ Arif Muhammad Khan
arif.khan@uos.edu.pk

✉ Chen Hong Li
chli@shou.edu.cn

- ¹ Shanghai Universities Key Laboratory of Marine Animal Taxonomy and Evolution, Shanghai Ocean University, Shanghai 201306, China
- ² Department of Biotechnology, University of Sargodha, Sargodha, Pakistan
- ³ Engineering Research Center of Environmental DNA and Ecological Water Health Assessment, Shanghai Ocean University, Shanghai 201306, China
- ⁴ Department of Entomology, University of Layyah, Layyah, Pakistan
- ⁵ University of Guelph, Guelph, Canada

Globally, elasmobranch populations have experienced dramatic declines due to increasing fishing pressure, advanced fishing technologies, and the high demand for shark products including fins, meat, liver oil, cartilage, and skin, with some species showing decline by more than 90% (Myers et al. 2007; Chapman et al. 2015; Moazzam and Osmany 2021; Pacoureau et al. 2021; Di Lorenzo et al. 2022). The global fin trade, historically a major driver of overfishing, has been complemented by rising meat consumption, with Brazil emerging as the largest shark meat importer worldwide (Kiyono 1996; Fatima et al. 2016). In response to these threats, international frameworks such as the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the Convention on the Conservation of Migratory Species of Wild Animals (CMS) have been established to regulate trade and protect migratory species. However, implementation remains inconsistent across range states (Javed et al. 2025). The Food and Agriculture Organization (FAO) has encouraged the development of National Plans of Action (NPOA-Sharks) to promote sustainable management, yet many shark-fishing nations, including Pakistan, have yet to implement effective plans (Gore et al. 2019).

Pakistan has historically ranked among the top shark-fishing nations globally, with elasmobranch landings peaking at over 51,000 tons in 2000 (Gore et al. 2019). The northern Arabian Sea, bordering Pakistan's 1050 km coastline, is a highly productive region due to nutrient-rich upwelling waters driven by seasonal monsoons, supporting a diverse elasmobranch fauna (Moazzam and Osmany 2021). However, shark and ray landings in Pakistan have declined markedly over the past two decades, with recorded landings decreasing from 54,959 metric tonnes in 1999 to 5793 metric tonnes in 2019 (Gore et al. 2019; Javed et al. 2025). Javed et al. (2025) recorded 1.99 million individual sharks representing 41 species from four fish landing sites, with 85% of these species listed as threatened (Critically Endangered, Endangered, Vulnerable, or Near Threatened) on the IUCN Red List. Moazzam and Osmany (2021) have provided comprehensive taxonomic accounts of Pakistan's shark fauna, documenting 79 species belonging to six orders. Despite these contributions, there is currently no targeted fishery for sharks in Pakistan; they are primarily caught as bycatch in trawl, gillnet, and longline fisheries targeting teleosts and crustaceans (Moazzam and Osmany 2022).

Accurate species identification is fundamental to fisheries management and conservation planning. However, morphological identification of elasmobranchs in Pakistan is complicated by the presence of morphologically similar species, overlapping distributions, and the fact that many specimens are processed (fins removed, carcasses discarded) before they can be identified (Fatima et al. 2016). Even experienced fisheries biologists struggle to distinguish closely

related species, particularly within the genera *Carcharhinus*, *Rhizoprionodon*, and *Scoliodon* (Javed et al. 2025; Siddiqui and Javed 2025). Psomadakis et al. (2015) provided a comprehensive field identification guide to the living marine resources of Pakistan, yet species-level identification remains challenging due to the absence of diagnostic morphological characters in juveniles and processed specimens.

DNA barcoding offers a powerful complementary tool that overcomes these limitations, enabling rapid and accurate species identification from any life stage or tissue type. This technique uses a short fragment of DNA known as Cytochrome oxidase subunit I (Hebert et al. 2003; DeSalle and Goldstein 2019). It is rapid and accurate, overcoming several limitations of traditional morphology-based identification, including difficulties arising from phenotypic similarity among closely related species, ontogenetic variation, damaged or processed specimens lacking diagnostic characters, and the identification of cryptic species. DNA barcoding data coupled with traditional morphology-based identification can serve as a powerful tool and provide valuable insights into species distribution and their uniqueness on a molecular basis. In areas such as Pakistani fish landing facilities, the DNA barcoding data of certain species have been explored (Karim et al. 2016; Amir et al. 2022; Khan et al. 2024; Sial et al. 2024; Zafar et al. 2024; Raza et al. 2025, 2026).

Taxonomic research on elasmobranchs in Pakistan has been conducted primarily through morphological approaches. Siddiqui and Javed (2025) provided a detailed taxonomic assessment of 41 shark species from Pakistani waters, supported by an identification key to discriminate similar-looking species. Moazzam and Osmany (2021, 2022) documented the species composition, commercial landings, distribution, and some aspects of biology for pelagic sharks, large demersal sharks, and medium-sized demersal sharks, respectively. Gore et al. (2019) provided the first account of the elasmobranch fishery in Balochistan, identifying 25 shark species and documenting a general collapse in landings since the turn of the century. Fatima et al. (2016) provided preliminary observations on elasmobranchs captured in Pakistan, noting the dominance of *Carcharhinidae* and *Dasyatidae* in landings. Despite these efforts, molecular studies on elasmobranchs from the Northern Arabian Sea remain scarce. While DNA barcoding has been applied to marine teleosts from the Pakistani coast (Javed et al. 2025), elasmobranchs have not been systematically barcoded. No COI-based reference library exists for elasmobranchs from Pakistani waters, and haplotype diversity has not been explored for any species from this region. This gap is particularly concerning given the high proportion of threatened species in landings, the unregulated nature of the fishery, and the lack of species-specific catch data for

management purposes (Fatima et al. 2016; Javed et al. 2025). The present study addresses these gaps by providing the COI-based identification for elasmobranchs from Pakistan.

The present study aimed to (1) use COI barcoding to confirm the species identity of elasmobranchs landed along the Pakistani coast; (2) assess sampling completeness using a species accumulation curve; (3) quantify intra- and interspecific COI-based genetic divergence; and (4) explore haplotype diversity in five species (*Chiloscyllium burmense*, *Carcharhinus sorrah*, *Rhizoprionodon oligolinx*, *Rhina ancylostoma*, and *Glaucostegus typus*). This study provides baseline molecular data on elasmobranch diversity from Pakistan and will support future research, conservation, and management of these ecologically important fishes in the region.

Materials and methods

Sample collection and site selection

A total of 28 specimens representing eight elasmobranch species (*C. burmense*, *Iago omanensis*, *C. sorrah*, *R. oligolinx*, *Rhizoprionodon acutus*, *Scoliodon laticaudus*, *R. ancylostoma*, and *G. typus*) belonging to 5 families and 3 orders were collected between 2020 and 2023 from daily fish landings at Karachi Fisheries Harbour (Sindh) and Gwadar Port (Balochistan) (Fig. 1). These two harbours were selected as they represent the two major commercial fish landing hubs along the Pakistani coast, handling the bulk of marine fish landings from Sindh and Balochistan provinces, respectively (Psomadakis et al. 2015; Javed et al. 2025). Sampling was conducted during multiple field visits spanning pre-monsoon (February–April), monsoon

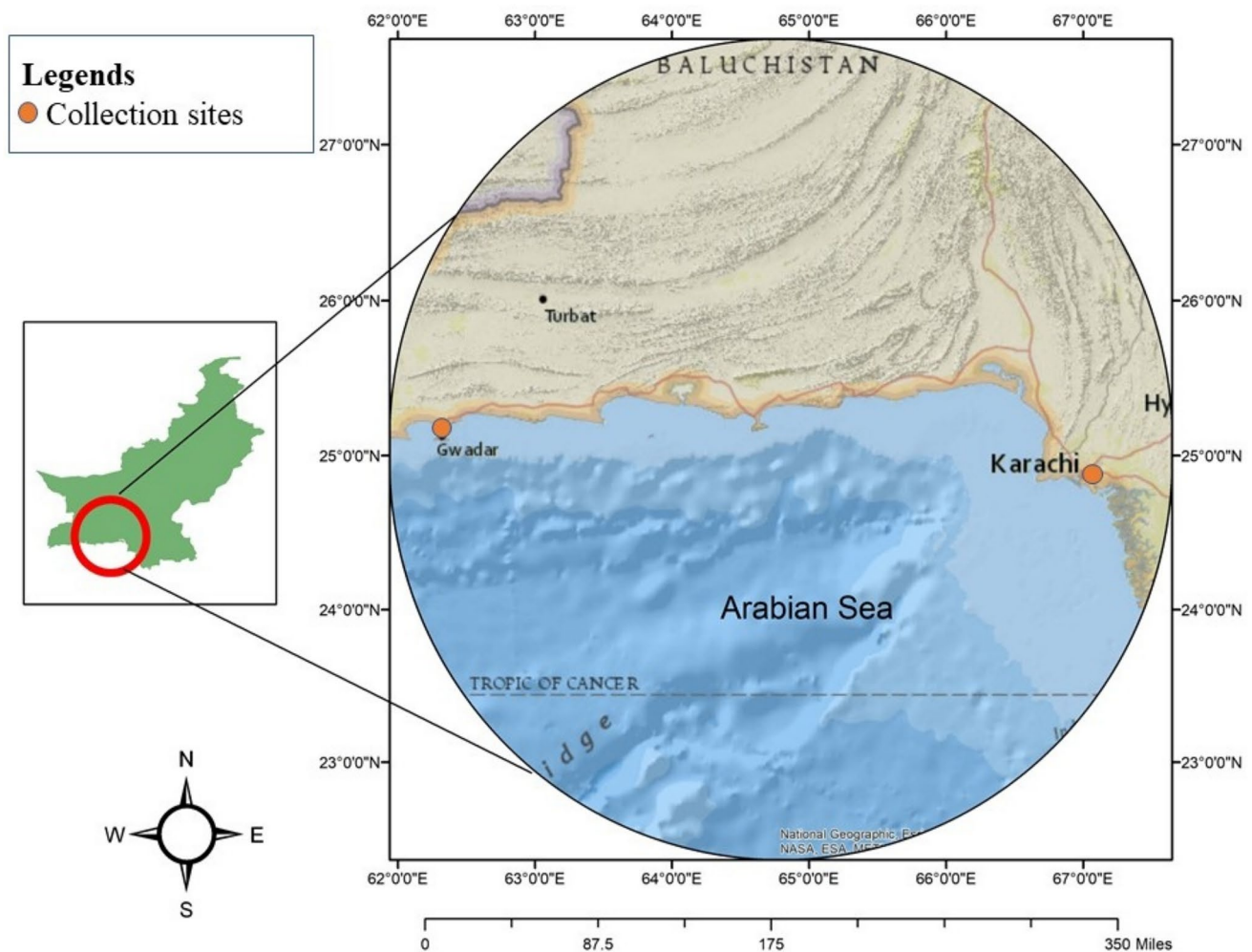


Fig. 1 The orange circle shows the exact collection sites of the specimens. Karachi Fisheries Harbour, Karachi, Sindh, Pakistan, and Gwadar Port, Gwadar, Balochistan, Pakistan

(May–September), and post-monsoon (October–January) periods; however, access during peak monsoon was sometimes restricted due to rough sea conditions. Information on fishing gear (gillnets, longlines, trawls) and capture locations was recorded where available from fishermen at the landing sites. After collection, specimens were transported to the fisheries laboratory at the Centre of Excellence in Marine Biology (CEMB).

Morphological identification

Each specimen was washed and identified based on a combination of morphometrics and specific patterns using the FAO field guide and other available identification keys (Pso-madakis et al. 2015), supplemented by Last et al. 2016 and Ebert et al. 2021. High-resolution photographs were taken for each species using a Canon EOS 1300D camera (18 megapixels). Thereafter, tissue samples (approximately 1 cm³) were excised from the dorsal musculature of each specimen under sterile conditions and preserved in 95% ethanol. Samples were stored in an ice box for 1–3 days during field trips before transport to the Department of Biotechnology at the University of Sargodha, where they were transferred to –20 °C until DNA extraction. Detailed information for all species is provided in Table 1. The exact locations for the collection of specimens are given in Fig. 1.

DNA extraction and sequencing of the DNA barcode region

Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Catalogue No. K0721) following the manufacturer's protocol. The extracted DNA was stored at –20 °C PCR amplification. The standard COI barcode region (~652 bp) was amplified using the universal fish primers FishF1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and FishR1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') (Ward et al. 2005). PCR reactions were carried out in a total volume of 25 µL containing 15.75 µL nuclease-free water, 2.0 µL MgCl₂, 2.5 µL dNTPs, 2.5 µL 10×PCR buffer, 0.25 µL of each primer, 0.25 µL Taq DNA polymerase, and 1.5 µL genomic DNA template. The thermal cycling profile consisted of an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Negative controls were included in all reactions to monitor contamination. PCR products were visualized on 1.5% agarose gels, and successfully amplified products (~650 bp) were purified using the Thermo Scientific GeneJET Gel Extraction Kit (Catalogue No. K0691). Purified PCR products, along with ethanol-preserved tissue samples, were sent to the Canadian Centre for DNA

Barcoding (CCDB), University of Guelph, for bidirectional Sanger sequencing. Raw sequence chromatograms were visually inspected using Geneious Prime 2021.1 (Biomatters, Auckland, New Zealand). Low-quality base calls, ambiguous nucleotides, and poorly resolved terminal regions were manually checked and trimmed. Only high-quality consensus sequences were retained for downstream analyses; no fixed length cutoff was applied, and trimming was based strictly on sequence quality assessment. All of the obtained sequences from sampled specimens were searched against the publicly available data using BLAST to get the best hit with a percentage identity of more than 99%. Morphological characters were subsequently rechecked to minimize potential misidentification. The final curated sequences were uploaded to NCBI GenBank and BOLD systems (Ratnasingham and Hebert 2007; Geer et al. 2010). Details of accession numbers and BOLD IDs are provided in Table 1.

Barcode gap, accumulation curve, and genetic distance analysis

After trimming and data filtering, the obtained sequences were aligned using MUSCLE alignment and then used for genetic distance using the K2P parameter in MEGA11, and the resulting values were converted into a heatmap using the R script provided in the supplementary material. The accumulation curve and barcode gap analyses were performed using all 28 sequences from the eight species (per-species sample sizes, 1–6 individuals; details are present in Table 1). Both analyses were performed on the BOLD systems workbench (accessible at <https://v4.boldsystems.org/>).

Haplotype network

The haplotype networks for five species (*R. ancylotoma*, *G. typus*, *C. sorrah*, *R. oligolinx*, and *C. burmense*) were constructed using the COI sequences from the sampled species and all available online sequences to visualize mitochondrial diversity and relationships among individuals. The details of the online sequences used are given in supplementary material (Table S1–5). These sequences were first aligned using the MUSCLE alignment in MEGA11; later on, DnaSP v6 (Rozas et al. 2017) was used to extract the haplotypes; the output from DnaSP was visualized in PopART (Leigh and Bryant 2015) with the TCS algorithm (Clement et al. 2002).

Phylogenetic tree construction

Overall, 29 COI sequences, including 28 sequences from sampled specimens and one outgroup sequence retrieved from NCBI, were used for phylogenetic analysis. A member of the order Chimaeriformes (*Callorhinchus milii*) was used as the outgroup to root the tree. The quality-filtered

Table 1 Detailed information for the collected Elasmobranchii species

Order	Family	Species name	Collection date	Bold systems ID	Sampling site	IUCN status	GenBank accession no
<i>Orectolobiformes</i>	<i>Hemiscylliidae</i>	<i>C. burmense</i>	2021-09-21	MAK-154A	Karachi	Vulnerable	PQ771365
<i>Orectolobiformes</i>	<i>Hemiscylliidae</i>	<i>C. burmense</i>	2022-10-01	MAK-154D	Karachi	Vulnerable	PQ771366
<i>Orectolobiformes</i>	<i>Hemiscylliidae</i>	<i>C. burmense</i>	2022-10-01	MAK-154E	Karachi	Vulnerable	PQ771367
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	2020-11-16	MAK-22A	Karachi	Least concern	PV065674
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	2020-11-16	MAK-22C	Karachi	Least Concern	PV065675
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	2021-09-21	MAK-155B	Karachi	Least Concern	PV065676
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	2022-10-01	MAK-219A	Balochistan	Least Concern	PV065677
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	2022-10-01	MAK-219B	Balochistan	Least Concern	PV065678
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	2022-10-01	MAK-219C	Balochistan	Least Concern	PV065679
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>C. sorrah</i>	2021-09-21	MAK-159A	Balochistan	Near Threatened	PQ771927
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>C. sorrah</i>	2023-03-01	MAK-159 J	Balochistan	Near Threatened	PQ771928
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>C. sorrah</i>	2023-03-01	MAK-159 K	Balochistan	Near Threatened	PQ771929
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>C. sorrah</i>	2023-03-01	MAK-159L	Balochistan	Near Threatened	PQ771930
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. oligolinx</i>	2021-09-21	MAK-124IA	Karachi	Near Threatened	PQ771940
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. oligolinx</i>	2021-09-21	MAK-124IB	Karachi	Near Threatened	PQ771941
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. oligolinx</i>	2021-01-01	MAK-124IIA	Karachi	Near threatened	PQ771942
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. oligolinx</i>	2021-01-01	MAK-191A	Karachi	Near Threatened	PQ771943
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. oligolinx</i>	2022-10-01	MAK-229A	Balochistan	Near Threatened	PQ771944
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	2022-10-01	MAK-229B	Balochistan	Vulnerable	PQ771945
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	2022-10-01	MAK-229C	Balochistan	Vulnerable	PQ771946
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	2022-10-01	MAK-229E	Balochistan	Vulnerable	PQ771947
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	2022-10-01	MAK-229F	Balochistan	Vulnerable	PQ771948
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	2022-10-01	MAK-229G	Balochistan	Vulnerable	PQ771949
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	2022-10-01	MAK-229H	Balochistan	Vulnerable	PQ771950
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>S. laticaudus</i>	2021-01-01	MAK-191B	Karachi	Near Threatened	PV055178
<i>Rhinopristiformes</i>	<i>Rhinidae</i>	<i>R. ancylostoma</i>	2021-01-01	MAK-189B	Karachi	Critically Endangered	PQ772031
<i>Rhinopristiformes</i>	<i>Glaucostegidae</i>	<i>G. typus</i>	2021-09-21	MAK-135A	Karachi	Critically Endangered	PQ772033
<i>Rhinopristiformes</i>	<i>Glaucostegidae</i>	<i>G. typus</i>	2021-09-21	MAK-135B	Karachi	Critically Endangered	PQ772034

sequences obtained after initial processing (as described in section “[Morphological identification](#)”) were aligned in MEGA11 using the MUSCLE algorithm with default parameters. The alignment was visually inspected to ensure accuracy prior to downstream analysis. The best-fit nucleotide substitution model was determined using the “Find Best DNA/Protein Models (ML)” tool implemented in MEGA11. Model selection was based on Bayesian information criterion (BIC), Akaike information criterion (AIC), and corrected AIC (AICc), and the general time reversible (GTR)

model was identified as the best-fitting model. Maximum likelihood (ML) phylogenetic analysis was performed under the GTR model, with rate variation among sites modelled using a gamma distribution (Γ) and invariant sites (I), as implemented in MEGA11. The robustness of the inferred phylogeny was assessed using 1000 bootstrap replicates. The resulting tree was visualized and annotated using the Interactive Tree of Life (iTOL) web tool, and the final tree with bootstrap support values was exported for presentation (Dinov et al. 2008; Tamura et al. 2021).

Results

Barcode gap analysis

The barcode gap showed clear COI-based separation among the species. Especially for *R. oligolinx* and *R. acutus*, we can see a clear barcode difference of 5.91% in Table 2. Moreover, species belonging to different families showed high barcode gap values.

In both maximum and mean intraspecific comparisons (Fig. 2A and B), the dots are present with respect to their barcode gap. Maximum intraspecific divergence values remained uniformly low across species, irrespective of sample size (Fig. 2C). Several taxa were represented by limited sample sizes, including singleton species, resulting in maximum intraspecific divergence values of zero.

Accumulation curve

The accumulation curve is based on the level of identification of unique taxonomic units, together with the increase in sampling effort (Fig. 3). The curve for species identification began to plateau at the 20th index, depicting adequate taxonomic coverage within the sampled dataset. The genus-level curve shows less coverage. However, the taxon curve (green) was elevated, suggesting a complete extraction of taxonomic information across different levels. The subfamily and the species curves were close to each other, showing consistent resolution.

Genetic distances

The pairwise genetic distances were interpreted in the form of a heatmap among eight elasmobranch species. The species belonging to the same genus, such as *R. acutus* and *R. oligolinx*, showed low genetic distances. Meanwhile, *R. ancyllostoma* and *G. typus*, which are genetically distinct from each other, showed darker colours on the heatmap chart and are isolated in the clustering (Fig. 4).

Haplotype networks

The haplotype analysis (Fig. 5A) illustrated four different haplotypes (Hap_1 to Hap_4) among the guitarfish individuals. The most widely distributed haplotype was Hap_1, and was present among the individuals from Australia and Papua New Guinea. The individuals from Bangladesh were represented by Hap_2; Hap_3 was present in Papua New Guinea, whereas the individuals from Pakistan were represented by Hap_4. The number of mutational steps separating the haplotypes reflects varying degrees of genetic divergence, with Hap_3 being separated from the remaining haplotypes by a comparatively larger mutational distance.

Three distinct haplotypes (Hap_1, Hap_2, and Hap_3) for *R. ancyllostoma* were identified through haplotype analysis across four geographical locations: Indonesia, Singapore, India, and Pakistan (Fig. 5B). Hap_1 was the most common haplotype and was shared among individuals from Indonesia and Singapore. Hap_2 contains individuals from India, whereas Hap_3 includes individuals from Singapore and Pakistan. The network also showed several mutational steps between haplotypes, depicting low to medium genetic divergence within the individuals.

Median-joining haplotype networks were also constructed for *C. sorrah*, *R. oligolinx*, and *C. burmense* (Fig. 6). *C. sorrah* comprised ten haplotypes (Hap_1 to Hap_10). Hap_1 was found in Tanzania, whereas Hap_5, Hap_6, and Hap_10 were unique to the UAE, Malaysia, and India, respectively. Hap_2 was shared among the UAE, India, and Bangladesh, while Hap_3 was shared by Madagascar, India, and Indonesia. Hap_4 was the most geographically widespread haplotype, occurring in Malaysia, the Philippines, Thailand, Vietnam, and Kuwait. Hap_7 was shared among Kuwait, Taiwan, and Australia, Hap_8 between Indonesia and the UAE, and Hap_9 among Pakistan, India, and Oman (Fig. 6A). *R. oligolinx* consisted of four haplotypes. Hap_1 was the dominant and most widespread haplotype, shared among Pakistan, Bangladesh, Indonesia, Sri Lanka, and the UAE. Hap_2 was unique to Malaysia and Hap_3 was shared

Table 2 Barcode gap analysis

Order	Family	Species	Nearest Species	Nearest neighbour	Distance to NN (%)
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>C. sorrah</i>	<i>R. oligolinx</i>	SUFIS339-21	9.06
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. acutus</i>	<i>R. oligolinx</i>	SUFIS638-23	5.91
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>R. oligolinx</i>	<i>R. acutus</i>	SUFIS699-23	5.91
<i>Carcharhiniformes</i>	<i>Carcharhinidae</i>	<i>S. laticaudus</i>	<i>R. oligolinx</i>	SUFIS339-21	10.26
<i>Carcharhiniformes</i>	<i>Triakidae</i>	<i>I. omanensis</i>	<i>R. oligolinx</i>	SUFIS412-21	12.52
<i>Orectolobiformes</i>	<i>Hemiscylliidae</i>	<i>C. burmense</i>	<i>R. oligolinx</i>	SUFIS638-23	20.31
<i>Rhinopristiformes</i>	<i>Glaucostegidae</i>	<i>G. typus</i>	<i>R. ancyllostoma</i>	SUFIS635-23	21.36
<i>Rhinopristiformes</i>	<i>Rhinidae</i>	<i>R. ancyllostoma</i>	<i>G. typus</i>	SUFIS362-21	21.36

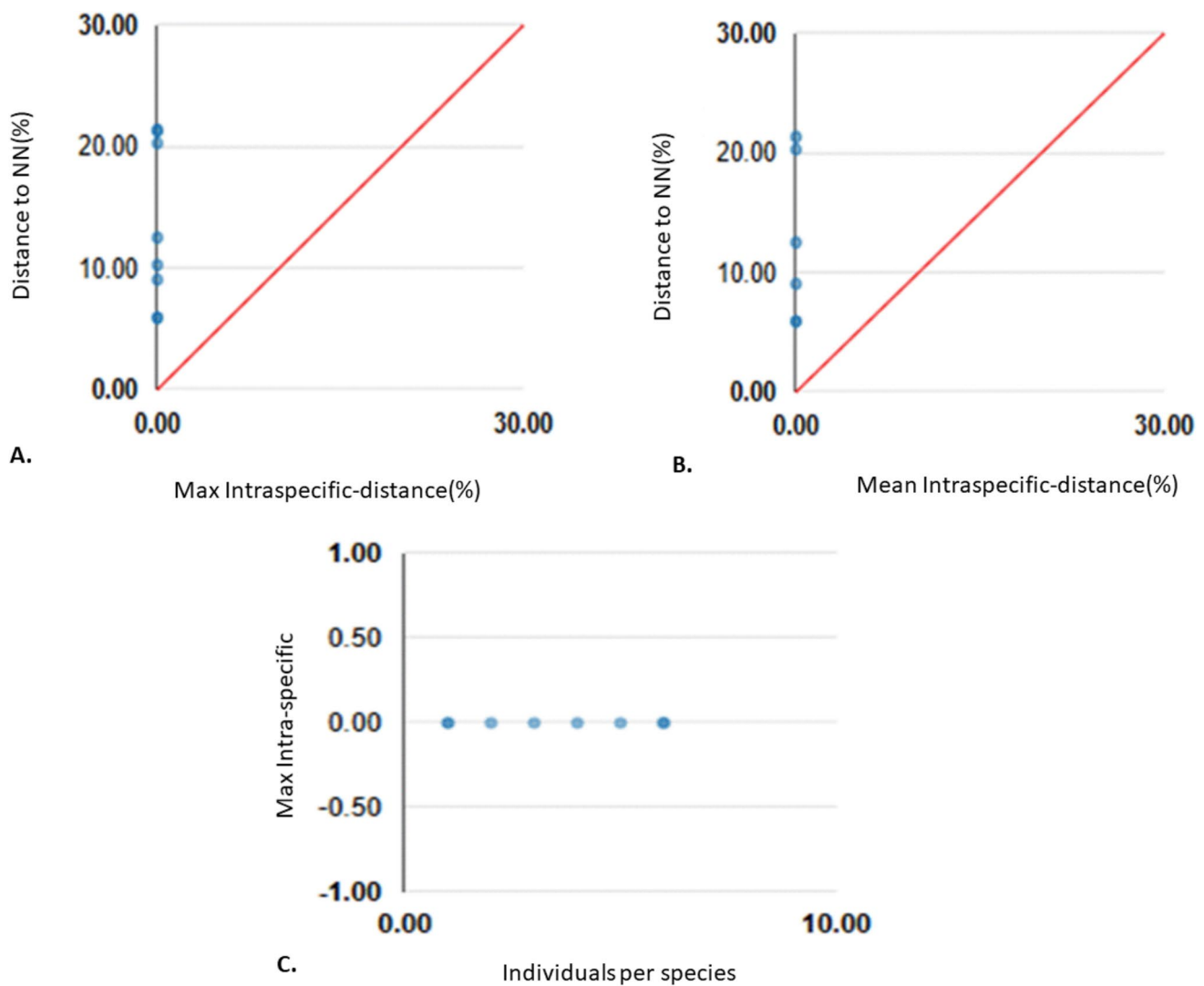


Fig. 2 Barcode gap analysis from sampled species. **A** Maximum intraspecific divergence vs. nearest neighbour (NN) distance. **B** Mean intraspecific divergence vs. NN distance. **C** The distribution of maxi-

mum intraspecific divergence values relative to the number of individuals sampled per species

between Malaysia and Indonesia, whereas Hap_4 was present in Indonesia (Fig. 6B). *C. burmense* exhibited two haplotypes. Hap_1 was shared between Pakistan and India, while Hap_2 was shared between India and Bangladesh (Fig. 6C).

Phylogenetic tree

The phylogenetic tree gave insights into the evolutionary relationships of the species (Fig. 7). The *Callorhynchus milii* species was used as an outgroup; bootstrap values ranged from 0.58 to 1.0, indicating variable levels of node confidence. The circular phylogenetic tree assigned specific clades to each species. *R. acutus* and *R. oligolinx* were well supported by a monophyletic cluster. Similarly, *C. sorrah*,

I. omanensis, and *S. laticaudus* were clustered in separate clades. Conversely, other taxa like *R. ancylostoma*, *C. burmense*, and *G. typus* were clustered under different clades.

Discussion

The present study used DNA barcoding to identify species, assess mitochondrial diversity, and provide baseline molecular data for elasmobranch biodiversity in Pakistani coastal waters. The study included several IUCN-threatened and two critically endangered species, highlighting the importance of continued research and conservation attention for these taxa. Considering the Critically Endangered and Vulnerable IUCN status of *R. ancylostoma*, *G. typus*, *C.*

Accumulation Curve Using All Data In Valid Groups

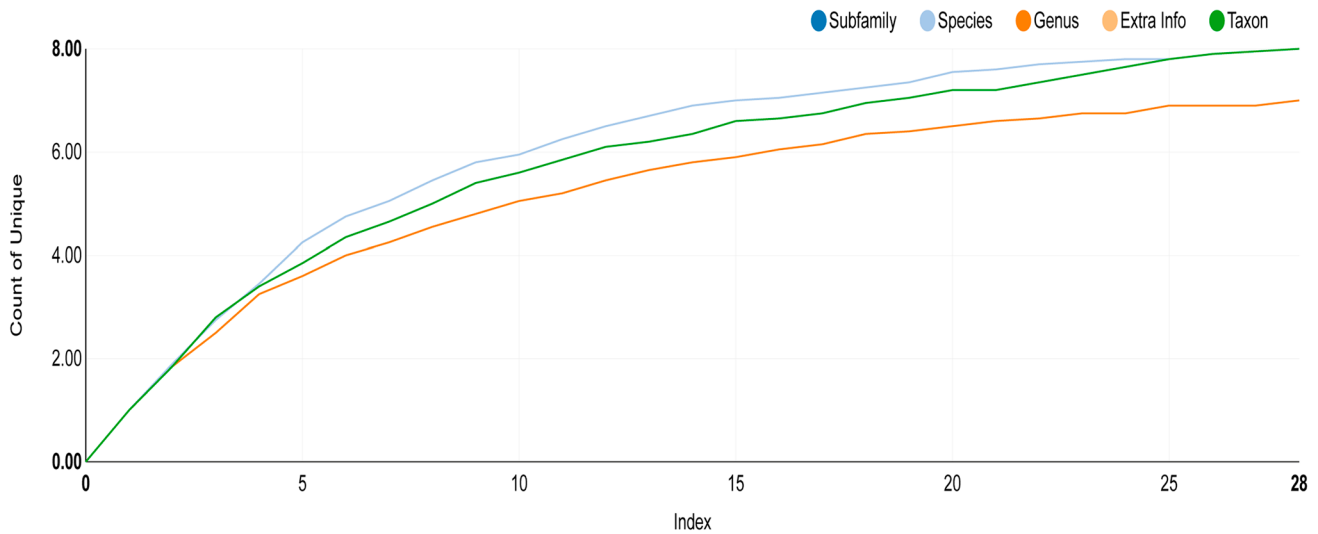


Fig. 3 The accumulation curve showing the number of taxonomic units identified on the y-axis and the effect of sampling effort on the x-axis. Each curve is represented by different colours for different taxonomic levels

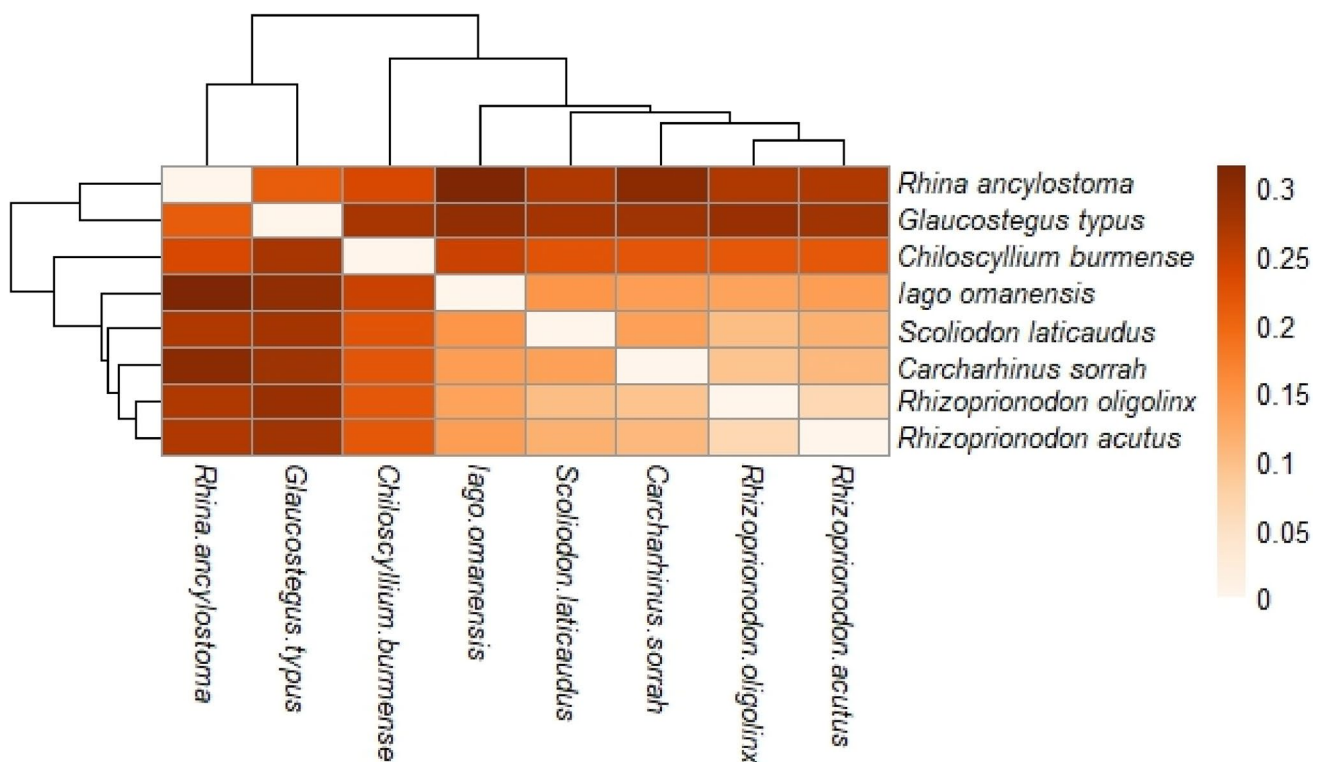


Fig. 4 Heatmap chart for the pairwise genetic distances. The scale bar according to the values is given on the figure. Lighter colour shows lower values and darker colour shows higher values

sorrah, *R. oligolinx* and *C. burmense*, the haplotype diversity of these taxa was analyzed. The research serves as a first molecular baseline for the Pakistani elasmobranchs and could be a paramount study for fishery resource conservation

and management (Fatima et al. 2016; Javed et al. 2025; Siddiqui and Javed 2025).

The presence of significant barcodes even in morphologically similar species can be seen from the barcode gap

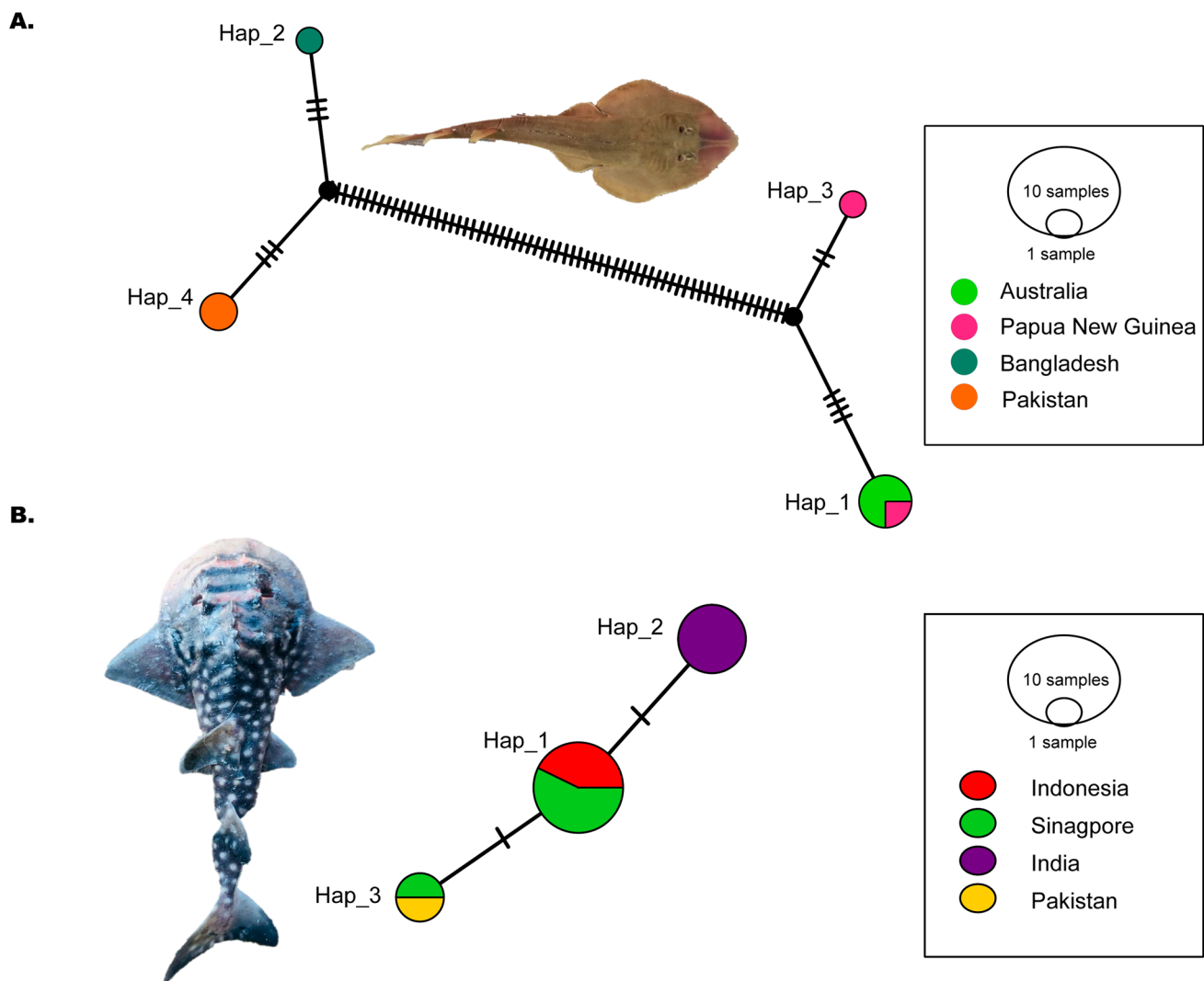


Fig. 5 Median-joining haplotype networks based on COI sequences for **A** *G. typus* and **B** *R. ancylostoma*. Circles represent unique haplotypes, with circle size proportional to the number of individuals sharing each haplotype. Colours indicate the geographic origin of samples, and hash marks on connecting lines represent mutational steps between haplotypes

analysis. The species of the genus *Rhizoprionodon* (*R. acutus* and *R. oligolinx*) are very similar in morphology; it is often challenging to differentiate between these potential species on a morphological basis. Therefore, providing a genetic basis to distinguish them is necessary. The main purpose of barcode gap analysis was to infer the COI-based boundaries between sampled species. Through these findings, it can easily be seen that these species, *Rhizoprionodon* (*R. acutus* and *R. oligolinx*), show a barcode gap of 5.91%, indicating a major difference between these two species. Moreover, the genetic distances and the phylogenetic tree analysis also separated these two species into separate clades. Although barcode gap analysis and COI-based genetic distance/phylogenetic results effectively distinguished some species in this dataset, their broader applicability should be interpreted with caution, as mitochondrial markers may not fully capture

species boundaries across all elasmobranch taxa due to factors such as incomplete lineage sorting, introgressive hybridization, or sex-biased dispersal (Sloan et al. 2017; Pazmiño et al. 2019; Roycroft et al. 2019). The accumulation curve suggests the effectiveness of DNA barcoding to capture the taxonomic diversity in Elasmobranchs, specifically at the species and subfamily levels. The saturation of these curves (Fig. 3) shows a reliable barcode dataset and enough sampling depth for most taxa. These findings demonstrated that DNA barcoding is a basic biodiversity monitoring tool (Chakraborty et al. 2021).

The COI divergence values observed in this study are consistent with those reported from major elasmobranch barcoding initiatives globally. Our mean intraspecific divergence (0.32%) closely matches values from Australia (0.37%, Ward et al. 2008), the broader Indian EEZ (0.32%,

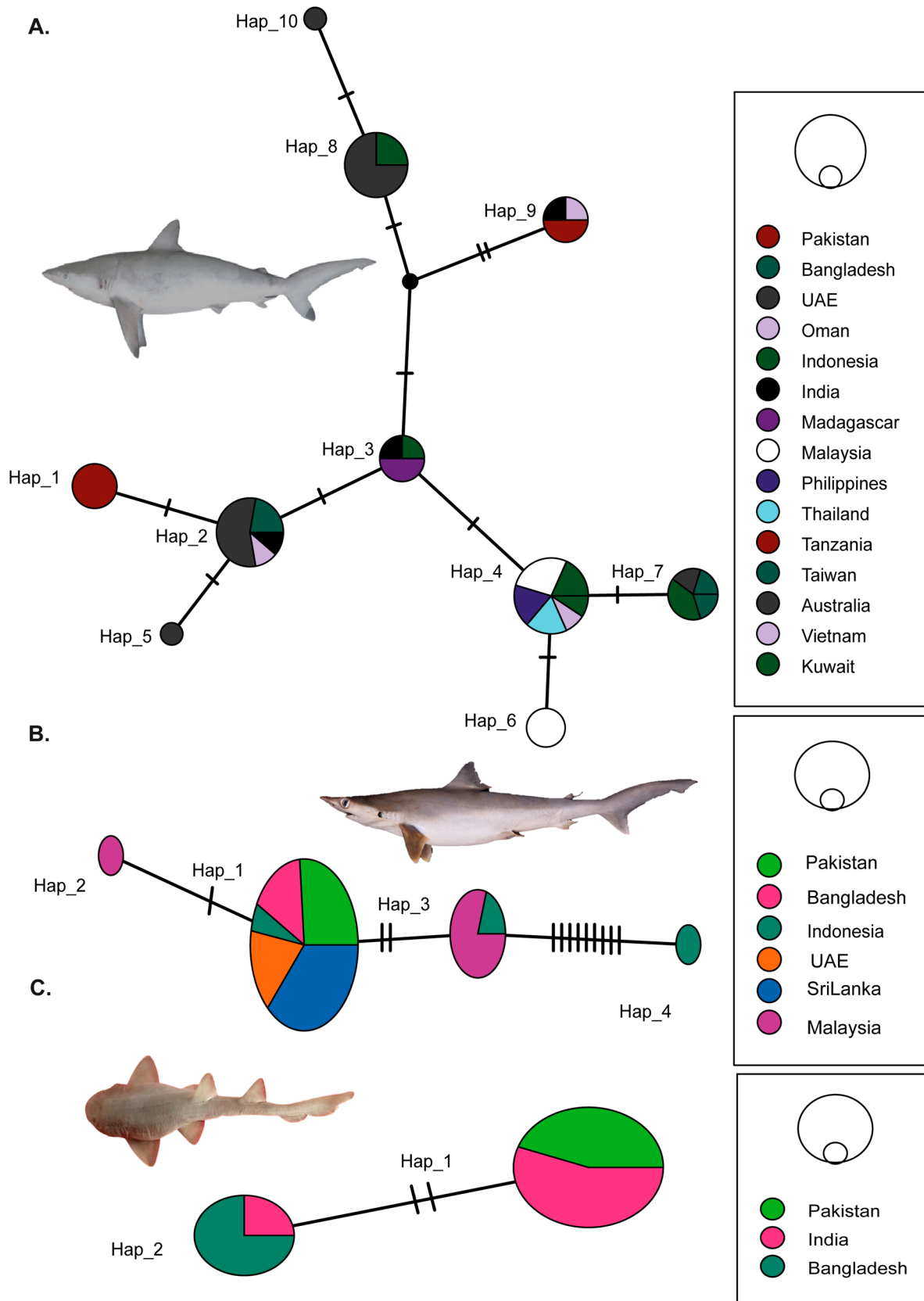


Fig. 6 Median-joining haplotype networks based on COI sequences for **A** *C. sorrah*, **B** *R. oligolinx*, and **C** *C. burmense*. Circles represent unique haplotypes, with circle size proportional to the number of individuals sharing each haplotype. Colours indicate the geographic origin of samples, and hash marks on connecting lines represent mutational steps between haplotypes

Bineesh et al. 2017), and the Mediterranean (0.29%, Cariani et al. 2017), reinforcing the low and predictable within-species variation of COI in elasmobranchs. Our mean congeneric divergence (6.73%) likewise aligns with these studies (range 6.73–7.48%), supporting the utility of DNA barcoding for species identification across geographic regions. Regarding the species-discrimination threshold, the 5.91% divergence between *R. acutus* and *R. oligolinx* substantially exceeds the conventional 2–3% threshold routinely applied for marine fishes (Ward et al. 2005), unequivocally supporting their status as distinct species. However, thresholds must be applied cautiously, as Ward et al. (2008) documented congeneric divergences as low as 0.45% between *Carcharhinus limbatus* and *C. tilstoni*, valid biological species that would be misclassified under a rigid threshold. Cerutti-Pereyra et al. (2012) similarly noted that the *Neotrygon kuhlii* complex exhibits up to 3.8% intraspecific divergence across geographic regions, potentially reflecting cryptic speciation rather than methodological failure. These observations underscore that while our COI data clearly discriminate the species in this study, thresholds should be applied flexibly and in concert with morphological and ecological evidence.

The different haplotype designs represented by the guitarfish (*G. typus*) indicate a significant increase in mitochondrial diversity among the studied individuals (Fig. 5A). The occurrence of Hap_1 in both Australia and Papua New Guinea may indicate historical or ongoing COI lineage sharing within the Indo-Pacific region; however, this interpretation remains tentative given the limited sample coverage. Conversely, the haplotypes Hap_2 in Bangladesh and Hap_4 in Pakistan are a result of a local COI divergence. The observed haplotype patterns suggest preliminary COI-based variation, which should be further evaluated using nuclear markers and larger, geographically balanced sample sizes. The haplotype diversity of *R. ancyllostoma* was delineated using COI. Hap_1 showed a broader distribution and was shared among individuals from Indonesia and Singapore. Hap_3 was identified in individuals from Singapore and Pakistan, indicating possible COI-based genetic connectivity; however, the limited Pakistani sampling precludes robust inference of contemporary genetic connectivity. Besides, the determination of mutational steps along each haplotype, illustrated in Fig. 5B, indicates low to moderate COI-based genetic divergence, which could be due to recent diversification.

The additional haplotype networks of *C. sorrah*, *R. oligolinx*, and *C. burmense* further showed contrasting patterns of COI-based mitochondrial divergence among elasmobranch species in the Indo-West Pacific. *C. sorrah* exhibited the highest haplotype diversity, with ten haplotypes distributed across multiple regions, suggesting preliminary COI-based historical connectivity and gene flow throughout its wide distribution. In contrast, *R. oligolinx* displayed four haplotypes, with a single dominant haplotype shared across much of its range and several geographically restricted haplotypes, indicating a combination of widespread COI-based maternal lineages and localized genetic variation. *C. burmense* exhibited only two haplotypes, both shared among neighbouring countries, reflecting comparatively low COI-based mitochondrial diversity, which may be associated with its more restricted distribution and limited dispersal capability. Although COI-based mitochondrial haplotype networks provide valuable insights into maternal lineage diversity and phylogeographic patterns, broader sampling across the species' distribution ranges and the inclusion of nuclear genetic markers would provide a more comprehensive understanding of population connectivity and evolutionary history.

Comparative haplotype data for *R. ancyllostoma*, *G. typus*, *C. sorrah*, *R. oligolinx*, and *C. burmense* in the Northern Arabian Sea are almost entirely absent, highlighting the novelty of our contribution. Bineesh et al. (2017) generated COI sequences for both species (*R. ancyllostoma* and *G. obtusus* from Kochi), providing regional reference points, but no published haplotype analysis exists from Pakistan, Iran, Oman, or the Arabian Gulf. The mutational steps connecting Pakistan and Singapore haplotypes in our *R. ancyllostoma* network (Fig. 5B) suggest possible long-distance COI-based connectivity, analogous to patterns observed in other batoids. Cerutti-Pereyra et al. (2012) found identical *G. typus* haplotypes across > 3000 km of Australian coastline, and shared *Pastinachus atrus* haplotypes across Australia, Indonesia, and Malaysia. Whether similar COI-based connectivity exists between Pakistan and Southeast Asian *R. ancyllostoma* populations cannot be determined from our limited sample; expanded geographic sampling incorporating nuclear markers will be required to resolve this question. Our study, therefore, provides a critical first molecular baseline for elasmobranchs in Pakistani waters, establishing reference data that will enable future assessments of population connectivity, fishery impacts, and conservation priorities in this understudied region.

Several limitations of the present study should be acknowledged when interpreting the haplotype and phylogeographic patterns. In particular, *S. laticaudus* and *R. ancyllostoma* were each represented by only a single individual from Pakistan, limiting our ability to infer within-population diversity or regional population structure for these

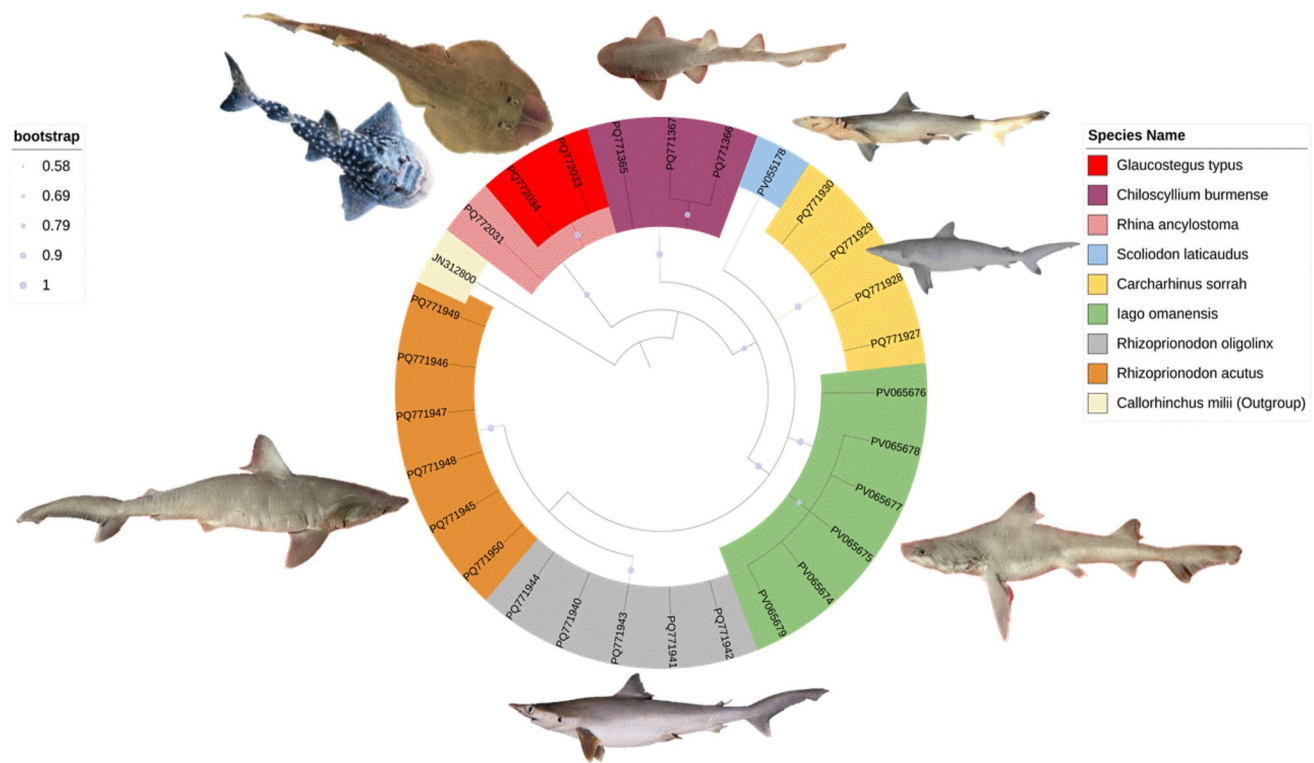


Fig. 7 Phylogenetic analysis of sequences generated in this study. Each species included in the tree is represented by a different colour. Nodes indicate bootstrap support, whereas the branch tips show the

corresponding NCBI accession numbers. Photographs of each species are displayed adjacent to the respective clades

taxa. In addition, the haplotype network for *R. ancylostoma* relied predominantly on publicly available COI sequences retrieved from NCBI/GenBank, resulting in uneven geographic representation among sampled regions. Consequently, the observed haplotype sharing patterns should be interpreted as preliminary evidence of COI variation rather than definitive indicators of population connectivity across the Indo-Pacific region. Expanded sampling from Pakistan and adjacent waters, together with nuclear genomic markers, will be necessary to robustly assess population structure and connectivity.

The phylogenetic analysis of the COI sequences showed evolutionary relationships of the studied species, and the supported clades are quite consistent with the well-known taxonomic classifications. It is worth noting that the representatives of the genus *Rhizoprionodon* (*R. acutus* and *R. oligolinx*) were grouped in one clade and supported by bootstrap values of between 0.58 and 1.0, which is reflective of close genetic and morphological relationships. Similarly, the grouping of *C. sorrah*, *I. omanensis*, and *S. laticaudus* within a single clade is consistent with their broader familial relationships; however, interpretation of the *S. laticaudus* placement should be considered cautiously because only one Pakistani specimen was available for analysis. On the contrary, the formation of three separate clades of species

that include *R. ancylostoma*, *C. burmense*, and *G. typus* shows their increased genetic and morphological separation, which can be explained by the fact that they belong to different families. These findings contribute to the study of COI-based phylogeny of elasmobranchs and support the use of more genetic markers to improve phylogenetic resolution (Gore et al. 2019).

These elasmobranch species were collected from daily fish landing facilities, which have no monitoring for endangered and Critically Endangered species, so immediate conservation strategies are needed. The implementation of catch reporting and monitoring programmes at local fish landing sites is crucial for gathering species-specific data and trade records (Fatima et al. 2016; Gore et al. 2019; Javed et al. 2025). This will help to identify the threatened species and to gain insights into the dynamics of the elasmobranch product market (Haque et al. 2023). Furthermore, the live release of threatened species, together with educational initiatives for fishermen on safe handling practices, could significantly reduce fishing mortality (Poisson et al. 2014; Gupta et al. 2020). The establishment of marine protected areas and no-fishing reserves, especially in the Arabian Sea, could offer additional protection to critical elasmobranch populations (Schärer-Umpierre et al. 2014). Moreover, it is also important to note that conservation managers face a scenario

where a percentage of described elasmobranch species can still be traded, making understanding and regulating trade in these species challenging (Prasetyo et al. 2023). Therefore, national programmes for the conservation and management and national plans of action should be strictly followed (Fatima et al. 2016; Gore et al. 2019; Javed et al. 2025).

Based on these results, it is crucial to have strict monitoring of the fish catch and management of elasmobranchs. Further research integrating multiple genetic markers and expanding geographic coverage will be important for refining our understanding of elasmobranch evolution and informing effective conservation policies (Stanton et al. 2019). Nevertheless, some phylogeographic interpretations remain preliminary due to limited sample representation for certain taxa.

Conclusions

In conclusion, this study provides critical insights into the genetic diversity, phylogenetic relationships, and conservation status of elasmobranch species in Pakistan. The application of DNA barcoding has proven effective in species identification and biodiversity monitoring, revealing unique COI-based genetic lineages and highlighting the vulnerability of threatened species. Given the unregulated harvesting practices observed at local fish landing facilities, the findings underscore the urgent need for implementing comprehensive monitoring programmes, promoting sustainable fishing practices, and establishing protected areas to safeguard elasmobranch populations and ensure the long-term health of marine ecosystems in the region.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12526-026-01699-1>.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All procedures involving animals were approved by the Ethical Committee of the University of Karachi (Approval No. IBC KU-260/2022).

Data availability COI barcode sequences generated in this study are available in GenBank (accession numbers PQ771365-PQ772034 and PV055178-PV065679; see Table 1 for details). All records are also available in BOLD. R scripts used for analysis are available in the supplementary file.

Author contribution MR: investigation, data curation, formal analysis, visualization, writing—original draft, writing—review and editing. SA: investigation, data curation, formal analysis, writing—review and editing. AMK: conceptualization, methodology, supervision, funding

acquisition, writing—review and editing. AAK: investigation, methodology, resources. CHL: formal analysis, methodology, visualization, writing—review and editing. MA: data curation, formal analysis, writing—review and editing.

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