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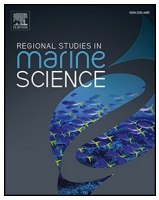

Preliminary characterization of coral reef diversity using environmental DNA in a hyper-diverse context☆

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ABSTRACT

Monitoring coral reef communities remains a scientific cornerstone of marine biodiversity conservation and management. Environmental DNA (eDNA) has recently emerged as a passive detection tool to enumerate organisms in dynamic marine systems. The utility of eDNA to provide meaningful results for coral reef conservation and management in Indonesia, a hyper-diverse and data-limited tropical area, however, remains poorly understood. In this study, we conducted seawater and sediment eDNA surveys in three regions of Indonesia and amplified them using a general metazoan primer. We found that eDNA revealed a diverse array of reef organisms overall (>11,000 amplicon sequence variants, or ASVs), including species of management importance (i.e., sharks and mollusks) that are otherwise missed by conventional surveying methods. Most fish and coral eDNA detections were verified by our concurrent visual surveys of those taxonomic groups, demonstrating the utility of these two methods as complementary approaches for broad-scale reef bioassessment. Almost half of the ASVs, however, were unclassified Eukaryotes. We estimated that with our eDNA protocol, 119–197 water samples and 259–375 sediment samples would be needed to recover the complete suite of operational taxonomic units (a genetic proxy for species) in each region. Despite not reaching the full saturation of potential diversity or taxonomic detection within regions, eDNA sampling revealed community compositions were regionally distinct, even though water and sediment samples differed in community structure and exhibited high heterogeneity. Our precursory examination of a taxonomically-broad eDNA survey in Indonesia highlights the short and long-term utility of the method as a confirmatory and complementary tool for biomonitoring, research, and conservation purposes. Nonetheless, more rigorous sampling, methodological refinements, and database curation are needed for eDNA to reach its full potential in Indonesia.

☆ We are saddened by the recent passing of our beloved co-author, Dr. Hawis Madduppa, who made this study possible.

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1. Introduction

The systematic data collection on species distributions and abundances remains an essential component of research for conservation, especially as unprecedented losses of biodiversity occur worldwide (Butchart et al., 2010). Coral reefs harbor one-third of the ocean's marine biodiversity, provide numerous ecosystem services, and are threatened from a variety of local and global anthropogenic stressors (Knowlton et al., 2010; Costello, 2015; Brandl et al., 2019). In many areas of the world, however, there are social, economic, and political barriers to comprehensive biodiversity inventories (Collen et al., 2008; Tolochko and Vadrot, 2021). To catalog species more efficiently, some researchers are testing environmental DNA (eDNA) metabarcoding, an emerging high-throughput sequencing methodology in which a diagnostic region of a gene (barcode) can be sequenced to identify multiple species at once from a single environmental sample. In high-diversity systems like coral reefs, it has been suggested that eDNA may be able to enumerate more species than is possible with visual observation or other, more conventional techniques of quantifying biodiversity (Miya, 2021; Richards et al., 2022).

Baseline eDNA studies have been conducted in several coral reef ecosystems, including the Red Sea (DiBattista et al., 2017), Northwest Australia (West et al., 2020; Alexander et al., 2020; Dugal et al., 2022), Florida Keys (Sawaya et al., 2019), Hawai'i (Nichols and Marko, 2019), Indian Ocean (Dunn et al., 2022) and Caribbean (Nguyen et al., 2020; Fernández et al., 2021). Studies have also been conducted across coral reef ecosystems in multiple ocean basins (Mathon et al., 2022). These studies collected seawater for eDNA and targeted organisms ranging from phytoplankton (Sawaya et al., 2019) to sharks (Dunn et al., 2022; West et al., 2020), as well as scleractinian corals (Nichols and Marko, 2019; Alexander et al., 2020; Dugal et al., 2022). When compared to visual surveys of fishes and corals, eDNA surveys generally had low to moderate overlap in species detections ($\leq$ 50% in most cases) and complemented existing species inventories by capturing sometimes more, though taxonomically unique, compositions of fish and coral species using both taxa-specific and broad primers (Alexander et al., 2020; Nguyen et al., 2020; Fernández et al., 2021; Dugal et al., 2022; Mathon et al., 2022).

Understanding what can and cannot be interpreted with coral reef eDNA sampling is a key component to larger discussions regarding marine eDNA's applicability in biodiversity conservation and management (Hansen et al., 2018; Ruppert et al., 2019; Huerlimann et al., 2020). Currently, there is a growing interest in expanding the scope and scale of eDNA technologies for fishes and other marine species (Davies et al., 2012; Miya, 2021; Ausubel and Stockle 2021; Ficetola and Taberlet, 2023). In fact, the third Research and Development area of the United Nations Decade of Ocean Science for Sustainable Development (2021–2030) includes piloting eDNA as a biotic component of the Global Ocean Observing System (Ryabinin et al., 2019). The vast majority of eDNA representatives advocating for such efforts, however, come from temperate Global North contexts where biodiversity infrastructure (e.g., labs, databases, and voucher specimens) are well-established (Hansen et al., 2018; Belle et al., 2019; Tolochko and Vadrot, 2021). Most biodiversity, however, is in the tropical Global South. Despite their high biodiversity, tropical marine ecosystems have not been a focus of Western biodiversity research and investment (Collen et al., 2008; Tolochko and Vadrot, 2021). Shifting paradigms and technologies for biodiversity monitoring may thus continue to leave out the perspectives and ecologies of those who belong to the most biodiverse places on Earth (Partelow et al., 2020).

An example of this asymmetry can be observed in Indonesia. While it is widely considered the epicenter of marine biodiversity (Allen and Erdmann, 2012), Indonesia has had relatively few studies on its coral reefs compared to other regions (Fisher et al., 2011). Located in the heart of the Coral Triangle, Indonesia has estimates of ~1,638 species of fishes and ~577 species of corals (Allen and Erdmann, 2012). While scientific interest in Indonesia's biodiversity goes back to early studies of biogeography with the center of origin, accumulation, and overlap hypotheses of the mid-20th century (e.g., Ekman, 1953; Ladd, 1960; Woodland, 1983), present biodiversity censusing efforts are still mainly done visually by a relatively low number of local and international researchers. More recently, however, there has been interest in using conservation genetics in the Indo-Pacific (Willette et al., 2014, von der Heyden et al., 2014).

We present the results from a baseline attempt to broadly survey marine animal biodiversity using eDNA from seawater and sediment samples on coral reefs in Indonesia. The results of this project are followed by some discussion on lessons learned about the sampling infrastructure required for continued eDNA biomonitoring in the area. We hypothesized that eDNA would exhibit regional patterns of alpha diversity and community compositions encompassing a broad taxonomic range using an easy-to-use and popular primer targeting metazoans. We expanded on recent coral reef eDNA studies in Indonesia that focused on fishes (Juhel et al., 2020; Marwayana et al., 2021; Gelis et al., 2021), Symbiodiniacaeae (Pratomo et al., 2022) or particular metazoan groups (i.e., chordates, mollusks, and echinoderms Madduppa et al., 2021) by analyzing the effect of eDNA substrate (water or sediment) on characterizing communities across a wide spatial and taxonomic range. In addition, we demonstrated the utility of using concurrently collected visual surveys of fishes and corals as a field benchmark for our eDNA detections, which helped validate taxonomic assignments of conspicuous and socio-culturally important groups in the face of incomplete reference databases and assignment errors (Juhel et al., 2020).

2. Methods

2.1. Study context

We conducted surveys between January 2018 - May 2019 in three regions of Indonesia: Lombok (July 2018), Misool (May 2019), and Waigeo (January 2018) (Fig. 1). We selected 20 sites that varied in socio-environmental characteristics. Each region has a combination of multi-gear and multi-species coral reef fisheries managed with MPAs and various gear restrictions (McKenna et al., 2002; Exton, 2010; Humphries et al., 2019; Campbell et al., 2020). SCUBA divers conducted eDNA collections and visual surveys of biodiversity concurrently at each site, along the same transects. All lab work (except for sequencing) was conducted in-country at Bogor Agricultural University on the island of Java.

2.2. Environmental DNA (eDNA) surveys

2.2.1. Sample collection

For each site, SCUBA divers collected a 4-L seawater sample at ~5 m depth and a surface sediment sample at ~10 m depth. Sampling was performed to maximize the utility of the collections across multiple simultaneous studies from field locations; thus, divers took the top >5 cm of sediment to ensure sampling of the oxic layer (Pawlowski et al., 2021; Brandt et al., 2021). Water and sediment samples were then filtered using a peristaltic pump through 12 μm and 0.4 μm polycarbonate filters 47 mm in diameter (Sterlitech). This study focuses on results derived from the 0.4 μm filters to focus on the size of target DNA (see Pratomo et al., 2022 and Borbee et al., 2022 for results from 12 μm filters, which captured larger planktonic cells). While all 4-L of seawater samples were filtered, sediment samples were shaken to suspend the sample then filtered until filters clogged which often occurred after 1–2 L (Borbee et al., 2022). Filters were cut in half, preserved in DNA/RNA Shield (Zymo Research, Irvine, CA), and stored at room temperature until transported back to the lab for DNA extraction. Tools were rinsed and sterilized with bleach or hot freshwater between samples (depending on local availability and access to reagents) (Kampmann et al., 2017), and regions were sampled in different months and

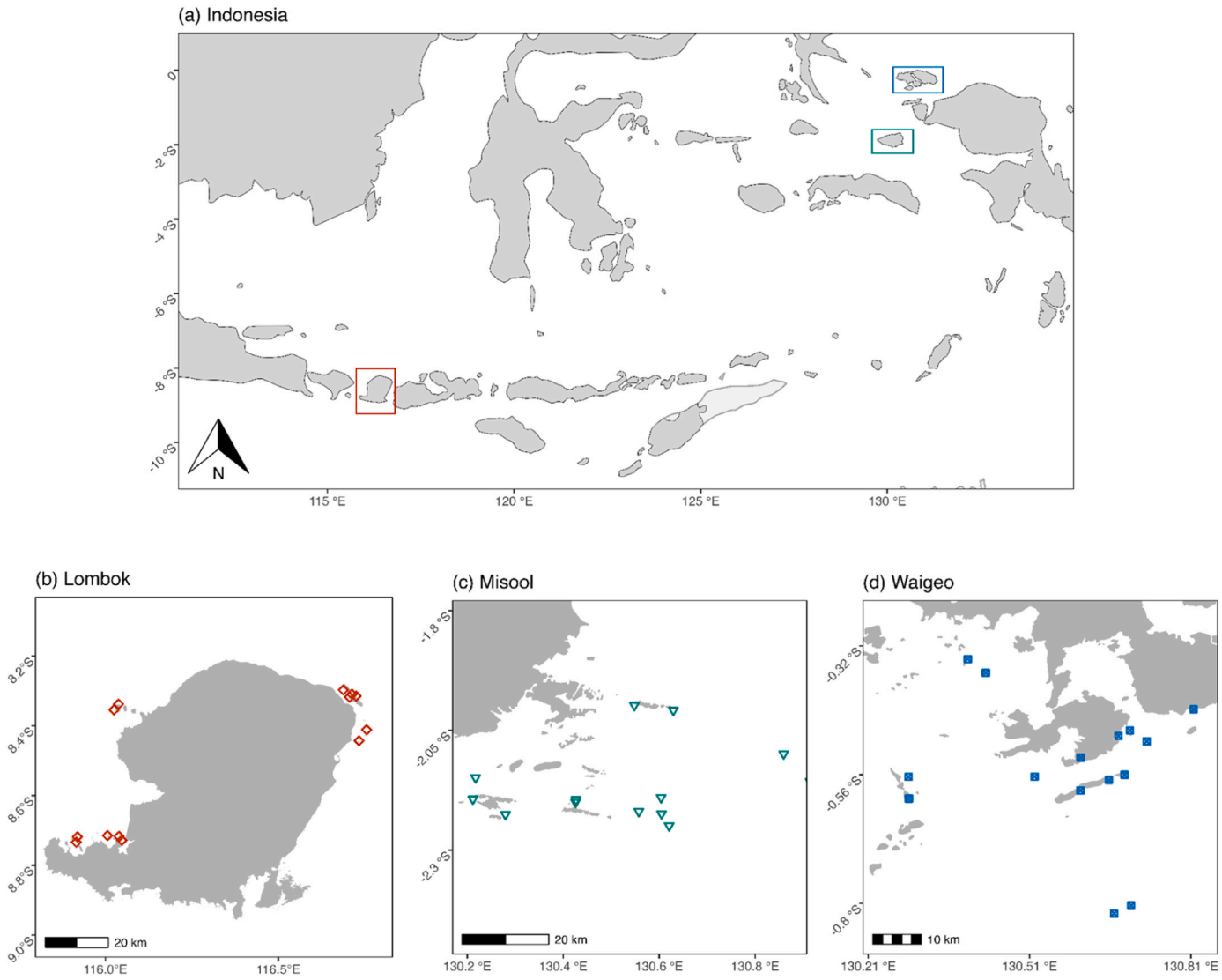

Fig. 1. Maps of (a) Indonesia with study regions boxed in region-specific colors used throughout the study, (b) Lombok (red diamond sites, n = 13), (c) Misool (green triangle sites, n = 13), (d) Waigeo (blue square sites, n = 15). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

years. Due to the remote nature of study sites and travel restrictions, proper field controls (e.g., sterile seawater samples) were sometimes not attainable. Thus, to be conservative, we treated sites within regions as replicates in eDNA analyses (described below) to account for the possibility of site-to-site contamination and the more general uncertainty around spatiotemporal variation and autocorrelation in eDNA datasets (Mathieu et al., 2020).

2.2.2. *DNA extraction, amplification, and sequencing*

DNA was extracted from filters with the ZymoBiomics DNA Miniprep kit (Zymo Research, Irvine, CA). DNA extracts were amplified with the m1COIintF/jgHCO2198 PCR primers, which target a 313 bp region of the cytochrome c oxidase subunit I (COI) gene (Leray et al., 2013). We chose this primer because of its ease of use, especially in benchmark tropical eDNA studies (Nguyen et al., 2020), ability to target a wide range of metazoans (multicellular eukaryotes), as well as its popularity as a taxonomic marker in reference databases (Andújar et al., 2018). Each 50 μL PCR reaction contained 25 μL of MyTaq Red Mix (Meridian Bioscience, Cincinnati, OH), 1 µL of each primer, 1 µL of DNA extract, and 22 µL of PCR water. A mock community was prepared with lab-cultured stramenopile lineages as a positive PCR control (see Borbee et al., 2022 for details) and DI water was used as negative controls. Amplification of the correct barcode was confirmed with gel electrophoresis before being sent off for 2×250 bp sequencing on an Illumina MiSeq with a version 2 MiSeq Reagent Kit (Illumina, San Diego, CA) at the Rhode Island Genomics and Sequencing Center in Kingston, Rhode Island (USA). The data for this study comes from partial subsets from two 2×250 MiSeq runs that were bioinformatically processed separately for run-specific quality control steps, then combined for taxonomic assignment.

2.2.3. *Bioinformatic processing and taxonomic assignment*

We trimmed sequencing adapters and primers with Trimmomatic (v. 0.39) (Bolger et al., 2014) and Cutadapt (v. 1.9.1) (Martin, 2011), respectively, and checked for successful trimming with MultiQC (Ewels et al., 2016). We also removed reads that were under 50 bp in length. We filtered, merged, inferred exact amplicon sequence variants (ASVs) with DADA2 (v. 1.18.0) (Callahan et al., 2016) in R (v. 4.0.3) (R Core Team, 2020) for each run separately, which allowed us to set specific parameters based on their unique error quality profiles. The ASV sequence tables were then combined for chimera removal and subsequent taxonomic assignment to detect species or families of interest. To assign

taxonomy at ≥97% identity to the ASVs, we used the CO1v4 database by Porter and Hajibabaei (2018), which contains COI sequences mined from GenBank and BOLD databases in April 2019, with a naive Bayesian classifier (Wang et al., 2007). Sequences that remained unassigned were compared against the NCBI NT database (retrieved May 2020) using BLAST (word size = 11; max e-value = 1e-20) and assigned taxonomy of the last common ancestor that is shared by 80% of all hits at ≥97% identity with BASTA (Kahlke and Ralph, 2019). After examining the taxonomic output from multiple cut-off values (ranging from 95% to 99%), we identified 97% as an appropriate threshold for taxonomic assignment that appropriately balanced trade-offs between assignment confidence and taxonomic resolution in our study system (Gold et al., 2021). In addition, we verified that taxonomic assignments to bony fishes (class Actinopteri) and sharks (class Chondrichthyes) had ranges in Indonesia with FishBase (Froese and Pauly, 2021). Because of low interspecies variation among stony coral species (Shearer and Coffroth, 2008), we examined coral detections at the family-level between eDNA and visual surveys, to be conservative.

For taxonomy-independent alpha and beta-diversity analyses, we clustered ASVs into operational taxonomic units (OTUs) at 97% similarity with VSEARCH (Rognes et al., 2016) and curated them (reducing the redundancy of co-occurring sequences for better richness estimates) with the LULU algorithm (Frøslev et al., 2017). We chose to use OTU clustering as a proxy for meta-genetic "species" for biodiversity analyses as a complement to our taxonomic assignment procedure using ASVs (a proxy for haplotypes) to account for the high intraspecies variability in the COI marker (Antich et al., 2021).

For both ASV and OTU count tables, we considered families of terrestrial or freshwater organisms in our dataset (i.e., humans, birds, cows, cockroaches) as contaminants and removed them (OTUs were assigned taxonomy with the CO1v4 database and naive Bayesian classifier for this sole filtering purpose). All input files and scripts can be found on Github (github.com/elaine-shen/Indo_eDNA_RSMS).

2.3. Visual surveys

We conducted visual surveys of corals and fishes to offer confirmatory support for the eDNA detections. To obtain fish counts, divers conducted underwater visual censuses by swimming along a 5×150-meter transect (750 m^2) on fore-reefs 3–10 m in depth. Fish were identified to the highest taxonomic resolution possible. The diver swam across the entire length of the transects twice: first to identify fish that are diver-shy, then those that are more neutral in response to human presence (Kulbicki, 1998). To obtain coral counts, another diver used the point-intercept method at 0.5-meter intervals along the same transect to identify the coral or benthos under the transect to the highest taxonomic resolution possible (Hill and Wilkinson, 2004).

2.4. Statistical analyses

For alpha diversity and community composition analyses, eDNA datasets were imported into R as count tables (species x site) with accompanying taxonomy tables and metadata into phyloseq (v. 1.27–6) (McMurdie and Holmes, 2013) and analyzed with vegan (v. 2.5–7) (Oksanen et al., 2020) and microbiome (v. 1.12.0) (Lahti et al., 2017). We generated rarefaction curves that estimated extrapolated and asymptotic richness, as well as measured regional sampling coverage using the iNEXT package (v. 2.0.2) (Hsieh et al., 2016). Extrapolated richness values are reliable up to double the observed sample size, whereas asymptotic richness represents a lower bound (an underestimation) of richness estimates (Chao et al., 2014). Taxa not identified to the species level (e.g., to only the genus or family level) were treated as a single additional pseudo-species in diversity analyses. We used Wilcoxon tests for comparisons of alpha diversity metrics among pairs of regions. Plots and maps were generated with ggplot2 (v. 3.3.5) (Wickham, 2016) and the package extension ggspatial (v. 1.1.5) (Dunnington, 2021).

To examine sampling design effects (i.e., region, date sampled) on the community composition of sediment and water eDNA samples, along with the full eDNA dataset, we performed permutational multivariate analysis of variance (PERMANOVA) on a Bray-Curtis distance matrix of Hellinger-transformed OTU counts (Legendre and Gallagher, 2001; Bagley et al., 2019). We chose PERMANOVAs for analysis of community compositions because they are less sensitive to correlation structures (in comparison to analysis of similarities, or ANOSIM, and Mantel tests) and generally robust to unbalanced sampling designs when dispersions are homogeneous (Anderson and Walsh, 2013). We used permutation tests for homogeneity of multivariate dispersions (PERMADISP2) in vegan to check that we were measuring location effects of groups rather than dispersion effects (Anderson, 2006; Anderson and Walsh, 2013). These distances were then visualized in an ordination using Principal Coordinates Analysis (PCoA). Analyses on eDNA ASVs subset to its metazoan and non-metazoan components, when possible, are available in the Supplementary Section. We also include summary information and analyses on the visual surveys of fishes and corals at the end of the Supplementary Section to help put our eDNA sampling effort in the context of conventional biodiversity surveys.

3. Results

The eDNA dataset had 92/120 seawater and sediment samples successfully sequenced, filtered, denoised, and checked for chimeras (5,303,326 raw reads to 2,897,874 high quality reads, 54.64% retained before removal of contaminant ASVs). Of these 92 samples, 82 were selected for downstream analysis because they had corresponding visual surveys of fishes to confirm eDNA detections (Fig. 1, Table 1). Negative controls and mock community PCR results conformed to our expectations and are further explained in Borbee et al. (2022). Out of 31,528 total ASVs, we retained 11,013 ASVs, which represented ASVs that were given a taxonomic assignment at ≥97% and were not contaminants (Table 1, Fig. 2). All ASVs were also clustered into 10,985 OTUs for

Table 1
Summary table of eDNA surveys by region and sample type, as measured by the number of samples successfully processed, observed species richness and abundances, as well as estimates of extrapolated and asymptotic richness estimates, sampling coverage (SC), and the number of samples needed for full SC from iNEXT.

	Lombok	Misool	Waigeo
Seawater eDNA			
# Seawater samples	13	13	15
Observed ASVs	3143	5874	4918
Filtered ASV abundance	235,152	325,574	686,973
Observed OTUs	943	1084	1282
Extrapolated richness estimate	1246.585	1491.491	1809.135
Asymptotic richness estimate	1556.946 ± 82.656	2073.538 ± 124.917	2499.302 ± 132.045
Filtered OTU abundance	232,669	317,473	682,671
SC of species richness (%)	87.54%	85.67%	79.55%
# samples needed for >99.9% SC of species richness	119	175	197
Sediment eDNA			
# sediment samples	13	13	15
Observed ASVs	6900	12,384	5161
Filtered ASV abundance	357,313	358,687	588,976
Observed OTUs	4474	5404	2668
Extrapolated richness estimate	7705.650	8817.869	4389.188
Asymptotic richness estimate	18,944.291 ± 850.071	16,633.975 ± 560.020	8950.654 ± 460.996
Filtered OTU abundance	356,521	349,722	587,300
Sample coverage (%)	43.38%	53.55%	55.23%
# samples needed for >99.9% SC of species richness	375	259	334

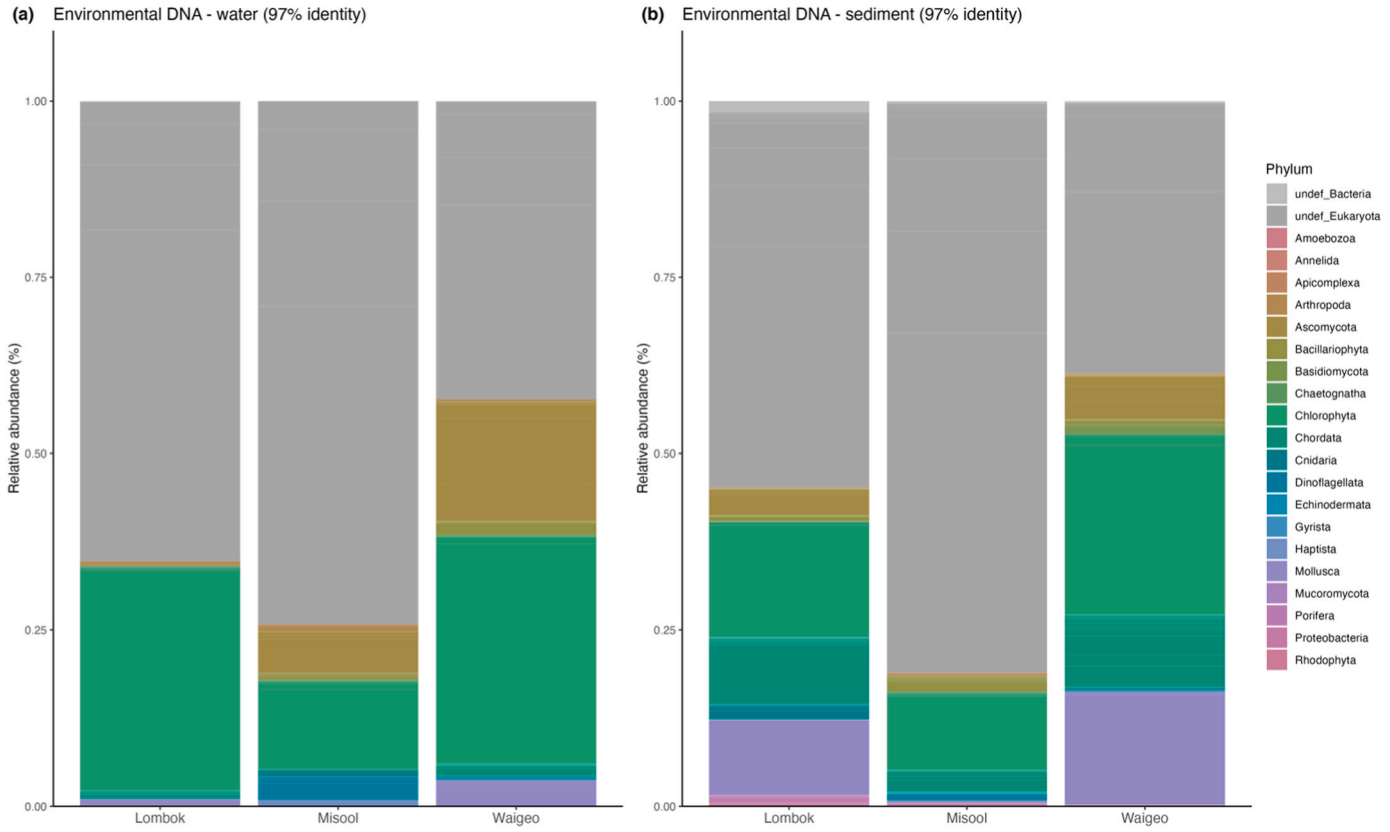

Fig. 2. Stacked relative read abundance bar plots for each region at the phylum level for (a) seawater eDNA ASVs and (b) sediment eDNA ASVs. Taxonomic assignment was conducted at 97% identity.

taxonomy-independent analysis (Table 2). Rarefaction curves indicate that sequencing depth was sufficient on a per-sample basis (Supplementary Section SI). To address potential site-to-site contamination, we aggregated sites together in our analyses so they represented regional replicates and report taxonomic detections at the regional level. Regionally, the observed eDNA sampling coverage for water samples was higher (>79% for all regions) than in sediment samples (>43% for all regions) (Table 1). Estimates for complete eDNA sampling coverage (≥99.9%) ranged from 119 to 197 water samples to 259–375 sediment samples per region (Table 1).

Table 2
PERMANOVA results for the full eDNA dataset and the eDNA dataset partitioned by sample type (water or sediment). Count data were Hellinger-transformed before Bray-Curtis matrices were generated for analysis. Asterisks represent p-values equal to or lesser than 0.0001 (****), 0.001 (***), 0.01 (**), and 0.05 (*).

	Term	DF	SS	R^2	F model	Pr (>F)	
eDNA, full	Region	2	1.2732	0.06089	2.5461	0.001	***
	Date	16	5.1274	0.24279	1.2817	0.003	**
	Sample type	1	0.2162	0.01024	0.8647	0.687	
	Residual	58	14.5023	0.68669			
	Total	77	21.1192	1.00000			
eDNA, water samples	Region	2	1.2665	0.16433	5.4111	0.001	***
	Date	16	4.1001	0.53199	2.1897	0.001	***
	Residual	20	2.3406	0.30369			
	Total	38	7.7072	1.00000			
eDNA, sediment samples	Region	2	1.4187	0.11513	2.6745	0.001	***
	Date	16	5.5990	0.45438	1.3194	0.001	***
	Residual	20	5.3046	0.43049			
	Total	38	12.3224	1.00000			

623 ASVs out of 11,013 total classified ASVs (5.56%) in the eDNA dataset were classified as metazoans. (Supplementary Table A1). Unidentified Eukaryotes at the phylum level comprised 48.99% of the total ASV dataset (Supplementary Table A2). Overall, the eDNA samples were dominated by green algae, fungi, and mollusk phyla, as well as other reef-associated phyla such as sponges, arthropods, and cnidarians (Fig. 2, Supplementary Table A2). The eDNA detected 143 fish ASVs belonging to 47 species or pseudo-species, 30 of which were confirmed to occur in our study regions with our visual surveys of fishes, and 17 of which were unique (Supplementary Table A3). In addition, we detected 13 species or morphospecies of sharks and rays (61 unique ASVs) detected in Lombok and Waigeo (primarily in sediment samples), three of which (*Alopias pelagicus, Carcharhinus brevipinna, Rhincodon typus,* 100% ID for all three) are classified as Endangered by the IUCN (Supplementary Table A4). These species were not observed by divers in our visual surveys. The four family-level eDNA detections of stony corals (and one order-level eDNA detection of a Scleractinian coral) were all confirmed to occur in our study regions with our visual surveys of corals (Supplementary Table A5). While the second and third most visually abundant stony coral families, Poritids and Pocilloporids, respectively, were also detected in the eDNA dataset, the most visually abundant stony coral family, Acroporids, was not detected in eDNA samples. Overall, the visual surveys of corals uniquely detected 15 families of stony corals.

Average site-level values and abundance-weighted diversity metrics among regions did not differ for water eDNA samples, except for Shannon's diversity between Misool and Waigeo (Fig. 3). In sediment eDNA samples, Misool on average had higher observed richness values, Shannon's diversity, and evenness than Waigeo ($p < 0.05$, Fig. 3). These patterns are generally consistent with alpha diversity comparisons of

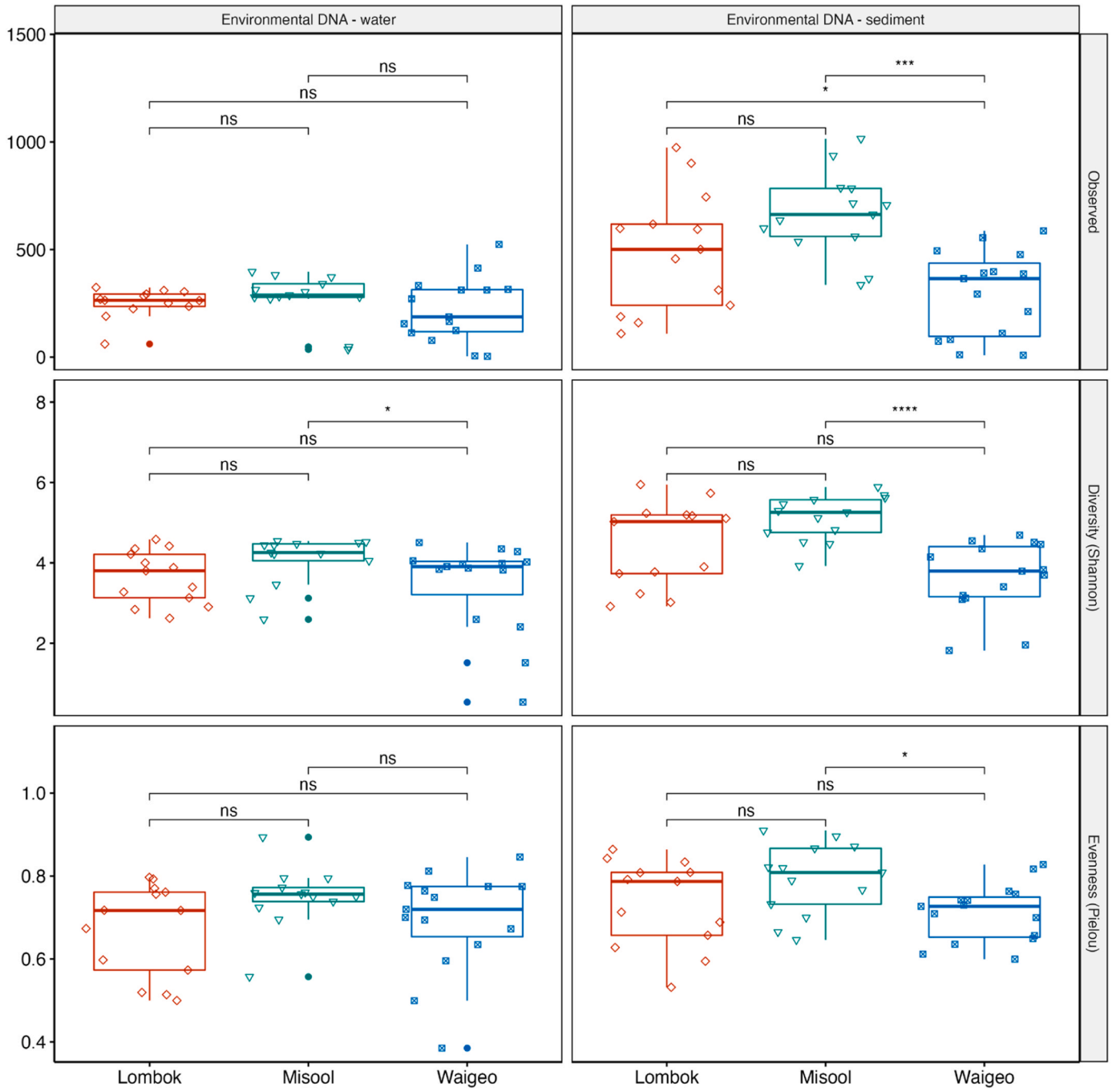

Fig. 3. Regional comparisons of alpha diversity metrics (rows) by eDNA substrate type (columns). OTU data was used for these comparisons. Mann-U Whitney tests were used on each pair of regions to test for significant differences. Asterisks represent p-values equal to or lesser than 0.0001 (****), 0.001 (***), 0.01 (**), 0.05 (*), and n.s stands for not-significant. Colored symbols are used to delineate regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

non-metazoan ASVs (Supplementary Fig. A2). Regional observed and extrapolated OTU richness estimates for seawater and sediment samples showed opposing patterns, with Waigeo having the highest observed and extrapolated richness estimates in water samples but the lowest richness estimates in sediment samples (Fig. 4). Asymptotic richness estimates confirm these trends, though Lombok is projected to overtake Misool in richness in sediment eDNA samples (Table 1).

The proportion of variation in community composition explained by sampling date (ranging from 24% to 53%) was higher than that explained by region (ranging from 6% to 16%) for the full eDNA dataset, eDNA water samples, and eDNA sediment samples ($p < 0.05$ for all variables and datasets) (Table 2). Moderate regional clusters were observed in PCoAs, with Misool's water and sediment eDNA samples more tightly clustered (with outliers) than those of Lombok and Waigeo (Fig. 5). The permutation tests for homogeneity of multivariate dispersions showed similar variances among regions for the eDNA datasets (Supplementary Table A6), verifying our tests measured location differences among region centroids and not variations in dispersions among regions (Fig. 5). When comparing eDNA sample types (water, sediment) on the full eDNA dataset and for each region, however, there were significant dispersion effects, meaning that the spread in variability in sediment and water samples differed ($p < 0.007$), except for in

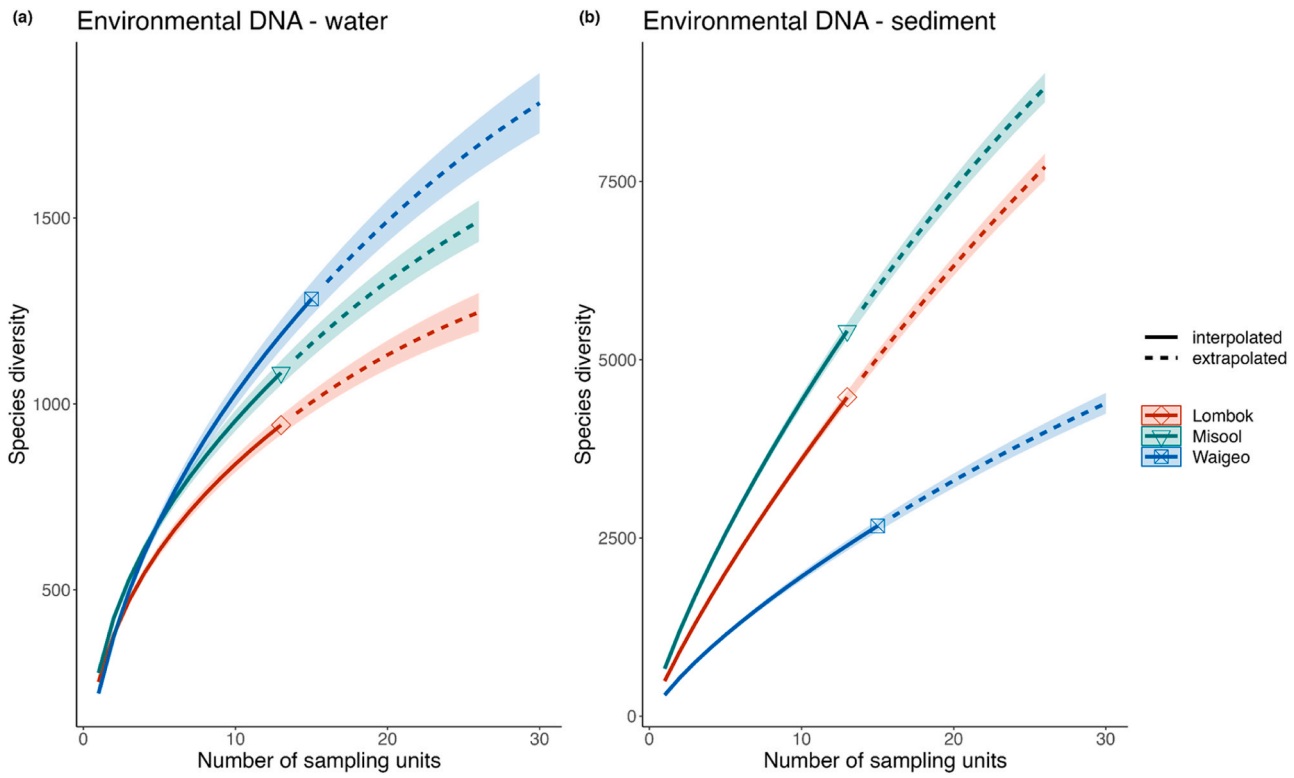

Fig. 4. Rarefaction curves for the acquisition of unique (a) OTUs in the eDNA water samples and (b) OTUs in the eDNA sediment samples. Solid lines indicate observed rarefied species richness while dotted lines represent extrapolated species richness values up to double the sample size. Shading around the lines represents the 95% confidence interval for the estimates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Waigeo ($p = 0.223$, Supplementary Fig. A3 and Table A7). The type of eDNA sample, however, was not significant in explaining variation among samples in the full eDNA dataset ($p = 0.572$, Table 2).

4. Discussion

We detected coral reef taxa and examined their patterns of alpha diversity and community compositions in three regions of Indonesia using eDNA metabarcoding, an emerging sequencing method to enumerate biodiversity. Despite sampling a subset of the total diversity within regions, we were able to uncover a wide range of metazoan and non-metazoan taxa that are relevant to the conservation and management of Indonesia's coral reef ecosystems. We predict that with more rigorous eDNA sampling and investment in local genetic infrastructure and capacity, including the refinement of regional genetic taxonomic databases, the preliminary regional comparisons of alpha diversity and community compositions we detected will be more robust and conform to known patterns of diversity within the Coral Triangle. Ultimately, we are optimistic that eDNA will be a useful complement to existing biomonitoring methods in this hyper-diverse part of the world.

Our eDNA sampling efforts of coral reefs across three regions of Indonesia demonstrated that seawater and sediment samples of eDNA amplified with a general metazoan COI primer were able to capture a wide taxonomic range of eukaryotes, from multicellular algae to endangered sharks and rays. Unassigned taxa at the phylum level, which comprised about half of our data, is equivalent to similar COI surveys of eukaryotes and metazoans in the Caribbean (58.2% unassigned in Bakker et al., 2019 and 53% non-metazoan sequences in Nguyen et al., 2020). Even in challenging tropical conditions, where some of our sites were far from human population centers and difficult to access, we were able to detect cryptic, small-bodied, rare, or elusive organisms and indicator species. In addition, despite not having access to appropriate field controls sometimes (such as sterile seawater or non-coral habitat samples), these detections were made possible by sterile techniques discussed above to suppress contamination concerns as much as possible, PCR positive and negative controls, a mock community sample generated from lab cultures, and subsequent conservative statistical treatment and interpretation of data (treating sites as regional replicates and confirming eDNA detections with visual survey results). We anticipate the logistical and infrastructural constraints we faced in this study will continue to be a challenge for eDNA practitioners attempting pilot projects in other remote or otherwise understudied marine environments.

Most fish and coral eDNA taxa we detected were confirmed by our concurrent visual surveys, suggesting that even imperfect eDNA detection still provides non-spurious taxonomic identifications. In addition, most of the fish eDNA taxa (33/47 species or genera-level assignments) from this study were also detected in Marwayana et al. (2021), a fish eDNA survey in Indonesia from 2017 that used the 12 S MiFish primer from Miya et al. (2015). Of the 14/47 detections that were not in Marwayana et al. (2021), six were visually confirmed by our visual surveys. A different study also estimated that 77.3% of fishes in the checklist for Bird's Head Seascape (an area of high biodiversity where our Misool and Waigeo regions are located) had voucher sequences for COI, in comparison to 24.5% for the 12 S rDNA region (Juhel et al., 2020). The coral families we detected visually (and with eDNA, since all our coral eDNA detections were confirmed visually), were consistent with similar comparative eDNA studies in Northwest Australia, though these studies used ITS2 primers (Alexander et al., 2020; Dugal et al., 2022). Interestingly, in these studies and our own, it appears that Acroporid corals, an ecologically important and prevalent group of stony corals in the Indo-Pacific, can be difficult to detect using eDNA with a single primer. These studies partially overcame this and other coral eDNA detection issues by "spiking" GenBank databases with voucher specimens (Dugal

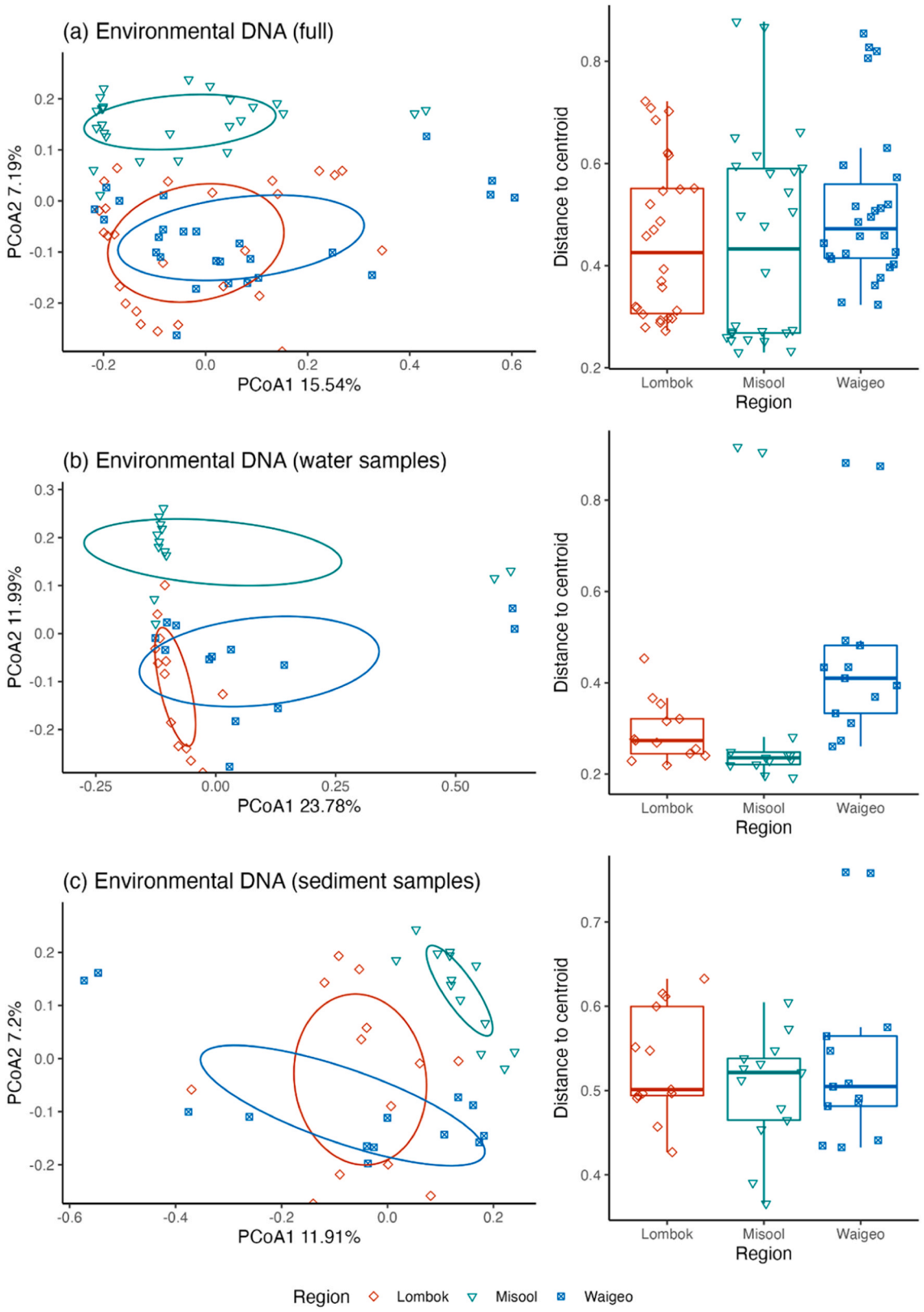

Fig. 5. Principal Coordinates Analysis of dissimilarities used in PERMANOVAs and boxplots showing results of permutation tests for homogeneity of multivariate dispersions for eDNA data. Numerical results are reported in Table 2 and Supplementary Table A7. Pairwise t-tests of group dispersions indicated that Lombok and Waigeo were statistically different in eDNA water samples (p= 0.05). Count data were Hellinger-transformed and distances were calculated using Bray-Curtis dissimilarities. Rows display (a) all environmental DNA OTUs, (b) eDNA water sample OTUs, and (c) eDNA sediment sample OTUs. Colored symbols are used to delineate regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2022) and modifying existing coral primers to bind to Acroporid sequences (developed in Alexander et al., 2020 and used in Dugal et al., 2022).

The general metazoan COI primer may thus offer confirmatory support and/or additional ecological context for eDNA sampling programs that also use taxa-specific primers targeting more conserved genetic regions (Stat et al., 2017; Ficetola and Taberlet, 2023). As previously mentioned, such taxa-specific eDNA studies of coral reef ecosystems include the use of 12 S primers or shorter COI primers for fish communities (Juhel et al., 2020; Miya, 2021) and ITS2 or 16 S primers for corals (Nichols and Marko, 2019; Alexander et al., 2020; Dugal et al., 2022). It is important to note, however, that these multi-primer eDNA assays have tended to rely on well-maintained and locally-available databases and voucher sequences to modify primer amplification efficiency and taxonomic assignment pipelines; the absence of such local genetic research infrastructure (as encountered here in this study) may impede the ability for scientists to troubleshoot and customize existing eDNA protocols to maximize the detection success of local species.

We found that eDNA alpha diversity results and comparisons of community composition are likely driven by a combination of (1) non-metazoan taxa, (2) sample type (water or sediment samples), and (3) under-sampling overall. Generally, bioinformatic analyses exclude non-target or unassigned taxa before analyses of community composition. Incorporating these sequences as OTUs, however, allowed us to conduct rigorous regional analyses with enough replicate sites, with each site containing 5,000 sequences after our stringent bioinformatics pipeline. This approach enables a holistic understanding of what the COI primer captures, which will be important as the primer grows in popularity in metabarcoding studies for its ability to biomonitor a wide taxonomic breadth (Andújar et al., 2018). We show that inclusion of non-target taxa leads to regional differentiation, rather than homogenization, of community compositions. Other studies have shown that these under-described COI sequences can drive differences in community compositions, suggesting their as-yet unknown ecological roles should not be overlooked in biodiversity studies (Bakker et al., 2019).

We collected water and sediment eDNA samples because they provided information on species assemblages at different timescales, with DNA decay rates slower in marine sediment than they are in seawater (Koziol et al., 2019; Sakata et al., 2020). Our data are congruent with other studies using the COI primer in showing that sediment eDNA recovers substantially higher amounts of diversity than seawater eDNA (Brandt et al., 2021). Distinct regional community compositions were revealed in our PERMANOVAs, especially in the full dataset (6% of variation explained) and in sediment samples (16% of variation explained). There were, however, significant differences in community variability (measured as heterogeneous dispersions) between water samples and sediment samples, both in aggregate and within the regions Lombok and Misool. Overall, water samples had more within-group spread than sediment samples, which suggests that they exhibit more heterogeneity and the community structure revealed by them is highly variable, which is consistent with other eDNA studies using seawater samples (Stauffer et al., 2021). This helps clarify why the effect of sampling date, a potential proxy for local biophysical conditions that affect eDNA degradation (sensu Barnes and Turner, 2016), explained more variation in the water samples (53%) than they did in the sediment samples (45%) and in the full eDNA dataset (24%). Our analysis shows the choice of eDNA substrate can not only affect conclusions about regional differentiation in community compositions (i.e., location effects), but the substrates themselves may exhibit various degrees of within-substrate multivariate scatter that should not be overlooked in analyses of beta diversity (i.e., dispersion effects, Anderson and Walsh, 2013).

Our sampling intensity and resulting under-sampling of coral reef diversity was analogous to other eDNA studies conducted around the same timeframe in Indonesia. Our sample coverage-based rarefaction analysis revealed upwards of 100 4-L water samples and 260 sediment samples would need to be taken to complete regional inventories of species that the COI primer could uncover with our lab protocols (>99.9% sample coverage). In the Raja Ampat Regency, located in Bird's Head Seascape (surveyed in our study as the Misool and Waigeo regions), Marwayana et al. (2021) estimated that >300 1-L water samples would be required to uncover all the fish diversity using the 12 S MiFish primer, while Juhel et al. (2020) predicted that 1,883 2-L water samples would be sufficient to uncover 95% of all the available OTUs, using a combination of 12 S and COI primers. These varied estimates suggest that asymptotic and rarefied estimates of diversity using eDNA are not just ecosystem-specific, but also dependent on primer amplification efficiencies, the number of replicates, and sampling volumes used (Kelly et al., 2019). In other words, species diversity estimates derived from eDNA may both be ecologically relevant, as well as dependent on the particular technical limits of various eDNA sampling methodologies (Stauffer et al., 2021). Future eDNA work may benefit from explicitly examining the effects of sampling intensity and varied lab protocols on local asymptotic diversity estimates. This could serve as a form of sensitivity analysis on the ecological conclusions derived from differences within and among different censusing methodologies, including between conventional censusing techniques and eDNA.

Returning to a point made above, another source of perceived under-sampling is under-detecting taxonomic groups due to incomplete reference databases, a major shortcoming raised previously by other eDNA researchers working in Indonesia (Juhel et al., 2020, 2022; Marwayana et al., 2021). For taxonomy-dependent biodiversity analyses, incomplete reference databases can affect estimates of compositional turnover because only a small portion of common species are recovered, erroneously suggesting there is low species turnover (Stauffer et al., 2021). In-silico PCR work on genetic database coverage of freshwater and marine fishes by Marques et al. (2020) showed that tropical species were poorly represented in the European Nucleotide Archive database for common metabarcoding primers (including two COI primers, though not the specific one used in our study). They found that tropical regions fared the worst in terms of database coverage for all primers studied. Indonesia had ~4–10% coverage of marine fishes for the COI primers studied, 24–27% coverage of marine fishes for the 12 S primers studied, and 16–44% coverage for the 18 S primers studied (Marques et al., 2020). We anecdotally observed that many of the taxonomic assignments to fishes were to groups commonly targeted by small-scale fisheries in Indonesia (i.e., Serranids and Lutjanids) (Humphries et al., 2019; Halim et al., 2020), suggesting that the current state of Indonesia's genetic databases and vouchering efforts may reflect taxa of socioeconomic importance rather than an ecologically representative subset of the overall marine biodiversity in the area. This observation complicates the aforementioned appraisal of COI databases as containing a large amount (>77%) of voucher sequences for fishes in local checklists (Juhel et al., 2020); more sampling and replication may uncover other fish taxa at more representative proportions to their population sizes. It is also worth noting that such societal preferences towards particular taxonomic groups are a feature of biodiversity studies and databases more generally (Troudet et al., 2017).

Although our study is a precursory examination of what eDNA metabarcoding may offer for bioassessment in Indonesia, it highlights the extraordinary potential for this method. In the short-term, we demonstrate that even a general COI primer can detect marine species of economic, cultural, or conservation importance. For example, seawater samples in Waigeo were able to detect sharks and rays more than in other regions, which are aligned with the high amount of conservation protections in the Raja Ampat region, including a ban of all shark and ray fisheries in 2012 (Andradi-Brown et al., 2021). Sediment samples of sharks (including 124 reads of the critically endangered broadnose wedgefish, *Rhynchobatus springeri* at 99% and 100% ID of two ASVs) can potentially support seawater eDNA detections by representing ecosystems that have historically supported these populations (Ellegaard et al.,

2020). In addition, because our primer was not limited to bony fishes, we were also able to detect 111 ASVs (127, 696 reads) of the small giant clam (*Tridacna maxima*, 97% ID), a species that has more recently been targeted by fishers in the Indo-Pacific as other giant clam stocks have crashed (Supplementary Table A8, Van Wynsberge et al., 2016). These detections can be further validated for appropriate interpretation through modeling the local biophysical processes that determine the persistence and degradation of eDNA, as well as the lab-based processes that affect amplification efficiencies (Barnes and Turner, 2016; Hansen et al., 2018; Collins et al., 2019; Mathieu et al., 2020). Thus, if given proper funding for increased sampling efforts, database curation, and local sequencing infrastructure, as well as appropriate social and ethical attention, broad eDNA surveys can complement fisheries stock assessments for a wide range of management objectives (Hansen et al., 2018; Ausubel and Stockle 2021; Miya, 2021).

As critical discussions about community-centered and equitable eDNA research paradigms emerge (Handsley-Davis et al., 2021; Shen et al., 2023), general primers like the one used in this study may offer a preliminary horizon-scan of what is possible for locally-driven research and inform priorities for capacity building efforts. This general primer can also be used in addition to other taxa-specific primers to reveal more about community dynamics (Stat et al., 2017; Ficetola and Taberlet, 2023). Indeed, researchers demonstrated this complementary and confirmatory approach in a separate study, which used the metazoan eDNA detections from this COI dataset to help fill in food-web dynamics (i.e., the presence of filter-feeders, such as bivalves, anthozoans, and sponges) known to affect protist communities (Borbee et al., 2022). Our COI dataset also helped Indonesian scientists explore fish compositions in Lombok as they related to local fisheries management regimes (Gelis et al., 2021), as well as inform additional eDNA sampling to understand the community dynamics of reef taxa that were relevant to their research interests and expertise (Madduppa et al., 2021).

Our pilot study illuminates some pragmatic on-the-ground lessons that will be useful for operationalizing eDNA research in Indonesia and similar study contexts in which biodiversity is high while relative genetic research infrastructure is low. While it may be logistically easier in the field to only collect seawater samples for eDNA (one could do this rapidly and without diving, for example), we found that it was critical to have concurrent visual surveys of conspicuous organisms to help validate our eDNA results. We recommend centering existing (generally visual-based) biodiversity censusing methods and determining how eDNA may complement them, rather than centering the eDNA technology itself, when designing a biodiversity study. This is because visual surveys will remain important in this region not only as a means to update local species checklists more generally, but also because they leverage the field-based knowledge and taxonomic expertise of local marine scientists and partner institutions. In terms of cost, it is important to consider that in similar low-income and high-biodiversity contexts, it will not necessarily be as expensive to implement labor-intensive visual surveys to detect species in comparison to other high-income areas where labor costs are higher (Sarkar and Chapman, 2021). Sarkar and Chapman (2021) further argued that employing local field researchers and technicians in these contexts can lead to positive conservation attitudes and provide a good salary away from more extractive industries. It may even be more expensive and less tractable (at least in the short-term) to implement eDNA-only monitoring programs in such contexts because much of the local financial investment towards such efforts (e.g., building local molecular labs and sequencing centers) currently does not exist in a robust-enough manner.

As a long-term goal for the eDNA community, future barcoding initiatives should not only prioritize tropical regions to make database coverage and biodiversity studies generally more equitable among different parts of the world (Collen et al., 2008; Huerlimann et al., 2020), but also ensure that additional efforts make genetic databases more well-rounded from an ecological or functional perspective. Indonesia is positioned to disproportionately benefit from such efforts due to its exceptionally high marine biodiversity and fisheries resources, as well as its concerted efforts to sustainably protect them (Andradi--Brown et al., 2021; Agung et al., 2022). International researchers working in Indonesia also cannot remove genomic DNA from the country due to strict permitting restrictions designed to combat biopiracy, thus a commitment to improving local capacity and genomic infrastructure will be critical to ensuring future eDNA research in this area (Rochmyaningsih, 2019). As articulated in-depth in a separate paper (Shen et al., 2023), only through intentional capacity building efforts that recognize existing sociopolitical power dynamics and ensure meaningful and inclusive participation of tropical Global South countries will eDNA's full suite of conservation benefits be accessible and equitably realized.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw sequence data are available on NCBI in the sequence read archive (SRA) under BioProject PRJNA107664. The scripts and code for analyses are publicly available on Github (github.com/elaine-shen/Indo_eDNA_RSMS).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.rsma.2024.103432.

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