Environmental DNA (eDNA) reveals endangered narrow sawfish across Indonesian Reefs

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Abstract. Environmental DNA or eDNA is a powerful method to uncover marine organisms in the seawaters. Furthermore, many marine species are difficult to determine in the sea waters because of their rare existence based on the visual census. In this study, we implemented environmental DNA to investigate the presence of the endangered species of narrow sawfish Anoxypristis cuspidata in Indonesia. Four liters of seawater samples were collected at six locations near the coral reefs ecosystem of Indonesia and filtered at 0.45 μm filter paper. DNA was extracted from the filter paper then Polymerase Chain Reaction (PCR) amplification was performed using the cytochrome c oxidase subunit I (COI) primer and analyzed by Next Generation Sequencing (NGS). The findings revealed that narrow sawfish exist in Indonesian waters, and it also simultaneously showed that environmental DNA could detect rare species. The environmental DNA approach to identifying narrow sawfish can provide reliable results and be used as a survey tool to protect endangered threatened and protected (ETP) species.

Keywords: Biomonitoring, coral triangle, endangered threatened, protected species

1. Introduction

Sawfishes consist of five common species that belong to the shark-like rays’ family and are found in low latitudes [1]. The habitats of sawfishes are usually in shallow and coastal waters of tropical to subtropical waters in seawater and even in freshwater [2], and the body size is quite large with a length that can reach 7 meters [3]. All sawfish species have degraded in the wild and are categorized as the most endangered species [4]. There are three sawfish species in the Pristidae family that are categorized into critically endangered species, and the other two species are listed as endangered species. Considering the lack of conservation efforts, it is still a high probability that both species may also be included as critically endangered species. One of the two endangered species is narrow sawfish Anoxypristis cuspidata, known as one of the existing sawfish in Indonesia. This species has been reported as a dominant species that were caught as bycatch in fisheries activity. Globally, the decline in sawfishes' population is due to bycatch and other causes of death (i.e. the international trade of sawfish body parts, especially in the fins) [5-6]. Meanwhile, the data of existences of narrow sawfish is still lacking in the wild and rarely reported.
A full perspective of the challenge ahead worldwide sawfish populations, and the means to apply methods for conservation of these species, is limited by the ability to accurately determine their remaining extant range due to their current rarity and occurrence in remote regions [7]. Confirming the presence of the species relies on the exact location of the organisms, which can prove challenging for low population species [8]. The most used survey on the ecological study to detect organisms in the wild is direct observation (i.e. underwater visual census) [9]. It is required to do an observation on the location where the organism has been reported to exist. In fact, this type of survey has a deficiency on the inaccuracy of survey time to appearance time of the organism, and it depends on the surveyor's subjective vision. Therefore, it does not guarantee the detection of rare species that are also present (i.e., sawfish). Another study by [10] showed the detection of sawfish by local ecological knowledge and identification of nurseries had been used in many regions, but it just determined the areas of historical and presences occurrence. An effective and reliable method is still necessary to detect the existence of sawfish as a rare existence organism.

Environmental DNA (eDNA) is being studied to supplement traditional monitoring tools to improve detection and robustness [11]. Environmental DNA takes on common values of molecular identification, especially in marine areas [12-18], by extracting genetic material from environment samples (i.e., water and sediment). This method was widely used to identify reef fish [16, 19]), marine mammals [12, 20], and rare species [21-22]. Investigation using environmental DNA to detect large-tooth sawfish Pristis pristis has been conducted in Australia's northern territory [7]. It showed the ability of environmental DNA designed to detect the occurrence of large-tooth sawfish where it was present in freshwater habitat by using cytochrome c oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA). This gene has a requirement character for determining the identity of a species [23]. This study aims to disseminate the environmental DNA as a powerful method to investigate narrow sawfish Anoxypristis cuspidata as endangered species in Indonesia seawaters. This would help to maintain the important role of environmental DNA in the conservation of threatened marine species.

2. Materials and methods

2.1. Collection and processing of eDNA samples

Environmental DNA seawater samples were taken directly underwater at six locations across Indonesia (figure 1) with 14 samples, at a depth of 8-9 meters, by diving using Self-Contained Underwater Breathing Apparatus (SCUBA). Four liters of seawater samples were filtered using a vacuum pump with 0.45 µm Pall Corporation sterilized filter paper. After the filtering process was completed, the filter paper was then put into a 2 mL cryotube filled with a 1.5 mL ZymoBIOMICS DNA/RNA shield. The DNA extraction process was carried out in this study using the ZymoBIOMICS DNA extraction kit produced by Zymo Research Corporation according to the manufacturer's guidelines given. DNA concentrations for each sample were quantified using Invitrogen Qubit dsDNA HS Assay Kit produced by Thermo Fisher Scientific following the manufacturer's guidelines given. The estimation of eDNA concentration was quantified using a qubit fluorometer and showed variability concentrate of extracted eDNA between 0.10 ng/uL to 34.60 ng/uL, the minimum concentration of extracted DNA was 0.10 ng/uL and 0.11 ng/uL respectively consisted in sample SE_6_2(Selayar) and GOR_1M(Gorontalo) meanwhile the maximum was 35.60 ng/uL consisted in sample SRB_MA(Seribu Island) (Appendix A).
Figure 1. Map of six locations of Environmental DNA (eDNA) seawater samples collection across Indonesian Reefs (Jakarta; Probolinggo, East Java; Selayar, South Sulawesi; Gorontalo; North Moluccas; Misool, West Papua).

2.2. Amplification and sequencing of eDNA samples

The fragment of mtDNA was amplified with the universal COI primer mICOlintF-adapt as forward: (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GGW ACW GGW TGA ACW GTW TAY C) and dgHCO2198-adapt as reverse (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTA AAC TTC AGG GTG ACC AAA RAA) [24]. Polymerase Chain Reaction (PCR) was conducted to amplify approximately 313 bp COI region [24]. The procedure was performed 0.6 μL of 10 nM for each forward and reverse primer, 25 μL of Taq polymerase (Bioline), 22 μL ddH2O and 1 μL samples template were included on PCR reaction. First, the pre-denaturation stage was conducted at 94°C for 1 minute. Secondly, it was denatured at 94°C for 30 seconds. Afterward, the annealing stage was conducted at 41°C for 10 seconds. Following that, the first extension was done at 72°C for 1 minute. The next step was repeating the second to the fourth stage for 30 cycles. Finally, the sample was post-extension at 72°C for 8 minutes.

The sequencing was conducted on the Illumina MiSeq using the MiSeq reagent kit 500 cycles according to the Illumina MiSeq 16S metagenomic sequencing library protocol. Dual indices and Illumina sequencing adapters from the IDT for Illumina - Nextera DNA Unique Dual Indexes, Set A (catalog number 20027213) (Illumina, San Diego, USA) were added to the target amplicons in a second PCR step using Kapa HotStart HiFi 2× ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, UK). Cycle conditions were 95 °C (3 min), then 9 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s), then a final extension of 72 °C (5 min). Libraries were purified using AMPure XP beads (LABPLAN; Naas, Ireland) according to the Illumina 16S metagenomic sequencing library protocol. Libraries were measured in quantity on a Qubit fluorometric. The barcoded amplicon libraries were combined in equal concentrations into a single pool according to their quantification measurement. The library pool was diluted and denatured according to the Illumina MiSeq library preparation guide. The amplicon library (10 pM) was spiked with 20% denatured and diluted PhiX Illumina control library version 3.

2.3. Data processing and analyses with mBRAVE

Bioinformatic for Initial quality filtering of reads and species identification was performed using The Multiplex Barcode Research And Visualization Environment (mBRAVE) online software pipeline (www.mBrave.net) [25]. The data was imported into mBRAVE using the run upload function and available within the mBRAVE (Project Code: MBR-SAWFISH) (Appendix B). Every parameter described here was retrieved from the mBrave platform and was available to the user (as last accessed in August 2020). For each mBrave run, the paired end merging of MiSeq reads required a minimum 20bp overlap between the forward and reverse reads while allowing up to 5 nucleotide substitutions. The front and end parts of the resulting sequences were trimmed for 30bp to remove tags and primer, then trimmed the total sequence length down to 500bp. Next, the data were filtered for sequences of lower average QV value than
and sequences shorter than 200bp. This filtering step allowed for max 2% nucleotides with >20 QV value and max 1% nucleotides with >10 QV value. Sequences fulfilling these criteria were dereplicated and clustered as Operational Taxonomic Units (OTUs) using a 2% similarity threshold. OTUs were taxonomically assigned using an initial 2% ID distance threshold to available publicity customize reference dataset on BOLDSystem (DS-PRIST) containing sawfish sequences data, with sequential decreases of 1%, 2%, 3%, 5%, 8%, and 10% to the ID threshold for genus, subfamily, family, order, class and phylum level identifications when no matches were obtained. After selecting parameter values, mBRAVE automatically applied the same parameters to every run in our reference dataset. Each run was categorized into six sets depending on sampling locations (Jakarta, Probolinggo, Selayar, Gorontalo, North Moluccas, and Misool). All sets were generated a run summary in mBRAVE, which was checked to ensure it aligned with expectations for the reference dataset. These summaries included sequence length distribution, GC composition distribution, run QV score distribution, and BIN vs OTU count.

3. Results

Environmental DNA has been successfully amplified and sequenced from 14 samples in total six sampling locations in Indonesia. After filtering by bioinformatics, the mean length of sequenced DNA ranges about 304.17-317 bp of mitochondrial cytochrome c oxidase subunit I (COI) region. All sequences were identified as *Anoxypristis cuspidata* (figure 2) based on custom libraries DS-PRIST on Barcode Of Life Data System (BOLDSystem) that contained three species of sawfish: *Pristis pristis*, *Pristis clavata*, *Anoxypristis cuspidata*.

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** Composition of reading count (green) and unique sequences (yellow) of *Anoxypristis cuspidata* across six locations in Indonesian reefs.

Each sample in locations pointed out variability in numbers of DNA unique sequences of narrow sawfish *Anoxypristis cuspidata* with mean similarity over than 99%. A total of 2062091 reads sequence obtained from Gorontalo has 13820 unique sequences of *Anoxypristis cuspidata*. This location has the highest percentage proportion of *Anoxypristis cuspidal* unique sequence than the others. Misool, North Moluccas, Selayar, Probolinggo, Jakarta respectively consisted of 1907405, 2197521, 664365, 695717, 1517753 of total reads and 3762, 2725, 2421, 478, 288 of the unique sequences (Table 1). The unique sequence referred to a similar sequence of a run after the analytical parameter filtered. Closely related, a comparison between reading count and unique sequence of *Anoxypristis cuspidata* (figure 2) showed major variations for each sampling location. Gorontalo has the most percentage of this composition by obtaining 13820/2006825
(unique sequences of *Anoxypristis cuspidata*/read count), followed by Selayar, Moluccas, and Misool, Probolinggo, and Jakarta. This study's overall number of reading counts varied by location due to the variable total number of eDNA samples obtained.

**Table 1.** Summary of species identified *Anoxypristis cuspidata* unique sequences along with their mean length (bp) from eDNA samples across six locations of Indonesian Reefs.

| Location      | Read Counts | Unique Sequences | Mean Length (bp) | Species Identified       | Means Similarity (%) |
|---------------|-------------|------------------|------------------|--------------------------|----------------------|
| North Moluccas| 2197521     | 2725             | 313              | *Anoxypristis cuspidata* | 99.47                |
| Misool        | 1907405     | 2285             | 313              | *Anoxypristis cuspidata* | 99.45                |
| Gorontalo     | 2006825     | 13820            | 313              | *Anoxypristis cuspidata* | 99.37                |
| Jakarta       | 1517753     | 288              | 313              | *Anoxypristis cuspidata* | 99.61                |
| Probolinggo   | 695717      | 478              | 313              | *Anoxypristis cuspidata* | 99.42                |
| Selayar       | 664365      | 2421             | 313              | *Anoxypristis cuspidata* | 99.42                |

4. **Discussion**

This study conducted an environmental DNA method as a genetic approach to identify narrow sawfish in Indonesian reefs. We found the existence of narrow sawfish *Anoxypristis cuspidata* in six locations across Indonesian reefs. The existence of narrow sawfish *Anoxypristis cuspidata* inferred from DNA material in seawater samples of the environment. All DNA material was successfully extracted refers to measured DNA concentration. All samples are successfully amplified and sequenced, then identified into species (*Anoxypristis cuspidata*) based on the nucleotide base sequences. The molecular approach delivers more dependable results by giving the percentage number of similarities to the database, ensuring correctness and confidence level. This study shows that all unique sequences of *Anoxypristis cuspidata* have a percentage number over 99% of similarity, giving more accurate identification of the data [26]. Utilizing molecular identification gives a chance to conduct an efficient survey and assessment on the identification of marine organisms by giving more accurate and reliable data. By conducting genetic approaches, enhancing species identification would be more convenient and accurate to detect organisms in marine areas.

The environmental DNA was very successfully used to identify more species in the environmental area [19] and is the most common method of inquiry for assessing biodiversity in aquatic environments compared to traditional methods [27-30]. The current method (i.e., direct observation) such as Underwater Visual Census (UVC), Remotely Operated Vehicle (ROV), hydrophone, photography was used to reveal nektonic marine organisms (e.g., fish and mammals) but has limited ineffectiveness on monitoring and identifying the high number of organisms. This kind of method depends on the surveyor's subjective vision and survey time. Observation based on morphological identification methods requires expertise and experience in identifying, and the phenotypic plasticity of taxa has the probability of leading to false identity [31], allowing a gap of reliable data. Otherwise, Environmental DNA appeared as a solution and was convenient to survey biodiversity assessment. This method brings more accurate identification results because it is based on a molecular approach. The molecular approach to identification has a high level of trust to determine organisms into species based on the sequence of nucleotide bases. The sequence gives information about species as a genetic code, and it is recognized as a barcode then identified on the database (e.g., National Centre of Biotechnology Information, Barcode Of Life Data System). The mitochondrial COI gene is commonly used as a species barcode since it is heavily conserved within organisms and common genetic pattern variability between different species [23]. This method attempts to find the limits of species classification, which corresponds to the difference between neighbors closest in a group [23, 32].

Two of five species of the sawfish family (Pristidae) are categorized into critically endangered species, and it is still a high probability that narrow sawfish *Anoxypristis cuspidata* may also be included as critically endangered species by considering the lack of conservation efforts. The narrow sawfish *Anoxypristis cuspidata* population was degraded then categorized as endangered species based on the International
Union for Conservation of Nature (IUCN) in 2013 [33]. Evaluation needs to be conducted in order to give more information and validation of this species’s existence.

By comparing the read count and unique sequence, high amounts of unique sequence read of Anoxypristis cuspidata were found in Gorontalo. It is still on the highlight that the overall number of reading counts in this study was varied from all locations. The unique sequence read could represent the relative abundance of the sampling location area; knowing the relative abundance would give insight into which location has a more specific organism (i.e., sawfish). However, Indonesia’s eastside area (Gorontalo, Selayar, North Moluccas, Misool) has a more recognized existence of Anoxypristis cuspidata than the westside area (Probolinggo, Jakarta). The westside of Indonesia, such as Probolinggo and Jakarta, are recognized as having anthropogenic impacts compared to other locations in this study. These two areas are located in Java Island, known as the island with the highest human population in Indonesia. Anthropogenic activities (i.e., fishing, transportation, recreation) put pressure on marine organisms in their habitat [34]. By comparison to the west side of Indonesia, the east side of Indonesia (Gorontalo, Selayar, North Moluccas, and Misool) have low anthropogenic activities based on the human population in the area.

The geographical distribution of threatened marine species (e.g., sawfish) is needed to be updated. However, it would be difficult to determine the geographic area of all individuals within a species, including those of decimated species, because the rareness makes them difficult to detect [4]. Recently, threatened marine organisms, particularly in endangered species: whale sharks [19], sawfish [7], marine skates [35], and marine fishes, have been reported success in the detection of the existences in aquatic environments by using environmental DNA. In linear to that, this study shows the environmental DNA in detecting the existence of sawfish Anoxypristis cuspidata. It disseminates environmental DNA as a powerful tool to investigate rare existing and endangered threatened marine species.

5. Conclusion
In conclusion, the environmental DNA was successfully used to identify rare species of narrow sawfish Anoxypristis cuspidata. This method would be an effective tool for investigating the extant range of rarity occurrence organisms (i.e., sawfish) in the role of enhancing and maintaining conservation of threatened marine species.

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Appendices
Appendix A. eDNA concentration showing the amount of DNA based on water samples.

| Location  | Sample Id | Extracted DNA Concentration(ng/μL) |
|-----------|-----------|----------------------------------|
| North Moluccas | MLK_HI     | 0.33                             |
| North Moluccas | MLK_PJ     | 2.07                             |
| North Moluccas | MLK_MA     | 2.58                             |
| North Moluccas | MLK_TI     | 0.9                              |
| Misool     | MSL_20     | 3.09                             |
| Misool     | MSL_3      | 1.21                             |
| Misool     | MSL_10     | 0.66                             |
| Gorontalo  | GOR_3M     | 0.11                             |
| Gorontalo  | GOR_PYT    | 1.42                             |
| Gorontalo  | GOR_1M     | 0.11                             |
| Jakarta    | SRB_MA     | 35.6                             |
| Jakarta    | SRB_TP     | 3.38                             |
Appendix B. Multiple Barcode Research and Visualization Environment (mBRAVE) project, analytical parameters, and datasets applied.

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