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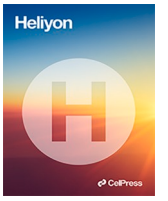

Research article

DNA metabarcoding unveils the hidden species composition in fish surimi: Implications for the management of unlabeled and mixed seafood products

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ABSTRACT

Fish surimi products are traditional foods primarily made from fish meat and may contain a complex species composition. In Taiwan, the abundant fishery resources and diverse fish species lead to local catches being widely used as ingredients in fish surimi products. However, due to growing market demand and increasingly scarce resources, some surimi products contain sensitive species, such as sharks, posing potential threats to the ecological environment and biodiversity. In this study, by applying metabarcoding techniques, we analyzed 120 fish surimi product samples from different brands and types throughout the four seasons in Taiwan's market. The main fish species identified included milkfish (*Chanos chanos*), dolphinfish (*Coryphaena hippurus*), Pomfret (*Taractes rubescens*), swordfish (*Istiophorus* spp.) and cartilaginous. Moreover, at least 37 species of cartilaginous fish, including 26 endangered species, were found. Through comprehensive and accurate species identification of surimi product ingredients, we unveiled the usage of sensitive species in products on the market. This finding is important for the surimi industry's quality control and market supervision. Furthermore, it can promote the sustainable use of Taiwan's fishery resources and protect biodiversity.

1. Introduction

Seafood comprises a variety of aquatic organisms used for food and has emerged as one of the main food commodities traded worldwide [1,2]. The demand for seafood has been consistently growing, with human consumption increasing by 3.1 % annually [3]. Over recent decades, the swift expansion of aquaculture production has substantially augmented the seafood supply, and it is anticipated to outpace capture fisheries production by 2030 [4]. However, considerable seafood still originates from capture fisheries [1,4,5]. Furthermore, the species diversity of seafood derived from capture fisheries can be more complex due to the inherent intricacy of aquatic biodiversity [6]. These catches may include species not usually consumed directly but often caught as bycatch. Taiwan is renowned for its rich marine biodiversity and resources, with over 3000 finfish species recorded in its waters, including more than 200 species of sharks [7,8]. Consequently, highly active coastal fisheries in Taiwan inevitably lead to the direct or indirect utilization of these abundant and diverse shark species [9–12].

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Fish surimi products, traditional foods primarily made from fish meat, often contain a diverse mix of specie. Among these, some surimi products contain sensitive species, including sharks, which have experienced population declines and possess vulnerable life history traits [13–15]. These sharks are sometimes used as the main ingredients, while in other instances, they might be unintentionally caught as bycatch and occasionally mixed into fish surimi products [13–15]. However, due to the growing market demand and increasingly scarce resources, sensitive species like sharks in surimi products pose potential threats to the ecological environment and biodiversity [14]. To address these concerns, it is of utmost importance to have accurate and reliable methods for identifying seafood species.

Traditionally, morphological characters have been used for the taxonomical identification of seafood species. While this approach is simple and inexpensive, it requires well-trained experts and may be difficult when morphological characters are removed, altered, or destroyed during processing, storage, or transport. Alternatively, DNA-based methods have proven effective for accurate seafood species identification, but they can be laborious and expensive when dealing with many samples [16,17]. Recently, DNA metabarcoding has emerged as a powerful tool for the simultaneous identification of multiple species in complex samples due to the rapid developments in Next generation sequencing (NGS) technology. This approach holds great potential for discovering marine biodiversity and identifying seafood species [18–21]. However, the choice of markers and primers in NGS and DNA metabarcoding is crucial and more complex, involving ensuring broad taxonomic coverage and avoiding amplification biases that can lead to incomplete or misleading results [22]. While NGS and DNA metabarcoding offer unparalleled depth and breadth of data, they also pose challenges in data analysis, requiring sophisticated bioinformatics tools to handle the vast amount of generated sequences and accurately identify species [22].

Historically, fish surimi products labeled only major species like milkfish and sailfish. Traditional methods prioritized quality control but lacked effective approaches for identifying the precise species composition [23–26]. In this study, we aim to identify species composition in fish surimi products from various sources in Taiwan using DNA metabarcoding. Our findings are expected to unveil the hidden species composition in fish surimi. Furthermore, this research can contribute to the surimi industry's quality control and market supervision, promoting the sustainable use of Taiwan's fishery resources and protecting biodiversity.

2. Materials and methods

2.1. Sample collection and DNA extraction

This samping was conducted in Taiwan's fish market and restaurant from April 2022 to March 2023. To reflect fish surimi product diversity, we sampled 120 from 30 sources with an interval of about three months (30/season; Fig. 1). Each sampling is taken at least 300 g of fish surimi product. Frozen samples were placed with ice until it is taken back to the laboratory. Approximately 0.2 g of each

Fig. 1. Seasonal availability and main species composition of various fish surimi. The fish species with the highest proportion in each sample was taken as the main species, while cartilaginous fishes were the sum of the species. The colored bars correspond to the main species used in the products. Pink: Cartilaginous; Green: Swordfish; Yellow: Dolphin fish Orange: Milkfish; Blue: Pomfret; Light Blue: Tarpor.

sample was individually collected in a 1.5 mL centrifuge tube. DNA extraction was conducted by PureDireX Genomic DNA Isolation Kit (Bio-Helix, NTPC, Taiwan). Moreover, DNA template quality was assessed through 0.8 % agarose gel electrophoresis. DNA concentration of each sample was checked by using the Qubit dsDNA HS Assay (Invitrogen, CA, USA)

2.2. DNA library preparation

The 12S rRNA region of mitochondrial gene was amplified by universal primers MiFish-U/E, which have been demostrated to be effective for the identification of fish species across a wide variety of taxa [27]. Each 20 μL PCR reaction contained 10 μL of 2x PCRBIO HS Taq Mix, 1 μL of each primer (10 μM), 7 μL ddH2O, and 1 μL of DNA extract. The following cycling conditions were used: 5 min at 95 °C (1 ×); 1 min at 95 °C, 30 s at 48 °C, and 45 s at 72 °C (38 ×); 5 min at 72 °C (1 ×). Three PCR replicates were amplified from each sample and then pooled for a single PCR cleanup with the QIAquick 96 PCR purification kit (Qiagen; 60 μL elution volume). Agarose (2 % w/v) gel electrophoresis was used to verify the amplification of samples. PCR products were pooled and quantified using Qubit dsDNA HS Assay before preparation for the library. The library was sequenced on the NovaSeq 6000 (with paired-end 150-bp reads, PE150) following the NovaSeq XP workflow. Library preparation, sequencing, and base calling were carried out by Genomics BioSci & Tech (http://www.genomics.com.tw/).

2.3. Data process

The overall quality of the Novaseq reads was inspected by FastQC (Babraham Bioinformatics). Adaptor sequence and low-quality tails in raw sequence data were trimmed (quality ≤10) by Trimmomatic 0.32 [28]. Sequencing reads were filtered to remove reads shorter than 150 bp. The remaining reads were merged using BBMerge algorithm with default parameter settings and reads [29]. The assembled reads were further demultiplexed by different index primer sets by Cutadapt 3.4 [30]. In order to remove reads with either ambiguous sites (Ns) or those showing unusual and too short lengths with reference to the expected size of the PCR amplicons. Primer clipping and lengths control of reads were also used Cutadapt 3.4 [30]. The pre-processed reads from the above pipeline were further dereplicated by using a 'derep_fulllengthzrusing' command in VSEARCH [31]. Keep only sequences with an abundance equal to or greater than 2. OTU clustering and chimera detection in dereplicated reads for each primer set was used "cluster_otus" command in USEARCH by default setting. All OTUs were subjected to local BLASTN searches against a fish mitogenome database MitoFish V3.86 [32]. The top BLAST hit with a sequence identity of more than or equal to 97 % and sequences larger than 100 bp was applied to species assignments of each OTU. Taxonomic assignment to the species level, and all the scientific names were checked to remove the synonyms by NomenMatch (http://match.taibif.tw/). The processed OTUs from all primer sets were built to the OTU table. The OTU

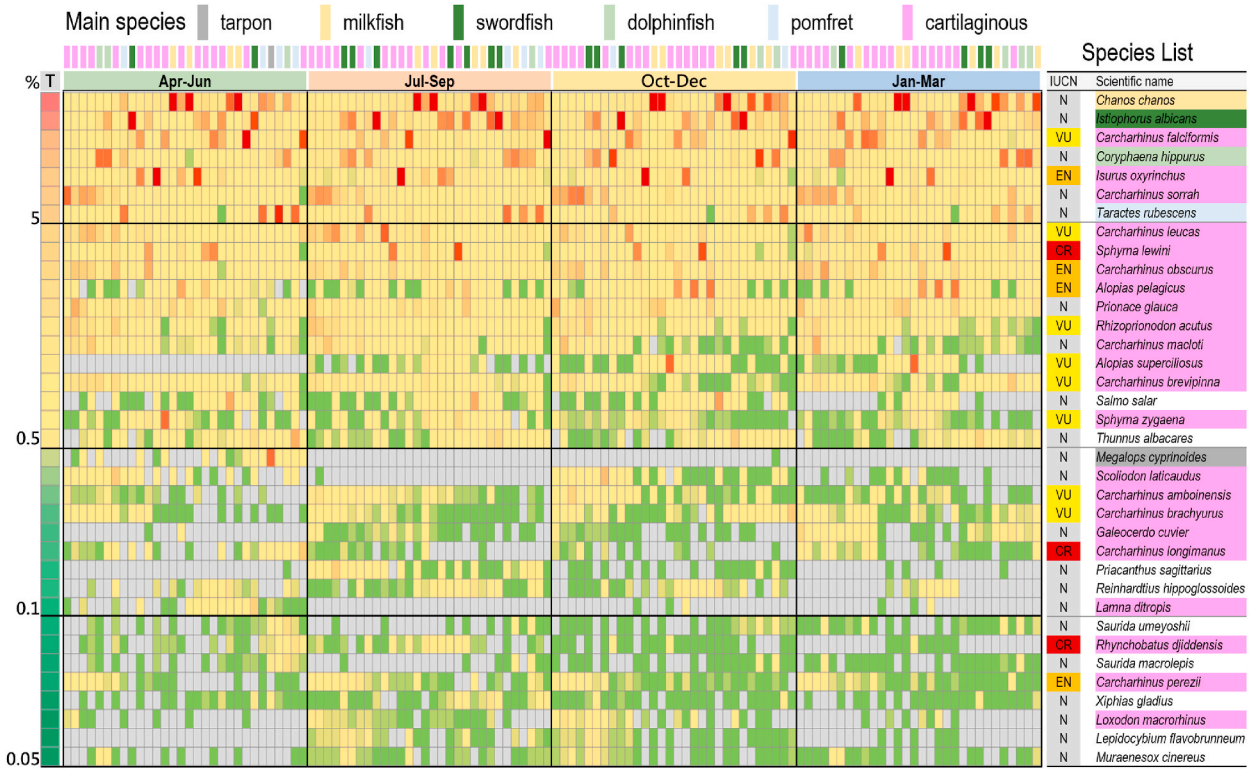

Fig. 2. Abundance of fish species (reads) from 120 fish surimi samples. The grey-colored boxes indicate the absence of species.

occurrences for each sample were performed "usearch_global" with a 97 % similarity threshold command in VSEARCH [31].

2.4. Statistical analysis

NGS reads in the OTU table for each sample were normalized and listed according to cumulative percentage across all samples. The relative abundance (from reads), taxonomic barplot, Alpha diversity (Abundance-based Coverage Estimator; ACE), Beta diversity (Bray–Curtis similarity with PCoA), and post hoc analysis were performed using MOCHI (https://mochi.life.nctu.edu.tw).

3. Results

3.1. Species identification and classification

A total of 73,040,450 merged reads were obtained from 120 fish surimi samples, ranging from 16,800 to 4,996,100 reads per sample, with an average of 608,670 reads. After processing the OTU table and removing duplicates and unidentified species, 35 species of sharks (class Chondrichthyes) and 110 species of other fishes (class Actinopterygii) were retained (Appendix S1). The Chondrichthyes were represented by three orders: Carcharhiniformes (29 species), Lamniformes (4 species), and Rajiformes (2 species). The Actinopterygii included fourteen orders: Anguilliformes (3 species), Argentiniformes (2 species), Aulopiformes (8 species), Beloniformes (1 species), Clupeiformes (1 species), Elopiformes (2 species), Gonorynchiformes (1 species), Mugiliformes (2 species), Osmeriformes (1 species), Perciformes (82 species), Pleuronectiformes (1 species), Salmoniformes (1 species), Scorpaeniformes (2 species), and Tetraodontiformes (3 species). For a detailed list of species, refer to Appendix Table S1. Th e number of species per sample ranged from 25 to 59, with an average of 40.1 species, including 13 to 29 cartilaginous species (Table S1). According to the IUCN Red List of Threatened Species (https://www.iucnredlist.org), 26 species identified are threatened, including 4 critically endangered (CR), 10 endangered (EN), and 12 vulnerable (VU) species (Fig. 3).

3.2. Species composition and ranking

A total of 38 species exceeded 0.05 % in cumulative percentage across all samples (Fig. 2). Among cartilaginous species, 19 species surpassed 0.01 % (Fig. 3). The fish species with the highest proportion in each sample was designated as the main species, while cartilaginous fishes were aggregated. The primary fish species identified included milkfish (*Chanos chanos*; 21/120 samples), dolphinfish (*Coryphaena hippurus*; 10/120 samples), pomfret (*Taractes rubescens*; 10/120 samples), swordfish (*Istiophorus* spp.; 18/120

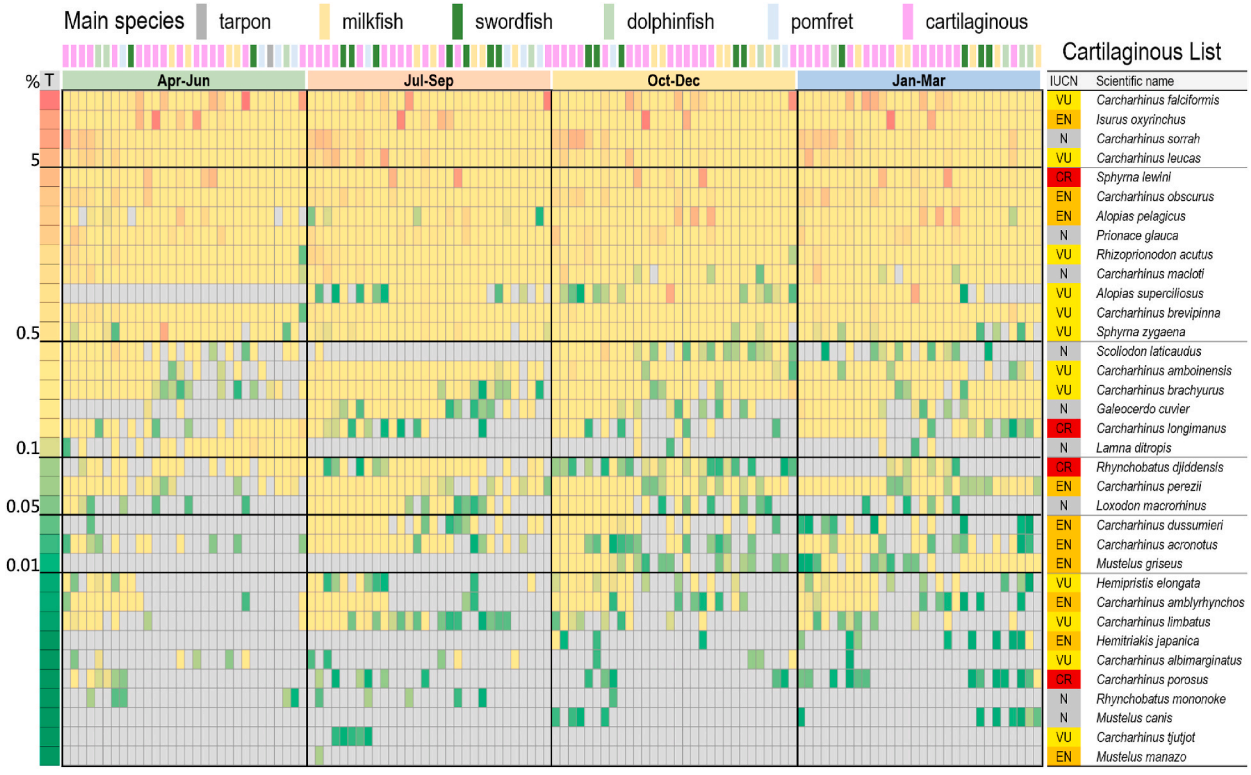

Fig. 3. Abundance of cartilaginous species from 120 fish surimi samples. The grey-colored boxes indicate the absence of species.

samples), tarpon (Megalops cyprinoides; 1/120 sample), and various cartilaginous fishes (60/120 samples) (Fig. 1). Consistency across four seasons was observed in fifteen sources (15/30) (Fig. 1). Among the 60 samples dominated by cartilaginous fish, 10 species exhibited the highest proportion in a single sample, including *Alopias pelagicus*, *Carcharhinus sorrah*, *Carcharhinus leucas*, *Carcharhinus falciformis*, *Carcharhinus macloti*, *Carcharhinus obscurus*, *Isurus oxyrinchus*, *Prionace glauca*, *Rhizoprionodon acutus*, and *Sphyrna lewini* (Fig. 4).

3.3. Species diversity and similarity

Alpha diversity was lowest in the April–June season group and within the milkfish group (tarpon were included in the milkfish group). The April–June group exhibited significant differences from the other seasonal groups (Figs. 5–6). Additionally, significant differences were observed between the milkfish-dolphinfish and dolphinfish-cartilaginous groups (Figs. 5–6). β-diversity revealed the distribution patterns of the samples (Figs. 7–8). The four seasonal groups did not form significant clusters ($p > 0.5$; pairwise PERMANOVA; Fig. 7), whereas the five main species groups formed significant clusters among all pairs ($p = 0.001$; pairwise PERMANOVA; Fig. 8).

4. Discussion

The present study employed DNA metabarcoding to analyze the complex composition of seafood products, specifically fish surimi. Unlike conventional DNA barcoding, which is suitable for single fish tissue analysis, DNA metabarcoding facilitates the simultaneous identification of multiple species within mixed fish products. This technique offers a significant advantage in detecting species diversity in processed seafood. Similar methodologies have been applied in various seafood studies. For instance, approximately 37.5 % of surimi products were found to be mislabeled using 16S rRNA and NGS [24]. The Nanopore system, which amplifies cytochrome *b* and COI sequences, successfully identified 21 commercial seafood species, including tuna, cod, flatfish, salmon, sardines, shrimp, and squid [21]. The mini-length COI region and NGS was used to identify 12 sea cucumber products [33]. Additionally, 16S rRNA and NGS were employed to detect mislabeling in 32 salmon products [34]. Mitochondrial cytochrome *b* and the control region, along with NGS, were used to identify six tuna species in canned tuna [35].

Although previous studies have employed mitochondrial COI, cytochrome *b*, 16S rRNA, and control region markers, we utilized 12S rRNA (Mifish E/U primers), commonly used in environmental DNA (eDNA) research (references 33–35). The Mifish system enabled us to identify 153 species from fish surimi samples. Indeed, Mifish has proven to be an effective molecular marker for detecting fish species in DNA metabarcoding and has been extensively utilized for studying fish biodiversity [12,34,36,37]. However, some studies have also highlighted limitations of Mifish in detecting fish species [12,34]. One primary reason is the lack of 12S rRNA sequence data for all fish species, which typically have COI data. While many unknown fish species lacking 12S rRNA data are expected, current NGS data can still be used to discover these species in the future. It is also worth mentioning that ND5 primer in the

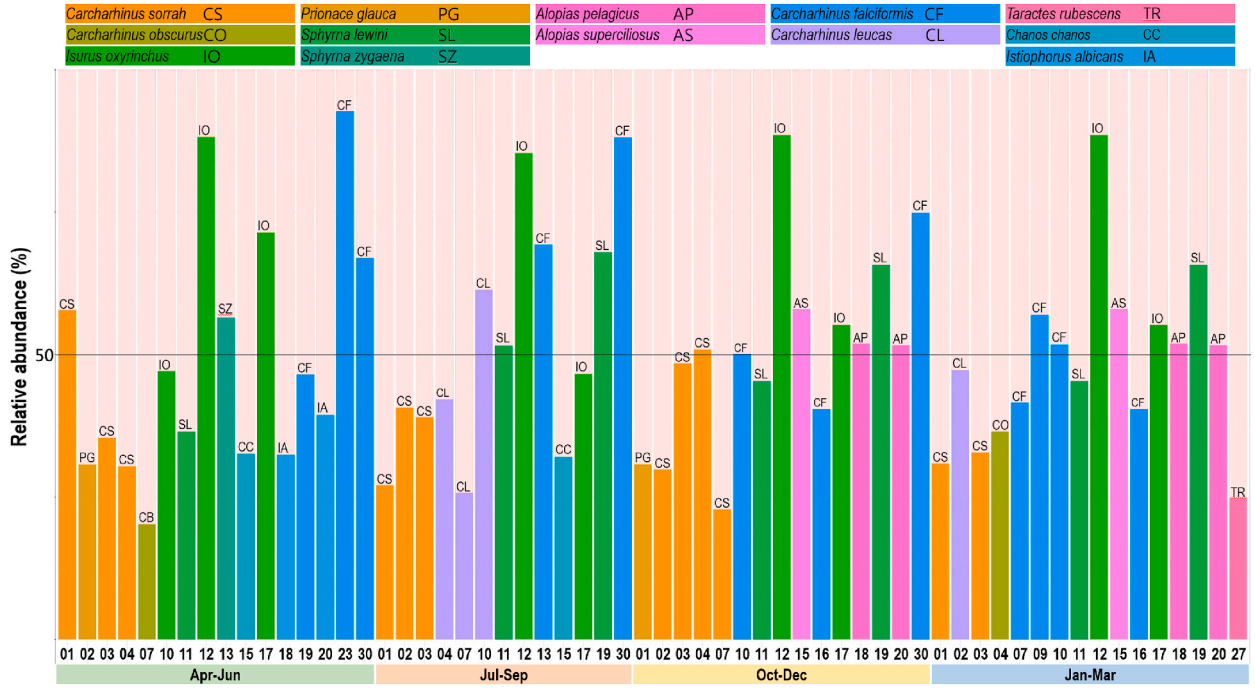

Fig. 4. Relative abundance of cartilaginous surimi samples.

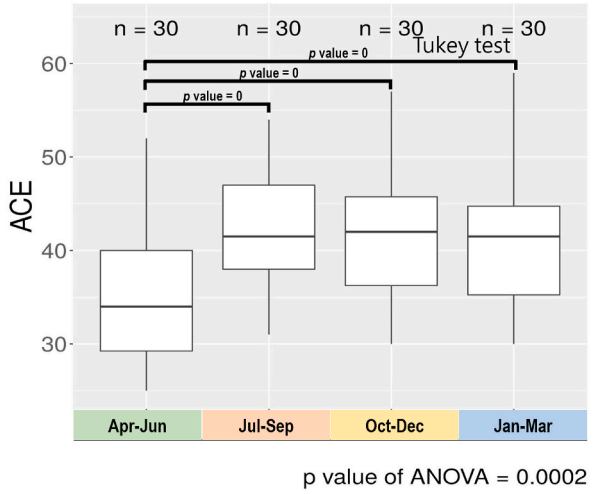

Fig. 5. Boxplot of Alpha-diversity for four-season groups. alpha-diversity was calculating by abundance-based coverage estimator (ACE).

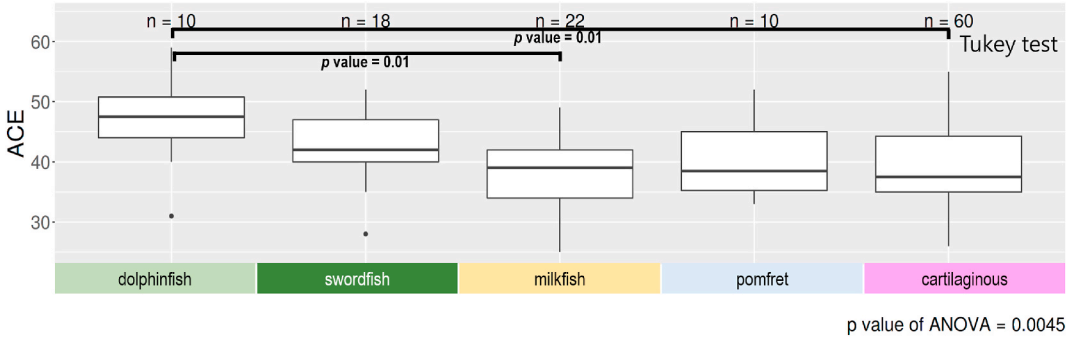

Fig. 6. Boxplot of Alpha-diversity for five main species groups. alpha-diversity was calculating by abundance-based coverage estimator (ACE). Tarpon were treated as milkfish group.

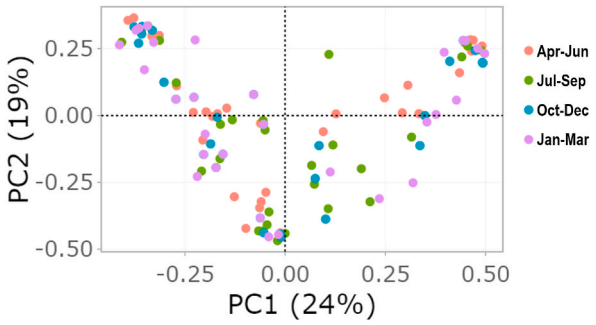

Fig. 7. Principal coordinate analysis (PCoA) of four-season groups. beta-diversity was calculating by Bray-Curtis similarity.

Mifish system is used to distinguish further some scombroidei species such as tuna and swordfish [38] and not utilized in the present study. Thus, the identification of swordfish in present study might represent more than one species. Considering the limit of cost for processing 120 samples, we opted to use only the Mifish E/U primers. Using a single universal primer pair is appealing because it can amplify a wide range of taxa, but the broader the taxonomic coverage, the less likely species-level identification becomes due to insufficient sequence resolution when priming sites are conserved across diverse groups [39]. Thus, multiple primer sets that target different genes can mitigate biases in amplification efficiency, sequence resolution, and reference data availability [24,39]. However, there are few empirical studies that have assessed markers for their complementary performance [12,21,39].

The diversity of fish species identified from the fish surimi samples in the present study was greater than expected. Many fish surimi

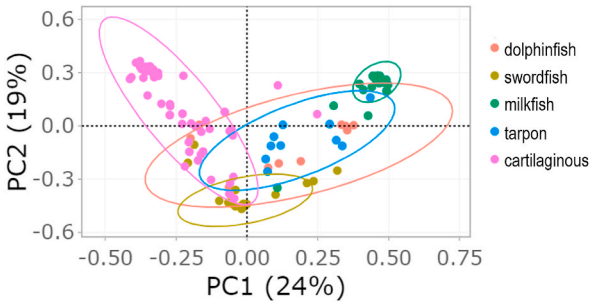

Fig. 8. Principal coordinate analysis (PCoA) of five main species groups. beta-diversity was calculating by Bray-Curtis similarity. Tarpon were treated as milkfish group.

claim the use of milkfish, dolphinfish, swordfish, and shark. Our analysis identified six major fish species from 120 samples: milkfish (*Chanos chanos)*, dolphinfish (*Coryphaena hippurus*), pomfret (*Taractes rubescens*), swordfish (*Istiophorus* spp.), tarpon (*Megalops cyprinoides*), and cartilaginous fish (Fig. 1). The presence of tarpon and pomfret was unexpected. Additionally, most samples contained fewer but abundant species obtained from local fisheries, commonly used as raw materials for fish surimi (Fig. 2). Among the six main species, cartilaginous fish was the primary ingredient in half of the fish surimi samples (60 out of 120), indicating that cartilaginous fish are a significant source for fish surimi production (Fig. 1). It is noteworthy that many consumers might not expect the surimi products they consume to be derived from cartilaginous fish, as these products have been transformed and do not resemble the more recognizable shark fins.

The gene fragments from fish surimi samples in this study were processed with relatively high sequencing depth, ranging from 16,800 to 4,996,100 reads, with an average of 608,670 reads per sample. Lower sequencing depth may fail to detect species with low abundance in fish surimi samples [12,40]. Some samples had significantly lower read counts, likely due to the freshness of the samples and subsequent processing (cooking or frying). DNA concentration decays over time, and its degradation rate varies with environmental conditions such as salinity, temperature, and pH [41,42]. Despite the boiling or frying of fish surimi, DNA from most samples remained detectable. Local restaurant fish surimi products are typically stored for 1–3 months. Consequently, we sampled each season to capture the diversity of the samples. Seasonal changes in sample composition were observed in half of the producers (15 out of 30; Fig. 1). This indicates that our seasonal sampling effectively reflects changes in the main species and shows that local producers adjust their choice of raw materials with the seasons (Fig. 1).

The number, composition, and abundance (reads) of fish species varied across the four seasons and among the main species groups (Figs. 5–8). Private fishery sources and preferences may influence the differences in fish species between samples from different producers and seasons. Seasonal variations in fish species can be attributed to the availability of species, as fishing seasons in Taiwan differ for various species [43]. Additionally, some samples reflect the instability of wild-caught seafood sources. For instance, the Apr–Jun samples, representing fish caught in Feb–Mar, showed minimal fish availability due to the Chinese New Year period, which significantly differed from other samples (Fig. 5). Furthermore, the samples from Jul–Sep exhibited changes, which we confirmed with the store were due to the impact of typhoons (Fig. 1).

Cartilaginous species (sharks and rays) have consistently garnered attention due to their declining abundance and vulnerable life history traits, such as slow growth rates and long generation times [44,45]. DNA barcoding and eDNA analysis of Chondrichthyan species have revealed at least 24 species involved in seafood consumption in Taiwan. In this study, we identified a total of 35 cartilaginous species in fish surimi samples from Taiwan (Fig. 3). The major sources were Carcharhiniformes and Lamniformes. At least ten species showed the highest proportion in a single sample, including *Alopias pelagicus*, *Carcharhinus sorrah*, *Carcharhinus leucas*, *Carcharhinus falciformis*, *Carcharhinus macloti*, *Carcharhinus obscurus*, *Isurus oxyrinchus*, *Prionace glauca*, *Rhizoprionodon acutus*, and *Sphyrna lewini* (Fig. 4). Collectively, Carcharhiniformes and Lamniformes species accounted for approximately 94 % of the shark species identified in the present study. It is important to note that producers' claims regarding the use of specific shark species may stem from inadequate species identification, potentially leading to an underestimation of the species utilized. Notably, 26 species, including 4 critically endangered (CR), 10 endangered (EN), and 12 vulnerable (VU) species, are now considered IUCN threatened species (Fig. 3). Among them, Isurus oxyrinchus (EN) and Sphyrna lewini (CR) were confirmed as frequently used species. Additionally, our detection of shark species revealed that unexpected species were sometimes consumed, such as *Rhynchobatus mononoke* and *Rhynchobatus djiddensis*. These species are generally not common catches, yet they were discovered in surimi products, suggesting that surveying surimi products can reflect the diversity of local fish catches and serve as a potential method to monitor minority species.

The diversity and complexity of Taiwan's surimi products pose challenges for food safety and labeling and are likely to face future controversies related to marine biodiversity protection [12]. Demanding transparent fish sources and excluding sensitive species is particularly challenging for producers primarily dealing with cartilaginous fish. Therefore, opting for single, predominantly farmed species, such as milkfish, or species that are relatively easier to document, like swordfish, offers more precise labeling and certification. Furthermore, mislabeling of surimi products can lead to the unintentional consumption of allergens or toxins, raising significant health risks for consumers [46].

Over the past decades, global marine biodiversity has experienced a rapid decline due to climate change, habitat destruction, and

overfishing [44,47,48]. The consumption of seafood is intrinsically tied to the conservation of marine biodiversity. To protect this biodiversity and sustain marine ecosystems, nations worldwide have tightened regulations on shark fishing and elevated the endangered status of numerous shark species [44,45,49]. Stringent controls and regulations now govern the trade and consumption of specific shark products. Traditionally, to cater to the Asian palate's fondness for shark fin soup, many sharks were de-finned and discarded, leading to international outcry and condemnation [10,11,44,50]. In Taiwan, post-2012, regulations have been implemented requiring sharks to be landed whole, and many restaurants have chosen to abstain from offering shark fin dishes [11,45]. However, in practice, the utilization of sharks has not ceased; fish surimi production has notably emerged as a method of use [12,51,52]. Our research has highlighted the underestimated presence of various shark species in Taiwan's fish surimi products, including some endangered ones. Refining and optimizing DNA metabarcoding for seafood authentication is imperative for future research in this domain. This includes developing and consolidating gene data (like 12S rRNA, COI, and complete mitochondrial DNA) to grant a richer insight into the range of fish species consumed via fish surimi products.

5. Conclusions

This study represents the first attempt to comprehensively examine the fish species diversity in traditional Taiwanese seafood, specifically fish surimi, using a DNA metabarcoding approach. Utilizing 12S rRNA DNA markers (Mifish) and high-throughput sequencing, we detected numerous cartilaginous species in most samples. Carcharhiniformes and Lamniformes were the primary sources across all samples. Notably, we identified four critically endangered (CR), ten endangered (EN), and twelve vulnerable (VU) species. Our findings suggest that the diversity of cartilaginous species used in fish surimi is significantly underestimated.

Data availability statement

The NGS datasets used in this study are available in online repositories. The repository names and accession numbers are as follows: NCBI (accessions: SAMN41402758, SAMN41402759, SAMN41402760, SAMN41402758).

Ethical approval

Ethics approval was not required for this research.

CRediT authorship contribution statement

Hung-Tai Lee: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cheng-Hsin Liao:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Te-Hua Hsu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36287.

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