Environmental DNA (eDNA) metabarcoding and fish visual census reveals the first record of *Doboatherina magnidentata* in the Philippines

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Abstract. *Bautista JA, Manubag JJ, Sumaya NH, Martinez JG, Tabugo SR. 2023. Environmental DNA (eDNA) metabarcoding and fish visual census reveals the first record of* Doboatherina magnidentata *in the Philippines. Biodiversitas 24: 3063-3072.* Biodiversity monitoring is the cornerstone for conserving marine fish species. However, classical methods, like the Fish Visual Census (FVC), are often limited due to sampling difficulties, the occurrence of rare and cryptic organisms, and reliance on a taxonomic expert for species identification. Recently, environmental DNA (eDNA) metabarcoding has been suggested as a non-invasive, powerful tool for biomonitoring. This study evaluates the eDNA approach as complementary tool for the FVC data in species detection and identification of important marine fishes from the marine sanctuary of Dalipuga, Iligan City, Philippines. The findings obtained through the eDNA approach provide insights into identifying significant fish species. Notably, the presence of the *Hippocampus kuda* Bleeker, 1852 (yellow seahorse), categorized as a vulnerable and threatened species, was detected. Additionally, the study identified *Herklotsichthys quadrimaculatus* Rüppell, 1837 (bluestripe herring), a native species to the Philippines that may pose potential risks to humans and the ecological balance. Furthermore, two demersal fish species, namely Large-scale whiting (*Sillaginops macrolepis* Bleeker, 1858) and Large-scale mullet (*Planiliza macrolepis* Smith, 1846), were also detected. The eDNA approach also delineated the morphologically cryptic fishes from Scaridae (parrotfishes) and Mugilidae (mullet fish) taxa to the species level. The highlight of this study was the detection of the new Indo-pacific atherinomorine fish species *Doboatherina magnidentata*, which to the best of our knowledge, was the first record in the Philippine marine waters. Despite the efficiency of the eDNA metabarcoding in fish species detection and identification, the viability of eDNA in the marine environment and biases of the primer limit this method. Thus, the classical method must complement the molecular approach for better taxonomic resolution and community analysis. Future studies were also recommended to use a multigene eDNA approach to improve taxonomic sensitivity and reduce primer biases.

Keywords: Cryptic species, *Doboatherina magnidentata*, eDNA, fish monitoring, marine fishes

INTRODUCTION

Fish biodiversity loss in marine environments remains a challenge in the 21st century. The continued impacts of climate change and various anthropogenic activities have resulted in an alarming decline in marine species richness and abundance. This, in turn, threatens the stability and health of many marine ecosystems and the global economy (Selig et al. 2014; Descombes et al. 2015). Hence, biodiversity monitoring is essential to the conservation and sustainability of many marine fishes. Fish monitoring must be sensitive and provide accurate data on species distribution and population size for conservation strategies to be effective. Fish monitoring traditionally relied on visual surveys and accounting of individuals through morphological characterization for data gathering of species (Dafforn et al. 2016).

In some cases, however, these techniques perform inefficiently due to sampling difficulties, which heavily depend on the weather, water conditions, and fish mobility (Thomsen and Willerslev 2015). The reduced accessibility and visibility of the environment often limit the efficiency of these methods (West et al. 2021). Additionally, rare and cryptic species can be problematic as they are naturally difficult to monitor (Pikitch 2018). These taxa are usually data deficient in terms of their species richness, range distribution, and population sizes (Niemiller et al. 2018), leaving databases with errors and incomplete checklists (Thomsen and Willerslev 2015). Moreover, visual surveys are also labor-intensive, costly, and time-consuming (Beng and Corlett 2020) and require taxonomic experts for morphological identification, which is rapidly declining nowadays (Sangster and Luksenburg 2015). All such limitations of traditional fish biodiversity monitoring demand an approach to supplement its constraints.

Environmental DNA (eDNA) can combat challenges associated with the fish visual census. By definition, eDNA is the extra-organismal genetic material suspended in the environment (Bohmann et al. 2014). Macro-organisms like fishes shed their DNA through feces, urine, mucus, blood, gametic secretions, scales, and sloughed tissues, while microorganisms may come from their entirety (Kelly et al. 2014; Barnes and Turner 2016). Information about the species, populations, and communities can be obtained by

retrieving DNA from environmental samples, such as water and sediment (Deiner et al. 2015). Thus, eDNA is proposed as a powerful tool for non-invasive monitoring and can provide complementary data for fish visual surveys.

The eDNA from water samples can be concentrated through filtration, which then can be extracted and subjected to a Polymerase Chain Reaction (PCR) and sequencing for species detection (Deiner et al. 2015). Particularly, the eDNA metabarcoding approach uses high-throughput Next-Generation Sequencing (NGS) platforms, allowing the simultaneous detection of multiple species (Beng and Corlett 2020). In this approach, the eDNA of a target taxon (i.e., fishes) is co-amplified using universal primers through PCR and affixed the amplicons with unique adapters and index sequences. The unique sets of index sequences enable massively parallel sequencing using NGS platforms with an output comprising millions of amplicon sequences from multiple sampling sites. A tentative list of species from each sampling site is then available after using bioinformatic analysis pipelines (Thomsen and Willerslev 2015; Sato et al. 2018).

Due to the cost-effectiveness and high sensitivity of eDNA metabarcoding, it has been applied to many aquatic studies for species detection, including freshwater environments (Evans et al. 2017; Nakagawa et al. 2018; Hallam et al. 2021), estuarine ecosystems (Zou et al. 2020), and marine waters (Miya et al. 2015; Yamamoto et al. 2017; Nester et al. 2020). Moreover, these studies demonstrated many fish species' successful detection and delineation. Furthermore, eDNA metabarcoding is a non-invasive tool to monitor rare and cryptic species (Goldberg et al. 2016). These organisms often elude fish visual census due to their low population size and patchy distribution (Pikitch 2018). Also, eDNA metabarcoding has useful applications in detecting the first occurrence of invasive species and the continued presence of a species thought to be extinct (Ruppert et al. 2019).

While eDNA metabarcoding offers promising applications for species monitoring, it is important to acknowledge that it is not a universal solution. Several challenges arise from using eDNA-based monitoring, including issues related to primer design, selectivity, DNA extraction, inhibition abatement, PCR methods, and the diverse nature of aquatic environments. Recent research has recognized and discussed these challenges (Van Driessche et al. 2023). However, despite these challenges, the potential of eDNA metabarcoding for marine wildlife conservation and monitoring is substantial. Hence, this study aimed to complement fish visual census by employing eDNA metabarcoding to detect and identify significant fish species within the marine protected area of Barangay Dalipuga, Iligan City, Philippines.

MATERIALS AND METHODS

Sampling site

Sampling was conducted in the marine sanctuary of Brgy. Dalipuga, Iligan City, Philippines (8.3065° N, 124.2699° E) (Figure 1) after obtaining prior informed consent and permits from the Local Government Unit (LGU) through Bantay DAGAT and the Department of Agriculture, Bureau of Fisheries and Aquatic Resources (DA-BFAR). Marine Protected Areas (MPA), such as this are the cornerstone of conservation and resource management for many marine species (Maestro et al. 2019). For this reason, the study had chosen the area for its significance in protecting Philippine marine fish species.

Fish Visual Census (FVC)

Fish Visual Census (FVC) was conducted following the protocol of Labrosse et al. (2002). Five members of the team surveyed the three sites (Figure 1). FVC focused solely on identifying fish species based on their presence or absence in the area. This approach aimed to validate and supplement the proposed eDNA method while providing supporting evidence for the obtained eDNA data.
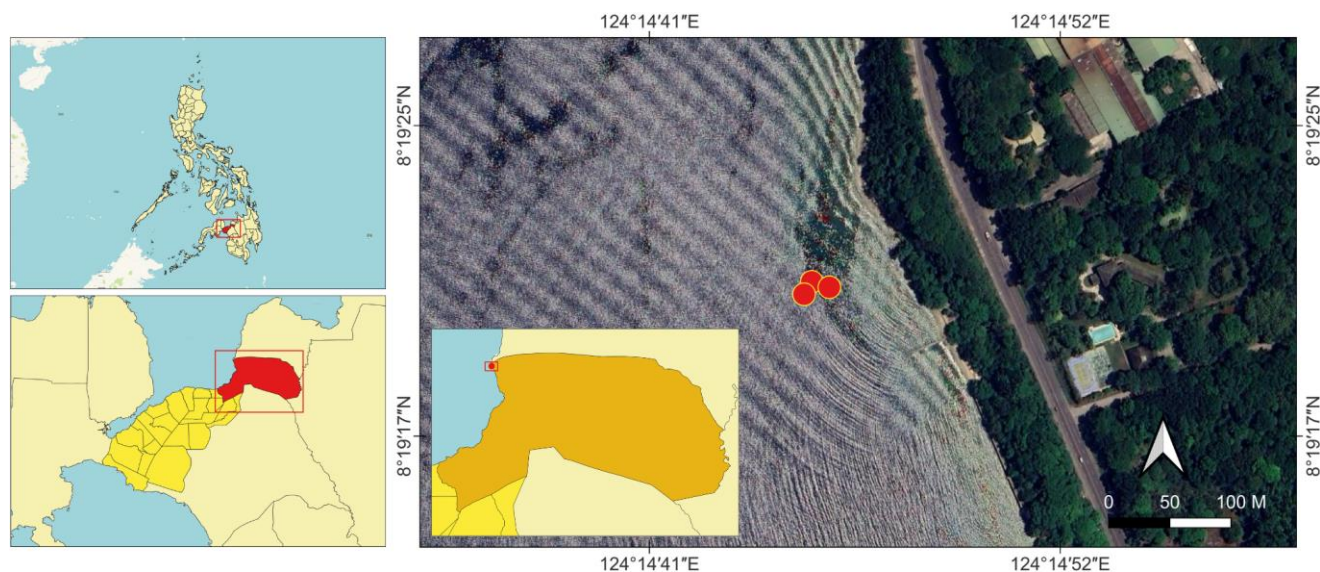

Figure 1. Map of the Brgy. Dalipuga, Iligan City, Philippines and showing the sampled sites of the Dalipuga marine protected area

Fish species were documented through snorkeling and scuba diving, and this survey was conducted once a month over a period of three months. The species were identified using the fish field guide by Gonzales (2013) and the Fishbase online database (https://www.fishbase.se/search.php) and consultation with experts in the field. Photographs were taken to aid in accurately identifying the fish species encountered during the surveys.

Collection of water samples for eDNA

Immediately after FVC, eDNA sampling was conducted. Water parameters (temperature, pH, salinity) were recorded to evaluate the viability of eDNA in the environment. Samples were collected from three sites with varying water depths (Site 1: 2-3m, Site 2: 4-5m, and Site 3: 4-6m) using bottles that were bleached and washed carefully with distilled water. A total of 20 L seawater samples were collected from each site. After collection, all seawater samples were filtered onsite using sterile 60mm Buchner funnel with a 50mm Polyethersulfone (PES) membrane (0.22 μm pore size). After filtering, membranes were placed in capped, sterile containers. Upon transit, the membranes were stored in an icebox at approximately -4°C to prevent eDNA degradation. Filter membranes were immediately brought to the Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation (CBSC), Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, for DNA extraction.

DNA extraction, amplification, and MiSeq sequencing

The eDNA from the water samples was extracted using HiPurA Water Purification Kit (HiMedia) following the manufacturer's protocol. Extracted eDNA was assessed by gel electrophoresis in Certified Molecular Biology Agarose gel (BIO-RAD) in 1X TBE buffer using Cleaver Scientific electrophoresis system (MSMINIONE). Gels were dyed with GelGreen (Ca, USA) (10,000x in water). The DNA samples were then sent to Macrogen, Korea for Metagenome Custom Amplicon Sequencing.

After quality check, 3 amplicon libraries were constructed using a custom primer set MiFish-U primer set (forward: GTCGGTAAAACTCGTGCCAGC and Reverse: CATAGTGGGGTATCTAATCCCAGTTG) based on the 12S mitochondrial DNA gene of fish species (Miya et al. 2015). PCR profile was as follows: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 98°C for 20 sec, annealing at 65°C, extension at 72°C for 15 sec, and with a final extension at the same temperature for 5 min. Sequencing was done on MiSeq 300bp PE.

Data preprocessing and taxonomic assignment

Data preprocessing and MiSeq raw reads were analyzed using the MitoFish pipeline version 3.89 (http://mitofish.aori.u-tokyo.ac.jp/mifish/) (Sato et al. 2018). The paired fastq files were uploaded to the pipeline and run through FastQC (Brown et al. 2017) for sequence quality assessment. Tail trimming was done via SolexaQA (Cox et al. 2010). Paired-end reads were merged using Fast Length Adjustment of Short Reads (FLASH) (Magoč and Salzberg 2011), and erroneous reads were removed. TagCleaner

(Schmieder et al. 2010) was used for primer sequence removal by allowing 3 base mismatches at maximum. Taxonomic assignment was done using NCBI Basic Local Alignment Search Tool (BLAST) (Camacho et al. 2009). Redundant sequences were merged into one sequence by keeping the count information. Remapping of low read-number sequences (<10) onto high read-number sequences (>10) was done at a given sequence-similarity threshold (99%), with the unmapped sequences being discarded. Blastn searches were conducted against MitoFish as a reference database with cutoff values of identity 97% and e-value 10^{-5}. Mitofish database (updated version 3.89 2023-04-08) was used for precise de novo annotations of fish mitogenomes. Then, species names of the top-hit sequences were retrieved. Moreover, the pipeline automatically analyzed and visualized biodiversity indices (Shannon, Simpson, Chao1). This provides a standardized measure of species diversity within a given ecosystem or community, providing insights into species' relative richness and evenness.

Phylogenetic analysis

To confirm the matched identities from eDNA data, a representative sequence was used to test for phylogenetic inference. Multiple sequence alignment of sequences was generated in MAFFT (Katoh et al. 2019). The best-fit evolutionary model was determined using jModel Test 2 on XSEDE based on AIC, AICc, and BIC values. Bayesian Inference was made using Mr.Bayes 3.2.2 on XSEDE (Ronquist and Huelsenbeck 2003) to infer phylogenetic relationships among species (available in the CIPRES Science Gateway v.3.3 Web Portal), using Markov chains sampled every 1000 generations. The analyses ran for 5,000 generations then Posterior Probabilities (PP) values expressed as probability percentage was calculated by Markov Chain Monte Carlo (MCMC) sampling. Bayesian Posterior Probability values (BIPP) over 0.95 were considered for BI trees, which were rooted with the outgroup. The obtained tree was viewed and edited using FigTree 1.4.0 software (Rambaut 2009).

RESULTS AND DISCUSSION

Fish visual census

The Philippines is recognized as one of the megadiverse countries in terms of fish species richness, housing over 3000 marine fish species in its waters (Allen 2015). This is in part of its contribution to the "Indo-pacific Coral Triangle", the epicenter of reef diversity (Go et al. 2015). However, the country is also considered a biodiversity hotspot due to climate change and various anthropogenic activities (Asaad et al. 2018). Thus, biomonitoring must always integrate the conservation and sustainable use of these marine resources. Based on the three sampling sites, at the time of sampling, a total of 31 fish species were documented in the marine sanctuary of Dalipuga, Iligan City based on Fish Visual Census (FVC), comprising 11 families: Pomacentridae, Acanthuridae, Chaetodonidae, Labridae, Lutjanidae, Scaridae, Synodontidae, Serranidae, Nemipteridae, Atherinidae, and Syngnathidae (Table 1). These species were found in marine environments as reef-

associated and forage fishes, with the exemption of *Pomacentrus brachialis* Cuvier, 1830 and *Hippocampus kuda* Bleeker, 1852, which can also be found in brackish waters. Despite the commercial importance in fisheries, most fish species were considered Least Concern (LC) according to the International Union for Conservation of Nature (IUCN) Red List status except *H. kuda* (highly commercial and vulnerable species) and *P. brachialis* (not evaluated). Meanwhile, *Scarus* sp. was not identified at the species level due to the cryptic morphological nature of the species (Liu et al. 2022). Moreover, the new Indo-pacific atherinomorine genus *Doboatherina* (*D. magnidentata*) was found in the area (Sasaki and Kimura 2019).

Table 1. List of fish species found in MPA of Dalipuga, Iligan City, Philippines through FVC

Family	Species	Common name	Water area	Habitat	IUCN red list status	Importance in fisheries
Pomacentridae	*Abudefduf vaigiensis*	Indo-Pacific sargeant	Salt water	Reef-associated	Least concern	Commercial
	Amblyglyphidodon curacao	Staghorn damselfish	Salt water	Reef-associated	Least concern	Minor commercial
	Amphiprion clarkia	Yellowtail clownfish	Salt water	Reef-associated	Least concern	Commercial
	Pycnochromis caudalis	Blue-axil chromis	Salt water	Reef-associated	Least concern	
	Chromis ternatensis	Ternate chromis	Salt water	Reef-associated	Least concern	Commercial
	Dascyllus trimaculatus	Threespot dascyllus	Salt water	Reef-associated	Least concern	Minor commercial
	Dascyllus reticulatus	Reticulate dascyllus	Salt water	Reef-associated	Least concern	Minor commercial
	Pomacentrus coelestis	Neon damselfish	Salt water	Reef-associated	Least concern	Commercial
	Pomacentrus moluccensis	Lemon damsel	Salt water	Reef-associated	Least concern	Commercial
	Pomacentrus vaiuli	Ocellate damselfish	Salt water	Reef-associated	Least concern	Commercial
	Pomacentrus brachialis	Charcoal damsel	Salt water, brackish water	Reef-associated	Not evaluated	
	Pomacentrus armillatus	Bracelet damselfish	Salt water	Reef-associated	Least concern	
	Centropyge vrolikii	Pearlscale angelfish	Salt water	Reef-associated	Least concern	Subsistence fisheries
Acanthuridae	*Zebrasoma scopas*	Twotone tang	Salt water	Reef-associated	Least concern	Commercial
Chaetodontidae	*Chaetodon baronessa*	Eastern triangular butterflyfish	Salt water	Reef-associated	Least concern	Commercial
	Chaetodon adiergastos	Philippine butterflyfish	Salt water	Reef-associated	Least concern	Commercial
	Chaetodon vagabundus	Vagabond butterflyfish	Salt water	Reef-associated	Least concern	Minor commercial
	Heniochos chrysostomus	Threeband pennantfish	Salt water	Reef-associated	Least concern	Minor commercial
Labridae	*Labriodes dimidiatus*	Bluestreak cleaner wrasse	Salt water	Reef-associated	Least concern	Commercial
	Thalassoma lunare	Moon wrasse	Salt water	Reef-associated	Least concern	Minor commercial
	Bodianus mesothorax	Splitlevel hogfish	Salt water	Reef-associated	Least concern	Commercial
	Epibulus brevis	Latent slingjaw wrasse	Salt water	Reef-associated	Least concern	
	Halichoeres argus	Argus wrasse	Salt water	Reef-associated	Least concern	Minor commercial
Lutjanidae	*Lutjanus decussates*	Checkered snapper	Salt water	Reef-associated	Least concern	Commercial
Scaridae	*Scarus* sp.					
Synodontidae	*Synodus variegates*	Variegated lizardfish	Salt water	Reef-associated	Least concern	Commercial
Serranidae	*Pseudanthias huchtii*	Red-cheeked fairy basslet	Salt water	Reef-associated	Least concern	Commercial
	Pseudanthias tuka	Purple anthias	Salt water	Reef-associated	Least concern	Commercial
Nemipteridae	*Scolopsis bilineata*	Two-lined monocle bream	Salt water	Reef-associated	Least concern	Subsistence fisheries
Atherinidae	*Doboatherina magnidentata*				Not Available	
Syngnathidae	*Hippocampus kuda*	Spotted seahorse	Salt water; brackish water	Reef-associated	Vulnerable	Highly commercial

Detected important fish species based on eDNA

For eDNA metabarcoding, sequences were subjected to Mitofish bioinformatic pipeline (http://mitofish.aori.u-tokyo.ac.jp/mifish/) for taxonomic assignment and data analyses. The three sites having varied depths were chosen in the marine sanctuary of Brgy, Dalipuga, Iligan City. Collected seawater samples reveal diverse fish species, with Site 3 having the most relative abundance (Figure 2A). This has been consistent with species diversity indices (Shannon, Simpson, Chao1) (Figures 2B, 2C, and 2D), indicating that Site 3 has the most fish assemblage in the area. This could be attributed to the location of the sites. Site 3 was the deepest (4-6m) area near the reef's edge towards open water. Deeper waters are less susceptible to disturbances and thus, provide refuge for many marine organisms (Pereira et al. 2018).

In contrast to the findings of FVC, a total of 24 fish species were found in the area based on eDNA metabarcoding approach, with 13 families comprising Pomacentridae, Sillaginidae, Zanclidae, Atherinidae, Clupeidae, Scaridae, Labridae, Lutjanidae, Mugilidae, Lethrinidae, Mullidae, Syngnathidae, and Balistidae (Table 2). Most of these fish species were found in marine environments, but can also be found in freshwaters (*Sillaginops macrolepis* Bleeker, 1858, *Herklotsichthys quadrimaculatus* Rüppell, 1837, *Ellochelon vaigiensis* Quoy & Gaimard, 1825, and *Planiliza macrolepis* Smith, 1846) and brackish aquatic ecosystems (*S. macrolepis, H. quadrimaculatus, Scarus dimidiatus* Bleeker, 1859, *E. vaigiensis, P. macrolepis,* and *H. kuda*). Reef fishes, such as most of these fish species, are important in reef ecosystems as they contribute directly and indirectly to the health of the ecosystem (Edwards et al. 2014).

According to the IUCN, most species were classified as Least Concern (LC) or have not been evaluated. However, FVC and eDNA detected the presence of *H. kuda*, an IUCN red-listed vulnerable species in high demand in the market. Although they are not used as human food, *H. kuda* and many species of the Syngnathidae taxa are heavily exploited for aquaria trade and Traditional Chinese Medicine (Hou et al. 2018; Foster et al. 2019). With their populations decreasing alarmingly, better biomonitoring and conservation intervention are essential for survival (Nester et al. 2020).
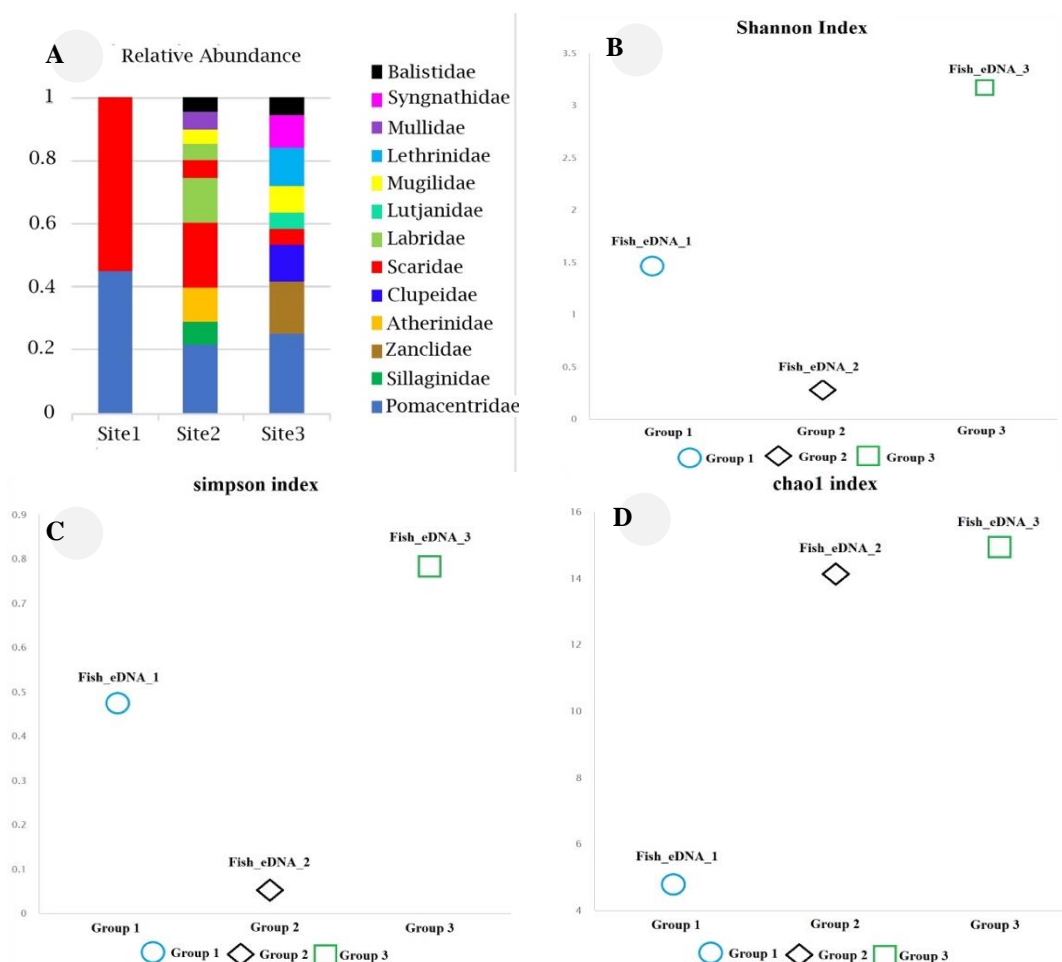

Figure 2. Relative abundance and Biodiversity indices of fish eDNA from the water samples of MPA of Dalipuga, Iligan City, Philippines. A. Relative Abundance, B. Shannon Index, C. Simpson Index, and D. Chao1 index. All indices were analyzed and visualized from the MiFish pipeline (http://mitofish.aori.u-tokyo.ac.jp/mifish/)

Table 2. List of fish species found in MPA of Dalipuga, Iligan City, Philippines detected through eDNA metabarcoding

Family	Species	Common name	Water area	Habitat	IUCN red list status	Importance in fisheries
Pomacentridae	*Abudefduf bengalensis*	Bengal sergeant	Salt Water;	Reef-associated	Least concern	Minor commercial
	Chromis xanthochira	Yellow-axil chromis	Salt Water;	Reef-associated	Not evaluated	
	Pomacentrus moluccensis	Lemon damsel	Salt Water;	Reef-associated	Not evaluated	
	Dascyllus albisella	Hawaiian dascyllus	Salt Water;	Reef-associated	Not evaluated	
	Chromis viridis	Blue green damselfish	Salt Water;	Reef-associated	Not evaluated	
	Chrysiptera unimaculata	Onespot demoiselle	Salt Water;	Reef-associated	Least concern	Minor commercial
Sillaginidae	*Sillaginops macrolepis*	Large-scale sillago	Fresh Water; Salt Water; Brackish Water;	Demersal	Not Evaluated	Minor commercial
Zanclidae	*Zanclus cornutus*	Moorish idol	Salt Water;	Reef-associated	Least concern	Subsistence fisheries
Atherinidae	*Doboatherina magnidentata*				Not Available	
Clupeidae	*Herklotsichthys quadrimaculatus*	Bluestripe herring	Fresh Water; Salt Water; Brackish Water;	Reef-associated	Least concern	Minor commercial
Scaridae	*Scarus dimidiatus*	Yellowbarred parrotfish	Salt Water; Brackish Water;	Reef-associated	Least concern	Commercial
	Scarus frenatus	Bridled parrotfish	Salt Water;	Reef-associated	Least concern	Commercial
	Scarus quoyi	Quoy's parrotfish	Salt Water;	Reef-associated	Least concern	Commercial
Labridae	*Epibulus brevis*	Latent slingjaw wrasse	Salt Water;	Reef-associated	Least concern	
	Xenojulis margaritaceus	Finspot wrasse	Salt Water;	Reef-associated	Least concern	Commercial
Lutjanidae	*Caesio caerulaurea*	Blue and gold fusilier	Salt Water;	Reef-associated	Least concern	Commercial
Mugilidae	*Ellochelon vaigiensis*	Squaretail mullet	Fresh Water; Salt Water; Brackish Water;	Reef-associated	Least concern	Commercial
	Planiliza macrolepis	Largescale mullet	Fresh Water; Salt Water; Brackish Water;	Demersal	Least Concern	Commercial
Lethrinidae	*Lethrinus ornatus*	Ornate emperor	Salt Water;	Reef-associated	Least concern	Minor commercial
Mullidae	*Parupeneus macronemus*	Long-barbel goatfish	Salt Water;	Reef-associated	Least concern	Commercial
Syngnathidae	*Hippocampus kuda*	Spotted seahorse	Salt Water; Brackish Water;	Reef-associated	Vulnerable	Highly commercial
Balistidae	*Melichthys vidua*	Pinktail triggerfish	Salt Water;	Reef-associated	Not evaluated	Minor commercial
	Balistapus undulatus	Orange-lined triggerfish	Salt Water;	Reef-associated	Not evaluated	Commercial

The eDNA approach also detected 3 species from the Scaridae family (see Table 2). Parrotfishes (family Scaridae) are often cryptic if based only on their sex and color patterns as they change during different life stages (Ebisawa et al. 2016). Furthermore, parrotfishes play a crucial role in the survival of reef ecosystems, as they consume benthic algae, participate in calcium carbonate cycling, and decompose coral skeletons into sediments (Russ et al. 2015). However, their populations have recently decreased due to overfishing as they are used as a human food resource (Edwards et al. 2014). Conservation measurements are necessary to ensure the sustainability of its wild populations and by extension, the health of the reef ecosystems.

Similarly, 2 mullet species (*E. vaigiensis* and *P. macrolepis)* from the Mugilidae taxa (see Table 2) were detected through eDNA. Mullet fishes are distributed in tropical, subtropical, and temperate regions across the globe, where they are ecologically and commercially important (Sunarni et al. 2021). However, most classical characterization and morphometric variability have poor diagnostic power or are limited when identifying these fishes (González-Castro and Ghasemzadeh 2016). Thus, it is quite impossible to accurately identify these fishes based on FVC.

There were also 2 demersal species namely Large-scale whiting (*S. macrolepis*) and Large-scale mullet (*P. macrolepis*), found in the MPA of Dalipuga, Iligan City. Demersal species are a major source of protein and are commercially valuable. For thousands of years, these fishes have comprised a large proportion of the marine harvest, providing nutrition for human consumption and contributing substantially to the economy (Riera and Delgado 2019).

Bluestripe herring (*H. quadrimaculatus*), considered native to the Philippines, was also detected through eDNA. Although this species is commercially valuable for its usage as fish bait (Oka and Miyamoto 2015), it can be potentially harmful to humans. The case study of Wu et al. (2014) reported palytoxin poisoning associated with the consumption of bluestripe herring. Patients suffered from hyperkalemia, hyperphosphatemia, acute kidney injury, and severe cardiac dysrhythmia due to palytoxin and other related compounds in the leftover fish. Thus, detecting and monitoring these fish species is necessary, especially in commercial trade.

Most importantly, a fish species belonging to the new Indo-pacific marine genus *Doboatherina* (see Table 2, Atherinidae family) was also detected. Four species were described as new to science and six species were described as members of the new genus according to Sasaki and Kimura (2019). Based on morphology, *Doboatherina aetholepis* (Kimura et al. 2002) was recorded to be found in the Gulf of Thailand, Philippines, Malaysia, Indonesia, and Papua New Guinea, while, *D. balabacensis* Seale 1910, (Seale 1910) was exclusively found in the Philippines. *D. yoshinoi* (Sasaki and Kimura 2019) was found on Yaeyama Island in Japan and Panay Island, Philippines. On the other hand, *D. magnidentata* (Sasaki and Kimura 2019) was known to be distributed along the Gulf of Thailand and the eastern coasts of Vietnam (Sasaki and Kimura 2019). However, the present study detected this novel species in the marine waters of the Philippines through FVC and eDNA. To our knowledge, this is the first record of *D. magnidentata* in the Philippines. Morphologically, wild *D. magnidentata* could be recognized by its white head and body with brilliant silvery sides, black fringed dorsal and dorsolateral scale pockets, and indigo-blue midlateral band with light yellow on the upper margin of the band (Figure 3). Particularly, this fish species has large and developed teeth on the premaxilla, vomer, and endopterygoids while other *Doboatherina* species have somewhat smaller and/or undeveloped features of these characteristics. In addition, the size range of *D. magnidentata* was around 58-76 mm (Sasaki and Kimura 2019). However, the fish species found in the MPA of Dalipuga, Iligan City size range were around 30-80 mm based on FVC, indicating that some fishes might be juveniles. The descriptions made by Sasaki and Kimura (2019) of *D. magnidentata* did not include its growth and biology thus, needs further studies to confirm this.

Moreover, obtained sequences through eDNA matched the identity *D. magnidentata* in the MitoFish database version 3.89 (http://mitofish.aori.u-tokyo.ac.jp/mifish/). To confirm this, a representative sequence from the generated eDNA data was tested for phylogenetic inference against reference sequences of *Doboatherina* species and an outgroup. Bayesian inference phylogenetic tree was constructed based on HKY+G as the best-fit evolutionary model for nucleotide substitution as determined by jModelTest2. The tree shows high Posterior Probability values (PP) expressed as probability percentages, indicating a high rate of recovery for species position in the generated monophyletic tree (Figure 3) where it verified the identity of the obtained eDNA as *D. magnidentata*. The length of the branches may represent the amount of evolutionary change or time but does not directly correspond to genetic distance.
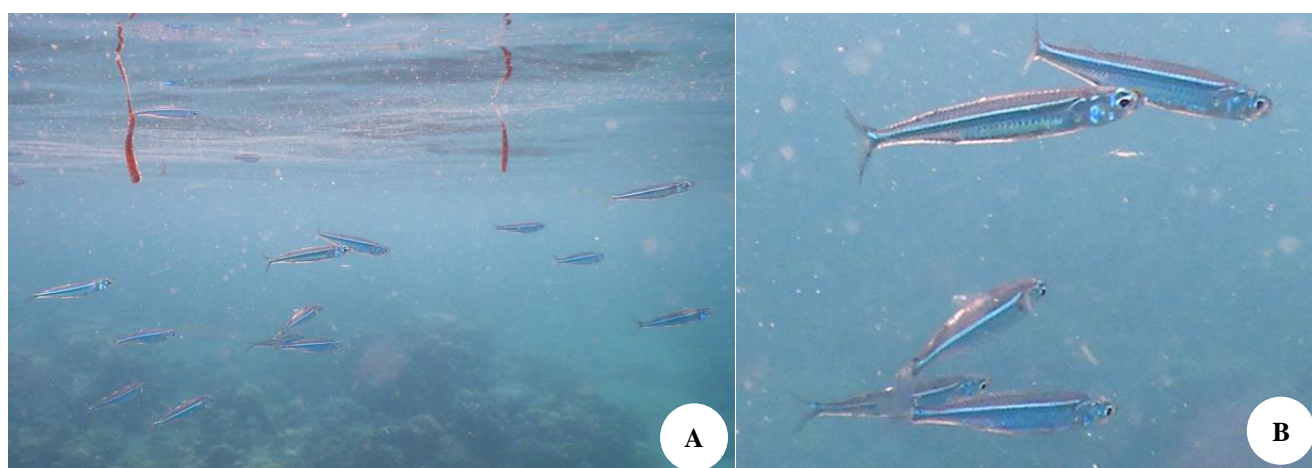

Figure 3. Images of *D. magnidentata* in their natural environment: A. School of fish (forage fishes), and B. Close-up shot of the fish found in the marine sanctuary of Brgy, Dalipuga, Iligan City, Philippines (photo by: Sharon Rose Tabugo)
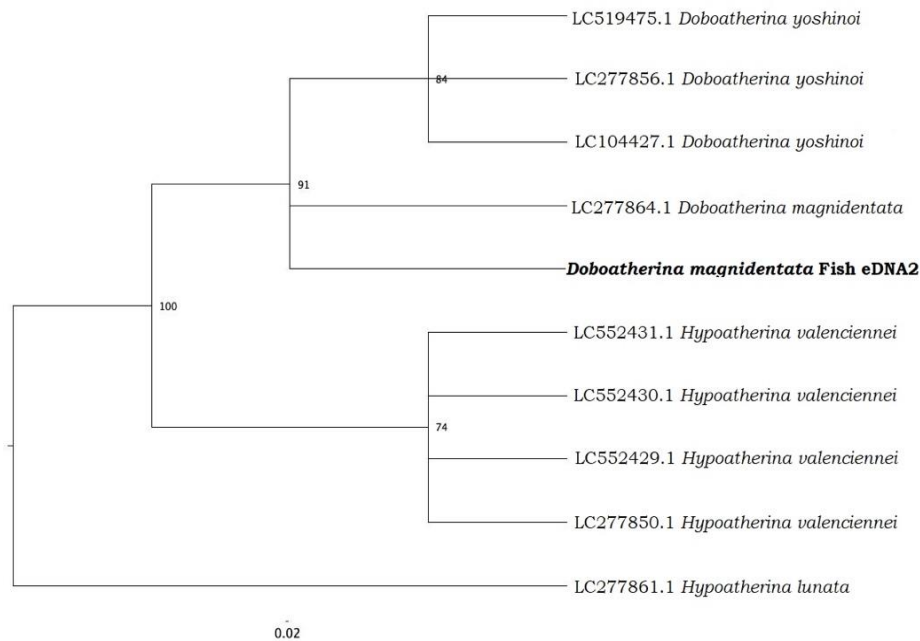
Figure 4. Phylogenetic consensus tree based on Bayesian inference analysis for *D. magnidentata* fish species detected from eDNA (Fish_eDNA2) Dalipuga, Iligan City, Philippines; Bayesian posterior probabilities are expressed as percent probabilities in nodes; *Hypoatherina lunata* (outgroup)

Most detections of eDNA were fish species that eluded the fish visual survey. However, unlike most eDNA-based studies, the eDNA metabarcoding approach also has not been able to detect most of the fish species that FVC found. This could be attributed to the decay of eDNA in the environment and the biases of the primer used. Degradation of eDNA begins the moment it is released into the environment, and its viability could vary from hours to days. Its longevity in the water is affected by temperature, pH, salinity, UV radiation, substrate, turbidity, and microbial activity (Strickler et al. 2015; Harrison et al. 2019). Water currents also affect the eDNA decay and dispersion in the environment due to exposure to sediment or biofilm precipitation which diminishes its initial concentration in the water column (Van Driessche et al. 2023). To maximize its recovery from environmental samples, primers for eDNA metabarcoding must amplify short fragments of the genetic material. Additionally, primers should be specific to the targeted taxa to avoid amplifying nontargeted groups in the environmental samples (Deiner et al. 2017). However, primer mismatches and base degeneracies are common during PCR amplification (Nester et al. 2020). Furthermore, genetic markers and primers vary across eDNA studies due to the differences in fish communities and their aquatic environments. Hence, using multiple primer pairs that vary in target genes (i.e. 12S, 16S, COI, Cytb) could improve the taxonomic sensitivity and avoid primer biases (Xiong et al. 2022).

Overall, utilization of the eDNA metabarcoding approach has provided complementary data for the limitations of the fish visual census. It detected multiple fish species that are ecologically and economically important, providing information that could improve species delineation, community assessment, biodiversity monitoring, and conservation measurements. However, the eDNA approach also has limitations. The decay of eDNA in the environment and primer biases affect the efficiency of this method. Thus, we recommend using a multigene eDNA approach alongside the classical fish monitoring methods for better taxonomic resolution and community analyses for future studies.

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