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SHARKlock holmes: Applications of DNA forensic in tackling illegal trade of sharks and rays in Southeast Asia Region

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Abstract. Globally, the market demand for fins, uncontrolled bycatch, and illegal fishing are all contributing to the sharp decline in shark and ray populations. Within two decades (2002–2021), ASEAN countries provided 19.4% of 15.06 million tons (2.9 million tons) of elasmobranch species landings, with Indonesia, Malaysia, and Thailand dominating. Moreover, ASEAN product variety, taxonomic uncertainty, and trade complexity make endangered species trade control challenging. The existence of legally protected and endangered elasmobranch species in the Asian market has been the subject of multiple investigations, which has brought attention to the inadequacies of present management and conservation strategies. The development of DNA-based methods for identifying elasmobranchs and present new approaches to combat the problem of illegal shark and ray fin trafficking, such as high-throughput sequencing (HTS) as an alternative to conventional DNA barcoding. Enhancing trade monitoring could provide persistent benefits for shark and ray resources in Southeast Asian ecosystems and populations, while also promoting a legal, sustainable, and traceable trade.

1 Introduction

Global habitat and wildlife degradation, including shark and ray extinction, is due to manmade pressures. Elasmobranchs' conservative life cycles make them susceptible to overfishing, and their extinction may impact the composition and functionality of ecosystems. Elasmobranch fisheries are capable of being responsibly managed, however overfishing and unauthorised, unreported captures result from the strong demand for liver

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oils, fins, and gill plates [1]. ASEAN Member State (AMS) provided 19.4% of 15.06 million tons (2.9 million tons) of elasmobranch species landings from 2002-2021, with Indonesia, Malaysia, and Thailand leading. While AMS provided 8.7% of 4.8 million tons (0.4 million tons) valued at 2.7 billion USD (13.2% of global elasmobranch trade at 21.1 billion USD), Singapore, Indonesia, Malaysia, and Vietnam were the largest contributors [2] (Figure 1).

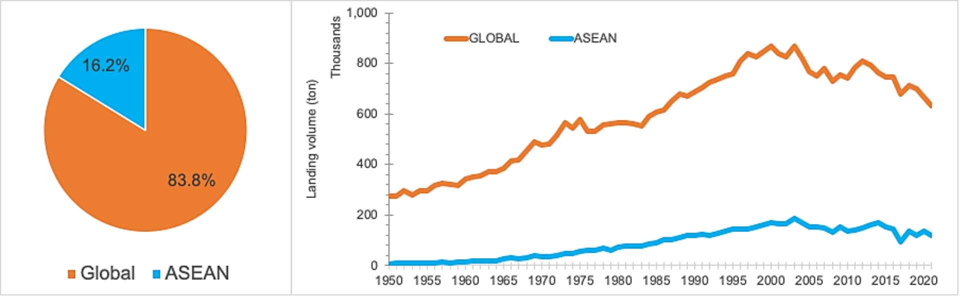

Fig. 1. Contribution of shark and ray landing volume of ASEAN Member State (AMS) and global catch [2]

Elasmobranch populations need monitoring and sustainable management, such as trade restrictions. High pricing can encourage non-compliance notwithstanding new international trade rules like CITES. In 2023, The number of CITES-listed species rose from 47 to 151, making trade biodiversity monitoring harder. Due to their varied use, value, and processing, elasmobranch trade regulation is complicated. Shark and ray species are similar in appearance and lack unique qualities in most derivative items; thus, traders may mislabel them. CITES implementation in Southeast Asia (SEA) is hard, CITES rules had a favorable influence in five of eight nations, a negative impact in two, and a persistent problem in protecting lawful commerce of these species [3].

Due to species identification difficulties, animal forensics uses genetic identification approaches [4]. DNA barcoding [5] and mini-barcoding [6] can be performed on both fresh and processed products, while rt-PCR [7], LAMP-based [8], and universal close-tube barcoding [9] can detect species in hours. However, conventional genetic methods used to identify traded species require analyzing individual tissue samples, which is a time-consuming process and limited to specific species. Recently, DNA metabarcoding-based techniques were developed to assess bulk samples at the same time, such as "shark-dust" [10]. This technique used high-throughput metabarcoding to screen the trade dust and waste of processing plant activities.

SEA Region have substantial contribution to landing and trade activities [2] and where potential illegal trade detected based on statistical discrepancy (red color) in the region [11] (Figure 2). Genetic-based monitoring tools could help to reduce the potency of misidentification and tackling illegal trade. Therefore, this project aims to examine the progression of DNA-based techniques utilized in the identification of elasmobranchs while also introducing novel strategies aimed at addressing the issue of illicit trafficking in shark and ray fins, from traditional DNA barcoding to the high-throughput sequencing (HTS) technique. High-throughput sequencing (HTS) approaches allow researchers to identify a diverse array of DNA sequences at the same time. This can be done by targeting a specific genetic region using metabarcoding or amplicon-based methods, or by sequencing all the genetic material in a single run, similar to DNA metabarcoding.

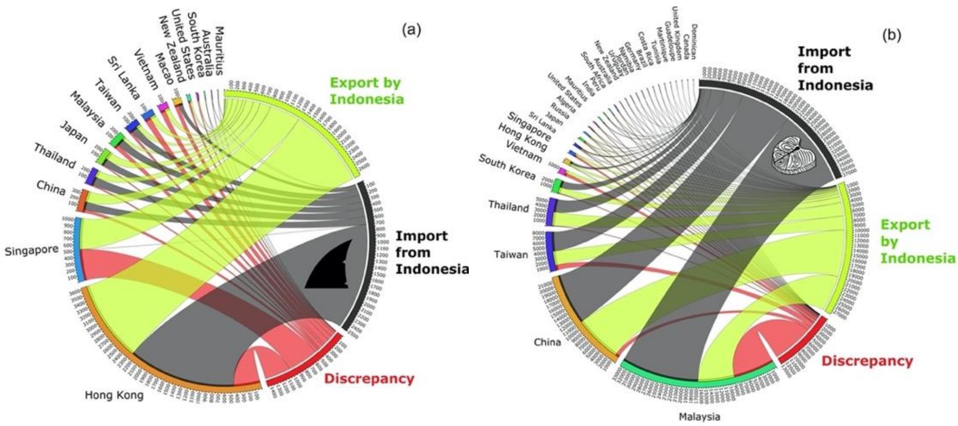

Fig. 2. The trade volume and disparity of shark fin (a) and meat (b) products between Indonesia and its primary trading partners, including AMS, in metric tonnes, during the period of 2012-2018 [11]

2 Single Species Detection

A total of 3,000 publications on DNA barcoding in diverse fields, including microbiology, food safety, biodiversity assessment, and wildlife forensics, were discovered (Figure 3). Publications about DNA barcoding gradually increased over the course of the year. The journals with the greatest articles about DNA barcoding published are PLoS One and Food Control [12].

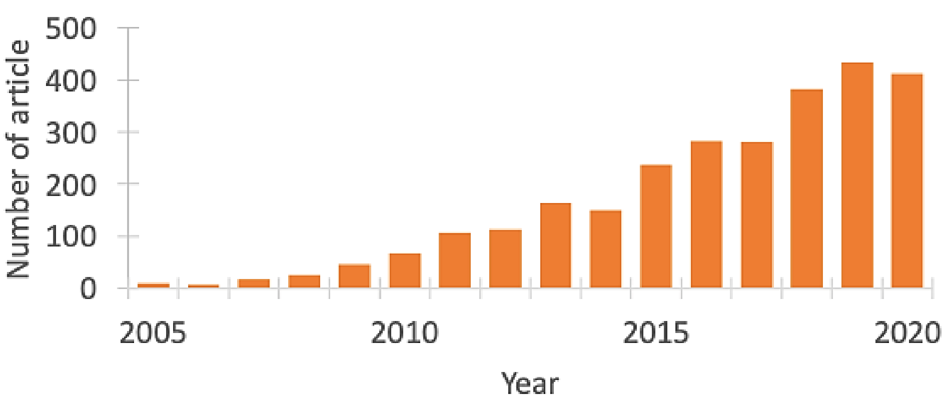

Fig. 3. Published article about DNA barcoding within 2005-2020 [12]

Visualization of most often used terms in titles, keywords, and abstracts of the retrieved revealed four clusters reflecting four study subjects (Figure 4). First cluster (red) focused in phylogeny, morphology and distribution. The second cluster (blue) concentrated on wildlife trade, fish and COI. Products challenges and detection clustered into third group (green), while species composition and ecosystem clustered into orange group (cluster 4th).

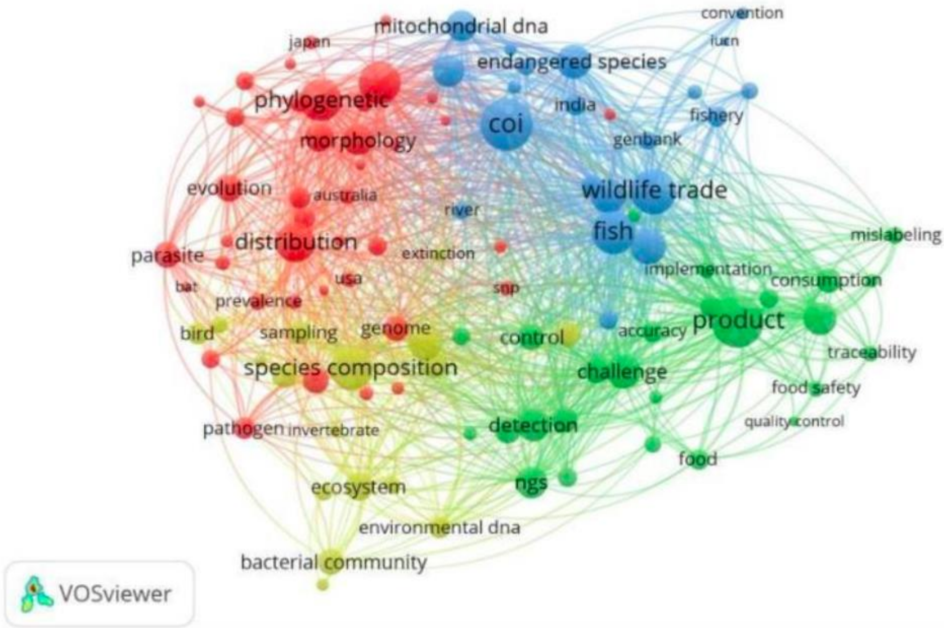

Fig. 4. Co-occurrence correlations between all keywords visualised in a network [12]

It is imperative to enhance and execute protective measures in order to avert further decline and the potential extinction of certain elasmobranch populations within our lifetime. Thus, precise identification of shark and ray species in traded products requires DNA-based procedures that are fast, sensitive, specific, and highly reproducible. The majority of law enforcement's endeavors have depended on traditional PCR amplification methods, such as PCR-multiplex assays utilizing primers particular to each species [13] or the arrangement of individual mitochondrial molecular markers using the Cytochrome Oxidase I (COI) [14-16]. A novel real-time quantitative PCR (qPCR) technique has been devised for the specific identification of nine shark species that are listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [17]. This method is useful because of its straightforwardness, rapidity (4 hours), and cost-efficiency ($0.94 USD for each sample) [7]. This method has limited capability in identifying only a small number of species, but it is the most effective PCR-based method for identifying a specific group of elasmobranchs. Finally, a universal single-tube assay known as FASTFISH-ID was created for the seafood sector to address the limitations of species-specific techniques 31. This technique utilizes LATE (Linear-After-The-Exponent) PCR to amplify a single strand of the complete 650bp COI barcoding area 32. It also employs fluorescent probes to selectively target two mini-barcode regions that exhibit significant intra-specific variability. The result is the generation of species-specific fluorescence signatures 31. The fluorescence signatures are compared to a cloud-based repository of authenticated specimen signatures.

COI is the most widely used molecular marker to identify elasmobranch, however hybridization, introgression, and insufficient lineage sorting prevent species-level classification [18]. *Carcharhinus obscurus* and *Carcharhinus galapagensis*, two shark species that have recently developed and are morphologically similar, could not be distinguished by the COI [18]. Despite recent divergence, hybridization makes it challenging to distinguish these species using mitochondrial DNA [19]. Due to misidentification of material from original specimens, GenBank and Life Data Systems' (BOLD) Barcode still offer inaccurate reference sequences [20].

Two or more markers evolving at different rates may help resolve taxonomic ambiguities. In particular, NADH2 marker evolves quicker than COI, making it a viable option for elasmobranch [21]. Many shark and ray species have NADH2 sequences in an internet database (https://sharksrays.org) with vouchers and photos. The utilisation of the mitochondrial DNA (mtDNA) multi-target region, such as 12S, COI and NADH2, can effectively prevent any uncertainties in identification, hence enhancing the overall success rate of identification. This approach proved effective for harvesting African woodland bushmeat [22]. Using a combination of multi markers, such as 12S, COI and/or NADH2, could be more effective in studying closely related species since it can cover a wider range of separation periods and evolution histories among elasmobranchs. In the coming years, the growing availability of whole elasmobranch mtDNA genomes on GenBank will facilitate species identification. Augmenting the quantity of mtDNA markers results in elevated expenses and time consumption for analysis, although it enhances the precision and resolution of species identification. There is a scarcity of research that has utilised 12S, COI and NADH2 for the purpose of identifying shark and ray fins [23].

Tissue sampling quality complicates molecular marker identification of elasmobranchs. Typically, processed elasmobranch items, including dry fins and meat, have degraded DNA, making it difficult for typical DNA barcode systems to amplify PCR targets [24]. Mini barcoding technique developed to minimize sequence size to better identify shark species [25]. For identifying processed shark fins, mini-barcoding works [25]. This method lowers the COI amplicon by 130 bp. Despite a 79% success rate, mini-barcoding failed to identify some threatened Carcharhinus shark species, such as *C. obscurus, C. longimanus*, *C. perezi*, and *C. galapagensis*. A PCR-multiplex developed by employing mini-barcoding at 150 and 200 bp, together with a full COI sequence (~650 bp) [7], to identify most traded shark species. While FASTFISH-ID failed to detect 3 species of 28 shark and ray species due to amplification and hybridization problem [9].

For the purpose of improving and standardising DNA-based methodologies for the identification of species and the monitoring of biodiversity resources, particularly in areas where illegal capture and trade are prevalent, it is crucial to improve global communication and coordinate research efforts. Even though there have been significant breakthroughs in the development of DNA-based approaches for identifying shark and ray species and providing law enforcement with more effective detection tools, there is still a need for additional collaboration and information sharing.

3 Bulk Detection

Single species detection demands the gathering and examination of individual specimens, which poses a substantial constraint when dealing with huge quantities of samples from many sites within a restricted time frame [11]. Advancements in next generation sequencing (NGS) have influenced the evolution of DNA barcoding [26] towards a technique called DNA metabarcoding (referred to as 'metabarcoding' henceforth), which enables the simultaneous identification of numerous taxa from a complex mixture [27]. Metabarcoding has been extensively used to analyse samples of environmental DNA (eDNA) - small bits of DNA left behind by organisms in water, soil, and air. This method efficiently complements and, in certain situations, exceeds established monitoring techniques [28, 29]. These advancements are enabling new uses in trade monitoring by analysing large quantities of mixes and addressing the shortcomings of current technologies.

It's still early days for HTS-based metabarcoding, but it's already been used to detect species in processed food [30]. Elasmobranchs typically utilise HTS for marine biodiversity surveys [28, 31], but not for market surveys or law enforcement purposes. The sole study that utilised High-Throughput Sequencing (HTS), specifically genome skimming, to determine shark species, analysed a wide range of molecular and nuclear barcodes (COI, NADH2, 16s rRNA, Internal Transcribed Spacer 2, 5.8S rRNA, and 28S rRNA) that are commonly employed for genetic identification purposes. By employing portable sequencing, we were able to rebuild the mitochondrial genome and determine the source of the seafood products [32]. Despite the current high cost of HTS-based species identification, its rapid data processing and extensive data storage capabilities make it a valuable tool for identifying various shark and ray species, particularly in processed elasmobranch products.

The most recent utilisation of HTS-based metabarcoding is the "Shark-dust" project, where we employed high-throughput sequencing to analyse trace DNA fragments found in trade dust and waste [10]. We conducted metabarcoding analysis on samples of 'shark-dust' gathered from seven processing factories in Java, Indonesia, which is the greatest site for shark landings globally. Through this analysis, we were able to identify 61 different taxa of sharks and rays, which represent half of all chondrichthyan orders. It is worth noting that more than half of these taxa could not be detected using tissue samples collected alongside the 'shark-dust' samples. Significantly, more than 80% of the sequences of shark dust originated from species in accordance with the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which is a global registry of endangered species. This approach has the potential to be a highly effective and efficient means of monitoring wildlife trading, while also being cost-effective (Figure 5).

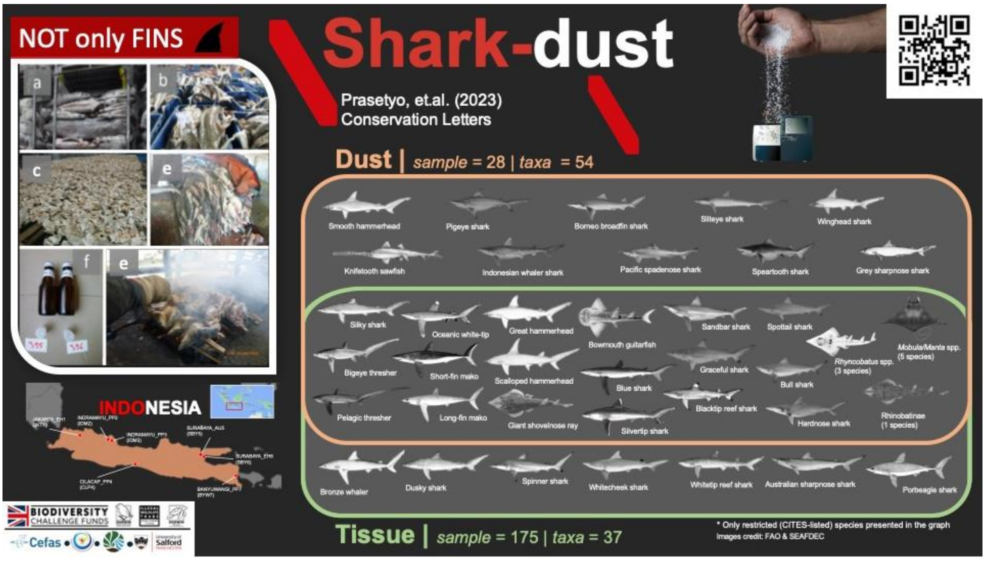

Fig. 5. Graphical abstract of shark-dust technique to detect traded species diversity using residues and scraps in processing plant [10]

4 Recommendation

In light of the expanding commerce in shark and ray fins, we advocate for the utilisation of DNA-based methods to aid in the conservation of elasmobranch species. The identification of a small fraction of shark and ray species that are transferred at their ultimate destination is inconsequential when contrasted to the higher risk posed by the illegal trading of fins. Hotspot identification and illegal trade detection are crucial for effective fisheries management and planning. The current understanding of the principal species implicated in illegal fin trade is limited based on recent studies [17, 25, 33]. However, a more extensive investigation is required to provide a thorough understanding of this issue. Otherwise, if no action is taken, there would be ongoing losses in population levels and a significant risk of extinction. Due to the ecological imbalance that will result from this, this will have a negative influence on the functioning of the ocean and will have an indirect impact on a large number of marine species that are found in lower trophic levels. The ability of elasmobranchs to adapt to a variety of settings is enhanced by their evolutionary resilience, which is indicative of the presence of a specific genetic feature. Sharks may derive advantages from these characteristics in terms of wound healing and the integrity of their genome [34]. Elasmobranch species possess numerous distinctive evolutionary traits, which can offer valuable biomedical and evolutionary knowledge [34]. Ultimately, we suggest allocating further resources to advance the primary uses of state-of-the-art DNA-based methods for monitoring the trade of sharks and rays, as well as implementing efficient conservation strategies to safeguard the survival of this significant group, particularly in the face of ongoing excessive fishing.

Hence, we propose the subsequent measures to surmount the barriers in shark and ray fin trade: (1) To determine the genetic identity of CITES-listed or suspected species, qPCR or PCR-multiplex techniques that rely on the 12S, COI or NADH2 regions can be employed. Nucleotide sequences can also rectify misidentification by the aforementioned methods. (2) DNA metabarcoding using short target fragment (12S and/or COI) is indicated for large-scale identification of unknown species, such as a group of species or heavily processed fins.

In order to effectively and economically identify shark products in trade, as stated in CoP18 Doc. 21.2, the CITES Secretariat promotes the development of skills and the exchange of knowledge among nations, including the use of genetic techniques. Compliance with CITES regulations has become increasingly challenging, particularly during the 2022 CoP CITES (CoP19) meeting, which included 151 species. This list encompassed more than 50 requiem sharks (*Carcharhinus* spp.), over 50 species of wedgefish and guitarfish, as well as thresher sharks, hammerhead sharks, manta rays, and freshwater stingrays. Elasmobranch populations have been decimated due to decades of excessive exploitation. However, in order for trade prohibitions to be effective, it is crucial to establish dependable and efficient monitoring methods that are also economically viable. The utilisation of a genetic strategy, specifically through the processing of sharks and rays, has the potential to effectively preserve elasmobranch populations on a global scale. Furthermore, this approach may serve as a source of inspiration for implementing similar approaches to combat various other forms of illicit wildlife trade.

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