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ORIGINAL RESEARCH

Fishing in the gene-pool: implementing trawl-associated eDNA metaprobe for large scale monitoring of fish assemblages

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Abstract Marine biodiversity monitoring in the Mediterranean's increasingly threatened ecosystems is crucial for effective ecosystem conservation and management. Here, we leveraged the Mediterranean International Trawl Survey program (MEDITS) to implement eDNA sampling through the recently tested 'metaprobe' procedure and characterize fish assemblages in three separate areas off the Italian coasts: Northern Adriatic Sea (NoAS), Ligurian and Northern Tyrrhenian Sea (LNTS), and Sardinian Sea (SaS). By combining the information from two homologous mitochondrial 12S metabarcodes—i.e., Elas02 and Tele02 targeting elasmobranchs and teleosts, respectively—we identified 108 species, over 60% of which overlapped with those caught by the trawl net. We produced an accurate reconstruction of fish community composition of the examined sites, reflecting differences in species assemblages linked with both geographic area and depth range. Metaprobe eDNA data consistently returned a biodiversity 'bonus' mostly consisting of pelagic taxa not captured through bottom trawl surveys, including rare

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and endangered taxa (e.g., elasmobranchs). Overall, the spatial characterisation of the assemblages across the surveyed areas was better delineated and more robust using eDNA metabarcoding than trawl data. Our results support the operationalisation of the metaprobe as a simple, inexpensive, versatile sampling tool, in association with pre-existing ship surveys, to overcome many of the limitations of marine data collection and strengthen marine management.

Keywords Environmental DNA · Fishing impacts · Fish communities · Marine biodiversity · Mediterranean Sea · Trawl surveys

Introduction

The persistent global increase of human pressure on ecosystems is accelerating habitat degradation, climate instability, and biodiversity loss across the planet (Mooney et al. 2009), making the quest for effective environmental monitoring and resource management more urgent than ever. In marine ecosystems, human impacts not only contribute to climate change and habitat degradation (He and Silliman 2019) but also provokes direct (for target stocks) and indirect (through accidental catches) effects on populations, species and communities, as a result of intense fishing pressure (Myers and Worm 2003; Yan et al. 2021).

The Mediterranean Sea is amongst the most impacted ecoregions globally, for its geographic characteristics as a semi-enclosed basin, and the high population density along its coasts (Amoroso et al. 2018). This is of particular importance as the Mediterranean Sea constitutes a global biodiversity hotspot (Myers et al. 2000); the Basin hosts ~ 18% of known marine species, a quarter of which are endemic (Bianchi and Morri 2000; Mouillot et al. 2011). Nowadays, Mediterranean ecosystems are undergoing significant transformations, and many species are experiencing population decline or even local extinction due to, among others, overfishing (Yan et al. 2021). This applies also to several megafaunal taxa accidentally by-caught during fishing activities, such as sharks and rays, marine mammals, sea turtles and seabirds. It is thus crucial to improve management plans to reduce fishing bycatch and expand habitat and species protection, especially for the most endangered ones (Fiorentino and Vitale 2021). However, effective marine management requires information on species distributions and abundance within ecosystems, but data collection from the oceans is still mainly based on traditional approaches such as capture-based techniques, hence limiting our understanding of marine biotas. In this context, the rise of environmental DNA (eDNA) is boosting biodiversity studies and enhancing our knowledge of species distribution. DNA collected from the environment represents a primary source of biological information in different habitats (Sigsgaard et al. 2020; Thomsen and Willerslev 2015); it is easier and cheaper to obtain, less invasive and often more accurate than pre-existing methods based on morphological identification. There is growing evidence of the potential implementation of eDNA for routine monitoring, and for the collection of data to inform marine management and policy decisions (Gilbey et al. 2021). However, a major bottleneck preventing broader use of eDNA remains the collection and concentration of DNA from large water volumes, a complex and time-consuming activity which requires sterile conditions and tools, hampering its use in scenarios where deck personnel are already engaged in other complex operations, such as manoeuvring a trawl net. To overcome these limitations, Maiello et al. (2022) recently developed a novel, inexpensive and easy sampling procedure, namely the 'metaprobe', that passively filtrates seawater and collects eDNA once placed inside the fishing net during trawling. DNA analysis from such samples has demonstrated its effectiveness in monitoring catch composition from trawlers, while contextually allowing the assessment of communities and ecosystems sustaining fishing activities (Maiello et al. 2023). Here, we opportunistically adopted the metaprobe sampling procedure at a large regional scale during scientific surveys in three distinct Mediterranean geographical sub-areas: Northern Adriatic Sea (NoAS), Ligurian and Northern Tyrrhenian Sea (LNTS), and Sardinian Sea (SaS). Using a combination of teleost- and elasmobranch-specific metabarcoding markers, we first compared the specificity of the two sets of primers, examining species assemblages returned by the two 12S metabarcodes. Secondly, we compared overall metaprobe metabarcoding detections with the species identified by the morphological inspection of net catches. Then, we investigated the accuracy of metaprobe data in portraying differences

in marine communities linked to sampling area and depth and explored the contribution of environmental gradients to explain the community structure returned by eDNA. Finally, for each site separately, we evaluated the per-species reads distribution as a semi-quantitative measure of abundance (Russo et al. 2021; Stoeckle et al. 2021). Our results suggest that associating metaprobe metabarcoding during surveys aboard scientific vessels has the potential to vastly upscale the range of ocean data collection, expanding our knowledge of both taxa targeted by fisheries, and rare and elusive endangered species, such as many elasmobranchs. This is of immediate relevance for the conservation and management of marine environments.

Material and methods

Sampling

Samples were collected in September 2021 from 21 sites, within the activities of the Mediterranean International Trawl Survey (MEDITS) framework, which annually generates information on the distribution and demographic structure of demersal populations of the Mediterranean Sea. Sampling hauls covered three areas: six hauls in the LNTS (GSA 9), 10 in the SaS (GSA 11), and five in the NoAS (GSA 17) (Fig. 1, Table S1). eDNA was gathered through the passive filtration of simple gauze rolls fixed to a 3D-printed sampler, the metaprobe (Maiello et al. 2022), which has already been shared with the scientific community (https://github.com/GiuliaMaiello/Metaprobe-2.0). To minimize contamination, we prepared 'metaprobe sampling kits' in a sterile laboratory: three sterile gauze rolls were secured with zip-ties to each metaprobe, which was then placed in a sterile ziplocked bag. On board the vessel, a metaprobe was placed inside the fishing net (codend mesh size of 20 mm) before the beginning of the hauls (which lasted between 30 and 60 min, over a stretch between 2 and 6 km). Immediately after the net was hauled on board and opened onto the deck, the metaprobe was located and processed. Wearing sterile gloves and using clean scissors and forceps, two out of the three gauze rolls were gathered from the metaprobe and stored in separate 50 ml sterile tubes with 99% ethanol and silica gel grains respectively. Additionally, two clean gauze rolls were opened on board and stored as field blanks to monitor the degree of contamination associated with the boat background. Samples were frozen at − 20 °C on board and subsequently in the laboratory until DNA extraction. Concurrently, on-board observers determined the species composition of each catch by visual inspection of the external morphology of the individuals in the net. For each site, the overall number of individuals and the total biomass of each species were recorded.

Laboratory procedures

DNA was extracted from the gauze rolls using the DNeasy Blood and Tissue Kit (Qiagen), following a slightly modified version of the Purification of Total DNA from Animal Tissues protocol. A small section (~ 2 × 2 cm) of each roll was cut into small pieces and then the remaining gauze was stored in the original 50 ml tube at − 20 °C. The pieces of gauze were incubated overnight at 56 °C with 540 µl of extraction buffer and 60 µl of proteinase K (20 µg/ml). DNA was subsequently extracted according to the manufacturer protocol, adjusting reagent volumes to the initial amount of extraction buffer, and concentrating the DNA through a DNeasy Mini spin column. Total DNA was finally eluted in 100 µl of elution buffer. Two extraction negatives (only reagents) were included to monitor the possibility and extent of contamination linked with extraction procedures and reagents.

DNA extractions from all the 42 rolls of gauze, along with two field blanks, two extraction controls, two PCR negative controls and one positive control (i.e., DNA of *Sebastes mentella*, a subarctic species absent in the Mediterranean Sea), were metabarcoded by targeting two homologous 12S ribosomal RNA fragments of the mitochondrial genome (Miya et al. 2015): we amplified a ~ 167 bp fragment using the fish-specific Tele02 primers (forward: 5'-AAACTC GTGCCAGCCACC-3'; reverse: 5'-GGGTATCTA ATCCCAGTTTG-3') and a ~ 171 bp fragment using the Elas02 primers (forward: 5'-GTTGGTHAAT CTCGTGCCAGC-3'; reverse: 5'-CATAGTAGGGTA TCTAATCCTAGTTTG-3') (Taberlet et al. 2018). Each forward and reverse primer was tagged with 8 bp indices, in order to univocally identify samples during demultiplexing and reduce the risk of cross-contamination and/or tag switching during Illumina

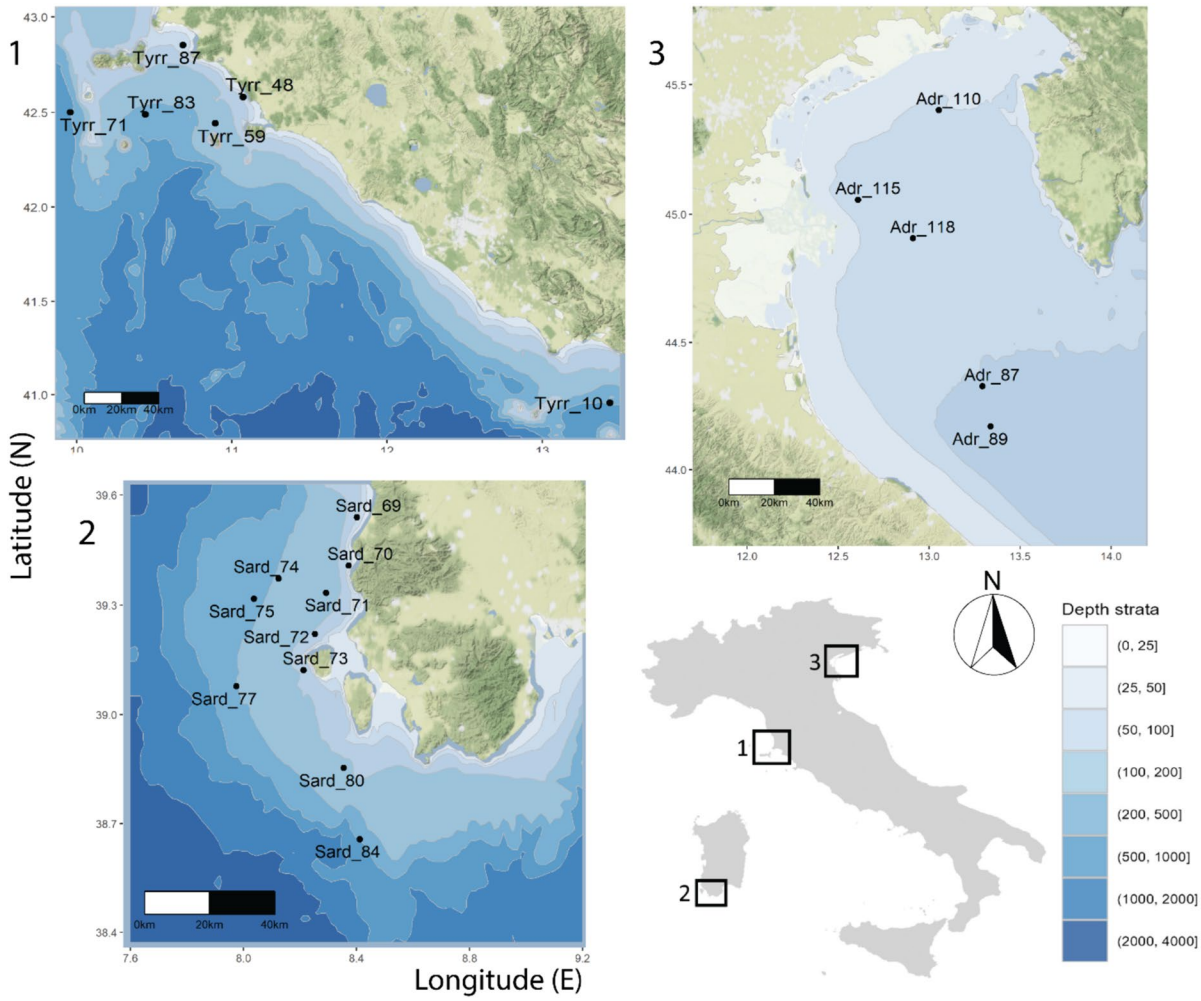

Fig. 1 Map of the 21 sampling sites: **1** Ligurian and Northern Tyrrhenian Sea (GSA 9), **2** Sardinian Sea (GSA 11), and **3** Northern Adriatic Sea (GSA 17). The map was created using the R-package GGMAP (Kahle and Wickham 2013)

sequencing. Each tag differed by at least three base pairs from other tags and was preceded by 2–4 degenerate bases (N) to increase sequence diversity during sequencing. Each sample was PCR-amplified in triplicate in 20 µl reactions containing 10 µl MyFi™ Mix (Meridian Bioscience), 0.16 µl of Bovine Serum Albumin (20 mg/ml, Thermo Scientific), 5.84 µl of UltraPure™ Distilled Water (Invitrogen), 1 µl of each forward and reverse primer (10 µM, Eurofins), and 2 µl of template DNA. PCRs were performed under the following thermocycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s, and a final elongation of 72 °C for 5 min for the Tele02 12S primers; and polymerase activation at 94 °C for 15 min, followed by 40 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and a final elongation of 72 °C for 5 min for the Elas02 12S primers. We then pooled triplicated PCRs and visualised samples on a 2% agarose gel stained with SYBRsafe (Invitrogen) to ensure the successful amplification of target fragments. PCR products were purified with Mag-Bind® TotalPure NGS magnetic beads (Omega Bio-tek Inc), adding a 1×ratio of magnetic beads to 30 µl of PCR products (Bronner et al. 2009). Purified DNA was quantified using a Qubit™ 4.0 fluorometer with the Qubit™ dsDNA HS Assay Kit (Invitrogen). Based on the total DNA concentration, samples were normalised

and pooled in equimolar concentration for library preparation. We performed end- repair, adapter ligation and library PCR amplification using the NEXTFLEX® Rapid DNA-Seq Kit 2.0 for Illumina® platforms (PerkinElmer) according to the manufacturer's protocol. We examined fragment lengths on an Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies); secondary products (e.g., adaptor dimers) were removed by another 1×ratio magnetic bead clean-up. Libraries were quantified using quantitative PCR (qPCR) on a Rotor-Gene Q (Qiagen) with the NEBNext® Library Quant Kit for Illumina® (New England Biolabs) and then diluted to 1 nM according to qPCR concentrations. Final libraries and PhiX Control were quantified using qPCR before sequencing. The two libraries were sequenced separately at 85 pM with 20% PhiX Control on an Illumina® iSeq™ 100 using the i1 Reagent v2 (300-cycle) (Illumina Inc.).

Bioinformatic processing

Bioinformatic procedures followed the OBITOOLS pipeline (Boyer et al. 2016). We first checked read quality with FASTQC (Andrews 2010) and trimmed low-quality ends for downstream analysis. ILLUMINAPAIREDEND was used to merge all paired reads showing a quality score > 40, and NGSFILTER to demultiplex samples based on their unique barcodes, allowing for a single base mismatch. We removed singletons, reads containing ambiguous bases, "N", and filtered reads for expected fragment lengths (129–209 bp for Tele02; 140–200 bp for Elas02 (Taberlet et al. 2018)) via OBIGREP, then dereplicated sequences via OBIUNIQ. Chimeras were detected and removed with UCHIME (Edgar et al. 2011) and the remaining sequences clustered into Molecular Operational Taxonomical Units (MOTUs) with SWARM (Mahé et al. 2015) setting the threshold to d = 3 for both primers.

Custom-made databases were created through in silico PCR against the EMBL database (Release version r143) implemented with ECOPCR. We obtained two 12S reference databases: one of 102,372 sequences for Tele02 and a second one of 92,011 sequences for Elas02. We first assigned taxonomy via ECOTAG and then manually checked the taxonomic assignment of ambiguous (e.g., non-Mediterranean taxa) and poorly resolved MOTUs (i.e., MOTUs that could not be unambiguously assigned to a genus or species level), searching against the NCBI database using BLASTn. Datasets were filtered retaining only sequences assigned to species or genus level showing > 98% identity match (Miya et al. 2015) and removing potential contamination noise taking advantage of field blanks and negative controls with the DECONTAM package in R (Davis et al. 2018), using the prevalence method with a threshold of 0.5. Singletons for every taxon at each sampling site were removed to avoid low-abundance false positives due to tag switching. We finally excluded from the final dataset non-target taxa (i.e., mammals, birds), retaining only teleosts and elasmobranchs for subsequent analyses.

Statistical analyses

We explored the actinopterygian/chondrichthyan detection efficiency of the two primers (i.e., Tele02 and Elas02), as despite their specificity both can amplify teleosts and elasmobranchs. We generated a Venn Diagram, using the VENNDIAGRAM package in R (Chen and Boutros 2011), to visualize differences in taxon composition between Tele02 and Elas02 datasets. For each primer set, the Actinopterygii/Chondrichthyes ratio was calculated, and differences in the proportions of species and reads assigned to the two fish groups were assessed by Pearson's chi-squared test and visualized through barplots. Furthermore, we explored community composition among sampling sites as returned by each of the two primers set through a non-metric multidimensional scaling (nMDS) with the 'metaMDS' function in the package VEGAN (Oksanen et al. 2018). The Multi Response Permutation Procedure (MRPP) (Mielke and Berry 1994) was then applied to compare the inter-site dissimilarities with the intra-site dissimilarities and test whether the portrayal of community structure across locations significantly differed between Tele02 and Elas02 datasets. Distance matrices were calculated using Bray–Curtis distance on a dataset including all the species detected by both primers (taxa identified by ethanol and silica gel were combined calculating the mean number of reads).

Venn Diagrams were also drawn to compare the overall taxa detection at both species and genus levels among metaprobe samples (merging all taxa identified by Tele02 and Elas02, and by ethanol and silica gel replicates) and the visual inspection of catches.

We then explored reads abundance distribution patterns at each of the 21 sampling sites separately for all the taxa as revealed by Tele02 and Elas02 together. We distinguished between the species that were caught by the fishing net and the visually unobserved 'bonus' species only detected through eDNA. Because of the biomass concentration in a reduced volume within the net, we expected the DNA of caught species to be more abundant in metaprobe-in-the-net samples compared to the genetic material of non-caught taxa (Maiello et al. 2023). We thus calculated the proportion of reads belonging to the most abundant caught species before the first 'bonus' species over the total number of reads for each sampling site.

We contrasted the species richness (number of taxa) with the sampling effort (number of sampling units), calculating accumulation curves for each sampling area and for each depth range separately with the iNEXT package (Hsieh et al. 2016). Depth ranges were identified based on distribution breaks (Fig. S1) corresponding to actual changes in the sea bottom morphology: shallow water (0–100 m), continental shelf (100–200 m), continental slope (200–400 m) and deep slope (> 400 m). We then explored community composition among the 21 sampling hauls using a semi-quantitative approach, as several recent studies suggested a significant quantitative meaning of eDNA data (Guri et al. 2023; Mariani et al. 2021). To prevent uneven sequencing yields among samples from unduly influencing community reconstruction, we square-root transformed number of reads for similarity matrices calculation. We performed a non-metric multidimensional scaling (nMDS) based on Bray–Curtis distance on the square-root transformed read abundance dataset combining all the taxa detected by Tele02 and Elas02 (calculating the mean value of read abundance for the common species) and keeping ethanol and silica gel replicates as separate. We combined datasets from the two markers using read abundances, as the two primers target homologous ribosomal RNA fragments and the relative proportions per sample in the two datasets were comparable. Polygons on the nMDS ordination represented sampling areas and depth ranges, respectively. Differences among sampling sites, sampling areas, and depth ranges were tested via a PERMANOVA test using the 'adonis' function in VEGAN with 9,999 permutations. Post-hoc differences among the three areas and the four depth ranges were assessed through the 'pairwise.adonis' function (9,999 permutations). We identified taxa (i.e., species or genera) associated with statistically significant differences among sampling groups (areas and/or depth ranges) using an indicator species analysis with the 'multipatt' function in the INDICSPECIES package (Cáceres and Legendre 2009).

Finally, we investigated the ecological value of the information provided by eDNA metabarcoding, compared or integrated with catch data, in terms of being able to characterise the area of origin (GSA) and the bathymetric layer. We first transformed catch and eDNA metabarcoding data into binary data in order to combine them. The 21 sampling sites were then randomly divided into two groups: the first ('training set') contained 80% of the sites and was used to train a series of Random Forest models to infer the origin of the samples in terms of GSA and bathymetric layer (Breiman 2001). The second group ('test set') contained the remaining 20% of the sites and was used to compare Random Forest predictions with the respective true values of GSA and bathymetric layer. The procedure was replicated 100 times and conducted considering: 1) only caught species; 2) only eDNA metabarcoding species; 3) all the species captured by both trawl and eDNA. Finally, to compare the performance of the three groups of trained Random Forests we computed the Cohen's K coefficient of agreement for each of the three sets of tests (i.e., only catch, only eDNA or both), using the 'kappa2' function in the package IRR. Results were visualised using confusion matrices.

All statistical analyses were conducted in R v4.0.5 (R Core Team 2023).

Results

Comparison between Tele02 and Ela02 libraries

High throughput sequencing resulted in a total of 3.7 million raw reads for the Tele02 library and 4.1 million for the Elas02 library. After bioinformatic analyses, taxonomic assignment, and data filtering, we obtained 1,878,487 reads (mean per sample = 44,726 ± 5,180 SE) for the Tele02 dataset (99.06% teleosts and 0.94% elasmobranchs) and 1,471,036 reads (mean per sample = 35,024 ± 2,499 SE) for the Elas02 dataset (93.61% teleosts and

6.39% elasmobranchs) (Fig. 2a; Table S2). A total of 93 taxa (84 teleosts and nine elasmobranchs) and 95 taxa (76 teleosts and 19 elasmobranchs) were retained in Tele02 and Elas02 final datasets respectively (Fig. 2a). The Actinopterygii/Chondrichthyes ratio returned by the two primer sets was significantly different in terms of read number ($\chi^2 = 75.97$, df = 1, $p < 0.05$), but not in terms of species number ($\chi^2 = 3.18$, df = 1, $p > 0.05$). Of the 108 taxa detected in total, 80 were shared between the two libraries, 15 were exclusive of Elas02 and 13 only identified by Tele02 (Fig. 2b). As expected, the majority of species only detected by Elas02 belonged to elasmobranchs (11 of the 15) while 12 out of the 13 species only present in Tele02 data were bony fishes.

The nMDS in Fig. 2c evidenced a coherent community structure among the two datasets, showing a strong affinity within each sampling site as returned by Tele02 and Elas02 independently. Consistently, the MRPP analysis revealed that the mean intra-site Bray–Curtis distance (between Tele02 and Elas02 data for the same site) was significantly lower than the inter-site one (0.39 and 0.72 respectively, and $p < 0.05$ in both cases).

Comparison between metabarcoding- and catch-inferred fish assemblages

Taxa composition inferred via metabarcoding generally matched with those visually identified in the catches, demonstrating the overall reliability of eDNA metabarcoding for caught taxa identification: 67 (48%) species and 65 (over 60%) genera were shared between eDNA and catch data, resulting in almost 70% of caught species (more than 80% when considering genera) being identified by metabarcoding

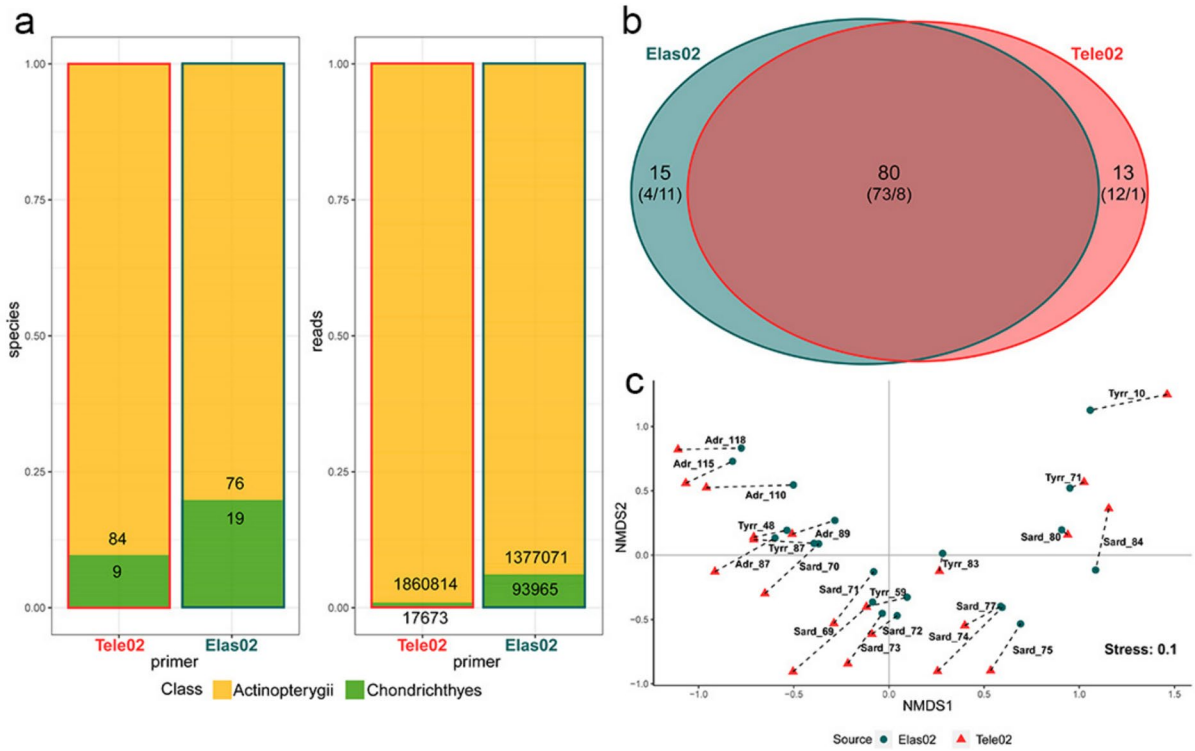

Fig. 2 Comparison between Elas02 and Tele02 primers **a** Barplots representing the number of species (left) and reads (right) of Actinopterygii and Chondrichthyes returned by each primer set. **b** Venn Diagrams of the taxa detected by Elas02 and Tele02. For each group the total number of species and the Actinopterygii/Chondrichthyes proportions are given. The names of the taxa in each group are reported in Table S6. Ellipse areas are proportional to the number of taxa. **c** Pattern of species assemblages across the 21 sampling sites, as returned by the non-metric multidimensional scaling (nMDS) with Bray–Curtis distance and based on eDNA metabarcoding data for Tele02 and Elas02. Dotted lines connect data from the same sites

(Fig. 3). Noticeably, 93% of the top 30 most abundant species present in the catch (accounting for 91% of caught biomass) were detected by eDNA metabarcoding. Gauze rolls also yielded a biodiversity 'bonus' of 41 species (26 genera) not caught by the fishing net. The pelagic/demersal ratio was significantly higher when comparing the metabarcoding data sub-set with the rest of taxa, both considering species ($\chi^2 = 10.88$, df = 1, $p < 0.05$) and genera ($\chi^2 = 10.00$, df = 1, $p < 0.05$).

Barplots in Fig. 4 showed a different relative abundance in terms of (transformed) number of reads between the caught species and the metabarcoding 'bonus' taxa, with the latter having generally a lower number of reads. This was confirmed by the proportions of reads associated with all the very abundant caught species before the first 'bonus' species; for all sampling sites, the proportion was over 50% of total reads, except for one Sardinian site (Sard_69) where two 'bonus' taxa (*Gymnammodytes* and *Dasyatis pastinaca*) were among the most abundant species (Table S3). The mean proportion of reads assigned to very abundant caught taxa was 74% with an associated standard deviation of 0.15.

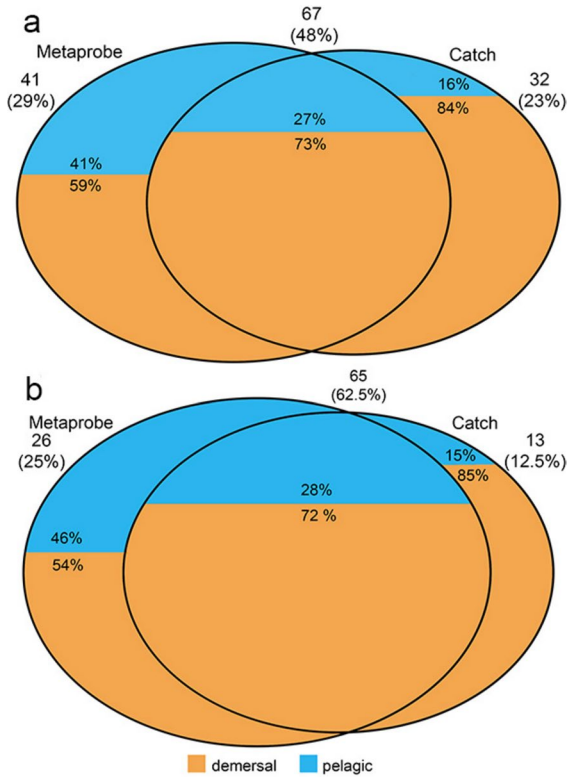

Fig. 3 Venn Diagrams of the species (**a**) and genus (**b**) detected through eDNA metabarcoding and morphological identification of catch. The names of the taxa in each group are given in Table S6. Each area is coloured based on the relative proportion of pelagic (blue) over demersal (orange) taxa (Froese and Pauly 2022). Diagram areas are proportional to the number of taxa

Ecological and environmental patterns

Species accumulation curves of sampling areas attested that the sampling effort (number of sampling units) adequately captured taxon diversity for each region separately (Fig. 5a). When contrasting taxon richness for each depth range with the number of sampling units, rarefaction curves did not reach a plateau for all the bathymetric ranges; increased sampling effort is needed to obtain a better representation of whole species diversity, especially for the [0-100 m) and the [100-200 m) ranges (Fig. 5b). The nMDS in Fig. 5c–d evidenced a strong intra-site affinity, further corroborated by PERMANOVA results which assigned 92.5% of the variance to sampling stations and only 0.4% contribution from replicates (Table 1a). Community structure was influenced by both sampling areas and depth ranges; polygons on the nMDS plot separated sampling sites according to the sea basin of origin (Fig. 5c) and revealed a clear depth gradient along the first axis (Fig. 5d). PERMANOVA results supported the observed influence of area and depth range on species composition (Table 1b). In post-hoc pairwise comparisons, substantial differences were recovered among all pairs of regions and depth ranges (Table S4). The indicator species analysis revealed that significant differences among sampling areas and depths corresponded to multiple species, designated as characteristic of each region and depth range because of their relative (over-)representation (Table S5).

The Random forest-based assessment further demonstrated the power of eDNA in characterising both the geographic area and the bathymetric layer of origin. Random Forests trained on only catch data always had the worst performance, with mean values of Cohen's K equal to 0.28 (GSA), and 0.79 (bathymetric range). In contrast, Random Forests trained on only eDNA metabarcoding data returned the best performance, with mean values of the Cohen's K equal to 0.83 (GSA), and 0.89 (bathymetric range).

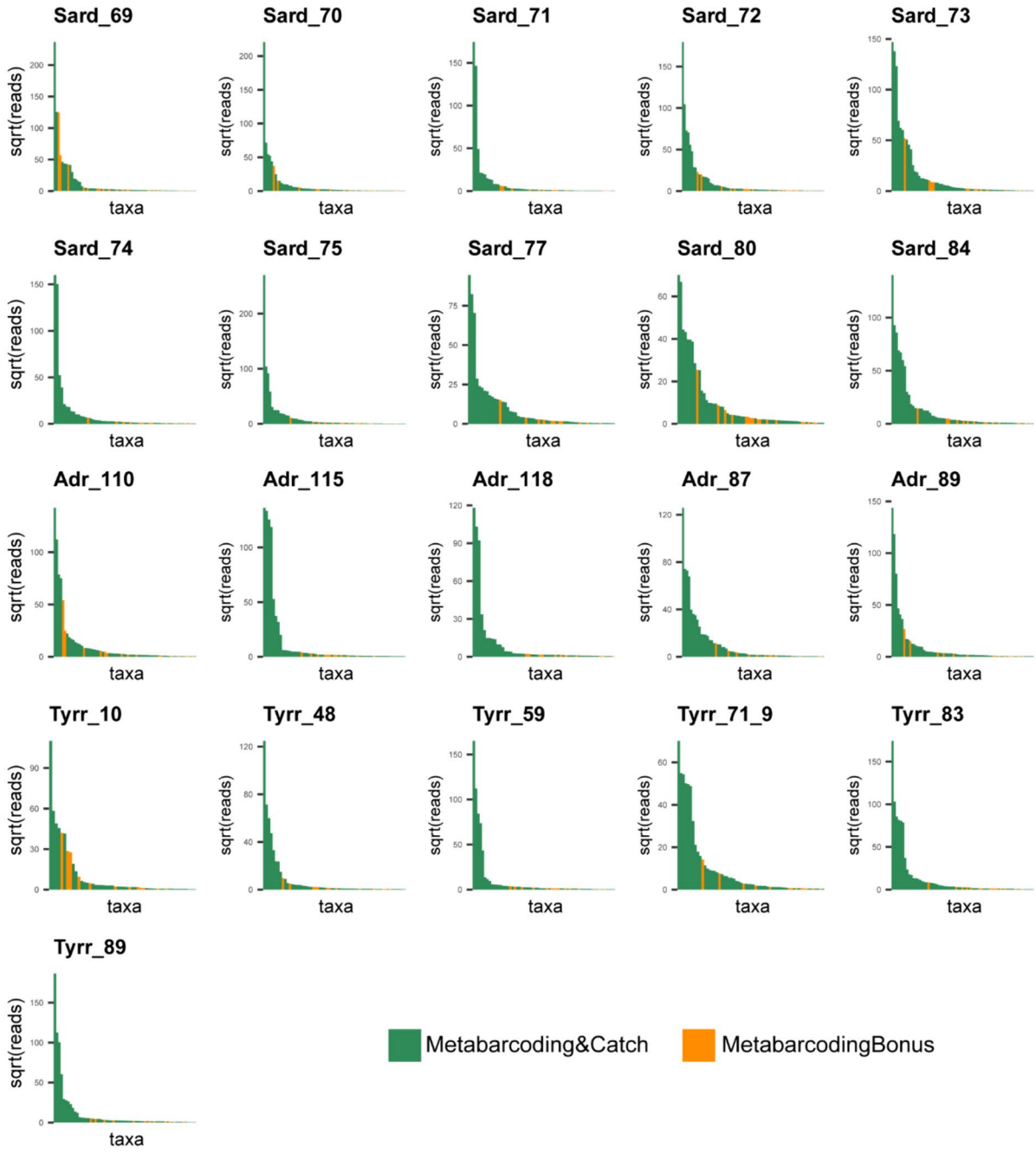

Fig. 4 Quantitative composition (square root-transformed read counts) of taxa detected by eDNA metabarcoding at each sampling site separately, colours are different for the taxa only identified by eDNA (MetabarcodingBonus) and the species also detected by visual inspection of external morphology (Metabarcoding&Catch)

Interestingly, the Random Forests trained on both catch and eDNA metabarcoding data had slightly lower mean values of the Cohen's K than those of the Random Forests trained on only eDNA metabarcoding. Confusion matrices corroborated this pattern (Fig. 6).

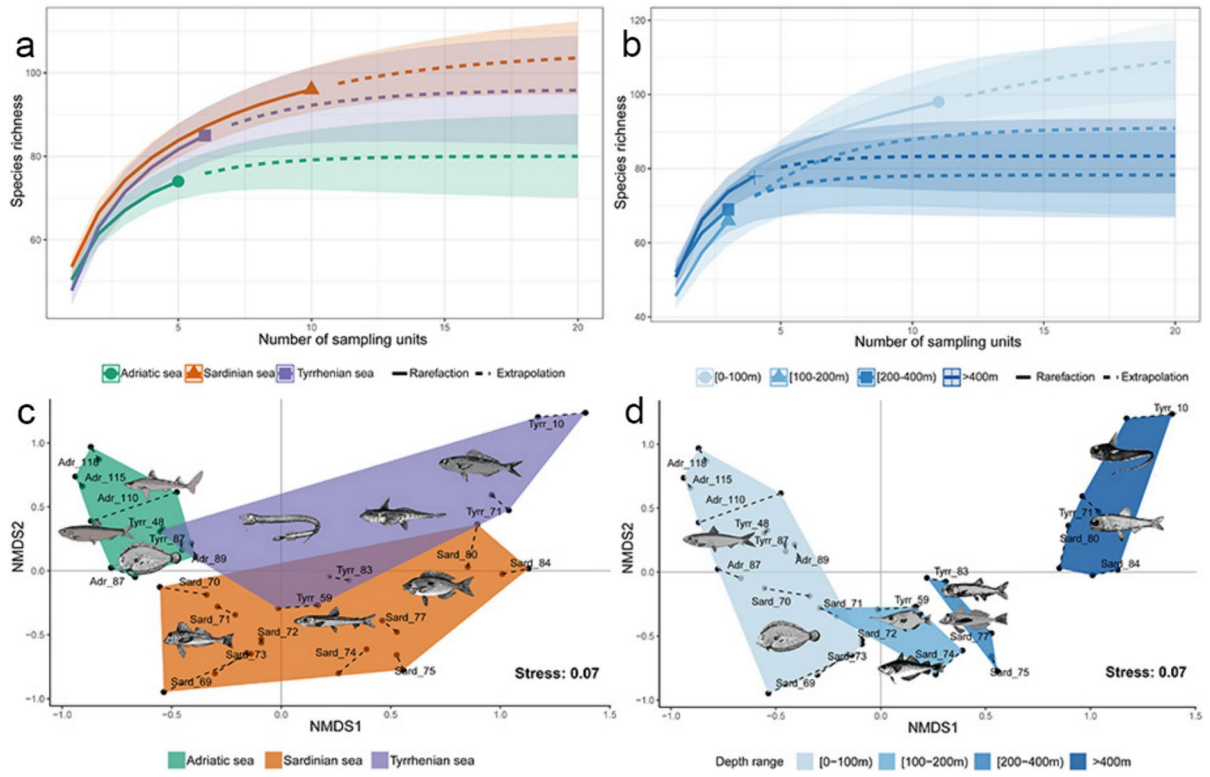

Fig. 5 Rarefaction curves of the total taxa detected in the 21 sampling sites by Tele02 and Elas02 12S. Curves are split based on the area (**a**) and the depth range (**b**). Pattern of the species assemblages across the 21 sampling sites, as returned by the non-metric multidimensional scaling (nMDS) with Bray–Curtis distance and based on eDNA metabarcoding data (combining Tele02 and Elas02). Dots indicate samples and are connected by a dashed line for each sampling site. Polygons are coloured according to the sampling area (**c**) and the bathymetric layer (**d**). Silhouettes represent the three (for areas) and the two (for depth ranges) top contributing species to each group differentiation, according to the indicator species analysis

Table 1 Results of PERMANOVA (Permutational Multivariate Analysis of Variance) analyses carried out to explore species composition changes in relation to: (a) sampling Site and Source (i.e., Ethanol and Silica); (b) Area and Depth range. Values are related to Bray–Curtis coefficients

a

	Df	SumsOfSqs	MeanSqs	F.Model	R^2	*p* value
Site	20	9.87	0.49	13.24	0.92	< 0.05
Source	1	0.05	0.05	1.35	0.004	0.19
Residuals	20	0.74	0.04		0.07	
Total	41	10.67			1.00	

b

	Df	SumsOfSqs	MeanSqs	F.Model	R^2	*p* value
Area	2	2.72	1.35	10.58	0.25	< 0.05
Depth range	3	3.33	1.11	8.64	0.31	< 0.05
Residuals	36	4.62	0.13		0.43	
Total	41	10.67			1.00	

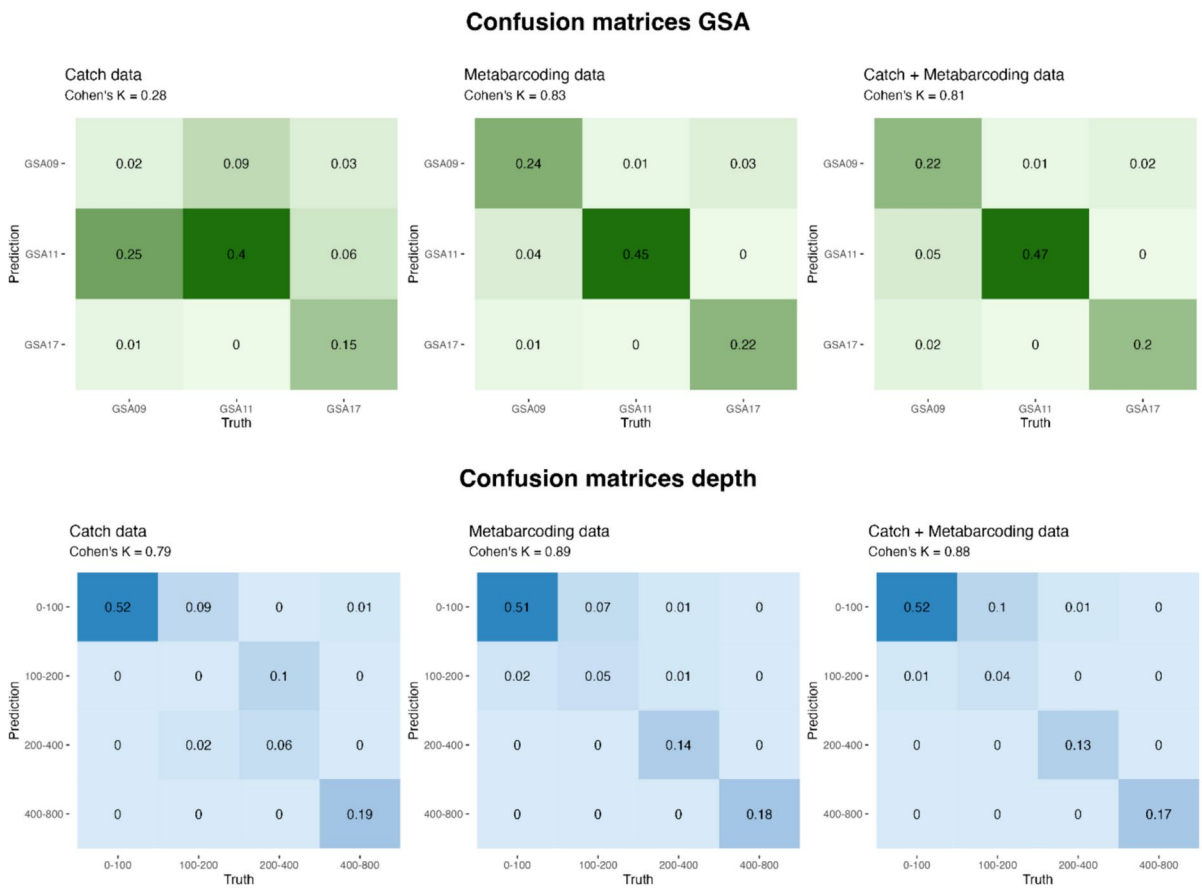

Fig. 6 Confusion matrices representing the comparison between observed and predicted (from Random Forest) values of the GSA (top set) and bathymetric range (bottom set) of randomly extracted sampling sites. The values inside each matrix correspond to the proportion of sampling sites (number of sampling sites over the total) assigned to each cell

Discussion

Marine biodiversity monitoring still largely involves 'traditional' methods based on the capture of organisms, which are invasive, involve hefty operational costs, and often yield noisy data. Environmental DNA metabarcoding, with its agile, accurate and efficient way of capturing biodiversity information in the ocean, has recently opened new opportunities for upscaling data collection (Gilbey et al. 2021), especially when accompanied by novel, low-cost, efficient alternatives to water capture, filtering, and storage (Maiello et al. 2022, 2023). Here, using a combination of two vertebrate-specific 12S markers, we accurately characterized fish communities in three basins around Italy, expanding the applicability range of metaprobes as a simple and non-disruptive way of sampling eDNA from trawling nets. In practice, the metaprobe approach single-handedly overcomes the limitations of both trawl surveys and classical eDNA approaches.

Despite subtle differences in the Actinopterygii/Chondrichthyes detection ratio between Tele02 and Elas02 metabarcodes (Fig. 2), the marker selection did not significantly influence the overall biodiversity: 74% of the species were shared among the two primer sets (Fig. 2b), and overall community structure across regions was consistent with the two markers (Fig. 2c), with intra-site dissimilarity (between Elas02 and Tele02 replicates of the same haul) being substantially lower than inter-site dissimilarity. However, we observed important differences in the taxa that were exclusively recovered by each of the two primers: 12 out of the 13 species only identified by Tele02 were

teleosts, and 11 of the 15 species exclusive of Elas02 were elasmobranchs, demonstrating barcode efficiency in detecting rare teleosts and elasmobranchs respectively. This is particularly relevant for elasmobranchs, which are globally threatened and influential for strategic conservation planning. For instance, among the species only identified by Elas02, sandy skate (*Leucoraja circularis*) is endangered, while shortfin mako (*Isurus oxyrinchus*) and blue shark (*Prionace glauca*) are critically endangered according to the Mediterranean IUCN red list (McCully et al. 2015; Sims et al. 2016; Walls and Soldo 2016). The detection of *I. oxyrinchus* and *P. glauca* in the Sardinian Sea is particularly noteworthy, as large predatory sharks have declined dramatically in abundance over the last two centuries in the Mediterranean Sea (Ferretti et al. 2009). Environmental DNA metabarcoding represents a valuable way to record movements and spatial and temporal hotspots of large pelagic sharks.

Almost 70% of caught fish species were detected by eDNA metabarcoding. However, the percentage of elasmobranchs and teleosts identified only by visual inspection and not by eDNA (23% of total species), was substantially higher than in Maiello et al. (2023) where, using a combination of COI and 12S barcodes, only 3% of the vertebrate species detected were exclusively returned by visual inspection of trawl catches. This supports the idea that COI metabarcoding, which often fails to generate satisfactory metazoan data in aqueous eDNA studies (Collins et al. 2019), can be very useful in the context of trawl net eDNA, where DNA concentration is substantially higher than in the naturally diluted seawater. Compared to the universal COI primers, the 12S metabarcode is known to have less complete reference databases and a lower taxonomic resolution (Collins et al. 2019)—which explains why taxonomic assignment at the genus level yields a more complete representation of captured taxa in the eDNA data (Fig. 3b). In contrast, 41 species were detected exclusively by eDNA metabarcoding; in this eDNA biodiversity 'bonus' sub-set the number of pelagic species was higher compared to the other sets (i.e., 'shared taxa' and 'catches only') where demersal species targeted by bottom trawlers prevailed (Fig. 3). This variation on the proportion of pelagic versus demersal species is in line with previous observations using this approach (Maiello et al. 2022, 2023). 'Bonus' species eDNA can be absorbed by the gauze during trawl descent/ascent or captured by the metaprobe from pelagic DNA sedimented on the sea floor. Of pelagic taxa exclusively found in metaprobe eDNA data, some were endangered elasmobranchs (e.g., *Isurus oxyrinchus*, *Prionace glauca*), while others (e.g., lanternfishes) can be informative indicators of ecosystem function. From the identification of species caught by the trawl net to the detection of endangered and/or keystone species, our results suggest that, even without the complement of the taxonomic power of COI, the use of a vertebrate-specific 12S metabarcode (especially Elas02) appears a valuable ecosystem assessment tool.

It is noteworthy that a moderate sampling effort is sufficient to recover most of the species diversity of each sampling area considered (Fig. 5a) and return trends that reflect expectations: for instance, the species richness was similar in the SaS and the LNTS, and higher than in the NoAS, which is known to be a marine biogeographic area with lower biodiversity compared to the rest of the Mediterranean (Gaertner et al. 2007). Furthermore, we were able to adequately reconstruct the overall quantitative β-diversity distribution, discerning between the 21 hauls and reflecting patterns of community structure influenced by sampling areas and depth ranges (Fig. 5c–d). Compared to qualitative approaches, the use of a semi-quantitative method (Bray–Curtis index) increased the discriminatory power of eDNA metabarcoding data (Guri et al. 2023), by mitigating the bias towards rare species that is inherent to presence/absence methods.

Species composition as returned by the metaprobe was significantly different among areas and depth ranges considered (Fig. 5c–d). The Adriatic Sea is the most distinctive of the three basins because of its geography and geomorphology, which limit exchanges with the rest of the Mediterranean Sea. The Adriatic Sea exhibited a higher number of distinctive species, according to indicator species analysis. Among those, there were small pelagic fishes typical of this region: *Sprattus sprattus*, *Scomber scombrus* and *S. colias*, *Engraulis encrasicolus*, main targets of north-central Adriatic Sea fisheries, as well as *Squalus acanthias*, a valuable commercial elasmobranch commonly caught by bottom trawl in the Adriatic Sea (Bargione et al. 2019), *Merlangius merlangus*, almost absent in the rest of the Mediterranean (Milić and Kraljević 2011), and *Myliobatis aquila*, a substantial fishery bycatch in the north-central Adriatic Sea (La Mesa et al. 2016). We found evidence of significant

differences also between the two contiguous Tyrrhenian and Sardinian basins and in the bathymetric distribution of species; all the depth layers examined had their own distinctive species. For instance, in 0–100 m waters, two shallow demersal (*Arnoglossus laterna* and *Serranus cabrilla*) and one epipelagic species (*Sardina pilchardus*) were identified. On the other hand, most of the > 400 m-depth species were typical deep-sea fishes commonly found as discards of Mediterranean deep-bottom trawlers (e.g., *Hymenocephalus italicus*, *Lampanyctus crocodilus*, *Nettastoma melanurum*).

The precision of 'metaprobe-in-the-net' eDNA metabarcoding data in mirroring species composition of each area (GSA) and bathymetric range was further demonstrated by Random Forest results. eDNA metabarcoding-trained Random Forests demonstrated greater accuracy compared to those trained on catch data. This is empirical evidence that eDNA data collected using the metaprobe are highly informative of the communities of origin, returning a more accurate description of the ecosystems exploited by fishing than even catch-based data. Additionally, the integration of eDNA and catch data does not provide better results than eDNA data alone; this could indicate that the metaprobe eDNA data alone are able to return the whole diversity necessary to characterise marine communities. Since ocean ecosystem monitoring programmes are essential for sustainable resource management, a future adoption of metabarcoding methods in scientific surveys and fishery-dependent data collection could represent an improvement in our ability to capture changes in the marine environment.

Further potential lies in the use of read abundance, as previous studies have identified a strong correlation between read numbers of taxa and their relative abundance or biomass (Russo et al. 2021; Shelton et al. 2022; Stoeckle et al. 2021). Here the distribution of (transformed) read abundance was coherent with expectation (Fig. 4); on average, 74% of total reads was associated with the group including all the most abundant caught species before the first 'bonus' ones appear in the list. This finding shows that the metaprobe-in-the-net approach does preferentially detect caught species. This is particularly relevant as it supports previous suggestions (Maiello et al. 2023) around using a read abundance probabilistic threshold to discriminate between the species caught and those representing an environmental bonus. This approach significantly reduces the reliance on ground-truthing eDNA metabarcoding inference with the visual inspection of the catch and enables broader use of the metaprobe in trawling-based monitoring without the necessity for expert taxonomists now crucial for the morphological identification of specimens in the net.

By generating extensive species inventories, the metaprobe approach provides insights into both pelagic and demersal components, accurately reflecting marine communities, and even detecting rare and elusive species. This simple and efficient DNA-based sampling tool can be conveniently utilized as an effective method for gathering crucial information about species status and distributions, without adding significant burden to on-board operations. The opportunity to use these novel methods with regular marine surveys paves the way for future strategies where management decisions are informed by data collected via increasingly sustainable and non-destructive approaches. From monitoring catches to mapping community distributions, including the detection of threatened and invasive species, all marine domains stand to benefit from the operationalisation of these tools.

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Author contributions G.M., P.C., S.M. and T.R. conceived the idea and designed the methodology; A.B., A.C., A.F., C.F., A.L. and P.S. collected the data; G.M., L.C., D.C., A.S., P.S., M.S., L.T., S.M. and T.R. analysed the data; G.M., S.M. and T.R. led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

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Data availability Raw sequencing data files can be accessed at https://doi.org/10.5061/dryad.s7h44j1g3. Bioinformatic pipeline, final datasets and R scripts used for statistical analysis and to generate figures are publicly available from https://github.com/GiuliaMaiello/Fishing-in-the-gene-pool.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any conflicts of interest. Author SM is Associate Editor for Reviews in Fish Biology and Fisheries and the peer-review process for this article was independently handled by another member of the journal editorial board.

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