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Review Article

DNA metabarcoding of trawling bycatch reveals diversity and distribution patterns of sharks and rays in the central Tyrrhenian Sea

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Conservation and management of chondrichthyans are becoming increasingly important, as many species are particularly vulnerable to fishing activities, primarily as bycatch, which leads to incomplete catch reporting, potentially hiding the impact on these organisms. Here, we aimed at implementing an eDNA metabarcoding approach to reconstruct shark and ray bycatch composition from 24 hauls of a bottom trawl fishing vessel in the central Mediterranean. eDNA samples were collected through the passive filtration of seawater by simple gauze rolls encapsulated in a probe (the "metaprobe"), which already showed great efficiency in detecting marine species from trace DNA in the environment. To improve molecular taxonomic detection, we enhanced the 12S target marker reference library by generating sequences for 14 Mediterranean chondrichthyans previously unrepresented in public repositories. DNA metabarcoding data correctly identifies almost all bycaught species and detected five additional species not present in the net, highlighting the potential of this method to detect rare species. Chondrichthyan diversity showed significant association with some key environmental variables (depth and distance from the coast) and the fishing effort, which are known to influence demersal communities. As DNA metabarcoding progressively positions itself as a staple tool for biodiversity monitoring, we expect that its melding with opportunistic, fishery-dependent surveys could reveal additional distribution features of threatened and elusive megafauna.

Keywords: biomonitoring, DNA metabarcoding, Elasmobranch, fisheries, "metaprobe", Mediterranean Sea, rays, sharks.

Introduction

Chondrichthyans, which include sharks, batoids (rays and skates), and chimaeras, are, irrespective of their preferred habitat or ecology, particularly vulnerable to anthropogenic impacts, especially to fishing activities (Stevens *et al.*, 2000; Dell'Apa *et al.*, 2012; Ramírez-Amaro *et al.*, 2020). In recent years, shark populations have suffered significant declines worldwide (MacNeil *et al.*, 2020; Pacoureau *et al.*, 2021; Walls and Dulvy, 2021) and this is largely due to direct or indirect pressures from capture fisheries (Dulvy *et al.*, 2021). The Mediterranean Sea is one of the most studied biodiversity hotspots on the planet, hosting 88 chondrichthyan species: 2 holocephalans, 38 rays and skates, and 48 sharks (Cariani *et al.*, 2017; Serena *et al.*, 2020; Supplementary Appendices S1 and S2). However, the actual occurrence of some species is still uncertain. The International Union for Conservation of Nature (IUCN; www.iucnredlist.org) listed nearly 40% of Mediterranean elasmobranchs as species at risk of extinction. The most impacted populations are large predatory sharks, whose abundance has decreased over the last century (Ferretti *et al.*, 2009; Leonetti *et al.*, 2020; Serena *et al.*, 2020).

Although none of the Mediterranean cartilaginous fish species is being directly targeted by fisheries, many of them are often trapped as bycatch of bottom trawl fishery, and longlines, including several endangered species (FAO, 2022). The most frequently caught taxa are skates (Rajidae), catsharks (*Scyliorhinus* spp. and *Galeus* spp.), and lantern sharks (*Etmopterus* spp.) (Cashion *et al.*, 2019; Follesa *et al.*, 2019). Often, fished chondrichthyans are not reported in official fishery statistics, leading to an underestimation of the impact of fishing on their populations. Furthermore, in some areas, their overall landings are often far outweighed by discarded catches (Cashion *et al.*, 2019), and the taxonomic resolution of catch reports is insufficient to gauge the actual population status and distribution of most chondrichthyan species (Cashion *et al.*, 2019). The Marine Strategy Framework Directive (MSFD; 2008/56/EC), which aims to achieve "Good Environmental Status" (GES) in European seas, emphasizes the maintenance of biodiversity in pelagic habitats for fragile species

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and key components of the ecosystem. As apex predators, sharks have a central role in this MSFD criterion and need well-structured monitoring programmes based on accurate, noninvasive, and reproducible methods. The recently adopted GFCM 2030 Strategy for sustainable fisheries and aquaculture in the Mediterranean and the Black Sea, also offers a common vision and guiding principles to achieve sustainable fisheries and aquaculture in the region. One of the targets of this strategy intends to establish effective area-based measures to minimize and mitigate impacts on vulnerable species (including elasmobranchs), sensitive habitats, and essential fish habitats to meet international spatial conservation targets (FAO, 2021). In the past, chondrichthyans were scarcely considered in fishery management, but in the last decade, their role as indicators of potential overfishing has become more prominent. For instance, in the Balearic Islands, the lesser spotted catfish (*Scyliorhinus canicula*) has been used as an indicator species for monitoring trawl fishery bycatch (Carbonell *et al.*, 2003).

Current methods for obtaining census data on cartilaginous fish are based on the visual identification of caught individuals, which is invasive and expensive, often species-selective and dependent on taxonomic expertise (Boussarie *et al.*, 2018). The recent development of environmental DNA (eDNA) offers a powerful, cost-effective, noninvasive alternative (Taberlet *et al.*, 2012; Thomsen *et al.*, 2012), which has been shown to be effective in tracking the presence of cartilaginous fish in various environments (Bakker *et al.*, 2017; Boussarie *et al.*, 2018; West *et al.*, 2021; Dunn *et al.*, 2022; Liu *et al.*, 2022). Spatial models are continuously developed for studying the impact of demersal fisheries, and the spatial origin of catches, using satellite-based information on fishing activities (D'Andrea *et al.*, 2020; Russo *et al.*, 2018, 2019); eDNA could enhance these models, being itself an important source of biological information. The vast expanse of the sea, however, still constitutes a barrier to the generation of species inventories at the scale and granularity that can significantly improve management practices. Here, we assessed the diversity of sharks, rays, and chimaeras inhabiting the central Tyrrhenian Sea (GFCM-Geographical Sub Area, GSA 9), employing an eDNA metabarcoding approach on a commercial bottom-trawl fishing vessel. Samples were gathered using the "metaprobe", a recently developed low-cost eDNA sampling tool that entails minimum disruptions of the fishers' activities (Maiello *et al.*, 2022). Since chondrichthyans are a typical bycatch component of trawlers, in the present study, we aimed at (i) contrasting species composition detected via eDNA metabarcoding analysis with catch data recorded aboard the fishing vessel, in order to test the possibility of using simple and cheap metabarcoding-based tools for more efficient monitoring of elasmobranch bycatch; and (ii) exploring the patterns of species distribution across sampling sites, in relation to some key environmental (i.e. sea bottom trawling depth and distance from the coast), ecological (i.e. alpha diversity), technical (i.e. percentage of nontarget reads), and anthropogenic (i.e. the different fishing effort exerted by the fleet of trawlers across the study area) variables.

The possibility of combining easy and inexpensive DNA metabarcoding-based sampling devices with the regular activities of fishers at sea to monitor not only target stocks but also bycatch and discarded species could add an increasing value to marine management, with a view to adding fishing vessels to the arsenal of observational platforms available to monitor the oceans.

Methods

Sampling procedures

eDNA samples were collected between July and August 2020 at 24 sites (H1–H24) in the central Tyrrhenian Sea (GFCM-Geographical Sub Area, GSA 9–western Mediterranean Sea) (Figure 1), aboard a commercial bottom trawl fishing vessel. Sampling locations covered various bathymetric layers, from shallower, edge-of-shelf areas with an average depth of 200 m, to the deep slope down to >800 m depth (Supplementary Appendix S3). For eDNA collection, we employed two different approaches. For each haul, we collected the water dripping from the net cod-end just after it was hauled on board (hereafter "slush"; Russo *et al.*, 2021). "Slush" water was placed in 50 ml sterile tubes and immediately frozen at −20°C. The second sampling method we used was custom-made rolls of gauze (1 g of cotton rolled in three sterile gauzes) tightly fixed by plastic cables tied inside a 3D-printed bespoke hollow sphere (henceforth "metaprobe"; Maiello *et al.*, 2022). The metaprobe was placed inside the net at the beginning of each haul and retrieved at the end of fishing operations, during the sorting of catches. Two gauze rolls were collected and placed in separate 50 ml sterile tubes containing 99% ethanol and silica gel grains, respectively, for genetic material preservation. Both tubes were frozen on board and maintained at −20°C until DNA extraction. To assess the influence of background contamination linked with the very presence of a fishing boat at sea, in 2 out of the 24 sampling sites, we collected marine water by dropping a bucket from the gunwale, from which 50 ml tubes were taken as field controls. The species composition of each haul was qualitatively determined by onboard visual sorting of net content.

Reference data for Mediterranean cartilaginous taxa

To improve molecular detection of cartilaginous taxa, we implemented an optimized reference database. We first downloaded an accurate and updated list of Mediterranean chondrichthyan species following the steps available at github.com/genner-lab/meta-fish-lib (Collins *et al.*, 2021). The list was then validated according to the recent literature (Serena *et al.*, 2020; F. Serena pers. comm.) and integrated by combining information from FishBase (Froese and Pauly, 2022), the IUCN Red List (www.iucnredlist.org), and Eschmeyer's Catalog of Fishes (researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp) (Supplementary Appendices S1 and S2). Of the 88 chondrichthyan species occurring in the Mediterranean Sea, 67 taxa had 12S ribosomal RNA sequences uploaded in public repositories; for those species, 12S sequences were retrieved from NCBI Genbank with the search term "12S OR 12S ribosomal RNA" and filtering the data by selecting "Animals" and "Mitochondrion". This resulted in 249581 sequences (accessed December, 2021). On the other hand, 21 chondrichthyan species lacked a 12S sequence in the nt NCBI Genbank public repository. To enhance the baseline for taxonomic assignment, we procured tissues for 14 of them using the voucher specimen repository of the ELASMOMED initiative (Cariani *et al.*, 2017), and generated the relevant mitochondrial sequences. Total genomic DNA (gDNA) was extracted from 1 to 5 voucher specimens for each species using 20 mg of tissue (fin or muscle) and applying the RBCBioscience® Tissue Mini Kit (Real Genomics®) according to the manufacturer's protocol. To validate the taxonomy,

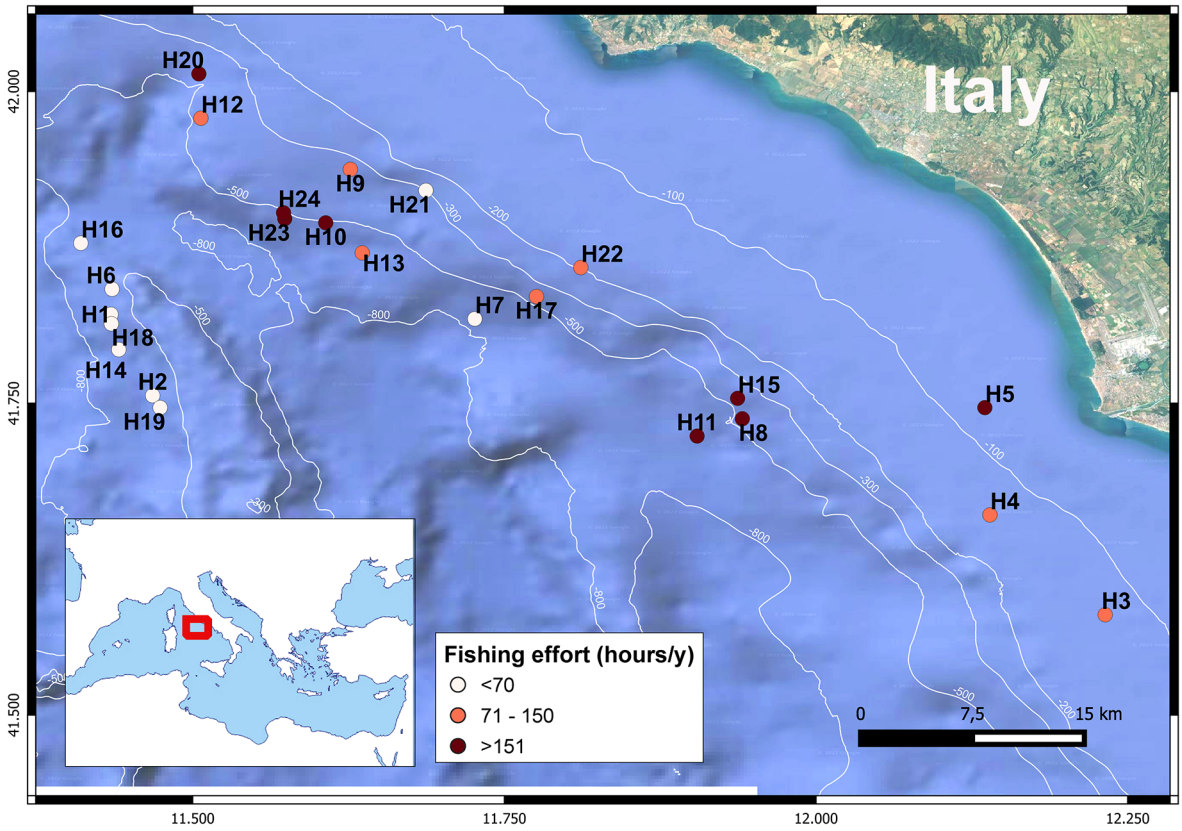

Figure 1. Map of the 24 sampling locations in the central Tyrrhenian Sea (FAO Geographical Sub Area, GSA 9–western Mediterranean Sea), with related fishing effort (hours of trawl fishing) and bathymetry layer (QGIS 3.4; Supplementary Appendix S3).

newly processed specimens were first processed for the mitochondrial Cytochrome oxidase subunit I (COI) marker with the FishF2-FishR2 primer set for skates and rays and FishF2-FishR1 for sharks (Ward *et al.*, 2005) and for NADH dehydrogenase subunit 2 (NADH2) for the taxa in which COI did not fully resolve the relationships (following Naylor *et al.*, 2012). After validation, samples were then PCR-amplified with the Aa22-PheF and Aa633-12sR primers (Collins *et al.*, 2021) to generate additional reference sequences for the 12S rRNA gene. All amplicons were enzymatically purified and then sequenced by Macrogen Europe BV. To visualize relationships among retrieved 12S sequences and the newly generated ones, we aligned them using the Multiple Alignment Using Fast Fourier Transform (MAFFT) tool (Katoh *et al.*, 2002) and built Neighbor-Joining trees using a p-distance model with 1000 bootstrap replicates (Felsenstein, 1985) in MEGA-X (Kumar *et al.*, 2018). Analyses were performed for COI and 12S separately (Supplementary Appendices S4, S5, and S6).

Laboratory processing of eDNA samples

For DNA extraction from the "metaprobe" rolls of gauze, half of each gauze roll was cut into small pieces and lysed overnight at 56°C with 400 µl of Extraction Buffer (0.5 M EDTA pH 8, 1 M urea) and 20 µl of proteinase K (100 μg ml^{-1}) (Malmström *et al.*, 2009). Approximately 150 μl of the lysed solution was transferred into QIAquick Spin Columns; the DNA was then purified by the QIAquick PCR Purification Kit (following the manufacturer guidelines) and finally eluted in 110 µl of Elution Buffer. "Slush" samples were filtered through cellulose filters (0.2 μm) with a vacuum pump before extraction, in order to concentrate the DNA. Total DNA was then extracted from the filters following the soil Mu-DNA extraction protocol (Sellers *et al.*, 2018). For both "metaprobe" and "slush" samples, we carried out PCR amplification by targeting a short 12S ribosomal RNA fragment of the mitochondrial genome (Miya *et al.*, 2015) using the Elas02 primers, specifically designed for elasmobranchs (Taberlet *et al.*, 2018). To distinguish samples and minimize PCR/sequencing cross-contamination, each primer pair carried unique 8 bp tags, the same for both forward and reverse primers. Each sample was amplified in triplicate, in a total volume mix of 20 µl, consisting of 10 µl MyFi™ Master Mix (Meridian Bioscience), 0.16 µl Bovine Serum Albumin (BSA; 20 mg ml^{-1}, Thermo Scientific), 5.84 μl of UltraPure™ Distilled Water (Invitrogen), 2 µl of forward and reverse primers (10 µM, Eurofins), and 2 µl of DNA template. PCR profile included: initial denaturation at 94°C for 5 mins, 40 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min, and a final extension step of 72°C for 5 mins. Both field and laboratory controls were included to monitor for possible contaminations at each step. Specifically, we had two seawater controls, four extraction negative controls, and a negative (i.e. reagents and UV-irradiated nucleotide-free H_2O in place of DNA template), a blank (i.e. only reagents), and a positive (i.e. the beaked redfish, *Sebastes mentella*, a subarctic species absent in the Mediterranean Sea) PCR controls. The three replicates of PCR products were pooled and checked on a 2% Agarose electrophoresis gel stained with SYBR safe to ensure the correct amplification of the target fragment. We performed PCR purification with a size selection magnetic

bead clean-up protocol (1×, Bronner *et al.*, 2009) on 30 µl of pooled PCR products. Cleaned PCR products were normalized to pool samples in equimolar concentrations, according to the total DNA concentration checked with a Qubit™ 4.0 fluorometer with a Qubit™ dsDNA HS Assay Kit (Invitrogen). Pooled samples were analysed using a TapeStation 4200 (Agilent, USA) to check DNA fragment size. Adapters were ligated using the NEXTFLEX® Rapid DNA-Seq Kit 2.0 by PerkinElmer (1 µg), and the library was finally quantified by qPCR using the NEBNext® Library Quant Kit for Illumina®, and sequenced at a 65 pM concentration with 10% PhiX control on an iSeq 100 platform using a 300-cycle i1 v2 kit.

Bioinformatics

For data analysis, we followed the OBITools metabarcoding pipeline (Boyer *et al.*, 2016). After the quality control of reads performed with FastQC, *illuminapairedend* was used to merge forward and reverse sequences retaining all paired-end alignments with a quality score >40. Then, samples were demultiplexed using *ngsfilter* and sequences were length filtered via *obigrep* to include only fragments in the expected length range (140–200 bp) (Taberlet *et al.*, 2018). *Obiuniq* was used to dereplicate reads and the *uchime* command in vsearch to remove chimeras. Sequences were clustered in Molecular Operational Taxonomic Units using SWARM v3.0 (Mahé *et al.*, 2015), with a clustering threshold of $d = 3$. The taxonomic assignment was performed using the Bayesian Lowest Common Ancestor taxonomic classification method (Gao *et al.*, 2017), against a custom 12S reference database created augmenting the nt NCBI Genbank 12S data with the newly obtained data for the additional 14 chondrichthyan species. A manual inspection was performed to validate the taxonomic assignment using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Datasets were finally filtered retaining only sequences showing >98% identity match (Miya *et al.*, 2015) and removing potential contamination noise taking advantage of field and laboratory controls with the "decontam" package in R (Davis *et al.*, 2018), based on the proportional abundance of reads and the prevalence model with a threshold of 0.5. Four field controls were processed to account for the level of contamination linked with sampling procedures during trawling activities, while extraction and PCR negative controls were assessed to monitor for laboratory contaminants.

Data analysis

For all downstream analysis, chondrichthyan species detected by "slush" water and "metaprobe" gauze samples were combined for each sampling site, to provide an overall eDNA metabarcoding output. To compare the overall composition of taxa obtained from eDNA and catches, we built Venn diagrams (bioinfogp.cnb.csic.es/tools/venny/) at both species and genus levels, combining data from all sampling sites. We then explored patterns of chondrichthyan distribution among the 24 sampling sites, performing a Nonmetric Multidimensional Scaling (NMDS) based on Jaccard distance on a presence–absence binary dataset of all the elasmobranchs detected by DNA metabarcoding. Estimates of diversity were evaluated by accounting for two environmental variables (depth and distance from the coast) and the impact of fishing activities (fishing effort), calculated as the mean total of yearly effort, in hours of trawling, over the last 5 years, in the range of 1 km radius around each haul, using Vessel Monitoring System data and applying the procedures described in Russo *et al.* (2011a, b, 2014, 2016). Despite the high correlation between depth and distance from the coast, we decided to keep these two environmental variables separate in the analyses in order to examine subtle complexities in sea bottom morphology, such as shoals, seamounts, or canyons (i.e. some distant sites targeted by fishers would be found on grounds shallower than some others closer to the coast). We included in the analysis two more variables that could influence elasmobranch beta-diversity distribution among sites: the alpha diversity (calculated as the total number of chondrichthyans species per each sampling site) and the percentage of nontarget (Actinopterygii) reads, as this could affect target (chondrichthyans) detection power (Gloor *et al.*, 2017; Silverman *et al.*, 2021). The percentage was calculated as the total number of actinopterygians reads over the total number of reads (including Actinopterygii and Chondrichthyes) for each sampling site. The NMDS analysis provided by the "metaMDS" function of the R package "vegan" (Oksanen *et al.*, 2018) was applied in order to explore the information from the distribution of chondrichthyan species across the 24 sampling sites. The NMDS was performed on the Jaccard distance matrix defined by the presence/absence of species at each sampling site. The NMDS allows assessing similarity/dissimilarity among samples of multiple taxa while being largely unconstrained by assumptions of multivariate normality and homoscedasticity. The effect of the five considered variables (i.e. depth, distance from the coast, fishing effort, alpha diversity, and percentage of nontarget reads) was then evaluated through environmental fit on the NMDS plot using the "envfit" function in the "vegan" R package. The function fits environmental vectors or factors into an ordination plot in order to maximize the correlation with corresponding variables: each arrow on the plot represents the gradient of a variable. With this approach, the environmental variables are the dependent variables explained by the ordination scores, and each dependent variable is analysed separately and the significance of fitted vectors is assessed using permutation of environmental variables.

To better visualize the effect of the two environmental components (i.e. depth and distance from the coast) and the fishing effort, we additionally drew polygons on the NMDS plot, partitioning variables into categories. Depth ranges were determined based on actual changes in sea bottom morphology (i.e. continental shelf ≤300 m, continental slope = 300–600 m, deep slope ≥600 m) and the distance from the coast gradient was split into three ranges (i.e. <11 km, 11–17 km, and >17 km). For fishing effort, categories were identified based on expert knowledge about the fishing footprint in the considered area (Russo *et al.*, 2019) (i.e. low ≤70 fishing hours year^{-1}, medium = 70–150 fishing hours year^{-1}, high ≥150 fishing hours year^{-1}).

Results

Reference data for Mediterranean cartilaginous taxa

We successfully sequenced the 12S of the 14 chondrichthyan species absent in the reference database of which tissues were available, which, added to the existing 67 taxa already deposited, bolstered our custom reference database with a to-

Table 1. List of chondrichthyan species recorded in the eDNA samples, annotated with total number of reads and total number of hauls containing the species (for detailed occurrence per hauls, see Supplementary Appendix S8).

Order	Genus	Species	Total reads	No. Hauls
Chimaeriformes	*Chimaera*	*Chimaera monstrosa*	13 884	17
Myliobatiformes	*Pteroplatytrygon*	*Pteroplatytrygon violacea*	5	1
Rajiformes	*Dipturus*	*Dipturus oxyrinchus*	7 556	14
Rajiformes	*Leucoraja*	*Leucoraja circularis*	9 264	21
Rajiformes	*Raja*	*Raja* sp.	170	4
Carcharhiniformes	*Carcharhinus*	*Carcharhinus falciformis*	182	1
Carcharhiniformes	*Carcharhinus*	*Carcharhinus leucas*	41	1
Carcharhiniformes	*Carcharhinus*	*Carcharhinus limbatus*	14	1
Carcharhiniformes	*Galeus*	*Galeus melastomus*	404 382	24
Carcharhiniformes	*Scyliorhinus*	*Scyliorhinus canicula*	6 834	23
Carcharhiniformes	*Scyliorhinus*	*Scyliorhinus stellaris*	13	1
Hexanchiformes	*Hexanchus*	*Hexanchus griseus*	1 959	19
Squaliformes	*Centrophorus*	*Centrophorus* sp.	163	2
Squaliformes	*Dalatias*	*Dalatias licha*	1 400	13
Squaliformes	*Etmopterus*	*Etmopterus spinax*	49 266	24

tal of 81 Mediterranean chondrichthyan species (Supplementary Appendix S1). The seven missing species are rare in the Mediterranean Sea and therefore not expected to introduce bias in the taxonomic assignment. The tree topologies generated with COI and 12S data largely overlap (Supplementary Appendices S4 and S5), thus supporting the suitability of the selected barcode for the identification of Mediterranean chondrichthyan taxa.

eDNA data processing and analysis

The Illumina sequencing run produced a total of 4400000 paired raw reads. After filtering, 2256366 reads were retained, of which 495133 belonged to chondrichthyans. The majority of other reads belonged to nontarget marine taxa, mostly teleosts, which were removed from our dataset because their identification was outside of the scope of this study. A mean of 889 reads was associated with extraction controls and no reads were present in PCR controls. Neither elasmobranch nor teleost species were identified as contaminants using the 0.5 prevalence threshold with "decontam". A total of 71 species of bony fish were identified (Supplementary Appendix S7), despite the set of primers being designed for elasmobranchs. The taxonomic assignment allowed the detection of 15 chondrichthyans taxa, 13 of which were identified at the species level and 2 down to the genus (Table 1; Supplementary Appendix S8). Among the recorded species, eight of them occurred in the majority of the hauls, while the remaining ones were associated with few or even a single sampling location (Table 1). The Venn diagram in Figure 2 showed that eight species were detected by both eDNA metabarcoding and catch samples. However, five species were detected only by eDNA, and two only by trawl samples. At an upper taxonomic level, the Venn diagram showed that all genera found in trawl catches were also identified by eDNA metabarcoding.

The NMDS returned a pattern (defined as the relative position of sampling sites) associated with a stress <5%, which is generally acknowledged as an excellent representation in reduced dimensions. The sampling sites are homogeneously dispersed around the axis origin, without separation of defined groups. However, the positions of the different species allow recognition of specific distribution patterns among the two NMDS axes (Figure 3). All the considered variables (i.e. depth, distance from the coast, alpha diversity, and percentage of nontarget reads) are significantly correlated with the data-point distribution on the NMDS plot, with the exception of fishing effort, which was only slightly over the level of significance (Table 2). Vectors evidenced a major influence on the first NMDS axis of all the variables except the alpha diversity, which had a "top-right" to "bottom-left" effect (Figure 3). Polygons showed that the first NMDS axis roughly separated samples along a right-to-left gradient of depth (Figure 3b) and distance from the coast (Figure 3c). The amount of fishing effort was also different along this axis: sampling sites of less-fished areas were located on the left, while sites from highly fished areas were mostly located on the right side (Figure 3d). Shallowest and proximal sampling sites are associated with the presence of nursehound catshark, skates of the *Raja* genus, and the pelagic stingray (*Pteroplatytrygon violacea*). At increasing depths and distances, sampling sites are characterized by the presence of small-spotted catshark (*Scyliorhinus canicula*), squaliform sharks of the genus *Centrophorus*, bluntnose sixgill shark (*Hexanchus griseus*), and sandy ray (*Leucoraja circularis*). Finally, species like the rabbitfish (*Chimaera monstrosa*), the long-nosed skate (*Dipturus oxyrinchus*), and the kitefin shark (*Dalatias licha*) were associated with high depths sampling sites located far away from the coast in an area with lower fishing effort. These species are associated with the left side of the NMDS ordination. The blackmouth catshark (*Galeus melastomus*), the most abundant species, and the velvet-belly lanternshark (*Etmopterus spinax*), normally prefer higher depths and farther distances, but they are close to the origin of the ordination because they were found in all the 24 hauls.

Discussion

This study represents the first eDNA study to specifically assess chondrichthyan diversity in the Mediterranean Sea in synergy with a commercial trawler, to explicitly examine the environmental and operational context that is pivotal to understanding threats and solutions to shark and ray conservation. In this way, the main novelty of this study is represented by the integration of eDNA collection (based on slush and metaprobe rather than water filtration and pumping) into the standard operation of trawlers. Overall, eDNA metabar-

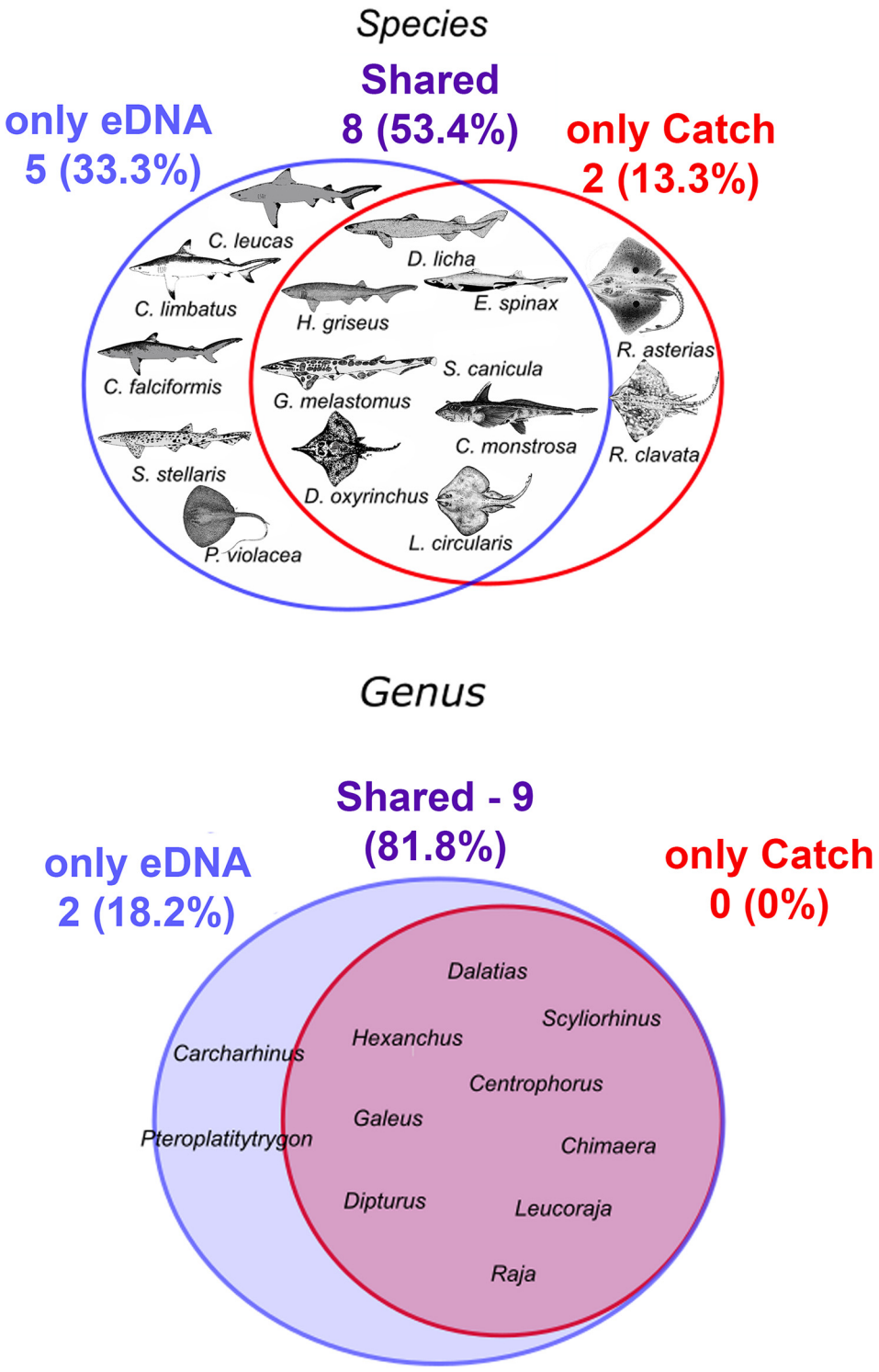

Figure 2. Venn diagrams of species and genera detected via metabarcoding of 12S DNA and/or revealed by catches. Further taxonomic information on the species are given in Supplementary Material. Drawing reproduced from FishBase (Froese and Pauly, 2022).

coding analysis detected 13 species and 2 additional genera of chondrichthyans, which is in the same order of magnitude as previous eDNA-based studies targeting Chondrichthyes in tropical areas, often with greater sampling efforts (Bakker *et al.*, 2017; Boussarie *et al.*, 2018; Mariani *et al.*, 2021; West *et al.*, 2021). This demonstrates that, despite long-term, chronic anthropogenic impacts, the Mediterranean Sea still harbours a considerable diversity of chondrichthyans, which should be a major consideration for initiatives aimed at protecting and recovering threatened species.

The most common species in terms of both prevalence and abundance (as expressed by the number of reads) across samples were the blackmouth catshark (*Galeus melastomus*) and the velvet-belly shark (*Etmopterus spinax*) (Table 1), demer-

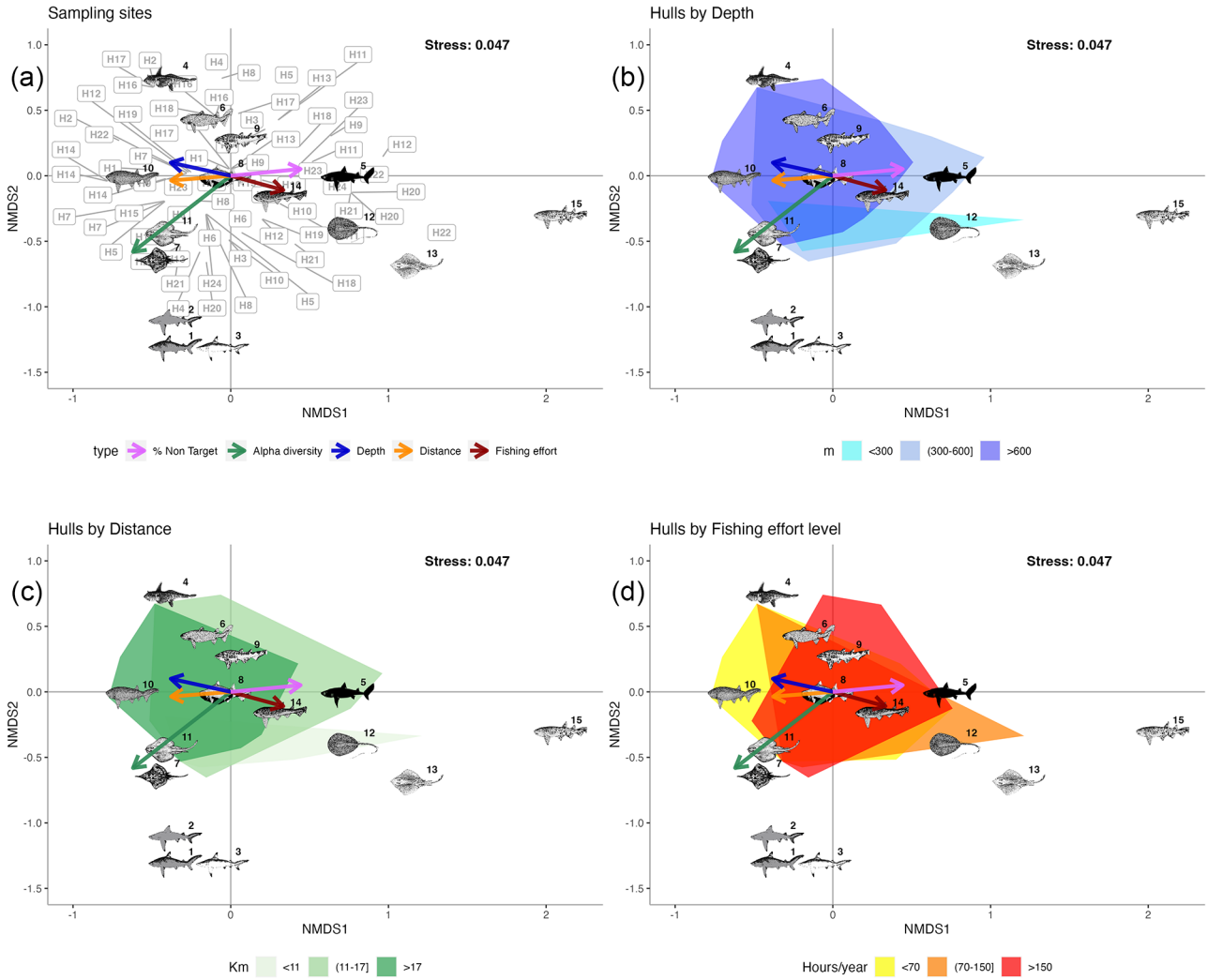

Figure 3. NMDS ordination with Jaccard distance matrix of species distribution in the sampling sites. Vectors represent gradients of the five considered variables (i.e. depth, distance from the coast, fishing effort, alpha diversity, and percentage of nontarget reads) as returned by environmental fit on the NMDS ordination. Sampling sites are grouped according to ranges of depth (b), distance from the coast (c), and fishing effort (d). Silhouettes represent the 15 elasmobranch taxa detected by eDNA metabarcoding. Specifically: (1) *Carcharhinus falciformis*; (2) *Carcharhinus leucas*; (3) *Carcharhinus limbatus*; (4) *Chimaera monstrosa*; (5) *Centrophorus* sp.; (6) *Dalatias licha*; (7) *Dipturus oxyrinchus*; (8) *Etmopterus spinax*; (9) *Galeus melastomus*; (10) *Hexanchus griseus*; (11) *Leucoraja circularis*; (12) *Pteroplatytrygon violacea*; (13) *Raja* sp.; (14) *Scyliorhinus canicula*; (15) *Scyliorhinus stellaris*.

Table 2. Results of the environmental fit on the NMDS, carried out to explore the influence of the five variable gradients considered (i.e. depth, distance from the coast, fishing effort, alpha diversity, and percentage of nontarget reads) on the species distribution among the 24 sampling sites.

	NMDS1	NMDS2	r^2	$pr(>r)$
Depth	−0.99	0.01	0.08	0.045*
Distance_coast	−0.99	0.01	0.09	0.0039**
Fishing_effort	0.95	−0.31	0.07	0.077
Alpha_diversity	−0.73	−0.68	0.43	0.001**
% non_Target_Reads	0.99	−0.11	0.12	0.017*

Significance codes: $*p < 0.05$; $**p < 0.01$.

sal species commonly caught by trawlers in the Mediterranean Sea (Cashion *et al.*, 2019; Follesa *et al.*, 2019; Leonetti *et al.*, 2020). Sequence read abundance was high also for the genus *Raja*, which could not discriminate between the Mediterranean starry ray (*Raja asterias*) and the thornback ray (*Raja clavata*) (both collected by the fishing net—Supplementary Appendix S5), confirming the limitations of the short 12S barcode, whose nucleotide variation is insufficient to distinguish some closely related species, as pointed out in previous studies (Cawthorn *et al.*, 2012; Collins *et al.*, 2019). We also detected eDNA of deep-sea gulper sharks (*Centrophorus* sp.) in the deep (~500 m) sites H12, H23, and H24, likely belonging to *Centrophorus uyato*: the taxonomy of this genus has been debated for decades, with the latest evidence indicating *C. uyato* as the only gulper shark species in the Mediterranean Sea (Kousteni *et al.*, 2021; White *et al.*, 2022; Bellodi *et al.*, 2022). Of particular interest is the presence of the sandy ray (*Leucoraja circularis*), which is listed in the IUCN red list as critically endangered, and in Annex II of the Specially Protected Areas and Biological Diversity in the Mediterranean Protocol of the Barcelona Convention.

Perhaps the most surprising detections were the three species of the genus *Carcharhinus*: silky shark (*Carcharhinus falciformis*), blacktip shark (*Carcharhinus limbatus*), and bull shark (*Carcharhinus leucas*). All the *Carcharhinus* species were detected in the same sample (H4), one of the shallowest sites, with an average depth of 133 m and a mean dis-

tance from the coastline of 6 km. The silky and blacktip sharks are epipelagic species that can approach the coastline, and have already been recorded in the Mediterranean Sea (Morey *et al.*, 2008; Garibaldi and Relini, 2012), though their presence was not expected, given the rarity of sightings in recent decades (Walls and Dulvy, 2021). The bull shark has never been recorded in the Mediterranean Sea: it is a tropical and subtropical shark that can cover great distances, moving between freshwater and seawater. As this finding was particularly unexpected, we cautiously considered the possibility of ambiguity with other closely related requiem sharks. The tree topology obtained when including all available 12S data of the genus *Carcharhinus*, not limited to Mediterranean species, confirms the clustering of our eDNA sequence with the other *C. leucas* sequences obtained from voucher specimens (Supplementary Appendix S6). Notwithstanding, 12S data for several *Carcharhinus* species are not available, thus hampering a comprehensive assessment. Based on phylogenetic reconstructions using more powerful sets of markers (Sorenson *et al.*, 2014), it may be possible to hypothesize that the detected sequence could belong to the bull shark's closest living relatives, but these (i.e. *C. acronotus*, *C. isodon*, and *Nasolamia velox*), given their rarity and distant, circumscribed Central-American distributions, would be certainly less likely than bull shark to make a foray into the Mediterranean. Lastly, bull shark tissue has been used, years prior to this study, as a positive control in another project, and despite the strictest contamination control procedures in our eDNA lab—and, importantly, the absence of bull shark reads in other samples and in the controls—we cannot completely rule out some trace contamination. Nevertheless, even considering every possible ambiguity and bias, given the increased records of Lessepsian species (Golani and Fricke, 2018), including tropical sharks (Tobuni *et al.*, 2016), we also argue that completely excluding the possibility of bull shark vagrancy in the Tyrrhenian Sea may also be hasty. Given the potential of eDNA analysis as a tool for the early detection of new species in the marine realm, we believe that all the eDNA findings that pass quality and contamination filters should be reported, cautiously, and with a thorough examination of all the possible underlying origins.

Overall, our results indicated that eDNA can effectively infer the composition of commercial bycatch of cartilaginous fish. The five species identified by eDNA metabarcoding but not detected by fishing survey methods, reflect the power of metabarcoding to identify rare species. Trawling gear can, through its physical action on the substrate, suspend and retain biological material from organisms that can be present in the environment, although they may not be caught, which can lead to the presence of DNA of species not present in the net (Russo *et al.*, 2021). Despite the high proportion of nontarget teleost species and reads, the relatively small number of chondrichthyan species, the moderate area investigated, and the risk of cross-contamination during the sampling due to the nonsterile conditions aboard the commercial fishing vessel, the results generated a clear pattern of community substructure. The significant influence of the percentage of nontarget (mostly teleost) reads on elasmobranch detections should be taken into account for future studies. It is well known that PCR efficiency can affect the proportion of sequences in the libraries, resulting in different proportions compared to the original samples (Deagle *et al.*, 2013; Shelton *et al.*, 2016, 2022; Hoshino *et al.*, 2021). PCR amplification efficiency bias can thus distort taxonomic compositions measured by metabarcoding from their true values in the field (Silverman *et al.*, 2021). Furthermore, the metabarcoding approach is based on the simultaneous evaluation of the whole biodiversity, target and nontarget taxa sequences are generated together; this means that the number of reads assigned to a certain taxonomic group is intrinsically dependent on the number of reads associated with all the others taxonomic groups (Gloor *et al.*, 2017). As 78% of our total reads were assigned to nontarget teleosts, we cannot rule out that the substantial amount of bony fish DNA could influence elasmobranch detection efficiency and hence the distribution of species reconstructed among sampling sites. Nevertheless, even considering this potential technical bias, chondrichthyan community structure among sampling sites fits with expectations linked to environmental knowledge as evident in the NMDS plots (Figure 3): pelagic and coastal species, such as *Raja* spp. or the pelagic stingray (*Pteroplatytrygon violacea*), were associated with the shallowest sites near the coastline, while bathyal species, like rabbitfish or sandy ray, were associated with deeper sites, in line with the ecological traits of the species. eDNA degrades quite quickly in the marine environment (Thomsen *et al.*, 2012; Sassoubre *et al.*, 2016; Jo *et al.*, 2017; Collins *et al.*, 2018), so if target species are distributed heterogeneously, the local release of their genetic material, will provide the opportunity to retrieve meaningful, spatially explicit information even at a small spatial scale (Jeunen *et al.*, 2019) and using a relatively small set of indicator species. Despite the complexities discussed above, eDNA metabarcoding clearly depicted cartilaginous fish distribution across a small area of the central Tyrrhenian Sea. The implementation of this approach in synergy with commercial fishing activities can provide important information about the bycatch of sharks and batoids (Cashion *et al.*, 2019) and can vastly expand our reach and capabilities for monitoring endangered megafauna and assisting strategies for their recovery. It is worth noticing that, in the near future, such approaches could be integrated into standard fishing activities for multiple purposes, including biodiversity assessments, catch control, and monitoring of threatened species.

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Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Authors contributions

L.A., A.C., A.F., G.M., S.M., and T.R. conceived the idea. P.C., G.M., T.R., A.S., and L.T. collected the samples. L.A., A.F., G.M., P.S., and L.T. carried out laboratory analyses. L.A., G.M., T.R., and P.S. led bioinformatic and ecological analyses. All authors discussed and interpreted the results. L.A., A.F., and G.M. drafted the manuscript, with contributions from S.M. and T.R. All authors approved the final version of the manuscript.

Data Availability Statement

Barcode data generated in this study are included in the "ELASMOMED Consortium" public project in the Barcode of Life Data system (BOLD, http://www.barcodinglife.org). All data included in the project can be reached via the Public Data Portal section https://www.boldsystems.org/index.php/Public_SearchTerms by using the search Keyword ELAMO).

Metabarcode data are included in the BioProject PRJNA911173.

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