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Nanopore sequencing facilitates screening of diversity and provenance of seafood and marine wildlife

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ABSTRACT

The soaring global demand for seafood has placed unprecedented pressure on fisheries, leading to the exploitation of vulnerable marine resources and data-deficient species. Over the past few decades, a surge of molecular methods has enabled identification of traded marine animal products that may be otherwise unrecognizable through morphological analysis. While universal DNA barcoding remains a powerful authentication and traceability tool, its application still requires lengthy procedures, established facilities, and assay development. In this study, we explored some of the uses and advantages of cutting-edge nanopore sequencing, a rapid and portable alternative method. We first tested the method to identify ten opportunistically sampled fishery products obtained in two different contexts: five fish fillets marketed in the UK, four shark specimens traded in Indonesia and one fish collected as part of a scientific survey. We present a full analytical workflow to produce accurate species identification based on direct, PCR-free long-read sequencing of the DNA extracted from each specimen. We then used the nanopore sequencing output to mine whole mitogenomes from samples of varying DNA quality. Finally, we used the extensive additional genomic information produced by the sequencing to pinpoint the geographic origin of two of the identified specimens for which robust baseline data existed. In the face of increasing threats to biodiversity, and the need to control exploitation and supply of fisheries and wildlife globally, this rapid and portable approach is poised to revolutionize the monitoring of seafood supply and the trade in endangered marine wildlife, contributing to the sustainable management of aquatic resources.

1. Introduction

The world's oceans are vast and teeming with life, providing a rich variety of resources for human consumption and other ecosystem services (Ehrlich and Ehrlich, 2008). Among these resources, seafood has emerged as an essential component of global food systems, supplying protein, micronutrients and fatty acids that are vital for human health (Golden et al., 2021), and increasing demand on this natural resource puts considerable pressure on marine populations and ecosystems (Ahmed et al., 2020). These living resources are also in demand as luxury or ornamental goods, which has led some taxa close to extinction (Mozer & Prost, 2023). Amongst these mounting challenges, ensuring the sustainability and traceability of marine organisms is crucial for the continued viability of these resources and this represents a considerable task for scientists, policymakers, and the industry.

Seafood products are traded on a global scale and various challenges have emerged, such as mislabelling, misidentification, and the need for tracing geographic origin (Jacquet & Pauly, 2008; Willette et al., 2021). The accurate identification of fishery products and the determination of their provenance are integral to safeguarding ocean biodiversity, promoting sustainable fisheries, and ensuring transparent market operations; yet, the task is made arduous by the huge diversity of traded species, complexity of supply chains, illegal activities, and lax governance (Cawthorn & Mariani, 2017; Miller et al., 2012; Temple et al., 2022). For instance, seafood products have been central to mislabelling disputes as the number of reported cases can reveal an alarming misrepresentation rate of over 50% (Xiong et al., 2018), coupled with ambiguities surrounding umbrella terms that permit the sale of multiple

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fish species under a single label, thereby hindering conservation efforts. The issue is further aggravated by the sale of severely data-deficient or endangered species, such as elasmobranchs, with biological characteristics that render them especially sensitive to overfishing (Pazartzi et al., 2019; Prasetyo et al., 2023). Furthermore, beyond identifying species, variability in stock status in spatially heterogeneous species means that the ability to trace fishery products to their source population is also a necessary task to achieve sustainability (Cusa et al., 2022; Naaum et al., 2021; Ogden & Linacre, 2015; Shum et al., 2017).

Plentiful methods have been developed to verify the authenticity of seafood products, each with its own set of advantages and constraints. Traditional approaches include morphological identification, which relies on examining physical features to distinguish species. However, this method is generally insufficient due to the high degree of similarity between related species and the loss of distinctive features after processing (Miller and Mariani, 2010). In recent years, molecular techniques like DNA barcoding have emerged as a powerful tool, reliably used to study the identity of seafood, particularly for species that are difficult to distinguish visually. This movement has led to improved practices in the sale of seafood and the implementation of more robust legislation (Mohanty et al., 2013). Nevertheless, universal DNA barcoding assays exhibit limitations in time constraints and portability. Real-time qPCR (Cardeñosa et al., 2019) and loop-mediated isothermal amplification (LAMP; Velasco et al., 2021) are rapid and portable, but necessitate a separate assay for each target species. Some recent advances in qPCR technology may twin assay universality with portability (Cardeñosa et al., 2019; Naaum et al., 2021), but are still reliant on the construction and curation of bespoke spectral reference databases and their use is limited to species identification. Moreover, DNA-based traceability tools for population assignment are available for some species but are very rarely applied due to their apparent complexity, the existing gaps in population genetic knowledge of most fished species, and the inadequate governance of trade flows.

Recently, there has been an increased interest in using Nanopore sequencing as an innovative and promising solution for seafood authentication (Voorhuijzen-Harink et al., 2019). This state-of-the-art technology offers swift and cost-effective whole genome sequencing, allowing researchers to access extensive genetic data and insights for a wide range of biological applications. For instance, Nanopore sequencing has enabled a variety of applications including DNA barcoding of biodiversity in the field (Menegon et al., 2017), genome skimming of complete mitochondrial genomes of threatened primates (Malukiewicz et al., 2021), and the identification of a shark individual at a landing site in India (Johri et al., 2019). These examples underscore the versatility of this tool, and especially its use in rapidly evaluating full mitogenomes from individuals, even for species with limited public genomic data. This effectively eliminates the need for prior knowledge on the species and for species-specific assays. By rapidly generating large sequencing data directly from extracted DNA, making species identification feasible, this technique also produces vast amounts of additional data that can then be used for other purposes, such as the screening of polymorphic regions to evaluate the geographic origin of specimens.

Here, we examine the performance and versatility of nanopore sequencing on a selection of seafood products opportunistically sampled from two different contexts (Fig. 1): the European seafood market and the Indonesian trade of shark products. To accomplish this, we go through the following steps: 1) we devise a bioinformatic strategy to evaluate the quality of nanopore reads and their suitability to whole-mitochondrial genome assembly; 2) we explore the effectiveness of nanopore long-read whole genome sequencing in identifying ten different marine animal products; and 3) we select two species to test how data can inform on stock provenance. Our findings show how such a

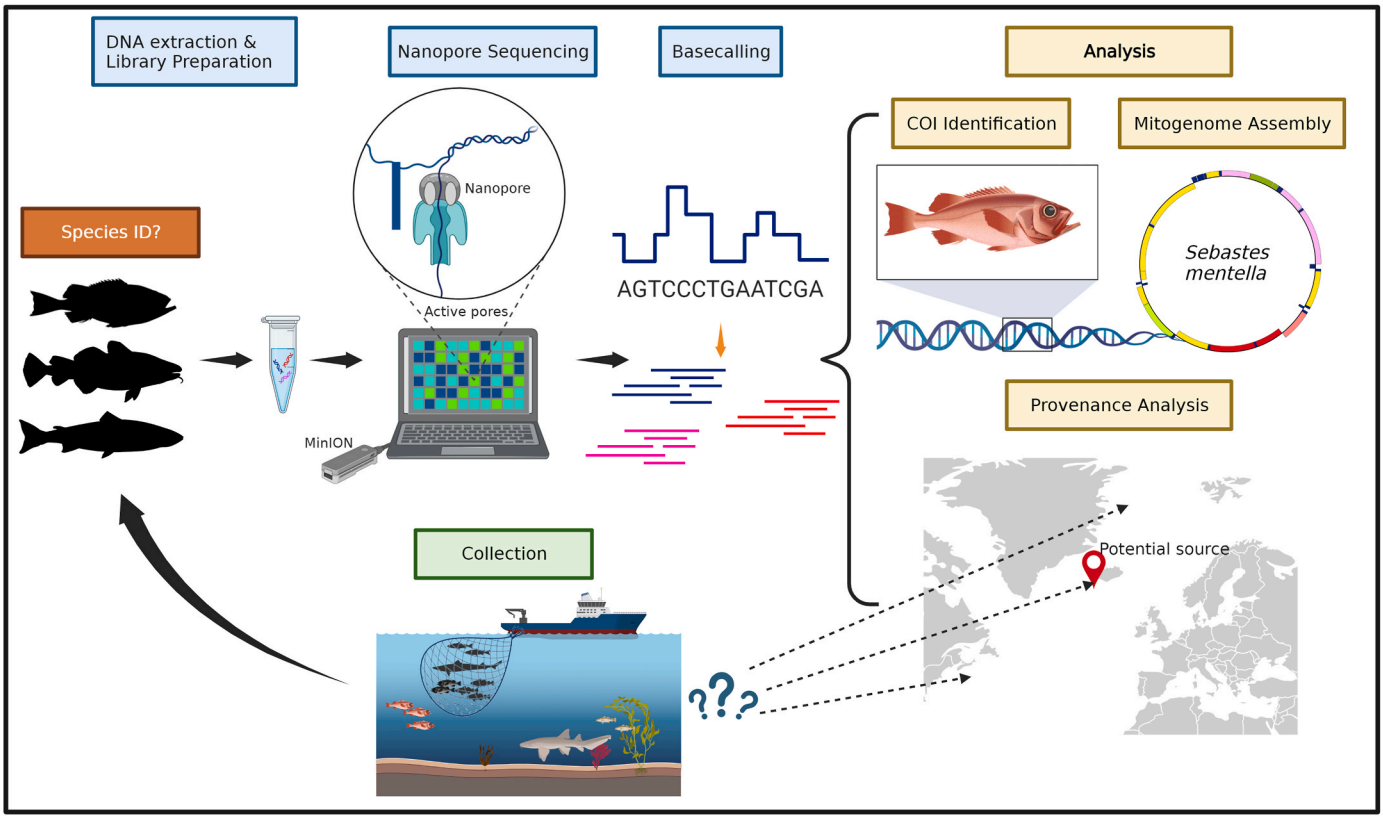

Fig. 1. Illustration of the process of nanopore sequencing of seafood, specimens collected from unknown locations. The process begins with the collection of seafood samples, followed by nanopore sequencing whole genome sequencing, which is then used for three main analyses: identification of the Cytochrome Oxidase I (COI) for species identification, assembly of mitogenomes, and a bespoke analysis of provenance to determine the geographical origin of the species. Created with BioRender.com.

universal, portable sequencing technology can usher a new era for the management and conservation of marine species.

2. Materials and methods

2.1. Sample collection

We obtained five teleost samples from seafood retailers in England and four shark tissue specimens from landing sites in Indonesia, plus one fish sample collected as part of Iceland's Marine and Freshwater Research Institute 2015 redfish survey (stored in RNAlater, see Table 1 for barcode number and sample). The fish samples were collected from muscle tissue present in fillets, and the shark samples were taken from fin tissue. Moreover, throughout January and February 2020, four shark and ray species were collected at various landing sites on Java Island, Indonesia. Tissue samples were collected fresh from full specimens. Those samples were preserved in 96% ethanol and stored at −20 °C. Genomic DNA was isolated either by employing a modified protocol using DNAzol Reagent (see supplemental information for details) or by adhering to the Mu-DNA tissue protocol (Sellers et al., 2018). The extraction yielding the highest quality DNA was subsequently selected for further use. DNA was assessed for quality and quantity using a Qubit 4 fluorometer with a Qubit dsDNA HS Assay kit and analysed on a 2200 TapeStation system with the Genomic DNA ScreenTape assay for integrity of high molecular weight gDNA.

2.2. Morphological identification

Five fish, Atlantic Mackerel (*Scomber scombrus*), Monkfish (*Lophius americanus*), Silver hake (*Merluccius bilinearis*), Atlantic Salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) were sampled at a fish monger in Manchester, UK and their species identification was confirmed by the fish monger. For the shark specimens, tissue samples were gathered from whole specimens and images of the specimens were recorded. These were visually identified by analysing distinct morphological characteristics, with the aid of specialistic identification keys, such as in the field guide to Indonesia sharks & rays (White et al., 2006). In addition, DNA of the shark samples were genetically characterised from Prasetyo et al. (2023) and were amplified with several cytochrome oxidase I (COI) primer sets, namely Fish02 primers (Ward et al., 2005) for Great Hammerheadand (*Sphyrna mokarran*) M13 forward primer for silky shark (*Carcharhinus falciformis*). Sanger sequencing of those two samples was outsourced to Macrogen Europe, while specimens of bamboo shark (*Chiloscyllium punctatum*) and broadnose wedgefish (*Rhynchobatus australiae*) were identified using a highly degenerated COI primer set (Wangensteen et al., 2018) using a high throughput barcoding (HTB) method (Prasetyo et al., 2023) with an Illumina MiSeq.

2.3. Nanopore library preparation and sequencing

Nanopore libraries were prepared from 1 μg of genomic DNA (gDNA) and following the protocol for the SQK-LSK109 ligation sequencing kit and EXP-NBD104 native barcoding expansion (Oxford Nanopore Technologies, ONT). In brief, DNA repair and end-prep was carried out by adding 3.5 μl NEBNext FFPE DNA Repair Buffer, 3.5 μl Ultra II End-prep reaction buffer, 3 μl Ultra II End-prep enzyme mix, 2 μl NEBNext FFPE DNA Repair Mix (New England Biolabs). Each sample reaction mix was incubated at 20 °C for 5 min and 65 °C for 5 min. The end repaired gDNA were further purified using magnetic beads before ligated to Nanopore barcodes by adding 500 ng of end-prepped DNA, 2.5 μl Nanopore barcodes to each sample and 25 μl Blunt/TA Master Mix (New England Biolabs), and then incubated at room temperature for 10 min. Barcoded gDNA were further purified using magnetic beads, pooled to 700 ng total DNA and ligated to the sequencing adaptors by adding 65 μl of gDNA, 5 μl Adapter Mix II (AMII), 20 μl NEBNext Quick Ligation Reaction Buffer (5X) and 10 μl Quick T4 DNA Ligase. Ligated and tethered libraries were purified by magnetic beads and eluted in 15 μl elution buffer (ONT). The sequencing flow cell was primed using 1000 μl of priming mix (Flush Tether and Flush buffer) following the recommended priming protocol (ONT). After priming, the 12 μl library was mixed with 37.5 μl Sequencing Buffer (SQB) and 25.5 μl Loading Beads (LB). The library was then loaded onto a Nanopore FLO-MIN106 (9.4.1 SpotON) flow cell and sequenced on a MinION Mk1B for 36 h.

Real time basecalling was performed with the MinIT and integrated ONT Guppy Barcoding Software v5.0.7 to produce the fastq files. The automatic real time division into passed and failed reads by the MinIT works as a quality check and removed reads with quality scores <7. The quality checked reads were demultiplexed and trimmed for adapters. Read quality statistics for raw data was visualised using custom python scripts.

2.4. Species identification - COI

The cytochrome oxidase I (COI) sequences records for Actinopterygii

Table 1
Details of seafood samples collected from retailers in England and Indonesia – Preservation, DNA extraction techniques, Identifications via NCBI and BOLD, and Initial Detection Times of COI during Nanopore Sequencing.

Sample ID	Category	Marketed as	Common name	Preservation	Collection	DNA extraction	BOLD >99%	BLAST >99%	Start time
1	Elasmobranch	*Chiloscyllium punctatum*	Brownbanded bamboo shark	ethanol	Indonesia	Mu-DNA	*Chiloscyllium punctatum*	*Chiloscyllium punctatum*	00:39.15
2	Teleost	*Sebastes mentella*	Beaked redfish	RNAlater	MFRI	DNAzol	*Sebastes mentella*/ *Sebastes* sp.	*S. mentella* (97.23%)/ *S. fasciatus* (97.10%)	18:44.57
3	Teleost	*Scomber scombrus*	Atlantic Mackerel	ethanol	England	DNAzol	*Scomber scombrus*	*Scomber scombrus*	00:20.27
4	Teleost	*Lophius piscatorius*	Monkfish	ethanol	England	DNAzol	*Lophius piscatorius*	*Lophius piscatorius*	02:33.30
5	Teleost	*Merluccius bilinearis*	Silver hake	ethanol	England	DNAzol	*Merluccius merluccius*	*Merluccius merluccius (98.71%)*	00:31.02
6	Teleost	*Salmo salar*	Atlantic Salmon	ethanol	England	DNAzol	*Salmo salar*	*Salmo salar*	00:18.14
7	Elasmobranch	*Sphyrna mokarran*	Great Hammerhead shark	ethanol	Indonesia	Mu-DNA	*Sphyrna lewini*	*Sphyrna lewini* (97.31%)	00:00:32
8	Elasmobranch	*Rhynchobatus springeri*	Broadnose wedgefish	ethanol	Indonesia	Mu-DNA	NA	*R. laevis* (91.82%)/ *R. australiae* (91.54%) **COII**	00:03:22
9	Elasmobranch	*Carcharhinus falciformis*	Silky shark	ethanol	Indonesia	Mu-DNA		*Carcharhinus falciformis* (95.17%)	00:41.12
10	Teleost	*Gadus morhua*	Atlantic cod	ethanol	England	Mu-DNA	*Gadus morhua*	*Gadus morhua* (98.45%)	00:54.12

Table 2
Nanopore sequencing statistics for each sample for total genomic sequencing reads and mitochondrial DNA reads.

	Sequencing statistics	1	2	3	4	5	6	7	8	9	10
Total genomic	Species identification	*Chiloscyllium punctatum*	*Sebastes mentella*	*Scomber scombrus*	*Lophius piscatorius*	*Merluccius merluccius*	*Salmo salar*	*Sphyrna lewini*	*Rhynchobatus springeri*	*Carcharhinus falciformis*	*Gadus morhua*
	Total number of genomic reads	117,616	140,198	76,157	96,157	81,112	59,613	68,808	50,666	61,991	70,196
	Mean genomic read length	811.5	2044.17	2246.92	1974	2209.9	2440.3	1644.4	1518.3	1025.7	1539.1
	Shortest read	123	138	108	96	132	96	137	145	108	93
	Longest read	37,506	93,877	55,092	49,869	69,607	63,106	31,370	47,626	27,565	30,702
	Average read accuracy	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
	Adjusted average read accuracy	93.76%	93.75%	94.10%	93.91%	93.42%	93.75%	93.30%	92.71%	93.16%	93.25%
	Average read quality score	20	20.1	20.22	20.12	19.40	19.86	19.46	18.88	19.18	19.26
	Adjusted average read quality score	11.74	11.76	11.91	11.80	11.48	11.70	11.23	10.67	11.21	11.42
	Average GC content	43.15%	41.64%	40.03%	40.28%	44.01%	43.58%	43.17%	43.99%	45.57%	44.74%
	Average N content	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	Mean genomic read quality	12	12	12.3	12.2	11.8	12	11.7	11.4	11.9	11.7
	Average read length N50	945	4115	3923	2839	3132	4338	2156.90	1882	1190	2695
mtDNA	Number of mtDNA Reads	54	92	2106	130	240	2817	26	10	15	29
	Average mtDNA read length (bp)	872.6	2874	1205	1202.70	1200.72	956	2996.30	1316.10	3017	2288.70
	Number of assembled contigs	N/A	4	1	4	N/A	1	1	N/A	N/A	1
	Longest contig length (bp)	N/A	15,053	16,533	2393	N/A	16,644	5118	N/A	N/A	14,426
	Average mtDNA sequencing depth	N/A	12x	142x	18x	N/A	154x	4x	N/A	N/A	4x
	COI sequence depth	25x	1x	212x	12x	18x	199x	18x	4x	12x	2x

and Elasmobranchii (4,208,167 records, accessed 10/08/2022) was retrieved from Genbank and a BLAST database was created. For each demultiplexed sample, reads were cross-referenced using BLASTn against the actinopterygii_elasmobranchii custom database to search for regions of sequence similarity for species identification and identify the COI sequence reads. COI sequence reads were aligned using MAFFT (Katoh & Standley, 2013) to obtain the consensus sequence which was then queried against the Barcode of Life Data Systems online (BOLD; www.barcodinglife.org; Ratnasingham & Hebert, 2007) using the species-level barcode database to identify each sequence. A threshold of 99% sequence similarities was used above which identification of samples was deemed reliable. Once a species match was established, existing reference mitogenomes for that species or a closely related species was obtained from Genbank. This was then compared against the original label information provided for each sample.

2.5. Mitogenome reconstruction

Mitochondrial reads of each sample were identified using an associated reference mitogenome previously established from the COI species identification (Table 2). FLYE v2.9.1 (Kolmogorov et al., 2019) was used to assemble the mitogenomes *de novo* using the read output. The reconstructed mitogenome sequence was annotated using a combination of methods: 1) a custom script to characterise the mitochondrial DNA completeness of the ONT reads against the reference mitogenomes; and 2) the CHLOROBOX web server (Tillich et al., 2017) with the following parameters: "circular sequence" option, mitochondrial DNA; tRNAscan-SE v2.0 in "Vertebrate Mitochondrial tRNAs" mode was enabled, and Server References from NCBI were selected including all RefSeqs using the species genera for each sample.

Barcode07 (later identified as *Sphyrna lewini* – see Results) was further employed for whole mitogenome comparisons against 34 mitogenomes of elasmobranchs sourced from NCBI. The phylogenetic relationships inferred from these whole mitogenomes were initially aligned using MAFFT (Katoh and Standley, 2013) and subsequently analysed with RAxML (Stamatakis, 2014) to generate the most robust phylogenetic tree. The analysis utilized 100 bootstrap replicates and used *Hexanchus griseus* as the outgroup. The radial phylogeny was visualised using iTOL (Letunic & Bork, 2007).

2.6. Case studies of species provenance

We examined a selection of samples to determine whether the DNA reflects genetically defined regions of origin. We screened publicly available data from GenBank to identify diagnostic genomic signatures that would allow point-of-origin testing and extracted ONT genomic reads from candidate samples identified as Beaked redfish *Sebastes mentella* and the Brownbanded bamboo shark *Chiloscyllium punctatum* for comparison (see results). We considered the *Sebastes* specimen (Barcode02) a reference as it was collected as part of a redfish stock assessment survey with known geographic provenance collected from the Irminger Sea below 500m and morphologically identified as a deep-pelagic *S. mentella*. Shum et al. (2017) characterised the phylogeographic distribution of *S. mentella* shallow- and deep-pelagic populations across the North Atlantic using the mitochondrial control region and the protein coding visual pigment rhodopsin. Therefore, we extracted these regions by creating a BLAST database using the raw ONT reads and queried reference data for the control region (D-loop) and rhodopsin sequences to identify ONT target regions. Once the ONT reads were extracted, the consensus sequence was corrected manually in MEGA11 (Tamura et al., 2021). For rhodopsin, the diagnostic amino acid at site 119 that separate shallow (valine) and deep (isoleucine) *S. mentella* populations was identified for reference data and the ONT rhodopsin reads were aligned using muscle in MEGA11. For D-loop, a network analysis with appropriate reference sequences was generated using a maximum likelihood phylogenetic framework (hapview, Salzburger

et al., 2011).

The Barcode01 sample was a fin tissue collected from the Karangsong fishing port, Indramayu, West Java, Indonesia and morphologically characterised by a clear white margin on gill slits as a Brownbanded Bamboo shark *Chiloscyllium punctatum*, and this identification was confirmed by COI analysis (see results). Lim et al. (2021) collected 135 samples of *C. punctatum* across five major coastal areas within three major Indo-Pacific regions, namely the Strait of Malacca, southern South China Sea and the Sulu-Celebes Sea. They screened the mitochondrial D-loop and dehydrogenase subunit 2 (ND2) genes to investigate population structure across the Sundaland region (Fahmi et al., 2021). Both regions were extracted from the raw ONT reads following the approach stated above. Following the original analysis from Lim et al. (2021), the D-loop and ND2 genes were concatenated and aligned to their reference data using muscle in MEGA11. A network was generated to visualise the relationship between haplotypes implemented in hapview.

3. Results

3.1. Quality assessment metrics

Nanopore long-read whole genome sequencing yielded a total of 911,658 reads, 753,510 of which passed quality control filtering. The passed reads were used for further analysis and the overall gDNA ONT sequencing metrics (as well as ONT mitochondrial reads) are summarised in Table 2 and Fig. S1. These metrics provide a comprehensive overview of the sequencing performance and data quality for the ten genomes from seafood products, enabling further downstream analysis and interpretation of the data. Of the total sequencing reads generated, an average of 9.1% was attributable to each of the ten samples studied. This share varied among the individual samples, with some contributing as low as 5.5% and others as high as 15% of the total reads. Despite the TapeStation showing HMW (>45 kbp) for most samples, the mean genomic read length was lower than expected between 811.5 bp–2440 bp. The adjusted average read quality score for Nanopore data ranged between 11.74 and 12 and this corresponds to an adjusted average read accuracy between 92.71% and 94.10%.

3.2. Species identification – COI

The cytochrome oxidase I (COI) gene was extracted from the ONT data to verify the species identity of fish collected in England and Indonesia. Table 1 provides an overview of each sample and the species identification results using the BOLD and BLAST databases with a >99% sequence similarity baseline. Results show that most samples accurately match the databases with samples IDs 01 (*C. punctatum*, brownbanded bamboo shark), 03 (*S. scombrus*, Atlantic Mackerel), 04 (*Lophius piscatorius*, Monkfish) and 06 (*Salmo salar*, Atlantic salmon) matching to their expected species designation with high similarity in both databases. However, the remaining samples exhibited multiple species hits or lower sequence similarity, such as sample 10, *G. morhua* (Atlantic cod) at 98.45% and sample 09, *C. falciformis* (silky shark), had a BLAST hit with 95.17%. Sample 05 (*Merluccius bilinearis*, silver hake) and 07 (*Sphyrna mokarran*, Great Hammerhead) matched to different expected species *M. merluccius* (98.71%, European hake) and *S. lewini* (97.31%, scalloped hammerhead) respectively, and indicate potential mislabelling or misidentification of the sample. COI identification was inconclusive for accurate species identification of sample 02, which was morphologically identified as *S. mentella* (beaked redfish), showing similarly high similarity to *S. mentella* (97.10%) and *S. fasciatus* (97.23%). Sample 08 (*Rhynchobatus springeri*, broadnose wedgefish) lacked COI information, so COII was utilized, revealing two low identity hits to *R. laevis* (91.82%) and *R. australiae* (91.54%).

3.3. Mitogenome assembly

Mitochondrial ONT reads were extracted from each marketed fish sample and using a reference-guided assembly approach. The completeness of each mitogenome was evaluated by calculating the number of contigs and coverage. For example, Barcode06 (*S. salar*) had a single contig with an average coverage of 154x (16,644bp), indicating high quality and complete assembly (Fig. S2). In contrast Barcode01 (*C. punctatum*) had 54 contig sequences, indicating a fragmented and incomplete assembly. Using a reference mitogenome for each sample, we annotated the gene content and organisation of the assemblies by characterising whether they contained typical numbers of protein-coding genes (PCGs), tRNAs, rRNA, and the D-loop for vertebrate mitochondrial genomes. For instance, Barcode06 (salmon) contained all complete 13 PCGs, 22 tRNAs, 2 rRNAs and D-loop genes arranged in the standard mitochondrial gene order (Table S2). Barcode01, on the other hand, contained 3 complete and 10 partial PCGs; 19 complete, 2 partial and 1 missing tRNA; 2 partial rRNA and partial D-loop genes (See Table S2 for the full list of metrics).

3.4. Phylogenetic analysis

The maximum likelihood analysis, incorporating the partial mitogenome contig (5,118bp) of Barcode07 successfully determined the species identity of the examined product (Fig. 2). In the phylogenetic tree, Barcode07 is aligned with NCBI accession number NC_022679.1, which corresponds as the scalloped hammerhead shark, *Sphyrna lewini*. The clade comprising Barcode07 and NC_022679.1 is grouped closely to other hammerhead shark species, *S. gilberti*, *S. tiburo*, and *S. mokarran*.

3.5. Species provenance

Based on the combined D-loop and rhodopsin analysis, the *Sebastes* sample (Barcode02) was determined to be sourced from a deep-pelagic *S. mentella* population collected in the central North Atlantic. The maximum likelihood network analysis of the mitochondrial D-loop including North Atlantic *S. viviparus*, S. *fasciatus*, *S. norvegicus* and *S. mentella* (shallow- & deep-pelagic populations) positioned the ONT *Sebastes* sample among the deep-pelagic *S. mentella* haplotypes (Fig. 3). Furthermore, the ONT rhodopsin sequence exhibited the amino acid Isoleucine at site 119 which is characteristic of deep-pelagic *S. mentella* (Shum et al., 2014, 2015).

The provenance of the Brownbanded bamboo shark (Barcode01) was shown to likely originate from the Malay Peninsula and evidence suggests that it may be more closely related to the population found along the Eastern Peninsula. The maximum likelihood network analysis identified a novel D-loop/ND2 haplotype linked to a common haplotype found in West and East Peninsular and Sarawak (Fig. 4). However, most haplotypes that group within this cluster originate from the East Peninsular.

4. Discussion

The vast diversity of species harvested from the oceans, and the intricacy of supply flows, present considerable obstacles in monitoring the trade of these commodities. Traditional COI barcoding and species-specific qPCR assays have been used for identification purposes; however, both approaches have operational constraints and, for the majority, are limited to resolving species authentication. Our results show that nanopore, PCR-free, long-read sequencing embodies the universality and portability required to achieve not only species identification of any organism, but also to unravel evolutionary relationships, and in some cases geographic provenance. The tool is portable and can be used anywhere in the world under a variety of contexts and through a combination of modest laboratory efforts, streamlined bioinformatics procedures, and the simultaneous generation of multi-gene information,

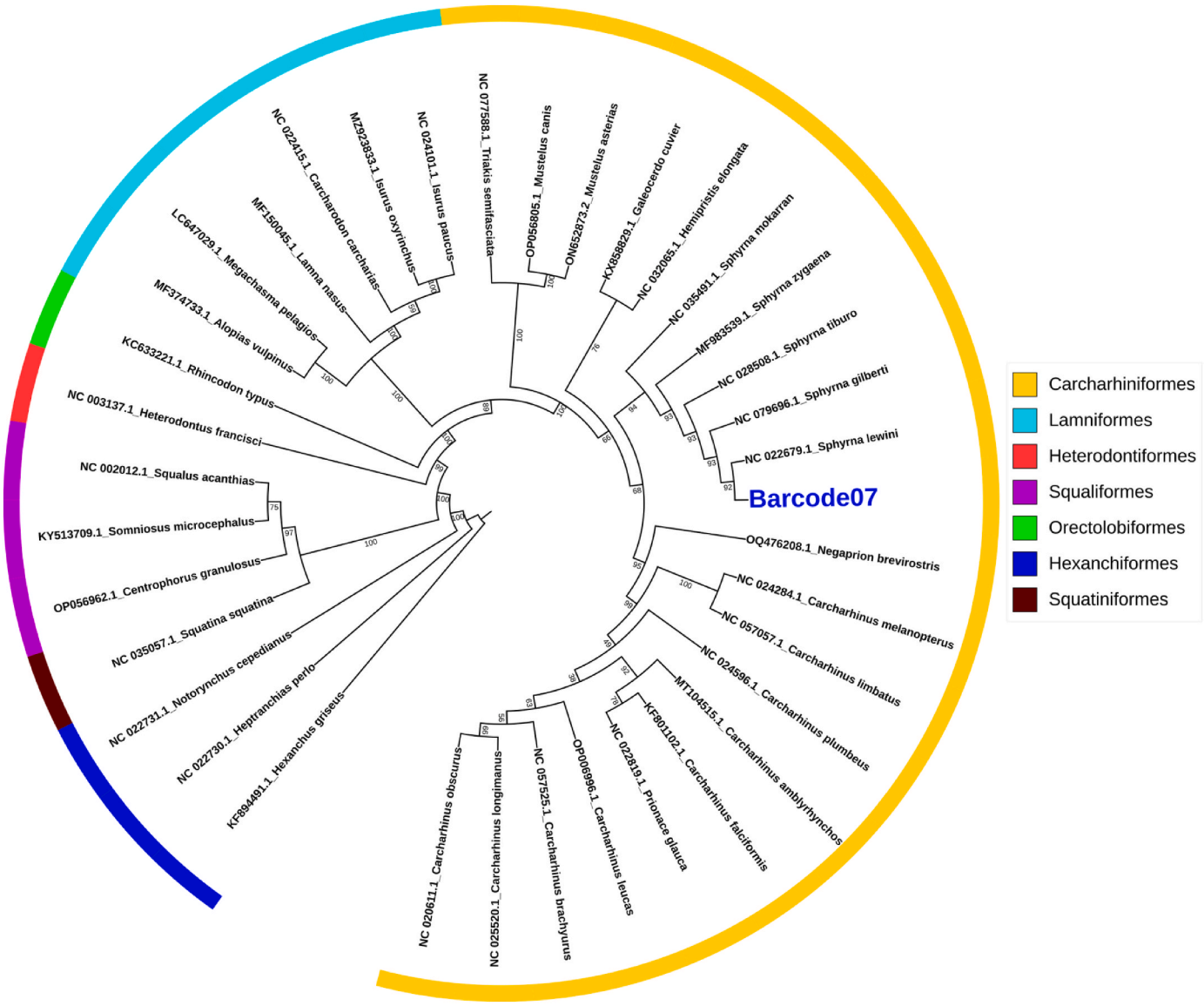

Fig. 2. Radial phylogenetic tree showing the evolutionary relationships among selected elasmobranchs, with Barcode07 (*Sphyrna lewini*, scalloped hammerhead shark) positioned among other hammerhead sharks. *Hexanchus griseus* (sixgill shark) is used as an outgroup. Bootstrap support values are displayed at the nodes.

thereby enabling a deeper exploration of the biology, ecology, and conservation status of these marine organisms.

4.1. Challenges and opportunities in seafood authentication

DNA barcoding is one of the most prominent molecular methods and has revolutionised species identification for seafood authenticity. The mitochondrial cytochrome-c oxidase I (COI) gene has become the most commonly used DNA barcode due to its high degree of variability among species and to its universal applicability across a wide range of taxa (Di Muri et al., 2018; Ward et al., 2005). This marker was the primary target for rapid species identification using our sequencing data, leading to reliable species identification in eight out of 10 specimens, but also proving inconclusive in two others. Of the robustly identified samples, two did not match the designated product name, potentially indicating instances of mislabelling and/or misidentification. Sample five was marketed as silver hake (*M. bilinearis*) which is commonly found along the Northwest Atlantic coastlines of Canada and the USA; but our molecular assessment identified this sample as European Hake (*M. merluccius*). The findings of Hake align with the broader patterns observed by Garcia-Vazquez et al. (2011). They found high rates of mislabelling in hake products, which signifies that hake is a particularly complex group of species to manage due to their similar morphologies and overlapping distributions. Sample seven, which was initially obtained and classified morphologically as a Great Hammerhead shark (*S. mokarran*) from an Indonesian market, was accurately identified as a Scalloped Hammerhead shark (*S. lewini*) through COI analysis. The probable cause of this discrepancy can be attributed to the challenging nature of distinguishing between these species when their fins are removed for commercial purposes, which is a practice frequently observed in such markets (Clarke et al., 2013).

The redfish sample exhibited inconclusive results in the COI analysis, displaying an ambiguous classification between *S. mentella* and *S. fasciatus*. Shum et al., 2017 previously demonstrated that DNA barcoding using COI for North Atlantic *Sebastes* species results in poorly resolved taxonomy, and they proposed utilising the mitochondrial control region (D-loop) as a more reliable marker for accurate species identification. Consequently, we leveraged the nanopore data to extract the mitochondrial control region, which allowed us to confidently assign the redfish sample to *S. mentella*, corroborating the initial morphological

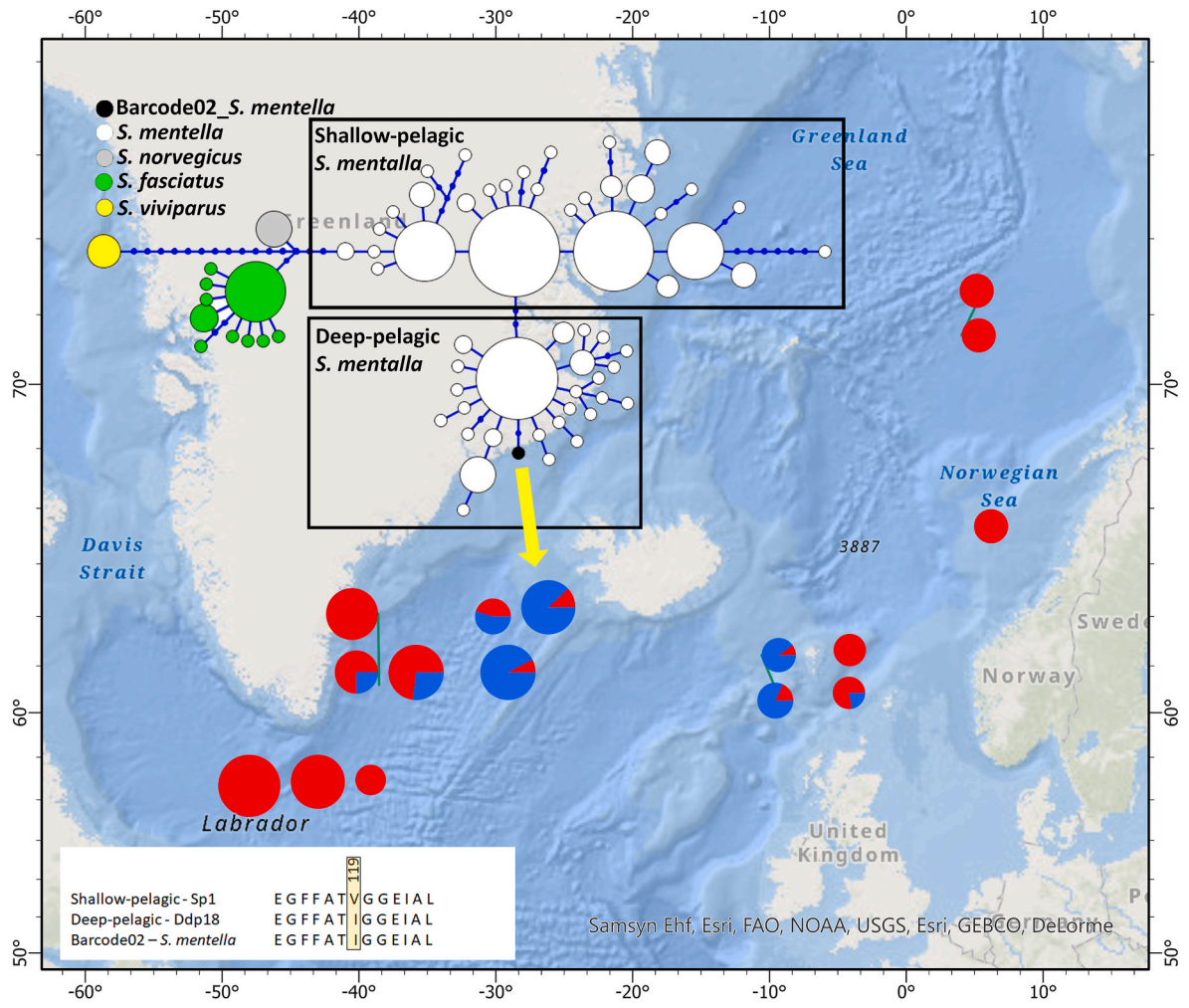

Fig. 3. Provenance Analysis and Genetic Diversity of Barcode02 (*Sebastes mentella*) - The figure illustrates the North Atlantic range of *S. mentella*, as reported by Shum et al. (2015). It includes a haplotype network that demonstrates the D-loop genetic diversity, placing Barcode02 within the deep-pelagic group captured southwest of Iceland (in black). An inset image at the bottom left highlights the single nucleotide polymorphism (SNP) at site 119 within the translated region of the Rhodopsin gene.

identification.

Another scenario pertained to the Broadnose wedgefish (sample eight), which exhibited the highest degree of DNA fragmentation, resulting in the absence of four mitochondrial protein-coding genes, including 16S, CYTB, and, most notably, COI. Consequently, we could not assess the species identification using COI. Employing COII, ND2 and 12S, we were confronted to ambiguous species classifications for two congeneric species, *R. laevis* and *R. australiae*, with confidence levels only around 90% identity. Thus, we were unable to confidently identify this sample using nanopore data alone. This sample was separately amplified using a short COI primer pair (Wangensteen et al., 2018), confirming its identity as *R. springeri*. The quality of input DNA in nanopore sequencing can directly affect the availability of key genes essential for specific applications or research questions. In this instance, despite the availability of other genes, the sample could not be identified due to the limitations of the existing database and the lack of information for this species. Even with the utilisation of the amplified COI, identification was made possible solely by relying on the single reference COI sequence for *R. springeri* available on GenBank (as of April 2023). This situation underscores the importance of enhancing and expanding databases to improve the accuracy and reliability of species identification methods, particularly for data-deficient species that are traded in markets.

4.2. Phylogenetics

While traditional methods such as COI analysis are widely employed for species identification, they can sometimes yield limited resolution, especially in cases of closely related species. In this context, the use of a longer fragment from the mitochondrial genome, can provide a more comprehensive genetic signature. Nanopore sequencing of long reads was harnessed to generate assemblies of the mitogenomes and has proven effective even with a partial mitogenome. This technique not only captures more extensive genetic information but also allows for a more nuanced phylogenetic placement of the sample. In the case of Barcode07, the identification of *S. lewini* was distinctly clarified using this longer fragment, highlighting this approach to take on heightened significance in the realm of seafood authentication and conservation biology. The generation of mitochondrial genome data, whether whole or partial, emerges as a powerful tool in instances where the identification based on shorter DNA fragments remains ambiguous or inconclusive. The ability to accurately discern species identity from even partial genomic data is not only a leap forward in the precision of species identification but also a crucial step in effective monitoring and regulation of the trade of endangered species such as most hammerhead sharks.

Our findings underscore the potential of mitochondrial genome analysis, facilitated by techniques like nanopore sequencing, to resolve

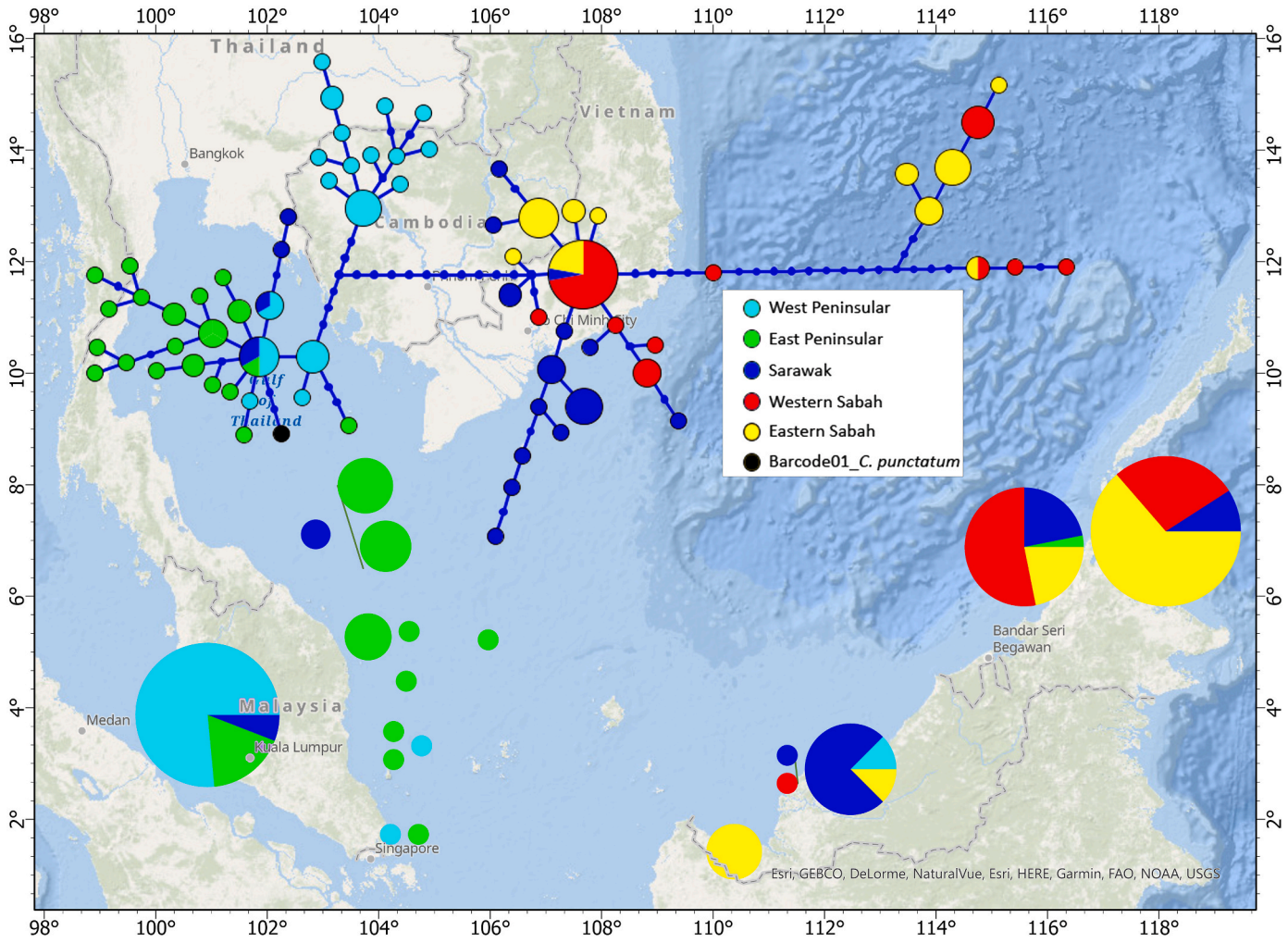

Fig. 4. Combined Provenance Analysis of Barcode01 (*Chiloscyllium punctatum*) using D-loop and ND2 - This figure illustrates the extensive provenance of this shark species across the vast regions of the Malay Peninsula and the South China Sea. It includes a haplotype network that highlights Barcode01(in black), and bubble plots derived from Lim et al. (2021).

uncertainties that often accompany shorter DNA fragment analysis. This enhanced resolution is instrumental in addressing challenges such as mislabelling in the seafood industry and illegal trade in conservation efforts. The scarcity of mitogenomic references has been a longstanding challenge for in-depth phylogenetic studies and new data points from diverse regions can start filling this critical gap. The inclusion of seafood samples can significantly diversify the existing genomic databases, thereby making public datasets more representative. Moreover, this surge in genomic information carries substantial implications for fisheries management. By providing a clearer picture of genetic diversity and population structure, we can create more effective conservation strategies, ensuring the sustainability and health of populations.

4.3. Species provenance

Fisheries operate virtually everywhere across the oceans; thus, determining the catch area of these globally traded animals is increasingly important for stock management, species conservation, and consumer awareness. DNA monitoring tools can enhance transparency in seafood supply and regulate marine wildlife trade. By identifying genetic markers unique to specific geographic regions, we can better understand the origin and distribution patterns of these species. As nanopore sequencing becomes more widely adopted, it has the potential to revolutionize our knowledge of the complex relationships between traded animals, their environments, and the global ecosystem. For instance, our *Sebastes* specimen served as a valuable reference, having been collected as part of a redfish stock assessment survey in the Irminger Sea at depths below 500m and morphologically identified as a deep-pelagic *S. mentella*. (Shum et al., 2015) investigated the phylogenetic history of *S. mentella* populations across the North Atlantic, revealing a deep-pelagic population in the central North Atlantic below 500m depth and a shallow-pelagic population widely distributed above 500m depth. These findings were subsequently corroborated with more extensive genomic screening by (Saha et al., 2017). The ability to accurately assign individuals to their population of origin has substantial implications for the management and conservation of these species, as certain populations may be more vulnerable to fishing pressures than others. For example, the deep-pelagic population of *S. mentella* is known to be less resilient to commercial harvesting compared to its shallow-pelagic counterpart. Nanopore data allowed harvesting control region and rhodopsin sequences to both gain better taxonomic detail and stock provenance, thereby simultaneously uncovering multiple insights into marine population dynamics and expanding the management and conservation value of the screening.

A pressing concern in the marine ecosystem is the unabated exploitation of sharks and rays, including species known to be threatened by extinctions and others for which we have insufficient information. Shark fisheries and trade face considerable controversy due to the widespread practice of finning, which often renders species identification based solely on fins unreliable. Consequently, DNA-based methods have

emerged as the most dependable approach for accurately identifying shark species, enabling better management and conservation of these vulnerable marine populations (Cardeñosa et al., 2022; Fields et al., 2018). In addition to species identification, the ability to leverage existing data and pinpoint the provenance of a sample serves as a game-changer in the field of marine conservation (Cusa et al., 2022). Nanopore technology is unique in its feature to produce large amounts of sequences that can be mined for multiple purposes, without requiring separate assays and protocols for different tasks. This is a feature that was also evident in the case of the bamboo shark (*C. punctatum*), a near-threatened shark species native to the Indo-Pacific region, including the western and eastern coastlines of the Malay Peninsula and the whole Indonesian archipelago. A recent study by (Lim et al., 2021) examined the distribution and genetic diversity of the bamboo shark in Malaysian waters, shedding light on the phylogeography of this species. Using their data as a reference, we were able to mine the nanopore data of our identified Bamboo shark and reveal a close genetic relationship with the peninsular haplotypes of the bamboo shark distribution, with a closer association with the eastern peninsular haplotypes, suggesting a potentially shared stock of bamboo shark population between Malaysia and Indonesia Fahmi.

4.4. Future impact and legacy of nanopore sequencing in seafood and marine wildlife monitoring

The accurate identification of fishery products is essential for safeguarding public health, promoting sustainable fisheries, and enforcing compliance with international regulations. Mislabelling and misidentification of seafood can lead to health risks (Sicherer et al., 2004; Jacquet & Pauly, 2008; Marko et al., 2014), depletion of marine resources (Marko et al., 2014), and the perpetuation of IUU fishing activities (Helyar et al., 2014; Cawthorn et al., 2018). While DNA tools like Illumina sequencing have transformed genomic analysis over the last couple of decades with their high-throughput and accurate short-read data, nanopore sequencing brings a unique set of desirable features to the table. These include high portability, real-time diagnostics (Prasetyo et al., 2022), lower start-up costs, and independence from bespoke reference data, enabling genome-wide data generation and a multi-purpose assay approach. Especially in the context of seafood identification, nanopore's ability to provide long-read sequencing facilitates a more direct and less reference-dependent analysis, which can be advantageous when dealing with non-model organisms or species with limited genomic resources.

Despite these impressive characteristics, several limitations must be overcome prior to full-scale operationalisation of nanopore technology. Firstly, in our study, we encountered sequencing error rate up to 8%, and were able to accurately identify specimens to species. Improvements in sequencing chemistry are expected to elevate nanopore accuracy to Q20, on par with Sanger sequencing, thus achieving the gold standard of established DNA barcoding platforms (Morrison et al., 2020). Secondly, nanopore sequencing analysis is more computationally intensive, depending on the research questions and depth of analysis required. This can involve additional hands-on bioinformatics. However, custom pipelines can be developed to simplify data analysis, making it more user-friendly. For example, the R environment offers a graphical interface for a plug-and-play approach to nanopore data analysis (Bolognini et al., 2019). Lastly, determining provenance relies on the availability and appropriateness of existing data, both reference and nanopore sequenced data. In our examples, mitochondrial genes were sufficient for identifying the point of origin. Nevertheless, more intricate data sets, such as those involving specific SNPs, may demand custom-tailored analysis, thereby escalating the bioinformatics complexity involved.

It should also be noted that, because of a more extensive use of nanopore long-read sequencing, it will become commonplace to obtain whole mitogenomes for a wide range of species. These resources will play a major role in expanding public sequence database for subsequent applications of DNA metabarcoding, whether it is for environmental DNA monitoring or for the screening of food mixtures. Overall, nanopore sequencing is well poised to play a major role in combating fraud and illegal, unreported, and unregulated (IUU) fishing practices. Its scalability and flexibility make it a valuable tool for addressing the challenges in seafood traceability and endangered wildlife trade, hence serving as a mighty tool for the sustainable management of our marine resources.

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Permit

Research permit no. 251/BRSDM/II/2020 issued by the Agency for Marine and Fisheries Research and Human Resources AMFRAD, the Ministry of Marine Affairs and Fisheries (MMAF), Republic of Indonesia.

Research ethics no. STR1819-45 issued by Science and Technology Research Ethics Panel, the University of Salford, United Kingdom.

Export permits no. 00135/SAJI/LN/PRL/IX/2021 (CITES-listed specimens) and 127/LPSPL.2/PRL.430/X/2021 (non-CITES-listed specimens) were granted under the authority of the Ministry of Marine Affairs and Fisheries (MMAF), Republic of Indonesia.

Import permit no. 609191/01-42 from the Animal and Plant Health Agency (APHA), United Kingdom.

Data accessibility statement

Raw Nanopore sequences are available on NCBI's SRA database BioProject ID: PRJNA1079683. Bioinformatics scripts are available at: https://github.com/shump2/Nanopore_seafood.

CRediT authorship contribution statement

Peter Shum: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marine Cusa:** Writing – review & editing, Methodology, Conceptualization. **Andhika Prasetyo:** Writing – review & editing, Methodology. **Stefano Mariani:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw Nanopore sequences are available on NCBI's SRA database BioProject ID: PRJNA1079683. Bioinformatics scripts are available at: https://github.com/shump2/Nanopore_seafood.

Python Scripts (Original data) (Figshare)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110382.

References

Ahmed, Z., et al. (2020). Moving towards a sustainable environment: The dynamic linkage between natural resources, human capital, urbanization, economic growth, and ecological footprint in China. *Resources Policy, 67*, Article 101677. https://doi.org/10.1016/J.RESOURPOL.2020.101677

Bolognini, D., et al. (2019). Nanor: A user-friendly R package to analyze and compare nanopore sequencing data. *PLoS One, 14*(5). https://doi.org/10.1371/journal.pone.0216471

Cardeñosa, D., et al. (2022). Two thirds of species in a global shark fin trade hub are threatened with extinction: Conservation potential of international trade regulations for coastal sharks. *Conservation Letters, 15*(5), Article e12910. https://doi.org/10.1111/CONL.12910

Cardeñosa, D., Gollock, M. J., & Chapman, D. D. (2019). Development and application of a novel real-time polymerase chain reaction assay to detect illegal trade of the European eel (Anguilla anguilla). *Conservation Science and Practice, 1*(5). https://doi.org/10.1111/csp2.39

Cawthorn, D. M., Baillie, C., & Mariani, S. (2018). Generic names and mislabeling conceal high species diversity in global fisheries markets. *Conservation Letters, 11*(5), Article e12573. https://doi.org/10.1111/CONL.12573

Cawthorn, D. M., & Mariani, S. (2017). Global trade statistics lack granularity to inform traceability and management of diverse and high-value fishes. *Scientific Reports, 7*(1), 1–11. https://doi.org/10.1038/s41598-017-12301-x, 2017 7:1.

Clarke, S. C., et al. (2013). Population trends in pacific oceanic sharks and the utility of regulations on shark finning. *Conservation Biology, 27*(1). https://doi.org/10.1111/j.1523-1739.2012.01943.x

Cusa, M., et al. (2022). A future for seafood point-of-origin testing using DNA and stable isotope signatures. *Reviews in Fish Biology and Fisheries, 32*(2), 597–621. https://doi.org/10.1007/S11160-021-09680-W/FIGURES/5

Di Muri, C., et al. (2018). Biodiversity defrosted: Unveiling non-compliant fish trade in ethnic food stores. *Biological Conservation, 217*, 419–427. https://doi.org/10.1016/J.BIOCON.2017.11.028

Ehrlich, P., & Ehrlich, A. H. (2008). Nature's economy and the human economy. *Environmental and Resource Economics, 39*(1), 9–16. https://doi.org/10.1007/s10640-007-9177-5. Springer.

Fahmi, et al. (2021). 'Population structure of the brown-banded bamboo shark, Chiloscyllium punctatum and its relation to fisheries management in the Indo-Malay region'. *Fisheries Research, 240*. https://doi.org/10.1016/j.fishres.2021.105972

Fields, A. T., et al. (2018). Species composition of the international shark fin trade assessed through a retail-market survey in Hong Kong. *Conservation Biology, 32*(2), 376–389. https://doi.org/10.1111/COBI.13043

Garcia-Vazquez, E., et al. (2011). High level of mislabeling in Spanish and Greek hake markets suggests the fraudulent introduction of African species. *Journal of Agricultural and Food Chemistry, 59*(2). https://doi.org/10.1021/jf103754r

Golden, C. D., et al. (2021). Aquatic foods to nourish nations. *Nature, 598*(7880), 315–320. https://doi.org/10.1038/s41586-021-03917-1, 2021 598:7880.

Helyar, S. J., et al. (2014). Fish product mislabelling: Failings of traceability in the production chain and implications for illegal, unreported and unregulated (IUU) fishing. *PLoS One, 9*(6), Article e98691. https://doi.org/10.1371/JOURNAL.PONE.0098691

Jacquet, J. L., & Pauly, D. (2008). Trade secrets: Renaming and mislabeling of seafood. *Marine Policy, 32*(3), 309–318. https://doi.org/10.1016/J.MARPOL.2007.06.007

Johri, S., et al. (2019). "Genome skimming" with the MinION hand-held sequencer identifies CITES-listed shark species in India's exports market. *Scientific Reports, 9*(1), 1–13. https://doi.org/10.1038/s41598-019-40940-9

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution, 30*(4). https://doi.org/10.1093/molbev/mst010

Kolmogorov, M., et al. (2019). Assembly of long, error-prone reads using repeat graphs. *Nature Biotechnology, 37*(5). https://doi.org/10.1038/s41587-019-0072-8

Letunic, I., & Bork, P. (2007). Interactive tree of life (iTOL): An online tool for phylogenetic tree display and annotation. *Bioinformatics, 23*(1), 127–128.

Lim, K. C., et al. (2021). Brown banded bamboo shark (Chiloscyllium punctatum) shows high genetic diversity and differentiation in Malaysian waters. *Scientific Reports, 11*(1). https://doi.org/10.1038/s41598-021-94257-7

Malukiewicz, J., et al. (2021). Genomic skimming and nanopore sequencing uncover cryptic hybridization in one of world's most threatened primates. *Scientific Reports, 11*(1). https://doi.org/10.1038/s41598-021-96404-6

Marko, P. B., Nance, H. A., & Van Den Hurk, P. (2014). Seafood substitutions obscure patterns of mercury contamination in patagonian toothfish (dissostichus eleginoides) or "Chilean Sea Bass". *PLoS One, 9*(8). https://doi.org/10.1371/JOURNAL.PONE.0104140

Menegon, M., et al. (2017). On site DNA barcoding by nanopore sequencing. *PLoS One, 12*(10). https://doi.org/10.1371/journal.pone.0184741

Miller, D., et al. (2012). Seafood mislabelling: Comparisons of two western European case studies assist in defining influencing factors, mechanisms and motives. *Wiley Online Library, 13*(3), 345–358. https://doi.org/10.1111/j.1467-2979.2011.00426.x

Miller, D., & Mariani, F. (2010). Smoke, mirrors, and mislabeled cod: Poor transparency in the European seafood industry. *Wiley Online Library, 8*(10), 517–521. https://doi.org/10.1890/090212

Mohanty, B. P., et al. (2013). Food safety, labeling regulations and fish food authentication. *National Academy Science Letters, 36*(3), 253–258. https://doi.org/10.1007/S40009-013-0139-X/TABLES/1

Morrison, G. A., et al. (2020). Nanopore sequencing of the fungal intergenic spacer sequence as a potential rapid diagnostic assay. *Journal of Clinical Microbiology, 58*(12). https://doi.org/10.1128/JCM.01972-20

Mozer, A., & Prost, S. (2023). An introduction to illegal wildlife trade and its effects on biodiversity and society. *Forensic Science International: Animals and Environments, 3*, Article 100064. https://doi.org/10.1016/J.FSIAE.2023.100064

Naaum, A. M., et al. (2021). 'Validation of FASTFISH-ID: A new commercial platform for rapid fish species authentication via universal closed-tube barcoding'. *Food Research International, 141*. https://doi.org/10.1016/J.FOODRES.2020.110035

Ogden, R., & Linacre, A. (2015). Wildlife forensic science: A review of genetic geographic origin assignment. *Forensic Science International: Genetics, 18*, 152–159. https://doi.org/10.1016/J.FSIGEN.2015.02.008

Pazartzi, T., et al. (2019). High levels of mislabeling in shark meat – investigating patterns of species utilization with DNA barcoding in Greek retailers. *Food Control, 98*, 179–186. https://doi.org/10.1016/J.FOODCONT.2018.11.019

Prasetyo, A. P., et al. (2022). Universal closed-tube barcoding for monitoring the shark and ray trade in megadiverse conservation hotspots. bioRxiv, p. 2022.11.30.518468 https://doi.org/10.1101/2022.11.30.518468.

Prasetyo, A. P., et al. (2023). Universal closed-tube barcoding for monitoring the shark and ray trade in megadiverse conservation hotspots. *iScience, 26*(7), Article 107065. https://doi.org/10.1016/J.ISCI.2023.107065

Ratnasingham, S., & Hebert, P. D. N. (2007). Bold: The barcode of life data system: Barcoding. *Molecular Ecology Notes, 7*(3). https://doi.org/10.1111/j.1471-8286.2007.01678.x

Saha, A., et al. (2017). Geographic extent of introgression in Sebastes mentella and its effect on genetic population structure. *Evolutionary Applications, 10*(1). https://doi.org/10.1111/eva.12429

Salzburger, W., Ewing, G. B., & Von Haeseler, A. (2011). The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. *Molecular Ecology, 20*(9). https://doi.org/10.1111/j.1365-294X.2011.05066.x

Sellers, G. S., et al. (2018). Mu-DNA: A modular universal DNA extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics, 2*(2), Article e24556. https://doi.org/10.3897/MBMG.2.24556

Shum, P., et al. (2014). Divergence by depth in an oceanic fish. *PeerJ, 2014*(1), e525. https://doi.org/10.7717/PEERJ.525/FIG-4

Shum, P., et al. (2015). Three-dimensional post-glacial expansion and diversification of an exploited oceanic fish. *Molecular Ecology, 24*(14), 3652–3667. https://doi.org/10.1111/MEC.13262

Shum, P., et al. (2017). Harnessing mtDNA variation to resolve ambiguity in "Redfish" sold in Europe. *PeerJ, 2017*(10), e3746. https://doi.org/10.7717/PEERJ.3746/SUPP-1

Sicherer, S. H., Muñoz-Furlong, A., & Sampson, H. A. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology, 114*(1), 159–165. https://doi.org/10.1016/J.JACI.2004.04.018

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics, 30*(9), 1312–1313.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*(7). https://doi.org/10.1093/molbev/msab120

Temple, A. J., et al. (2022). Illegal, unregulated and unreported fishing impacts: A systematic review of evidence and proposed future agenda. *Marine Policy, 139*, Article 105033. https://doi.org/10.1016/J.MARPOL.2022.105033

Tillich, M., et al. (2017). GeSeq - versatile and accurate annotation of organelle genomes. *Nucleic Acids Research, 45*(W1). https://doi.org/10.1093/nar/gkx391

Velasco, A., et al. (2021). A new rapid method for the authentication of common octopus (Octopus vulgaris) in seafood products using recombinase polymerase amplification (rpa) and lateral flow assay (lfa). *Foods, 10*(8). https://doi.org/10.3390/foods10081825

Voorhuijzen-Harink, M. M., Hagelaar, R., van Dijk, J. P., Prins, T. W., Kok, E. J., & Staats, M. (2019). Toward on-site food authentication using nanopore sequencing. *Food Chemistry, X*, 2.

Wangensteen, O. S., et al. (2018). DNA metabarcoding of littoral hardbottom communities: High diversity and database gaps revealed by two molecular markers. *PeerJ, 2018*(5). https://doi.org/10.7717/peerj.4705

Ward, R. D., et al. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences, 360*. https://doi.org/10.1098/rstb.2005.1716, 1462.

Ward, R. D., et al. (2005). DNA Barcoding Australia's Fish Species Philosophical Transactions Of The Royal Society B DNA barcoding Australia's fi sh species. *Hebert Source: Philosophical Transactions: Biological Sciences DNA Barcoding of Life Trans. R. Soc. B, 360*(360).

White, W. T., et al. (2006). *Economically important shark and rays*. Australian Centre for International Agricultural Research.

Willette, D. A., et al. (2021). The last mile challenge: Certified seafood and federal labeling laws out of sync at the end of the supply chain in Los Angeles, California. *Marine Policy, 125*, Article 104380. https://doi.org/10.1016/J.MARPOL.2020.104380

Xiong, X., et al. (2018). Multiple fish species identified from China's roasted Xue Yu fillet products using DNA and mini-DNA barcoding: Implications on human health and marine sustainability. *Food Control, 88*, 123–130. https://doi.org/10.1016/J.FOODCONT.2017.12.035