Environmental DNA

ORIGINAL ARTICLE OPEN ACCESS

Understanding the Current Use of Environmental DNA in Southeast Asia: Promoting Accessibility through Networking and Capacity Building

Tracking Fin-Prints on Reef Flats: A Genus-Specific eDNA Metabarcoding Assay for Epaulette Sharks (*Hemiscyllium* spp.) With Field Validation in Raja Ampat

Danang Ambar Prabowo[1] | Edy Setyawan[2] | Abdy Wunanto Hasan[3] | Ronald Mambrasar[4] | Andhika Prima Prasetyo[5] | Sutikno Sutikno[5] | Muhammad Farrel Ewaldo[6] | Orgenes Ambafen[7] | Nesmus Saleo[7] | Mochamad Iqbal Herwata Putra[8] | Mark V. Erdmann[9,10]

[1]Research Center for Biosystematics and Evolution, Research Organization for Life Sciences and Environment, National Research and Innovation Agency (BRIN), KST Soekarno, Cibinong, Jawa Barat, Indonesia | [2]Elasmobranch Institute Indonesia, Denpasar, Bali, Indonesia | [3]Focal Species Conservation Program, Ocean and Science Department, Konservasi Indonesia, Denpasar, Indonesia | [4]Papua Program, Konservasi Indonesia, Kaimana, Papua Barat, Indonesia | [5]Research Center for Applied Zoology, Research Organization for Life Sciences and Environment, National Research and Innovation Agency (BRIN), KST Soekarno, Cibinong, Jawa Barat, Indonesia | [6]Infectious Disease and Immunology Research Center, Indonesia Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia | [7]Raja Ampat Marine Protected Area Management Authority (BLUD UPTD Pengelolaan Kawasan Konservasi di Perairan Kepulauan Raja Ampat), Waisai, Papua Barat Daya, Indonesia | [8]Focal Species Conservation Program, Ocean and Science Department, Konservasi Indonesia, Jakarta, Indonesia | [9]Re:Wild, Austin, Texas, USA | [10]ReShark, Auckland, New Zealand

Correspondence: Danang Ambar Prabowo (dana011@brin.go.id) | Edy Setyawan (edysetyawan@gmail.com)

Received: 15 September 2025 | **Revised:** 26 March 2026 | **Accepted:** 1 April 2026

Keywords: conservation monitoring | cryptic species | eDNA metabarcoding | elasmobranch | *Hemiscyllium* | NADH dehydrogenase subunit 4 | Papua | primer design

ABSTRACT

Environmental DNA (eDNA) metabarcoding is a powerful tool for monitoring elusive marine species, but its effectiveness is constrained by incomplete reference databases. This limitation is especially evident for epaulette sharks (*Hemiscyllium*), a genus of small benthic sharks endemic to the Indo-Australian Archipelago. Six of the nine species occur in eastern Indonesia and are fully protected under national law, yet monitoring their distribution remains challenging using traditional methods. To address this gap, we developed the first genus-specific eDNA metabarcoding assay for *Hemiscyllium*, targeting the mitochondrial NADH4 gene. Laboratory validation confirmed that the new ES-200ND4 primer amplified *Hemiscyllium* DNA in simulated eDNA experiments, even at low concentrations (<11 ng/μL). Field application in Raja Ampat detected the endemic *H. freycineti* at six of the seven sampling locations. The ES-200ND4 primer demonstrated taxonomic specificity, with all 55 ASVs generated from field samples assigned exclusively to *H. freycineti* (mean genetic distance: 0.78%), while the universal 12S marker (elas02) exhibited non-specific amplification but detected a broader range of marine taxa, including *H. freycineti* (17 ASVs), *Carcharhinus* sp. (1 ASV), and numerous phytoplankton groups. Notably, eDNA detection at Dayan, where daytime visual surveys recorded no sharks, underscores the method's capacity to reveal species presence when traditional approaches fall short. Overall, this study presents the first genus-specific eDNA metabarcoding assay for *Hemiscyllium* spp. and the first application of NADH4 as a metabarcoding marker in marine environments. Finally, this scalable assay supports Indonesia's protective legislation for *Hemiscyllium* and offers a transferable framework for monitoring other data-limited, cryptic, or threatened marine taxa.

Danang Ambar Prabowo and Edy Setyawan should be considered joint first author.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2026 The Author(s). *Environmental DNA* published by John Wiley & Sons Ltd.

1 | Introduction

The epaulette or walking sharks of the genus *Hemiscyllium* Müller & Henle 1838 are small, bottom-dwelling sharks in the order Orectolobiformes (carpet sharks), specifically belonging to the family Hemiscylliidae. They are endemic to the shallow coastal waters throughout the Indo-Australian Archipelago and northern Australia. Their distinctive "walking" motion, powered by their pectoral and pelvic fins, enables them to navigate complex benthic habitats, including coral reefs, mangroves, seagrass beds, and tidal pools, mainly to search for prey. Their limited mobility and restricted geographic ranges make them particularly vulnerable to habitat degradation, climate change, and fisheries bycatch (VanderWright et al. 2022). Six of the nine *Hemiscyllium* species are found in eastern Indonesia, with three classified as 'Vulnerable' (*H. galei*, *H. henryi*, and *H. strahani*) and two as 'Near Threatened' (*H. freycineti* and *H. halmahera*) on the IUCN Red List (Erdmann and Dudgeon 2024). Conversely, *H. trispeculare*, found in the Aru Islands and extensively across western and northern Australia, is currently classified as 'Least Concern'. However, preliminary genetic evidence suggests that the Aru population may represent a distinct species, warranting future reassessment of its conservation status (Dudgeon et al. 2020). In response to these conservation concerns, the Indonesian government issued Ministerial Decree No. 30 of 2023, granting full legal protection to all *Hemiscyllium* species (Indonesian Ministry of Marine Affairs and Fisheries 2023). However, the effective implementation of this protection remains a challenge due to incomplete data on their fine-scale distribution ranges and population sizes.

To date, efforts to monitor these sharks have relied on traditional survey methods such as nighttime reef walks, snorkeling, and scuba diving. These approaches are labor-intensive and logistically challenging, can disturb sensitive habitats, and have limited spatial coverage, often yielding sparse data relative to the effort required. They also expose researchers to risks posed by venomous organisms and, in some regions, by saltwater crocodiles (Jutan et al. 2018; Nebuchadnezzar et al. 2023; Widiarto et al. 2020).

Environmental DNA (eDNA) analysis offers a transformative alternative for wildlife management, enabling non-invasive species detection and ecosystem monitoring by detecting genetic material in environmental samples (Lopez et al. 2025; Yates et al. 2023; Çevik and Çevik 2025). This approach is particularly valuable for studying elusive or threatened groups, such as elasmobranchs, where traditional surveys often fall short (Boussarie et al. 2018; Miya 2022).

However, the effectiveness of eDNA metabarcoding for these taxa is often constrained by the lack of comprehensive reference sequences for standard mitochondrial markers like COI, 12S, and 16S rRNA (Fontes et al. 2024). Without a robust reference database, species-level identification is unreliable, limiting the potential of eDNA for conservation planning. This limitation is especially evident for *Hemiscyllium*. At the outset of this study, the mitochondrial NADH dehydrogenase subunit 4 (NADH4) gene was the only marker with complete reference sequences for all nine species (Dudgeon et al. 2020), rendering universal elasmobranch primers ineffective for species-level discrimination within this genus. Consequently, this gap made NADH4 the sole viable candidate for developing a targeted, genus-specific eDNA assay for *Hemiscyllium*.

In this study, we present the first genus-specific eDNA metabarcoding assay for *Hemiscyllium* and the first application of NADH4 as a metabarcoding marker in a marine environment. This assay was developed and validated through a combination of laboratory experiments and field surveys conducted in Raja Ampat, a global biodiversity hotspot recently designated as an Important Shark and Ray Area (ISRA) (Jabado et al. 2024) and home to the endemic *H. freycineti* (Raja Ampat Epaulette Shark) (Erdmann and Dudgeon 2024; Dudgeon et al. 2020; Setyawan et al. in review). This region faces increasing anthropogenic threats from fisheries, coastal development, and resource extraction, underscoring the urgent need for effective monitoring tools (Mangubhai et al. 2012). Our specific objectives were to: (1) design primers targeting a short, variable region of NADH4 capable of discriminating among all nine *Hemiscyllium* species; (2) validate primer performance on tissue-derived DNA and simulated eDNA samples; and (3) assess the field applicability of the new assay. Finally, we discuss the limitations of this approach, its broader implications for marine biodiversity monitoring, and provide practical recommendations for applying this assay in future epaulette shark surveys.

2 | Materials and Methods

2.1 | Primer Design

Primer design followed three key criteria: (1) an amplicon length of 200–350 bp to accommodate fragmented eDNA and the sequencing platform (Illumina MiSeq) (Riaz et al. 2011), (2) providing sufficient interspecific variation for species-level differentiation (Miya et al. 2015), and (3) conserved terminal regions (20–30 bp) flanking the targeted amplicon to ensure consistent amplification across all species (Elbrecht and Leese 2017; Valentini et al. 2016).

To identify a suitable NADH4 region for eDNA metabarcoding of *Hemiscyllium* species, we retrieved 17 sequences from the NCBI GenBank database (Table 1) and aligned them using the MUSCLE (Edgar 2004) feature in MEGA X (Kumar et al. 2018). We identified a single ~200 bp amplicon region flanked by two conserved terminal regions (21 bp each) (Figure 1) for PCR primer binding. The alignment also revealed 21 variable nucleotide positions within this amplicon region, providing sufficient polymorphism for species-level discrimination of *Hemiscyllium*.

A novel primer pair (ES-200ND4; Table 2) was subsequently designed, comprising the forward ES-200ND4_F (5′–CCCTTATTGCTTACTCCTCAG–3′) and reverse ES-200ND4_R (5′–GGTTTGTTAGAAGTCATCAGG–3′), to amplify the targeted hypervariable region (Figure 1). Primer properties were assessed using NetPrimer (Premier Biosoft, San Francisco, CA, USA) and PCR Primer Stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html), yielding melting temperatures of

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 | List of DNA sequences of *Hemiscyllium* spp. acquired from GenBank and used for the ES-200ND4 primer design.

Species	Acc. no. (NADH4)	Acc. no. (12S)
Hemiscyllium freycineti	MF740831.1 MF740830.1 MF740829.1	PQ043201.1
Hemiscyllium galei	MF740843.1	PQ043202.1
Hemiscyllium halmahera	MF740834.1 MF740833.1 MF740832.1	PQ043204.1
Hemiscyllium henryi	MF740835.1	PQ043205.1
Hemiscyllium strahani	MF740836.1	PQ043208.1
Hemiscyllium trispeculare	MF740840.1 MF740839.1 MF740838.1	PQ043209.1
Hemiscyllium ocellatum	MF740846.1 MF740845.1 MF740844.1	PQ043207.1
Hemiscyllium michaeli	MF740842.1	PQ043206.1
Hemiscyllium hallstromi	MF740841.1	PQ043203.1

62°C (forward) and 60°C (reverse), with G/C contents of 47.6% and 42.9%, respectively.

2.2 | Primer Validation

The new ES-200ND4 primer was validated on genomic DNA extracted from dorsal fin tissue of three *H. freycineti* specimens (WS76, WS81, WS101) collected in Raja Ampat, Indonesia (Figure S1a; Supporting Information), as tissue samples from other *Hemiscyllium* species were not available at the time of this study. DNA extraction was performed using the Quick DNA Miniprep Plus Kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocols. PCR was performed in 25 μL reactions containing 2 μL of DNA extract, 1 μL of each primer, 12.5 μL MyTaq 2× HS Red Mix, and 8.5 μL nuclease-free water. Thermocycler conditions included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

In addition to the ES-200ND4 primer, we amplified the same genomic DNA samples using two additional primer sets (Table 2). First, the ND4F-LeuR primer (Arèvalo et al. 1994; Inoue et al. 2001) served as a positive control to confirm successful amplification of tissue-derived DNA targeting the NADH4 gene (amplicon size ~800 bp). However, this long-amplicon primer was not intended for the eDNA samples, as DNA fragmentation would preclude amplification of such a long target. Second, the 12S-elasmobranch primer (elas02; expected amplicon size ~200 bp) (Miya et al. 2015;

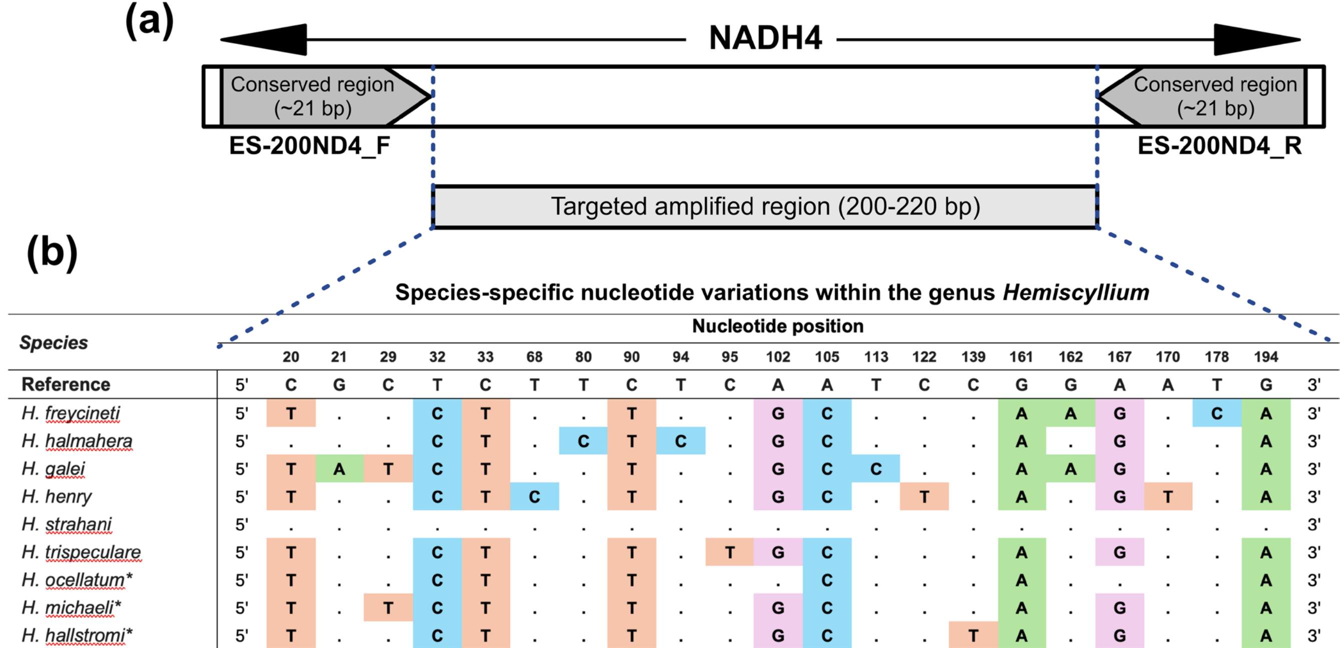

FIGURE 1 | Schematic diagram of the epaulette shark NADH4 gene showing ES-200ND4 primer annealing sites (a) and species-specific nucleotide variations within the genus *Hemiscyllium* (nine species) (b), with nucleotide differences across species highlighted at specific positions. Dots (·) indicate regions of exact nucleotide matches with the reference sequence.

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 | Amplification primers used in this study.

Primes (Gene)	Sequence (5′—3′)	Length	References
ES-200ND4 (NADH4)			
ES-200ND4_F	CCCTTATTGCTTACTCCTCAG	200–220 bp	This study
ES-200ND4_R	GGTTTGTTAGAAGTCATCAGG		
ND4F-LeuR (NADH4)			
ND4-F	CACCTATGACTACCAAAAGCTCATGTAGAAGC	~800 bp	Arèvalo et al. (1994)
H12293-Leu-R	TTGCACCAAGAGTTTTTGGTTCCTAAGACC		Inoue et al. (2001)
elas02 (12S rRNA)			
elas02-F	GTTGGTHAATCTCGTGCCAGC	~180 bp	Miya et al. (2015),
elas02-R	CATAGTAGGGTATCTAATCCTAGTTTG		Taberlet et al. (2018)

Taberlet et al. 2018) was initially used to generate the missing 12S rDNA reference sequence for *H. freycineti* for taxonomic annotation and phylogenetic analysis, and to benchmark the taxonomic resolution of the eDNA metabarcoding process.

PCR conditions for the ND4F-LeuR primer were identical to those for the ES-200ND4 primer. The elas02 primer protocol involved an initial denaturation at 94°C for 15 s, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min (Maiello et al. 2024). All runs included negative controls, and amplification success was confirmed via 1% agarose gel electrophoresis.

2.3 | Environmental DNA (eDNA) Simulation

To evaluate the efficacy of the ES-200ND4 primer for detecting *Hemiscyllium* DNA in seawater, we prepared three sterilized DURAN bottles (coded A, B, and C), each containing 1 L of GF/F-filtered seawater for eDNA simulation (Figure S1b; Supporting Information). Bottle A received a tissue fragment from *H. freycineti* specimen WS189, Bottle B from specimen WS290, and Bottle C was spiked with 75 μL of mixed DNA extracts from the Primer Validation experiment (Section 2.2). Each bottle was subsequently hand-shaken for ~10 s and incubated at room temperature for varying durations to simulate eDNA release and persistence: Bottle A (> 12 h) to test detection following extended environmental exposure, Bottle B (~1 h) to simulate recent DNA release, and Bottle C (~2 h) as an intermediate between recent release and extended environmental exposure. After incubation, seawater from each bottle was filtered through 0.45 μm mixed cellulose ester (MCE) membrane filters using a 500 mL PALL magnetic filtration system (Nihon PALL Ltd., Tokyo, Japan) and a 10 L vacuum pump (JoanLab Equipment Co. Ltd., Zhejiang, China). Filters were aseptically folded using sterile tweezers, placed into sterile 2.0 mL cryotubes containing 1.0 mL DNA/RNA Shield (Zymo Research Corp., Irvine, CA, USA), and stored in the dark at ambient temperature (~26°C) for 2 days before DNA extraction.

DNA was extracted using the ZymoBiomics DNA Miniprep Plus Kit (Zymo Research Corp., Irvine, CA, USA) under a pre-sterilized laminar flow hood (wiped with 10% bleach and 70% ethanol, then irradiated with UV for 10 min) to minimize contamination. Bead-beating lysis was performed in a TOMY Micro Smash MS-100 disruptor (4000 rpm, 2 × 3 min cycles). PCR amplifications were performed as described in Section 2.2, but without the ND4F-LeuR primer. Amplification success was confirmed via 1% agarose gel electrophoresis.

2.4 | Sanger Sequencing, Phylogenetic Analysis, and Genetic Distance Estimation

Following confirmation of amplification success in previous steps, DNA extracts were submitted for Sanger sequencing (PT. Genetika Science Indonesia; https://ptgenetika.com). Sequences were assembled and aligned with *Hemiscyllium* spp. and all available Orectolobiformes sequences from GenBank (Table S1; Supporting Information) using MUSCLE (Edgar 2004) implemented in MEGA X (Kumar et al. 2018). Two *Carcharhinus* species were included as outgroups. The final NADH4 alignment comprised 198 bp across 59 taxa, and the 12S alignment comprised 176 bp across 40 taxa.

At the time of initial analysis, 12S sequences for all nine *Hemiscyllium* species were not publicly available; therefore, preliminary phylogenetic analyses were performed solely with sequences generated in this study. However, as of 2025, the complete mitochondrial genomes of all *Hemiscyllium* species (Dudgeon et al. 2020) have been released to the public in GenBank, and these sequences (Table 1) have now been incorporated into the 12S phylogenetic analyses presented in this report.

Phylogenetic analyses were conducted using maximum likelihood (ML) and neighbor joining (NJ) methods with 1000 bootstrap replications. Uncorrected pairwise genetic distances (*p*-distances) were calculated in MEGA X to assess sequence divergence among the newly generated sequences (intraspecific) and between these sequences and those of other species or genera (interspecific) of the order Orectolobiformes. The assembled partial sequences from primer validation and eDNA simulation have been deposited in GenBank with Accession Numbers PX317577–PX317585.

2.5 | Field Survey and Sampling Locations

Field surveys were conducted from December 11–22, 2024, across seven sampling locations in the Raja Ampat Archipelago

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

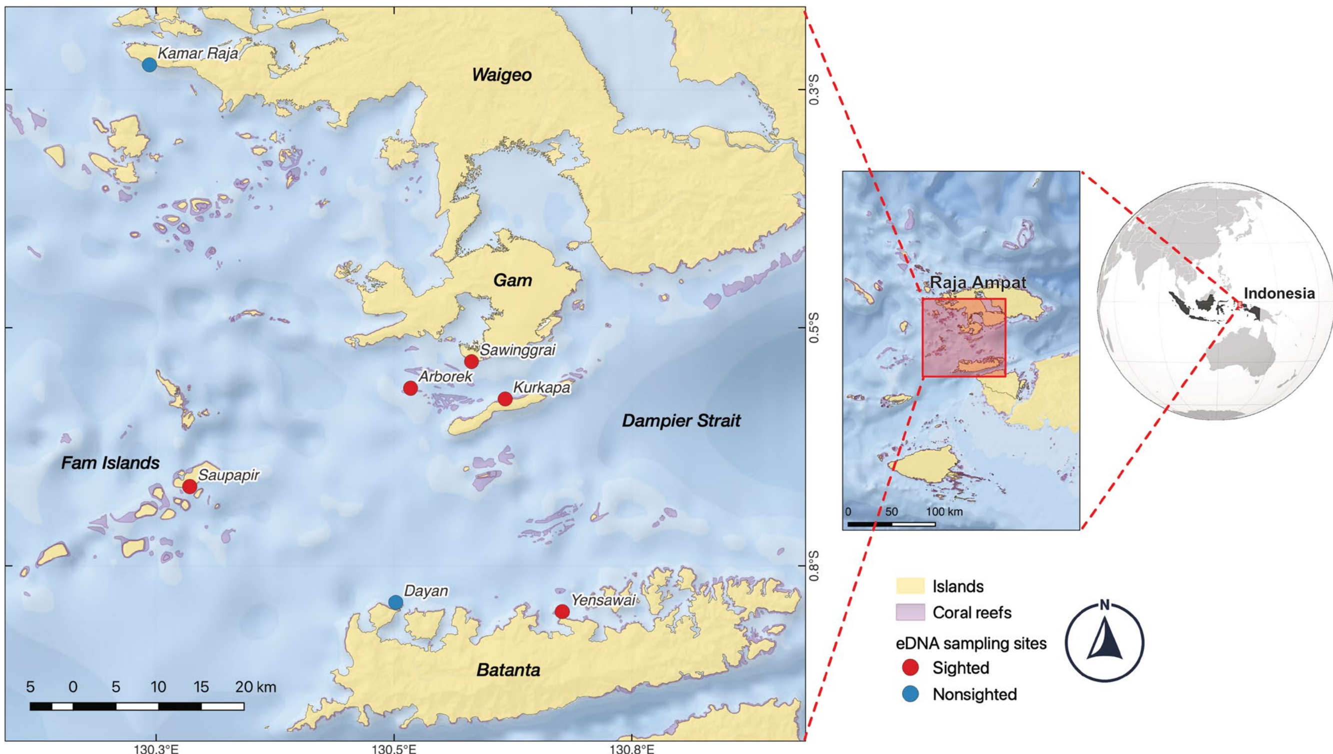

FIGURE 2 | Environmental DNA (eDNA) sampling locations in Raja Ampat, showing locations where the Raja Ampat Epaulette Sharks were sighted (red dots) and not sighted (blue dots).

(Figure 2), with Arborek Island serving as the primary study location, home to a known population of >600 *H. freycineti* individuals (Setyawan et al. in review), also locally known as 'Kalabia' or 'Mandemor' (Jabado et al. 2024). Sampling targeted previously surveyed microhabitats (Arborek Island, Sawinggrai, Kurkapa, and Yensawai) and new locations where the local community has reported shark sightings (Kamar Raja, Saupapir, and Dayan).

Nightly visual surveys of approximately 2h were conducted during low tide across reef flats, rubble zones, and seagrass meadows. Epaulette sharks were located using LED torches, and data on GPS location, sex, maturity, total length, and weight were recorded for each encountered individual (Setyawan et al. in review). Due to logistical constraints, only daytime sampling was conducted at Dayan.

2.5.1 | eDNA Sampling

Before sampling, all non-electrical equipment (e.g., buckets, containers, hoses, etc.) was sterilized with 10% bleach, rinsed with mineral water, and dried. At each location, triplicate samples of 3L seawater were collected within a 1–2m radius of epaulette shark sightings, and the samples were pooled into 10L foldable plastic containers. Blind or field-negative controls were collected at randomly selected points at each location, without visual confirmation of sharks nearby. Due to the considerable distances between location (Figure 2), only one location was visited per night to ensure adequate time for sample processing. In total, 18 eDNA samples were collected across seven locations (Table 4).

2.5.2 | Sample Filtration and eDNA Processing

Seawater samples were filtered within 6–12h of collection using 0.45μm MCE filters, either on land or aboard the vessel, following the filtration steps in Section 2.3. All samples were kept cool and in the dark until filtration to minimize DNA degradation. Filters were preserved in DNA/RNA Shield and stored in the dark at ambient temperature. DNA extraction and PCR were performed as in Section 2.3, with modified reaction volumes consisting of 4μL template DNA, 1μL of each primer, 12.5μL MyTaq 2× HS Red Mix, and 6.5μL nuclease-free water. Due to funding limitations, we prioritized samples that were positive for both primers, ensuring confident species-level detection while acknowledging the conservative bias this introduces (see Section 4.5).

2.6 | High-Throughput Sequencing (HTS)

Library preparation and HTS were performed by an external service (PT. Genetika Science Indonesia). DNA quantity and purity were assessed using NanoDrop 2000 and Qubit dsDNA HS Assay Kit. PCR amplification used Phusion Plus PCR Master Mix (F631L) with ES-200ND4 and elas02 primers under conditions described in Section 2.2. Libraries were prepared by the service provider using standard Illumina protocols, indexed with unique dual-index adapters, and pooled at equimolar concentrations. HTS was conducted on the Illumina MiSeq platform, generating 300bp paired-end raw reads (PE300). The raw HTS dataset is available in the NCBI Sequence Read Archive (SRA; BioProject ID PRJNA1321526).

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

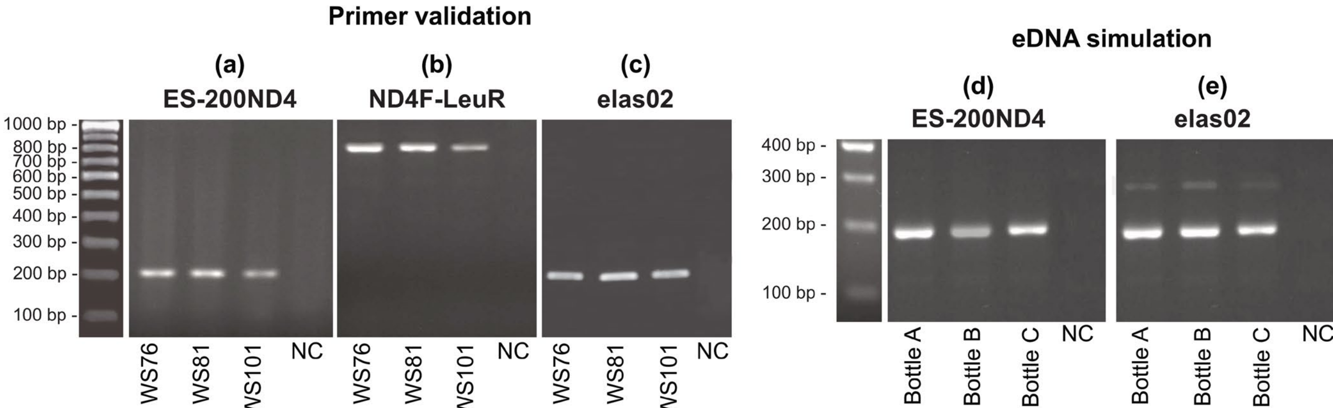

FIGURE 3 | Agarose gel electrophoresis of amplicons from primer validation (a–c) and eDNA simulation (d–e) experiments using the newly designed ES-200ND4 (a, d), ND4F-LeuR (b), and elas02 (c, e) primers. The experiments involved three Raja Ampat Epaulette Shark (*H. freycineti*) specimens for the primer validation step (WS76, WS81, and WS101) and three simulated conditions for the eDNA simulation step (Bottle A, B, and C). Negative controls are coded as 'NC'.

2.7 | Data Processing and Bioinformatics Analyses

Demultiplexed paired-end raw reads for each of the NADH4 and 12S rRNA datasets were processed in QIIME2 ver. 2023.7 (Bolyen et al. 2019). Adapter and primer sequences were removed using *cutadapt* (Martin 2011). Quality filtering, read merging, and chimera removal were performed with the DADA2 plugin (Callahan et al. 2016), and the remaining high-quality reads were assigned to amplicon sequence variants (ASVs). Taxonomic annotation was performed using a custom-trained QIIME2 classifier based on the MIDORI2 (GB264) reference databases for NADH4 and 12S (Leray et al. 2022), with an 80% identity threshold for genus-level assignment. Downstream analysis and visualizations were conducted in RStudio (Posit Team 2025), implementing R v4.4.3 (R Core Team 2024) and R packages, such as *phyloseq* (McMurdie and Holmes 2013) and *ggplot2* (Wickham 2016).

2.8 | Post-HTS Phylogenetic Analyses

Species-level identities of *Hemiscyllium*-annotated ASVs from HTS were confirmed using phylogenetic analyses identical to those described in Section 2.4, with genetic distances calculated to quantify divergence among the sequences, species, or genera. Alignments comprised 198 bp (NADH4 ASVs, 105 taxa) and 176 bp (12S ASVs, 51 taxa). All newly generated ASV sequences are provided in Data S2 and S3.

3 | Results

3.1 | Primer Performance in Laboratory Experiments

The primer validation experiment showed successful and consistent amplification of the target region in all epaulette shark genomic samples using the new ES-200ND4 primer, producing fragments of ~200 bp, within the desired amplicon range (200–350 bp) (Figure 3a). Parallel amplifications with the ND4F-LeuR primer also produced the expected ~800 bp long fragments (Figure 3b), while the elas02 primer produced ~200 bp products (Figure 3c), further validating primer performance and DNA template quality. Negative controls showed no amplification, confirming the absence of contamination.

DNA extracted from membrane filters of the eDNA simulation experiment yielded varying concentrations: 57.21 ng/μL (Bottle A), 10.78 ng/μL (Bottle B), and 21.70 ng/μL (Bottle C). The ES-200ND4 primer successfully amplified the targeted amplicon size in all treatments, yielding a clear band at ~200 bp (Figure 3d). In contrast, the elas02 primer produced double bands in all positive samples, including the expected ~200 bp targeted fragment and a fainter ~280 bp band, suggesting possible off-target amplification (Figure 3e). All negative controls remained blank, confirming the absence of contamination.

The phylogenetic positions of the Sanger sequences generated during the primer validation experiment (9 for NADH4 and 6 for 12S) are shown in Figure 4. In general, the organization of species and genera within the order Orectolobiformes based on NADH4 and 12S largely confirmed the findings of Boyd and Seitz (2021) and Dudgeon et al. (2020). Notably, all nine species of *Hemiscyllium* formed a monophyletic clade with high bootstrap supports, sister to the *Chiloscyllium*. Notably, all Sanger sequences generated in this study were placed within the *H. freycineti* clade (Figure 4), with minimal interspecific genetic divergence from reference sequences of this species: 0.00%–2.53% (avg. 0.55%) for NADH4 and 0.00%–2.35% (avg. 0.49%) for 12S (Table 3). The intraspecific distances among the Sanger sequences were within this range. Subsequently, their interspecific distances to other *Hemiscyllium* species were higher, ranging from 1.01% to 6.06% (avg. 2.58%) for NADH4 and 1.15% to 4.12% (avg. 2.12%) for 12S. Interspecific genetic distances to other species or genera of the order Orectolobiformes (i.e., *Chiloscyllium* spp., *Rhincodon typus*, *Ginglymostoma cirratum*, *Nebrius ferrigineus*, *Stegostoma tigrinum*, *Orectolobus* spp., and *Cirrhoscyllium*

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

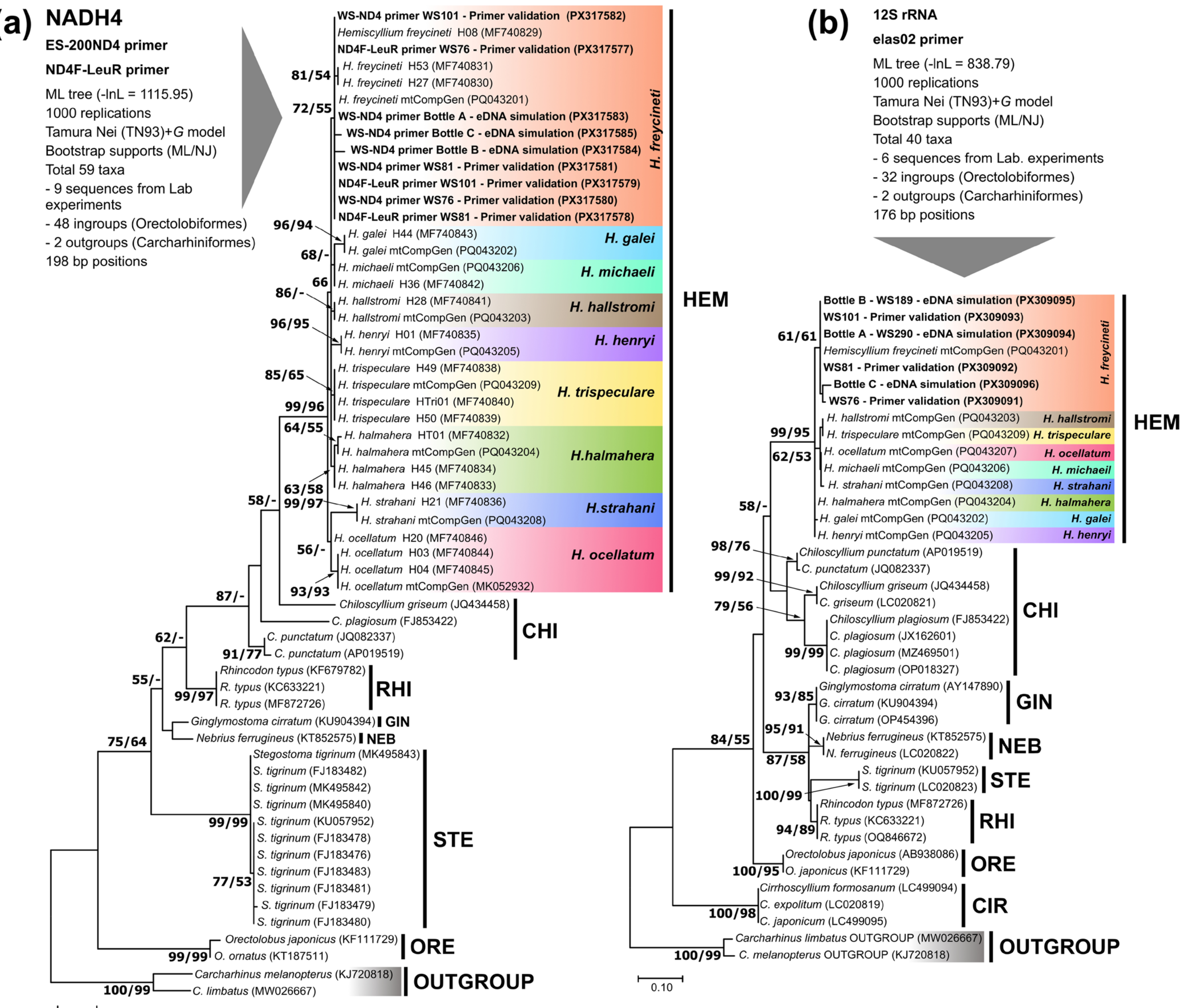

FIGURE 4 | Maximum likelihood phylogenies of NADH4 (a) and 12S rRNA (b) gene sequences, showing the placement of sequences generated from the primer validation and eDNA simulation experiments (in bold) among nine species of the genus *Hemiscyllium*. Reference sequences from other Orectolobiformes (grouped by three-letter codes of their genus for estimating the genetic distances) and outgroup taxa (*Carcharhinus limbatus* and *C. melanopterus*) are included. Branch nodes show bootstrap support (> 50%) for ML and neighbor-joining analyses (NJ). GenBank Accession Numbers are presented in parentheses.

spp.) were substantially higher, ranging 10.61%–20.20% and 9.77%–19.65% for NADH4 and 12S, respectively (Table 3).

3.2 | Field Sampling and Amplification Outcomes

Field surveys recorded varying numbers of epaulette sharks across locations, with the highest sightings being at Saupapir (26 individuals), whereas at Kurkapa, Sawinggrai, and Yensawai, 16 individuals were sighted, respectively (Table 4). No sharks were sighted at Kamar Raja (due to heavy rain) and Dayan (due to daytime sampling).

DNA concentrations from field-collected filters ranged from 3.89 to 63.21 ng/μL (Table 4), with higher yields generally associated with better amplification success. PCR amplification success varied between the primers, with positive bands most frequently observed in samples with DNA concentrations > 10 ng/μL and in those collected near visually confirmed shark sightings, especially for the ES-200ND4 primer, except for Dayan, where no epaulette shark was encountered. The elas02 primer produced positive bands in more samples (13 out of 18) than the ES-200ND4 primer (8 out of 18) (Table 4 and Figure S2; Supporting Information). All negative controls remained amplification-free, confirming the absence of contamination. Although the elas02 primer showed better amplification success, the lack of comprehensive 12S reference sequences for *Hemiscyllium* at the time of this study required us to use the ES-200ND4 primer as a filter to confirm that the targeted DNA of this shark was successfully detected for both primers (i.e., dual-positive confirmation). Based on this criterion, eight samples showing positive amplification with both primers (i.e., ES-Arb6, ES-Arb7, ES-Arb8, ES-Kur1,

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 | Uncorrected pairwise genetic distances (*p*-distance; %) between the newly generated sequences from the primer validation and eDNA simulation experiments and representative Orectolobiformes species or genera, including the outgroup, based on NADH4 and 12S markers. The number of sequences compared against all experiments is indicated as ∑seq. The *p*-distances were calculated from the alignments used for phylogenetic reconstructions in Figure 4. The complete pairwise distance matrix is provided in Data S4.

		NADH4 genetic distance (%)				12S rDNA genetic distance (%)		
Sequence comparison	**∑seq**	**Min**	**Max**	**Avg**	**∑seq**	**Min**	**Max**	**Avg**
Intraspecific								
Within all experiments (intraspecific)	9	0.00	2.53	0.56	6	0.00	2.35	0.90
Interspecific								
All experiments vs. *H. freycineti*	4	0.00	2.53	0.55	1	0.00	2.35	0.49
All experiments vs. *H. halmahera*	4	1.01	3.03	1.64	1	1.15	2.35	1.45
All experiments vs. *H. henryi*	2	2.02	3.54	2.30	1	1.15	2.35	1.45
All experiments vs. *H. galei*	2	2.53	4.04	2.81	1	2.72	2.94	2.02
All experiments vs. *H. strahani*	2	5.05	6.06	5.22	1	2.30	2.94	2.79
All experiments vs. *H. trispeculare*	4	1.01	2.53	1.29	1	1.72	4.12	2.22
All experiments vs. *H. michaeli*	2	1.01	2.53	1.29	1	2.30	3.53	2.60
All experiments vs. *H. hallstromi*	2	1.01	2.53	1.29	1	1.15	4.12	1.83
All experiments vs. *H. ocellatum*	4	1.52	4.04	2.55	1	2.30	3.53	2.60
All experiments vs. *Chiloscyllium* (CHI)	4	10.61	16.16	12.44	8	9.77	12.64	11.21
All experiments vs. *Rhincodon* (RHI)	3	15.66	16.67	15.82	3	13.22	13.79	13.65
All experiments vs. *Ginglymostoma* (GIN)	1	12.12	13.64	12.40	2	13.22	14.20	13.78
All experiments vs. *Nebrius* (NEB)	1	12.12	13.13	12.29	2	10.92	11.18	10.96
All experiments vs. *Stegostoma* (STE)	11	15.66	16.67	15.82	2	16.09	16.67	16.54
All experiments vs. *Orectolobus* (ORE)	2	18.18	20.20	18.71	2	11.49	13.53	12.22
All experiments vs. *Cirrhoscyllium* (CIR)	NA	—	—	—	3	18.92	19.65	19.44
All experiments vs. *Carcharhinus* (Outgroup)	2	24.24	26.77	25.17	2	19.54	21.18	20.39

ES-Saw1, ES-Sap1, ES-Day1, and ES-Yen1) were selected for high-throughput sequencing (HTS) (Table 4).

3.3 | HTS and ASV Analysis

Illumina Miseq of the eight dual-positive samples yielded nearly 1.6 million raw reads (Table S2; Supporting Information). After quality filtering (Q-score ≥ 30), a total of 743,093 NADH4 reads and 714,878 12S reads remained. The species accumulation curve plateaued at approximately 25,000 reads per sample (Figure S3; Supporting Information), indicating sufficient sequencing depth to capture most detectable diversity. The amplicon read lengths ranged from 198 to 242 bp (avg. 220 bp) for NADH4 and 180–251 bp (avg. 212 bp) for 12S, consistent with the Sanger sequencing results. Processing with the DADA2 pipeline yielded 55 ASVs for NADH4 and 269 ASVs for 12S. Subsequently, all 55 ASVs generated with the ES-200ND4 primer were taxonomically annotated at the genus level as *Hemiscyllium* (Figure 5a), confirming the primer's specificity for this genus. In contrast, the elas02 primer detected a broader range of marine organisms (Figure 5b), including *Hemiscyllium* (17 ASVs), one ASV of *Carcharhinus*, and various phytoplankton groups.

3.4 | Phylogenetic Confirmation and Genetic Distances of *Hemiscyllium* ASVs

Phylogenetic analysis of the *Hemiscyllium*-annotated ASVs from both markers confirmed their placement within the *H. freycineti* clade, with clear separation from other *Hemiscyllium* species and broader Orectolobiformes representatives (Figure 6). Genetic distance analyses provided quantitative support for these phylogenetic placements (Table 5). Intraspecific distances among the 55 NADH4 ASVs generated from field samples were 0.00%–2.53% (avg. 1.02%). Their interspecific distances to the reference sequences of *H. freycineti* ranged from 0.00% to 2.02% (avg. 0.77%), with 10 ASVs showing identical matches (0.00% divergence). Subsequently,

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 | Environmental DNA (eDNA; $n = 18$) sample details, including the number of sharks sighted, total DNA extraction yields, and agarose gel electrophoresis validation results during the initial quality checking step.

Sample	Location	Time/condition of sampling	Shark sighting	Shark count	DNA conc. (ng/μL)	Purity		Electrophoresis	
						A260/280	A280/230	NADH4	12S
ES-Arb1[b]	Arborek	DT, under homestay	No	0	23.13	2.1	2.2	−	+
ES-Arb2[b]	Arborek	NT, under homestay	No	0	8.32	2.4	2.3	−	+
ES-Arb3	Arborek	NT, near main bridge, LT	Yes	2	4.67	1.9	1.8	−	−
ES-Arb4	Arborek	NT, LT	Yes	2	11.34	1.9	1.9	−	+
ES-Arb5-MCS[b]	Arborek	DT, manta ray CS	No	0	43.73	1.8	1.9	−	+
ES-Arb6[a]	Arborek	NT, LT	Yes	9	63.21	1.8	1.9	+	+
ES-Arb7[a]	Arborek	NT, LT	Yes	1	21.34	1.9	2.0	+	+
ES-Arb8[a]	Arborek	Incubation water	Yes	1	24.85	2.1	2.1	+	+
ES-Kur1[a]	Kurkapa	NT, LT	Yes	16	23.72	2.0	2.0	+	+
ES-Kur2[b]	Kurkapa	NT, LT	No	0	5.43	4.2	2.1	−	−
ES-Saw1[a]	Sawinggrai	NT, LT	Yes	16	26.93	2.1	2.0	+	+
ES-Saw2[b]	Sawinggrai	NT, LT	No	0	4.11	3.3	2.8	−	−
ES-Kam1[b]	Kamar Raja	NT, LT, HR	No	0	3.89	2.0	2.3	−	−
ES-Sap1[a]	Saupapir	NT, LT	Yes	26	19.66	2.1	2.0	+	+
ES-Sap2[b]	Saupapir	NT, LT	No	0	13.99	2.9	2.6	−	+
ES-Day1[b,a]	Dayan	DT, under the bridge	No	0	46.33	2.1	2.3	+	+
ES-Yen1[a]	Yensawai	NT, LT	Yes	16	23.67	2.0	1.8	+	+
ES-Yen2[b]	Yensawai	NT, LT	No	0	5.26	2.1	2.2	−	−

Abbreviations: CS, cleaning station; DT, daytime; HR, heavy rain; LT, low tide; NT, nighttime.
[a]Indicate samples showing positive PCR amplifications using both primers (i.e., ES-200ND4 and elas02) and selected for HTS processing.
[b]Denote blind samples.

distances between ASVs and other *Hemiscyllium* species were consistently higher (1.01%–6.06%; avg. 2.54%), while interspecific distances to other Orectolobiformes species or genera were on an order of magnitude higher (10.10%–20.20%; avg. 15.01%) (Table 5).

Similar patterns were observed for the 17 ASVs detected by the elas02 primer (Table 5). Intraspecific distances among these 12S ASVs ranged 0.00%–2.30% (avg. 1.30%), while interspecific distances to the reference sequences of *H. freycineti* were 0.00%–1.15% (avg. 0.78%), with 13 ASVs showing identical matches. Comparisons with other *Hemiscyllium* species yielded intraspecific distances of 1.15%–4.02% (avg. 2.79%). As with the NADH4 dataset, the largest genetic distances were observed between the species or genera of Orectolobiformes (9.77%–20.35%, avg. 14.23%), with intermediate distances to *Chiloscyllium* spp. (9.77%–13.79%, avg. 11.59%).

4 | Discussion

4.1 | Primer Specificity and Performance

In this study, we developed and validated the first genus-specific eDNA metabarcoding assay for epaulette sharks (*Hemiscyllium* spp.), targeting the mitochondrial NADH4 gene. The ES-200ND4 primer exhibited strong genus-level specificity throughout our validation pipeline. The primer consistently amplified *Hemiscyllium* DNA across template concentrations as low as 10.78 ng/μL (laboratory experiments) and 19.66 ng/μL (field samples), demonstrating sensitivity appropriate for environmental applications where target DNA is often scarce or degraded (Yates et al. 2023). The lower amplification success rate of the ES-200ND4 primer in field samples (8 of 18) compared to elas02 (13 of 18) may reflect differences in primer binding efficiency or target DNA availability between the two markers.

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

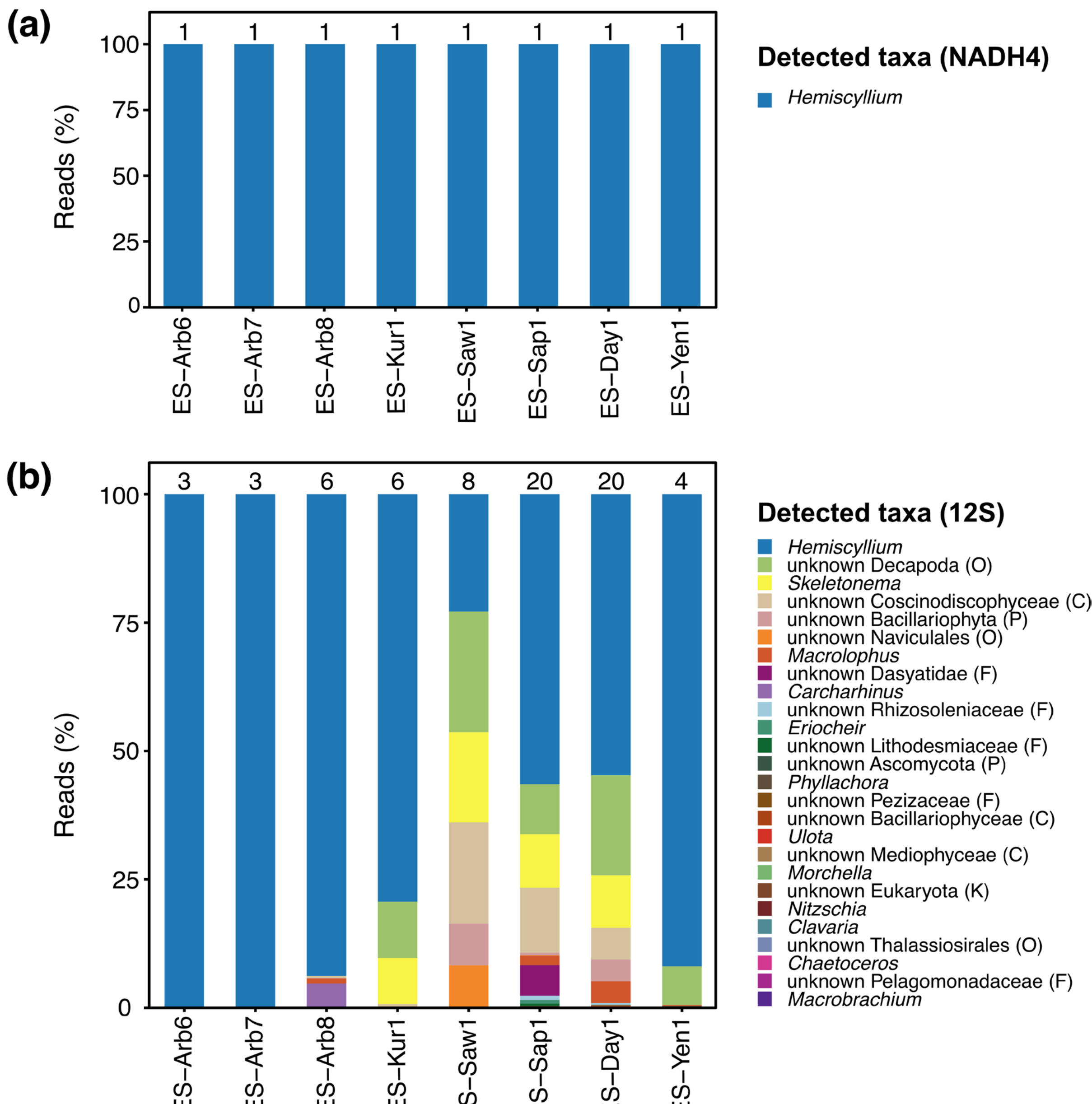

FIGURE 5 | Taxonomic composition of the NADH4 (a) and 12S rRNA (b) HTS dataset at the genus level detected by the ES-200ND4 and elas02 primers. Numbers above the bars indicate the total count of unique taxa (genus level) detected in each sample. Letters in brackets indicate the first letter of the nearest taxonomic ranks (i.e., Kingdom to Family) annotated for the ASVs.

As a single-copy protein-coding gene, NADH4 is inherently less abundant than the multi-copy ribosomal 12S gene, and its longer amplicon (~200–220 bp vs. ~180–200 bp) may be more susceptible to degradation in environmental samples, potentially reducing detection probability when target DNA is present at very low concentrations. Critically, the specificity of this primer was confirmed by HTS of field samples, in which all 55 NADH4 ASVs were assigned exclusively to *Hemiscyllium*. This contrasts sharply with the universal elas02 primer, which detected a broad spectrum of marine taxa, including *Hemiscyllium* (Figure 5b). This comparison illustrates the fundamental trade-off between sensitivity and specificity in eDNA primer design (Collins et al. 2018; Hansen et al. 2018). While broad detection is valuable for overall biodiversity assessments (Miya 2022), it complicates targeted monitoring when reference sequences for the target genus are incomplete. The absence of non-target amplification with ES-200ND4, even in samples containing diverse marine communities, confirms that our primer design successfully targets a ~200 bp hypervariable region containing 21 variable nucleotide positions (Figure 1) diagnostic for species discrimination, flanked by conserved sequences unique to *Hemiscyllium*.

4.2 | Species-Level Resolution, Phylogenetic Confirmation, and Methodological Context

The ability to accurately discriminate among *Hemiscyllium* species is essential for conservation applications. Species of *Hemiscyllium* are thought to have highly restricted geographic

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

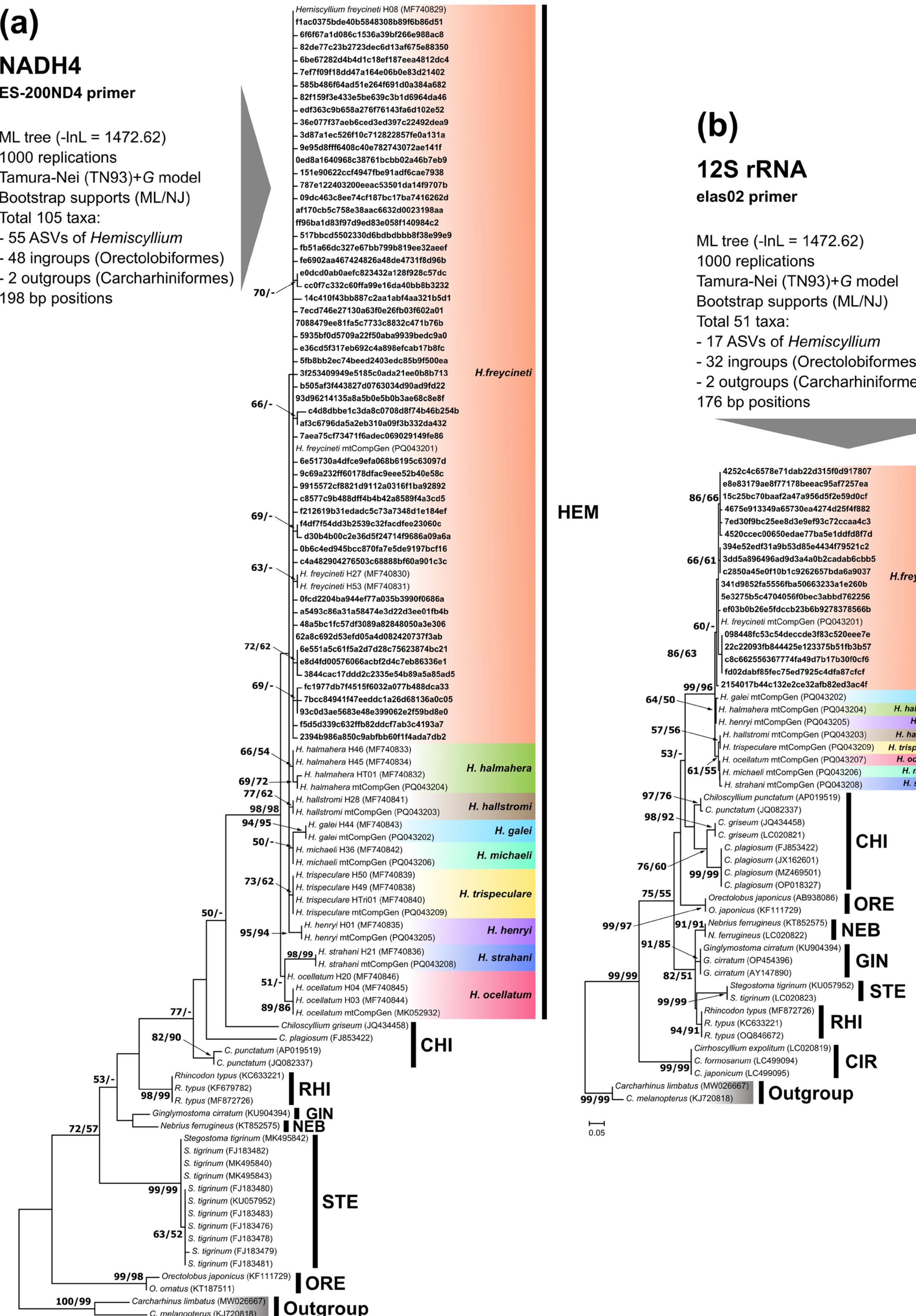

FIGURE 6 | Legend on next page.

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

FIGURE 6 | Maximum likelihood phylogenies of NADH4 (a) and 12S rRNA (b) gene sequences, showing the placement of ASVs annotated as *Hemiscyllium* (in bold) among nine species within this genus. Reference sequences from other Orectolobiformes (grouped by three-letter codes of their genus for estimating the genetic distances) and outgroup taxa (*Carcharhinus limbatus* and *C. melanopterus*) are included. Branch nodes show bootstrap support (> 50%) for ML and neighbor-joining analyses (NJ). The ASV IDs are QIIME2-generated UUIDs (Universally Unique Identifiers). All ASVs are listed in Data S2 and S3.

TABLE 5 | Uncorrected pairwise genetic distances (*p*-distance; %) between the newly generated ASV sequences from eDNA metabarcoding analysis and representative Orectolobiformes species or genera, including the outgroup, based on NADH4 and 12S markers. The number of sequences compared against all the ASVs is indicated as ∑seq. The genetic *p*-distances were calculated from the alignments used for phylogenetic reconstructions in Figure 6. The complete pairwise distance matrix is available in Data S4.

Sequence comparison	∑seq	NADH4 genetic distance (%)			∑seq	12S rDNA genetic distance (%)		
		Min	Max	Avg		Min	Max	Avg
Intraspecific								
Among ASVs (intraspecific)	55	0.00	2.53	1.02	17	0.00	2.30	1.30
Interspecific								
ASVs vs. *H. freycineti*	4	0.00	2.02	0.77	1	0.00	1.15	0.78
ASVs vs. *H. galei*	2	2.53	4.04	3.04	1	1.72	2.87	2.43
ASVs vs. *H. michaeli*	2	1.01	2.53	1.52	1	2.30	3.45	3.01
ASVs vs. *H. strahani*	2	4.55	6.06	5.52	1	1.72	4.02	3.52
ASVs vs. *H. hallstromi*	2	1.01	2.53	1.52	1	1.72	2.87	2.50
ASVs vs. *H. henryi*	2	2.02	3.54	2.53	1	1.15	2.30	3.45
ASVs vs. *H. trispeculare*	4	1.01	2.53	1.52	1	1.72	2.87	2.50
ASVs vs. *H. halmahera*	4	1.01	3.03	1.90	1	1.15	2.30	1.86
ASVs vs. *H. ocellatum*	4	1.01	4.04	2.77	1	2.30	3.45	3.01
ASVs vs. *Chiloscyllium* (CHI)	4	10.10	16.16	12.68	8	9.77	13.79	11.59
ASVs vs. *Rhincodon* (RHI)	3	15.15	16.67	16.10	3	12.64	14.94	14.03
ASVs vs. *Ginglymostoma* (GIN)	1	11.62	13.64	12.56	2	12.64	15.03	14.06
ASVs vs. *Nebrius* (NEB)	1	11.62	13.13	12.55	2	10.34	12.07	11.19
ASVs vs. *Stegostoma* (STE)	11	16.16	18.18	17.33	2	15.52	20.35	16.77
ASVs vs. *Orectolobus* (ORE)	2	17.68	20.20	18.83	2	11.49	13.22	12.37
ASVs vs. *Cirrhoscyllium* (CIR)	NA	—	—	—	3	18.02	20.35	19.63
ASVs vs. *Carcharhinus* (Outgroup)	2	23.74	26.77	25.37	2	18.97	21.84	20.81

distributions (Erdmann and Dudgeon 2024; Dudgeon et al. 2020), yet their true ranges and potential distributional overlaps remain poorly resolved. Detecting a *Hemiscyllium* species outside its previously known range can directly inform the expansion and adaptive management of protected areas by providing evidence for more accurate distribution mapping.

A necessary first step toward such discrimination is to identify genetic markers with sufficient variation to resolve species boundaries. The decision to use the less popular NADH4 gene for developing an eDNA metabarcoding assay in this study was primarily driven by the availability of complete reference sequences for all *Hemiscyllium* species at the outset of this work.

While mitochondrial NADH dehydrogenase genes, including NADH2 and NADH4, have been widely used in phylogenetic and population genetic studies of elasmobranchs (Naylor et al. 2012; Dudgeon et al. 2020) and other vertebrates (Andreu-Sanchez et al. 2021; Caballero et al. 2015), their application specifically for eDNA metabarcoding in marine systems has remained unexplored. In terrestrial systems, NADH4 has been used in targeted eDNA applications for detecting snake species (Schumer et al. 2019; Galbraith et al. 2021), supporting its extension to marine systems in the present study.

Building on this foundation, we next assessed whether the NADH4 marker, amplified with the new ES-200ND4 primer,

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

provides sufficient genetic resolution for *Hemiscyllium* by analyzing intra- and interspecific genetic distances. The observed intraspecific distances of our sequences generally fall within the range (0.00%–2.12%) reported for within-species mitochondrial variation in elasmobranchs (Naylor et al. 2012), though population-level inferences would require a dedicated study design. When compared to the phylogenetic distance estimates reported by Dudgeon et al. (2020), in which interspecific distances among *Hemiscyllium* species based on the full NADH4 gene ranged from 0.0% to 1.0% (avg. 0.2%), our results appear substantially higher (1.01%–6.06%; avg. 2.54%) (Table 5). This discrepancy is not surprising, as our primer was specifically designed to target a ~200 bp hypervariable region within the NADH4 gene. While Dudgeon et al. (2020) calculated distances across the full gene length (~678 bp), which includes both conserved and variable sites, our 198 bp amplicons captured a subset of positions enriched for phylogenetic signal, resulting in higher observed divergence among species. This was confirmed by phylogenetic analysis, which showed that all 55 NADH4 ASVs from field samples clustered unequivocally within the *H. freycineti* clade (Figure 6a). Collectively, these results confirm that the ~200 bp amplicon generated by the ES-200ND4 primer provides sufficient species-level resolution for differentiating members of the genus *Hemiscyllium*.

However, because taxonomic assignments based solely on bioinformatic algorithms can be unreliable for short eDNA reads, often due to limited phylogenetic signal or incomplete reference databases (Blackman et al. 2023; De Santiago et al. 2025; Mathon et al. 2021), we complemented initial taxonomic assignments with phylogenetic analyses (Figures 4 and 6). For this validation step, we used a reference dataset restricted to *Hemiscyllium* species. This approach provided an additional layer of validation, ensuring that species-level identifications were not merely artifacts of algorithmic inference. Instead, they were supported by explicit phylogenetic placement relative to verified reference sequences. The concordance between taxonomic annotations and phylogenetic placement further reinforces confidence in the species identifications reported herein. To our knowledge, this represents the first application of NADH4 as an eDNA metabarcoding marker in marine environments and the first for any elasmobranch. This demonstrates that markers traditionally reserved for phylogenetics can be repurposed for conservation monitoring when reference gaps are filled. Such a strategy is applicable to other data-poor taxa as genomic resources continue to expand.

4.3 | Field Validation and the Complementary Value of eDNA

Field application of the ES-200ND4 primer across seven locations in Raja Ampat detected *H. freycineti* DNA at six locations, demonstrating the assay's robustness under realistic survey conditions. Detection success was generally associated with visual confirmation of Raja Ampat Epaulette Sharks (Saupapir, Kurkapa, Sawinggrai, and Yensawai), where multiple individuals were observed (Table 4). A key advantage of the eDNA approach was demonstrated at Dayan, where daytime visual surveys recorded no sharks, but eDNA analysis confirmed their presence. This detection, along with the observation that positive amplifications with the ES-200ND4 primer occurred predominantly in samples collected near visually confirmed sharks, reinforces the assay's specificity for detecting target species DNA. While the exact source of this eDNA signal remains unclear, this detection corroborates anecdotal reports from local communities and highlights the value of eDNA for validating traditional ecological knowledge, a pattern observed in studies of other cryptic marine fauna (Yates et al. 2023). This finding highlights the complementary relationship between traditional and eDNA survey methods: while nocturnal reef walks remain valuable for collecting demographic data (e.g., size, sex, maturity, photographic identification) (Setyawan et al. in review), they are constrained by timing, weather, and logistical challenges in remote locations. eDNA methods, by contrast, can detect species presence from samples collected during daytime hours, in inclement weather, or in areas where night work poses unacceptable risks. The successful detection at Dayan, explained by the nocturnal behavior of *Hemiscyllium* species (Allen et al. 2016; Dudgeon et al. 2020), suggests that *H. freycineti* may be more widely distributed across Raja Ampat's reef flats than visual surveys alone would indicate.

The field results also reinforced the sensitivity-specificity trade-off observed in laboratory validation. The universal elas02 primer detected *Hemiscyllium* DNA in more samples (13 of 18) than the ES-200ND4 primer (8 of 18) (Table 4), consistent with its broader reactivity. However, this increased sensitivity came at the cost of specificity, as elas02 amplified numerous non-target taxa (Figure 5b), whereas ES-200ND4 amplified *Hemiscyllium* exclusively (Figure 5a). This trade-off supports the dual-primer strategy introduced in Section 4.1: using elas02 for sensitive initial screening and ES-200ND4 for confirmatory species-level identification.

However, linking eDNA detection to local population abundance remains challenging. The comparison between visual shark counts and eDNA-derived metrics illustrates the complexity of relating molecular detection to local abundance (Figure 7). For instance, the location with the highest visual count (Saupapir, 26 unique individuals) did not yield correspondingly high ASV richness or read numbers. In contrast, another location with fewer sightings (Kurkapa, 16 unique individuals) produced the highest NADH4 ASV richness. These discrepancies likely reflect factors known to influence eDNA recovery, including differential DNA shedding rates, hydrodynamic transport, temporal mismatches between instantaneous visual surveys and time-integrated eDNA signals, and microhabitat variation in eDNA persistence (Collins et al. 2018; Yates et al. 2023). This complexity underscores that while eDNA is a powerful tool for detecting species presence, quantitative comparisons with visual abundance estimates require careful interpretation and, ideally, calibration through controlled experiments.

4.4 | Implications for Conservation Monitoring

The Indonesian government's decision to grant full legal protection to all *Hemiscyllium* species (Indonesian Ministry of Marine Affairs and Fisheries 2023) represents a landmark commitment to elasmobranch conservation. However, protected status alone cannot ensure species persistence without

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

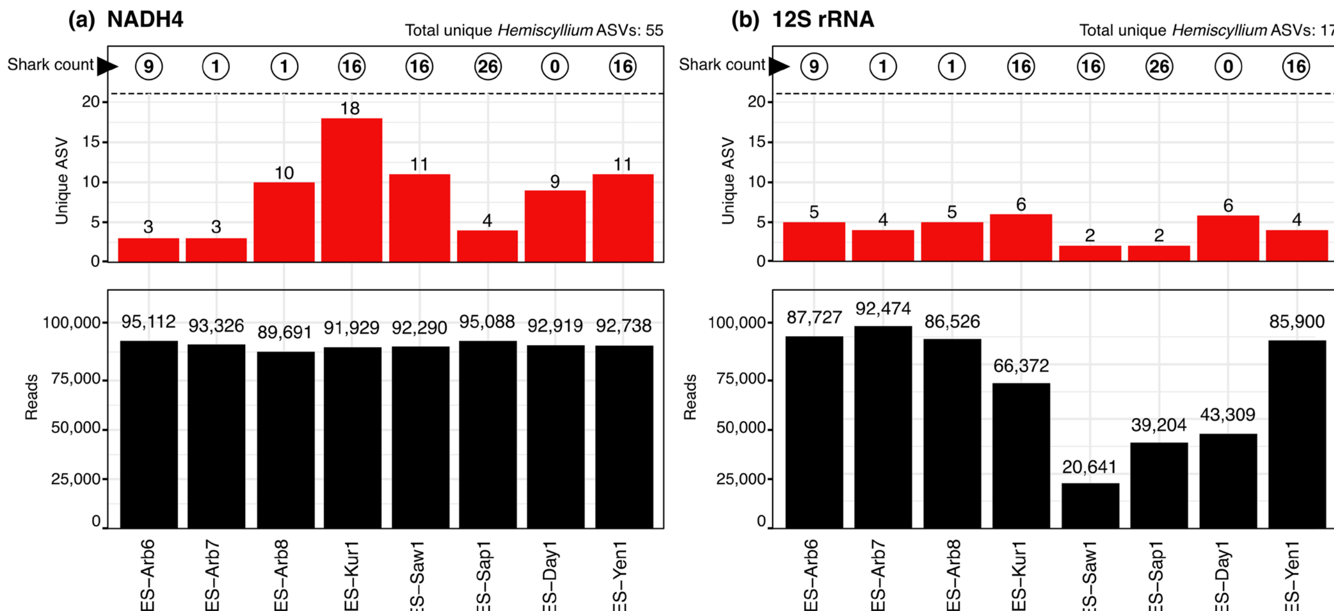

FIGURE 7 | The number of unique ASVs (red bars) and total read count (black bars) annotated as *Hemiscyllium* within the NADH4 (a) and 12S rRNA (b) HTS datasets. Numbers in circles indicate the number of Raja Ampat Epaulette Sharks (*H. freycineti*) sighted per location.

effective monitoring to inform management actions (Lopez et al. 2025). Beyond its immediate application to *H. freycineti* in Raja Ampat, this assay has broader potential for documenting the distribution of rarer and more threatened congeners across the genus range (Erdmann and Dudgeon 2024). The ES-200ND4 primer, designed against all nine *Hemiscyllium* reference sequences, should be capable of detecting any member of the genus, pending field validation with additional species. Such applications could also help resolve taxonomic uncertainties, such as the status of the Aru population of *H. trispeculare* relative to its Australian counterparts (Dudgeon et al. 2020). Detection of *Hemiscyllium* species outside their previously known ranges, as our Dayan finding demonstrates, can directly inform the expansion and adaptive management of Marine Protected Areas (MPAs) by providing evidence for more accurate distribution mapping.

More broadly, our approach provides a generalizable template for developing targeted eDNA assays for other data-poor, cryptic, or threatened taxa where universal markers prove inadequate. The strategy is straightforward: identify a marker with complete reference sequences for the target group, design short-amplicon primers flanking a hypervariable region, and validate progressively from laboratory experiments to field applications. As genomic resources continue to expand, with complete mitochondrial genomes becoming available for an increasing number of species, this approach becomes increasingly feasible for diverse taxa. Such tools enable conservation geneticists to monitor species that have long eluded detection, addressing an urgent need to meet global biodiversity targets and prevent extinctions amid ongoing environmental threats.

4.5 | Limitations and Future Directions

Several limitations of this study should be considered when interpreting results and planning future applications. First, the lower amplification success rate of ES-200ND4 in field samples (8 of 18) compared to elas02 (13 of 18) suggests sensitivity may be constrained by the single-copy nature of the NADH4 gene, potentially leading to false negatives at location with low population densities. Future studies could address this by increasing replication, filtering larger water volumes, concentrating eDNA extracts (Hunter et al. 2019), or developing complementary assays targeting multi-copy markers such as COI, 12S, or 16S rRNA using the now-available complete mitochondrial genomes of *Hemiscyllium* spp. (Dudgeon et al. 2020).

Second, while our sampling strategy prioritized locations with known shark presence, appropriate for initial assay validation, this approach may bias detection success upward. The Dayan finding demonstrates that detection is possible without a priori knowledge, suggesting that systematic surveys across randomly selected locations, coupled with occupancy modeling (Tyre et al. 2003), would provide more robust distribution estimates and should be a priority for future research.

Third, variable amplification success across locations with similar shark abundances highlights the influence of environmental factors on eDNA persistence and transport. While the strong Dayan signal confirms that eDNA can persist for hours after animals depart, the precise decay dynamics under local conditions remain uncharacterized. Controlled mesocosm experiments investigating shedding rates and decay dynamics (Evans et al. 2016; Spear et al. 2021) for *Hemiscyllium* would strengthen quantitative inference and enable calibration with traditional survey data.

The recent public release of complete mitochondrial genomes for all nine *Hemiscyllium* species (Dudgeon et al. 2020) opens additional opportunities beyond the present study. These resources will enable systematic in silico evaluation of existing elasmobranch universal primers (e.g., MiFish-U, MiFish-E, 16S-Fish; Miya and Sado (2019)) to identify which reliably amplify *Hemiscyllium* DNA. Complementary wet-lab validation could

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

then benchmark ES-200ND4 against the best-performing universal alternatives, quantifying trade-offs in sensitivity, specificity, and taxonomic resolution. Complete genomes also enable exploration of environmental RNA (eRNA) approaches for assessing population health and recent activity (Pochon et al. 2017).

Finally, our decision to sequence only dual-positive samples, necessitated by funding constraints, may have excluded locations where *Hemiscyllium* was present but detectable only by elas02, introducing conservative bias. Future studies with larger budgets should sequence all samples positive for either marker to enable direct comparison of detection rates and assessment of false-negative rates for ES-200ND4. By acknowledging these current constraints, subsequent studies can build on this framework to further refine non-invasive monitoring tools for data-poor taxa.

5 | Conclusion

This study presents the first genus-specific eDNA metabarcoding assay for epaulette sharks (*Hemiscyllium* spp.) and the first application of NADH4 as a metabarcoding marker in marine environments. The ES-200ND4 primer demonstrated absolute genus-level specificity, with all 55 field-detected ASVs exclusively assigned to *Hemiscyllium*, and sufficient sensitivity to detect target DNA. Field validation in Raja Ampat confirmed the assay's robustness, detecting the endemic *H. freycineti* at six of seven locations. Critically, eDNA detection at Dayan, where daytime visual surveys recorded no sharks, demonstrates the method's power to reveal species presence when traditional surveys fail due to behavioral or logistical constraints, confirming that eDNA complements rather than replaces visual surveys. By providing a non-invasive, scalable monitoring tool, this assay directly supports the implementation of Indonesia's protective legislation for all *Hemiscyllium* species. Although validated only in Raja Ampat, the endemic home of *H. freycineti*, the primer was designed against all nine reference sequences and should be applicable to other congeners across the genus range, pending field validation. More broadly, our approach provides a generalizable template for developing targeted eDNA assays for other data-poor, cryptic, or threatened marine taxa for which universal markers are inadequate, contributing to the non-invasive methods urgently needed to meet global biodiversity goals.

Author Contributions

D.A.P. and E.S. conceptualized the study and drafted the original version of the manuscript, therefore should be considered as joint first authors. D.A.P., A.P.P., S.S., and M.F.E. performed the laboratory work, including formal analysis, validation, and visualization. D.A.P., E.S., A.W.H., R.M., A.P.P., S.S., M.F.E., O.A., and N.S. were involved in data investigation and curation. M.I.H.P. and M.V.E. managed and supervised the project, with funding secured by M.I.H.P., M.V.E., and E.S. All authors reviewed and approved the final version of the manuscript prior to submission.

Acknowledgments

We would like to thank BLUD UPTD Pengelolaan Kawasan Konservasi di Perairan Kepulauan Raja Ampat (the Raja Ampat MPA management authority) that fully supported our research. We would also like to thank YatchAid Global and Zoran Selakovic, the SV Seahawk owners: Adam and Gisela Alpert and the SV Seahawk's wonderful crew for their support during the Operation Swimway expedition in Raja Ampat. We extend a warm thanks to Urias Tuhumena and Demas Fiay for their support with Yaswal. Also, thanks to the field support team who ensured boats and logistical supplies were always available: Timore Kristiani, Hanrika Leimena, Julius Thonak, Marselinus Uskono. We also thank the Raja Ampat local community members who assisted us during the fieldwork: Bapak Deny Mambrasar, Bapak Hironimus Bani from Arborek and Aksim from Saupapir village. We thank Research Center for Biosystematics and Evolution and the Genomic Laboratory of BRIN for their support with molecular facility. Finally, this research was supported by a 2025 Pew Marine Fellowship awarded to Edy Setyawan.

During the preparation of this manuscript, the author(s) used DeepSeek to enhance language, clarity, and readability. Portions of the graphical abstract were generated using NotebookLM and subsequently edited manually with Affinity (by Canva). After using these tools, the author(s) thoroughly reviewed and revised the content as necessary, and took full responsibility for the published work.

Funding

The research was generously funded by MAC3 Impact Philanthropies, Yacht Aid Global, Foundation Segrè, and Pew Charitable Trusts.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw HTS data supporting the conclusions of this article will be made available by the authors upon publication from the NCBI SRA BioProject ID: PRJNA1321526. The Accession Numbers of new DNA sequences generated in Primer validation (PX317577–PX317585) and eDNA simulation experiments (PX309091–PX309096) are available from NCBI GenBank upon publication. New ASV sequences used for phylogenetic tree reconstructions in Figure 6 are supplemented in the Data S2 and S3.

References

Allen, G. R., M. V. Erdmann, W. T. White, et al. 2016. "Review of the Bamboo Shark Genus *Hemiscyllium* (Orectolobiformes: Hemiscyllidae)." *Journal of Ocean Science Foundation* 23: 51–97. https://doi.org/10.5281/zenodo.164197.

Andreu-Sanchez, S., W. Chen, J. Stiller, and G. Zhang. 2021. "Multiple Origins of a Frameshift Insertion in a Mitochondrial Gene in Birds and Turtles." *GigaScience* 10, no. 1: giaa161. https://doi.org/10.1093/gigascience/giaa161.

Arèvalo, E., S. K. Davis, and J. W. Sites Jr. 1994. "Mitochondrial DNA Sequence Divergence and Phylogenetic Relationships Among Eight Chromosome Races of the *Sceloporus grammicus* Complex (Phrynosomatidae) in Central Mexico." *Systematic Biology* 43: 387–418. https://doi.org/10.1093/sysbio/43.3.387.

Blackman, R. C., J. C. Walser, L. Rüber, et al. 2023. "General Principles for Assignments of Communities From eDNA: Open Versus Closed Taxonomic Databases." *Environmental DNA* 5, no. 2: 326–342. https://doi.org/10.1002/edn3.382.

Bolyen, E., J. R. Rideout, M. R. Dillon, et al. 2019. "Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2." *Nature Biotechnology* 37: 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Boussarie, G., J. Bakker, O. S. Wangensteen, et al. 2018. "Environmental DNA Illuminates the Dark Diversity of Sharks." *Science Advances* 4: eaap9661. https://doi.org/10.1126/sciadv.aap9661.

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Boyd, B. M., and J. C. Seitz. 2021. “Global Shifts in Species Richness Have Shaped Carpet Shark Evolution.” *BMC Ecology and Evolution* 21, no. 1: 192. https://doi.org/10.1186/s12862-021-01922-6.

Caballero, S., S. Duchêne, M. F. Garavito, B. Slikas, and C. S. Baker. 2015. “Initial Evidence for Adaptive Selection on the NADH Subunit Two of Freshwater Dolphins by Analyses of Mitochondrial Genomes.” *PLoS One* 10, no. 5: e0123543. https://doi.org/10.1371/journal.pone.0123543.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. “DADA2: High-Resolution Sample Inference From Illumina Amplicon Data.” *Nature Methods* 13: 581–583. https://doi.org/10.1038/nmeth.3869.

Çevik, T., and N. Çevik. 2025. “Environmental DNA (eDNA): A Review of Ecosystem Biodiversity Detection and Applications.” *Biodiversity and Conservation* 34, no. 9: 2999–3035. https://doi.org/10.1007/s10531-025-03112-y.

Collins, R. A., O. S. Wangensteen, E. J. O'Gorman, S. Mariani, D. W. Sims, and M. J. Genner. 2018. “Persistence of Environmental DNA in Marine Systems.” *Communications Biology* 1: 185. https://doi.org/10.1038/s42003-018-0192-6.

De Santiago, A., T. J. Pereira, T. J. Ferrero, et al. 2025. “Persistent Gaps and Errors in Reference Databases Impede Ecologically Meaningful Taxonomy Assignments in 18S rRNA Studies: A Case Study of Terrestrial and Marine Nematodes.” *Environmental DNA* 7, no. 2: e70080. https://doi.org/10.1002/edn3.

Dudgeon, C. L., S. Corrigan, L. Yang, et al. 2020. “Walking, Swimming or Hitching a Ride? Phylogenetics and Biogeography of the Walking Shark Genus *Hemiscyllium*.” *Marine and Freshwater Research* 71: 1107–1117. https://doi.org/10.1071/MF19163.

Edgar, R. C. 2004. “MUSCLE: Multiple Sequence Alignment With High Accuracy and High Throughput.” *Nucleic Acids Research* 32: 1792–1797. https://doi.org/10.1093/nar/gkh340.

Elbrecht, V., and F. Leese. 2017. “Validation and Development of COI Metabarcoding Primers for Freshwater Macroinvertebrate Bioassessment.” *Frontiers in Environmental Science* 5: 11. https://doi.org/10.3389/fenvs.2017.00011.

Erdmann, M. E., and C. L. Dudgeon. 2024. “Walking (epaulette) Sharks.” In *The Global Status of Sharks, Rays, and Chimaeras*, edited by R. W. Jabado, A. Z. A. Morata, R. H. Bennet, et al., 2053–2066. IUCN SSC Shark Specialist Group.

Evans, N. T., B. P. Olds, M. A. Renshaw, et al. 2016. “Quantification of Mesocosm Fish and Amphibian Species Diversity via Environmental DNA Metabarcoding.” *Molecular Ecology Resources* 16: 29–41. https://doi.org/10.1111/1755-0998.12433.

Fontes, J. T., K. Katoh, R. Pires, P. Soares, and F. O. Costa. 2024. “Benchmarking the Discrimination Power of Commonly Used Markers and Amplicons in Marine Fish (e) DNA (Meta) Barcoding.” *ARPHA Preprints* 5: e128777. https://doi.org/10.3897/mbmg.8.128646.

Galbraith, E., C. Santamaria, M. Hoffman, and A. Gainsbury. 2021. “Environmental DNA Detection Method From Soil Samples for Eastern Indigo Snakes (*Drymarchon couperi*). Society for Integrative and Comparative Biology. Annual Meeting.” https://sicb.org/abstracts/environmental-dna-detection-method-from-soil-samples-for-eastern-indigo-snakes-drymarchon-couperi/.

Hansen, B. K., D. Bekkevold, L. W. Clausen, and E. E. Nielsen. 2018. “The Sceptical Optimist: Challenges and Perspectives for the Application of Environmental DNA in Marine Fisheries.” *Fish and Fisheries* 19: 751–768. https://doi.org/10.1111/faf.12286.

Hunter, M. E., J. A. Ferrante, G. Meigs-Friend, and A. Ulmer. 2019. “Improving eDNA Yield and Inhibitor Reduction Through Increased Water Volumes and Multi-Filter Isolation Techniques.” *Scientific Reports* 9, no. 1: 5259. https://doi.org/10.1038/s41598-019-40977-w.

Indonesian Ministry of Marine Affairs and Fisheries. 2023. “Ministerial Decree 30/2023 on the Full Protection of Walking Sharks (*Hemiscyllium* spp.) in Indonesian Waters.”

Inoue, J. G., M. Miya, K. Tsukamoto, and M. Nishida. 2001. “A Mitogenomic Perspective on the Basal Teleostean Phylogeny: Resolving Higher-Level Relationships with Longer DNA Sequences.” *Molecular Phylogenetics and Evolution* 20, no. 2: 275–285. https://doi.org/10.1006/mpev.2001.0970.

Jabado, R., E. García-Rodríguez, P. Kyne, et al. 2024. *Asia: A Regional Compendium of Important Shark and Ray Areas*. IUCN SSC Shark Specialist Group. https://doi.org/10.59216/ssg.isra.2024.r9.

Jutan, Y., A. Retraubun, A. Khouw, V. Nikijuluw, and J. Pattikawa. 2018. “Study on the Population of Halmahera Walking Shark (*Hemiscyllium halmahera*) in Kao Bay, North Maluku, Indonesia.” *International Journal of Fisheries and Aquatic Studies* 6, no. 4: 36–41.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. “MEGA X: Molecular Evolutionary Genetics Analysis Across Computing Platforms.” *Molecular Biology and Evolution* 35: 1547–1549. https://doi.org/10.1093/molbev/msy096.

Leray, M., N. Knowlton, and R. J. Machida. 2022. “MIDORI2: A Collection of Quality Controlled, Preformatted, and Regularly Updated Reference Databases for Taxonomic Assignment of Eukaryotic Mitochondrial Sequences.” *Environmental DNA* 4: 894–907. https://doi.org/10.1002/edn3.303.

Lopez, M. L. D., N. Acharya-Patel, M. J. Allison, A. Migneault, S. Trilesky, and C. C. Helbing. 2025. “From Environmental DNA Into Actionable Biodiversity Data: Towards the Implementation of the Kunming-Montreal Global Biodiversity Framework.” *Journal of Heredity*: esaf068. https://doi.org/10.1093/jhered/esaf068.

Maiello, G., A. Bellodi, A. Cariani, et al. 2024. “Fishing in the Gene-Pool: Implementing Trawl-Associated eDNA Metaprobe for Large Scale Monitoring of Fish Assemblages.” *Reviews in Fish Biology and Fisheries* 34, no. 4: 1293–1307. https://doi.org/10.1007/s11160-024-09874-y.

Mangubhai, S., M. V. Erdmann, J. R. Wilson, et al. 2012. “Papuan Bird's Head Seascape: Emerging Threats and Challenges in the Global Center of Marine Biodiversity.” *Marine Pollution Bulletin* 64: 2279–2295. https://doi.org/10.1016/j.marpolbul.2012.07.024.

Martin, M. 2011. “Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads.” *EMBnet.Journal* 17: 10–12. https://doi.org/10.14806/ej.17.1.200.

Mathon, L., A. Valentini, P. E. Guérin, et al. 2021. “Benchmarking Bioinformatic Tools for Fast and Accurate eDNA Metabarcoding Species Identification.” *Molecular Ecology Resources Resour* 21, no. 7: 2565–2579. https://doi.org/10.1111/1755-0998.13430.

McMurdie, P. J., and S. Holmes. 2013. “Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data.” *PLoS One* 8: e61217. https://doi.org/10.1371/journal.pone.0061217.

Miya, M. 2022. “Environmental DNA Metabarcoding: A Novel Method for Biodiversity Monitoring of Marine Fish Communities.” *Annual Review of Marine Science* 14: 161–185. https://doi.org/10.1146/annurev-marine-041421-082251.

Miya, M., and T. Sado. 2019. *Multiple Species Detection Using MiFish Primers*. Vol. 2, 55–92. Environmental DNA sampling and experimental manual version.

Miya, M., Y. Sato, T. Fukunaga, et al. 2015. “MiFish, a Set of Universal PCR Primers for Metabarcoding Environmental DNA From Fishes: Detection of More Than 230 Subtropical Marine Species.” *Royal Society Open Science* 2: 150088. https://doi.org/10.1098/rsos.150088.

Naylor, G. J. P., J. N. Caira, K. Jensen, K. A. M. Rosana, W. T. White, and P. R. Last. 2012. “A DNA Sequence-Based Approach to the Identification of Shark and Ray Species and Its Implications for Global Elasmobranch

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Diversity and Parasitology." *Bulletin of the American Museum of Natural History* 367: 1–262. https://doi.org/10.1206/754.1.

Nebuchadnezzar, A., D. G. Bengen, I. W. Nurjaya, N. M. N. Natih, B. Subhan, and A. Baksir. 2023. "Population Structure of the Halmahera Walking Shark (*Hemiscylium halmahera*) Endemic Species in Morotai Island Sea, North Maluku, Indonesia." *Egyptian Journal of Aquatic Biology and Fisheries* 27: 377–393. https://doi.org/10.21608/ejabf.2023.292366.

Pochon, X., A. Zaiko, L. M. Fletcher, O. Laroche, and S. A. Wood. 2017. "Wanted Dead or Alive? Using Metabarcoding of Environmental DNA and RNA to Distinguish Living Assemblages for Biosecurity Applications." *PLoS One* 12: e0187636. https://doi.org/10.1371/journal.pone.0187636.

Posit team. 2025. *RStudio: Integrated Development Environment for R*. Posit Software, PBC. http://www.posit.co/.

R Core Team. 2024. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/.

Riaz, T., W. Shehzad, A. Viari, F. Pompanon, P. Taberlet, and E. Coissac. 2011. "ecoPrimers: Inference of New DNA Barcode Markers From Whole Genome Sequence Analysis." *Nucleic Acids Research* 39, no. 21: e145. https://doi.org/10.1093/nar/gkr732.

Schumer, G., E. C. Hansen, P. J. Anders, and S. M. Blankenship. 2019. "Development of a Quantitative Polymerase Chain Reaction Assay and Environmental DNA Sampling Methods for Giant Gartersnake (*Thamnophis gigas*)." *PLoS One* 14, no. 9: e0222493. https://doi.org/10.1371/journal.pone.0222493.

Setyawan, E., R. Mambrasar, A. Hasan, et al. (*in review*) "An assessment on the Biology, Demographics, and Habitat Preferences of the Raja Ampat Epaulette Shark (*Hemiscyllium freycineti*) in the Dampier Strait, Raja Ampat, Indonesia." *Frontiers in Fish Science*.

Spear, M. J., H. S. Embke, P. J. Krysan, and M. J. Vander Zanden. 2021. "Application of eDNA as a Tool for Assessing Fish Population Abundance." *Environmental DNA* 3, no. 1: 83–91. https://doi.org/10.1002/edn3.94.

Taberlet, P., A. Bonin, L. Zinger, and E. Coissac. 2018. "Marine Environments." In *Environmental DNA: For Biodiversity Research and Monitoring*, edited by P. Taberlet, A. Bonin, L. Zinger, and E. Coissac. Oxford University Press. https://doi.org/10.1093/oso/9780198767220.003.0013.

Tyre, A. J., B. Tenhumberg, S. A. Field, D. Niejalke, K. Parris, and H. P. Possingham. 2003. "Improving Precision and Reducing Bias in Biological Surveys: Estimating False-Negative Error Rates." *Ecological Applications* 13, no. 6: 1790–1801. https://doi.org/10.1890/02-5078.

Valentini, A., P. Taberlet, C. Miaud, et al. 2016. "Next-Generation Monitoring of Aquatic Biodiversity Using Environmental DNA Metabarcoding." *Molecular Ecology* 25, no. 4: 929–942. https://doi.org/10.1111/mec.13428.

VanderWright, W. J., C. L. Dudgeon, M. V. Erdmann, A. Sianipar, and N. K. Dulvy. 2022. "Extinction Risk and the Small Population Paradigm in the Micro-Endemic Radiation of Epaulette Sharks." In *Imperiled: The Encyclopedia of Conservation*, edited by D. A. DellaSala and M. I. Goldstein, 752–762. Elsevier. https://doi.org/10.1016/B978-0-12-821139-7.00130-6.

Wickham, H. 2016. "Data Analysis." In *ggplot2: Elegant Graphics for Data Analysis*, 189–201. Springer International Publishing.

Widiarto, S. B., I. Wahyudin, H. Sombo, et al. 2020. "Populasi Hiu Berjalan, Kalabia (*Hemiscyllium freycineti*), di Perairan Misool, Kabupaten Raja Ampat." *Aquatic Science Management* 8: 15–20. https://doi.org/10.35800/jasm.8.1.2020.30597.

Yates, M. C., E. Furlan, B. Thalinger, H. Yamanaka, and L. Bernatchez. 2023. "Beyond Species Detection—Leveraging Environmental DNA and Environmental RNA to Push Beyond Presence/Absence Applications." *Environmental DNA* 5: 829–835. https://doi.org/10.1002/edn3.459.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supplementary Figures S1-S3 and Tables S1-S2. **Figure S1:** Schematic diagram of laboratory experiments for the eDNA metabarcoding. **Figure S2:** Agarose gel electrophoresis of PCR products from extracted eDNA samples ($n = 18$). **Figure S3:** Species accumulation curves showing the relationship between sequencing depth and taxonomic richness. **Table S1:** List of reference sequences used in phylogenetic analyses of the NADH4 and 12S rDNA genes. **Table S2:** Sequence reads composition of the HTS datasets derived from the NADH4 and 12S. **Data S2:** Sequences of *Hemiscyllium freycineti* (n=55 ASVs) generated from the NADH4 metabarcoding and used for phylogenetic tree reconstruction (Figure 6a). **Data S3:** Sequences of *Hemiscyllium freycineti* (n=17 ASVs) generated from the 12S rDNA metabarcoding and used for phylogenetic tree reconstruction (Figure 6b). **Data S4:** Excel file listing the detailed uncorrected pairwise genetic distances (*p*-distance) between species and ASVs of Orectolobiformes.

26374943, 2026, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.70281 by Edy Setyawan - Nat Prov Indonesia , Wiley Online Library on [23/05/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License