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ORIGINAL ARTICLE

An environmental DNA metabarcoding approach versus a visual survey for reefs of Koh Pha-ngan in Thailand

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Abstract

Information on diversity indices and abundance of individual species is crucial for the assessment of ecosystem health, especially for endangered ecosystems as coral reefs. The application of environmental DNA (eDNA) to monitor coral biodiversity is, however, just beginning to come into focus for marine biologists. In this study, an eDNA metabarcoding approach of seawater samples in three different reefs on Koh Pha-ngan, Thailand, was compared with simultaneously collected visual census data. In addition, differences in read abundance and number of genera detected between daytime and nighttime eDNA samples were examined, and a local coral barcode reference database ($n = 23$ genera; COI gene) was constructed to improve assignment of eDNA reads to the genus level. As a technical extension of existing assays, two methods for library construction were compared: a commercial kit and in-house developed fusion primers. Combining eDNA metabarcoding and visual data, 29 different genera of scleractinian corals from 14 families were detected. In addition, a log-linear correlation was found between the abundance of eDNA reads and visually determined relative coral cover at the genus level, suggesting a predictive relationship between eDNA reads and coral cover. Results also showed diurnal variation between day and night samples in the number of eDNA reads, purported to relate to the activity phases of corals. The use of uniquely labeled fusion primers, gave comparable results to a commercially available library preparation kit. Especially with frequent use, fusion primers can be very cost-effective, and therefore a consideration for large-scale studies. Using a custom reference database of 89 sequences from coral tissue samples of 23 different coral genera produced better results than querying against NCBI GenBank, highlighting the importance of locally optimized databases. We consider these results important for establishing eDNA as a complementary tool to visual surveys to track changes in coral diversity and cover.

KEYWORDS
biodiversity, biomonitoring, COI, coral reefs, eDNA, metabarcoding, Scleractinia

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1 | INTRODUCTION

Information on diversity indices, such as species richness, and abundance of individual species are of great importance to better understand ecosystems and their resilience to changing environmental conditions (Margules & Pressey, 2000; Richards, 2013). Especially for coral reef structures created by ecologically functional and endangered species groups like hermatypic corals, this knowledge is crucial to correctly assess the ecosystem's health status (Richards, 2013). Knowledge of the baseline diversity of reef-building corals in healthy reefs is the necessary foundation for continuous monitoring to detect temporal changes, as well as their cause and consequences (Donner et al., 2005; Graham et al., 2015). Traditional monitoring of scleractinian coral biodiversity is labor-intensive and based on visual surveys; subsequent species identification is based on morphological characteristics, requiring a high level of taxonomic expertise to obtain accurate results (Hill & Wilkinson, 2004; Richards & Hobbs, 2014; Veron & Stafford-Smith, 2000; Wallace, 1999). Regardless of expertise, species may go unobserved and, in many cases, only coral cover is assessed as it is logistically challenging to intensively survey large areas of coral reef (Brown, 2004; West et al., 2020). Due to the obvious limitations of coral reef monitoring, biodiversity and especially coral composition is poorly or insufficiently documented in many reef structures worldwide (Richards, 2013; Veron et al., 2015; Wagner et al., 2020).

Such documentation problems can be overcome by complementary use of classical field ecological methods and genetic methods such as sequencing of environmental DNA (eDNA). DNA in the environment can come from multiple sources, such as whole organisms, biological secretions, or even free molecules. It is collectable directly from the water column, benthic sediments, or fecal and gut collections (Berry et al., 2017; Koziol et al., 2019; Rodríguez-Ezpeleta et al., 2021; Taberlet et al., 2012). In recent years, eDNA has emerged as a breakthrough approach to surmount the challenges of biodiversity monitoring and management; it has been used to successfully detect invasive, rare, cryptic, and bioindicator species (Bohmann et al., 2014; Bolte et al., 2021; Deiner et al., 2017; Mariani et al., 2021; Rees et al., 2014; Ruppert et al., 2019).

eDNA metabarcoding expands on the principles of traditional molecular barcoding (Hebert et al., 2003) by applying next-generation sequencing to mixed template samples and is increasingly popular for monitoring marine ecosystems (Alexander et al., 2020; Bohmann et al., 2014; Rees et al., 2014; West et al., 2020). Often, a single or a small number of universal DNA primer pairs are used, designed to cover a relatively broad range of taxa rather than a single group (Jeunen et al., 2019; Leese et al., 2021; Macher et al., 2018; West et al., 2021). However, metabarcoding with universal primers can lead to biases, as PCR (polymerase chain reaction) amplification can be uneven across taxa due to differences in primer efficiency (Elbrecht & Leese, 2015; Fonseca, 2018; Leese et al., 2021). This can be circumvented with taxon-specific primers that restrict amplification to target taxa. This allows for more accurate species diversity to be detected and even for abundance estimates (Bakker et al., 2017; Kutti et al., 2020; Nichols & Marko, 2019; Rourke et al., 2022; Thomsen et al., 2016).

Qualitative detection of specific taxa using eDNA is increasingly popular while little is still known about quantitative relationships of relative abundance of taxa and eDNA (Bakker et al., 2017; Elbrecht & Leese, 2015; Rourke et al., 2022; West et al., 2021). Theoretically, there should be a correlation between the biomass of studied taxa and the resulting amount of eDNA reads in environmental samples. So far though, information on specific eDNA shedding rates is limited to a handful of species and can differ drastically between taxonomic groups or sampling locations (Andruszkiewicz et al., 2021; Klymus et al., 2015; Nichols et al., 2022; Wood et al., 2020). Furthermore, studies are needed to expand our knowledge of eDNA replenishment, transport, and degradation in aquatic environments (Andruszkiewicz et al., 2021; Jeunen et al., 2019, 2020; Koziol et al., 2019; Nichols & Marko, 2019; Rourke et al., 2022). Although eDNA metabarcoding has been widely used to survey biodiversity in different taxonomic groups (Mariani et al., 2021; Rourke et al., 2022; Ruppert et al., 2019; West et al., 2020), its use for monitoring coral biodiversity is just beginning (e.g., Alexander et al., 2020; Dugal et al., 2022; Ip et al., 2022; Nichols & Marko, 2019; Shinzato et al., 2021; West et al., 2021).

We aim to determine if eDNA abundance correlates with relative abundance of corals based on percent cover from visual surveys at study sites with high coral biodiversity such as the reefs of Koh Phangan in the Gulf of Thailand. Therefore, we used a metabarcoding primer pair published by Nichols and Marko (2019) that amplifies a mitochondrial DNA gene (COI: cytochrome oxidase-1) and has already been established for abundance analyses of coral eDNA on reefs in Hawaii. To improve detection of coral species, a local coral database was created from tissue samples collected directly at the reefs of Koh Pha-ngan. Since many coral reef taxa extend their tentacles only at night to feed, we hypothesize that higher DNA release may occur at night. Therefore, we additionally focused on comparing eDNA samples collected during the day and at night to detect diurnal variations in the number and amount of coral operational taxonomic units (OTUs), reflecting temporally different activity patterns of coral taxa. Additionally, as a technical extension, we compare two library preparation methods: a commercial kit for the preparation of the amplicons and a two-step PCR method (Elbrecht & Steinke, 2019) using custom-made fusion primers consisting of incorporated flow-cell adaptors, P5, P7, Ilumina TrueSeq CD Index sequences and sequencing primers.

2 | MATERIAL AND METHODS

2.1 | Study site and data collection

Visual surveys, coral tissue collection and seawater sampling for eDNA metabarcoding were conducted from July to September 2019 at the three sampling sites, Haad Khom, Haad Salad and Haad Yao, along the northwestern shore of Koh Pha-ngan, in the

Gulf of Thailand (Figure 1). For visual surveys at each of the three sites, six 30 m transects were aligned perpendicularly to the shore at 5–10 m intervals. Photos of a frame (0.25 m^2) were taken every 2 m on alternating sides along a transect. Thus, 16 quadrats per transect were photographed. Scleractinia were visually identified from the photographs to genus-level using the Indo Pacific Coral Finder (Kelley, 2016) and analyzed using 50 random points per quadrat (for a total of 800 points per transect) in CPCe (Kohler & Gill, 2006) for an estimate of coral cover and biodiversity. Statistical analyses were computed in RStudio (RStudio Team, 2020).

To build a genetic reference database, tissues and photos were taken of 138 different coral colonies (34 genera; 15 families; Table S1 and Figure S1) from five sampling sites (Figure 1). Corals were identified to genus-level using the Indo Pacific Coral Finder (Kelley, 2016) and the Corals of the World online database (Veron et al., 2021: http://www.coralsoftheworld.org); taxonomic nomenclature was assigned based on the currently accepted nomenclature in the World Register of Marine Species (WoRMS; http://www.marinespecies.org, accessed November 2021). Genomic DNA of the coral tissues was extracted using the Qiagen DNeasy Blood & Tissue Kit (Quiagen, Hildesheim, Germany) according to the manufacturers protocol. COI reference sequences of around 400 bp (HICORCOX_F1: 5'-GAACAAGGRGCKGGBAC-3′ and HICORCOX_R2: 5'-GCAACAAAAGTYGGKATTAT-3′, Nichols & Marko, 2019) were amplified using 6.25 µl VWR Go Taq Master Mix (VWR, Darmstadt, Germany), 1 µl extracted DNA template, 3.75 µl nuclease free water and 0.25 µl of the forward and reverse primer (10 pmol/µl). Subsequently, the unincorporated primers and nucleotides were removed with ExoSAP (GE Healthcare, Solingen, Germany) digestion and the samples were sequenced at the Ruhr University Bochum Sequencing Service (Bochum, Germany).

Water samples were collected between 24 July 2019 and 3 August 2019 (midday and night-time) and again from 28 August 2019 to 1 September 2019 (midday). Collection was performed

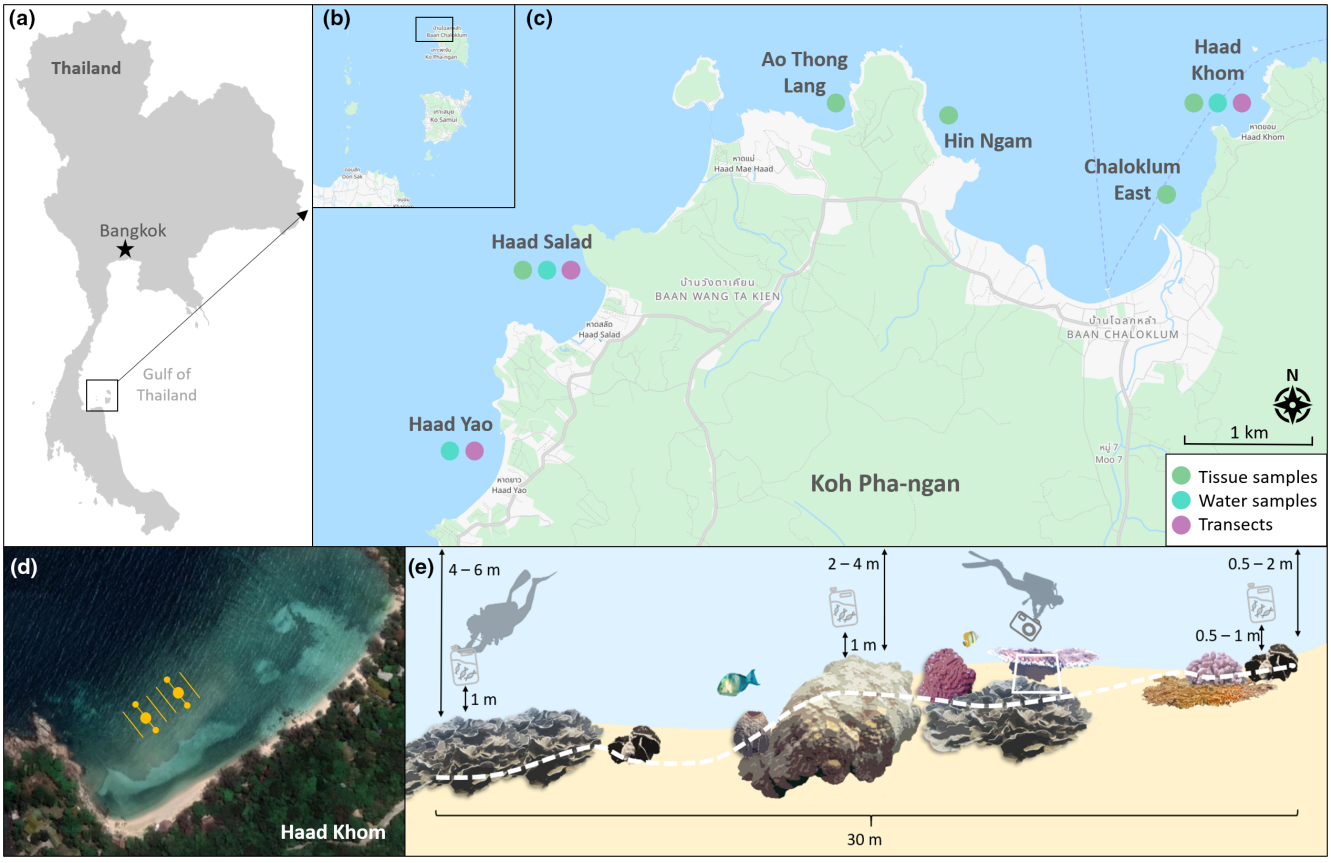

FIGURE 1 (a) Location of the study area in Thailand. (b) Position of the sampling sites on Koh Pha-ngan. (c) Position of the sampled reef sites on the north-west coast of Koh Pha-ngan. Type of sample indicated by color (green = tissue sample + photo of a coral colony; blue = water samples for filtering; violet = photo transects) (© OpenStreetMap). (d) Exemplary sampling scheme for transects and water samples at Haad Khom. Yellow lines indicate position of the 30 m transects laid perpendicular to the shore. Yellow points indicate position of water samples (© Google earth). (e) Schematic overview of the sampling procedure along a transect. Every 2 m along, a frame (0.5 × 0.5 m) was placed next to the 30 m transect in a checkerboard pattern on the reef and photographed, resulting in a total of 16 photos per transect. Transects were spaced 5–10 m apart. Water samples were typically collected 1 m above the reef unless the water was shallower. One sample was taken at the beginning of the transect in shallow water on the reef crest, while two water samples were taken in the middle and one at the end of the transect on the reef slope. An additional mixed seawater sample was prepared in the laboratory from all four water samples taken from one reef. One month later, three additional transects were placed laterally offset from the previous transects and water samples were again collected in the middle transect.

three times per sampling site: at noon, 12 hours later on the same day and 4 weeks later during daytime. Samples of 6 L each were collected with sterile containers at depths between 1–6 m by scuba divers, on one of the surveys transect per site. During the day (at noon), one sample was taken at the middle of the inner transect, one at the shallow end of the transect and one at the deep end of the transect (Figure 1). At night, three samples were taken at the middle of the transect. Immediately after collection, seawater samples were placed in the dark on ice and processed within 2 hours of collection. Collection blanks of 1 Liter tap water were additionally taken into the field and afterwards handled like all other samples to exclude contamination during transport (Turner et al., 2015).

2.2 | Water filtration, eDNA extraction and library preparation

Seawater filtration took place in a lab provided by COREsea located on Koh Pha-ngan and was separated from all other molecular work. The filtration was conducted using a vacuum pump and a vacuum-safe Erlenmeyer flask (Thermofisher, Dreieich, Germany). Water samples were inverted and homogenized, and 2 liters of seawater from each of the three samples per collection site and date were filtered. A fourth sample was taken by mixing 1 L seawater from each of the three samples together and filtering a 2 liter subsample of this mix. For the filtration, sterile analytical test filter funnels were used (Nalgene, Thermofisher, Dreieich, Germany; 47 mm diameter; 0.22 µm pore size). All filters were handled with sterile forceps at each step and all equipment was sterilized in a 10% bleach solution before and after use. Surfaces were sterilized using 10% bleach solution and 100% ethanol. Negative controls were used at different steps to monitor contamination. Besides collection blanks, extraction blanks were also generated during the filter extraction. The resulting 54 filters were preserved individually in absolute ethanol and stored at −20 °C. All molecular working steps, including eDNA extraction, PCR and library preparation, were separately conducted in our (sterile) molecular laboratories at Ruhr University Bochum, Germany, to minimize the probability of sample cross contamination. Filter extractions were performed under a sterile work bench, with all surfaces sterilized with 10% bleach solution and UV light. DNA from the filters was extracted using the Qiagen DNeasy Blood & Tissue Kit plus an additional bead beating step and minor adjustments (Nichols & Marko, 2019) to the extraction protocol.

HICORCOX_F1 and HICORCOX_R2 (Nichols & Marko, 2019) were used to amplify a ~ 400 bp sequence from the COI mitochondrial gene and PCR products were adjusted to 80 ng each. Libraries were prepared using the NEBNext® Ultra II DNA Library Prep Kit for Illumina® (New England Bio Labs, Frankfurt a. M., Germany). Libraries of collection and extraction blanks and of four samples did not amplify, so in total libraries from 29 water samples were sent away for sequencing (Table S2).

Libraries from a subset of 16 water samples were prepared with fusion primers. Fusion primers were designed by adding Illumina adapters and inline barcodes, as described by Elbrecht and Leese (2015) and Elbrecht and Steinke (2019) to increase per-base pair sequence diversity during sequencing and allow for a two-step PCR protocol. Primers CoralFusion_F1 and CoralFusion_R2 (Figure S2) used to amplify a ~ 700 bp amplicon using the Q5® TaqMaster Mix (Promega, Walldorf, Germany) included 0.5/0.25 units of Taq, 1 µl extracted DNA template, 12.5 µl 1× supplied PCR buffer, 0.25 µM of forward and reverse primer and 8.74 µl of H_2O. Amplifications were done using a Flexcycler (Analytik Jena AG, Jena, Germany) with the following cycling protocol: initial denaturation at 94°C for 300 s was followed by 15 cycles at 95 C for 30 s, 60 C for 30 s and 72 C for 120 s and a final elongation of 72 C for 600 s. PCRs were run in duplicate per sample resulting in 50 µl PCR product and all samples were cleaned up using the MAGBIO High Prep selection beads (400–700 bp; 0.55× ratio) (MAGBIO, Kraichtal, Germany).

All libraries were measured on QIAxcel Advanced (Qiagen, Hildesheim, Germany) to assess size distribution of DNA samples (560–700 bp). DNA concentrations were finally measured with a Qubit fluorometer (Thermo Fisher Scientific), and samples were equimolar pooled for sequencing. In total, 81 libraries, 54 prepared with NEBNext Ultra II and 27 prepared with fusion primers (see Table S2) were equimolar pooled and sent to Macrogen (Seoul, South Korea) for sequencing on an Illumina MiSeq platform.

2.3 | Bioinformatics

Libraries were demultiplexed from the sequencing service of Macrogen (Seoul, South Korea) and analyzed using Geneious Prime (Kearse et al., 2012). Illumina reads were paired, quality controlled, trimmed via BBduk (all adapters, > Q30; Kmer length 27; minimum 100 bp), and merged using BBmerge (high merging rate). Sequences were deduplicated with Dedupe (kmer seed length 37) and error correction and read normalization of the deduplicated reads were conducted using the plugin BBNorm (aggressively; target coverage level = 100). UCHIME (Edgar, 2016) and VSearch v2.8.4 (Rognes et al., 2016) were used to detect chimeric reads within the pre-processed sequence data. Clustering of amplicon sequence variants into OTUs was completed using the open-source platform GALAXY Europe (Afgan et al., 2018; https://www.galaxy.eu), using QIIME (Caporaso et al., 2010) with the function "pic_otus.py" and VSEARCH with a 97% similarity threshold. OTUs were curated post-clustering using QIIME (pick_rep_set.py; method = first). The resulting QIIME-derived OTUs were exported for reference database taxonomic alignment. OTUs were compared and aligned, using Basic Local Alignment Search Tool (BLASTn) v2.2.31; (Altschul et al., 1990), with reference material from Genbank, an open-source nucleotide reference database within the National Center for Biotechnology Information (NCBI). Alignment was completed using a high-performance cluster

supercomputer (Ruhr University Bochum), which returned the five best-matched alignments for each OTU based on the expected value for a ≥ 97% identity match. Alignments were validated manually using MEtaGenomeANalyser (MEGAN v.6.12.0) Community Edition (Huson et al., 2016), into which blast files were imported directly using relaxed 'lowest common ancestor' parameters (minimum bit-score\50, minimum support percent removed). All OTUs within the scleractinian node with a percent identity below this threshold were re-BLASTed individually using the BLASTn function in Geneious. OTUs were additionally mapped to the Midori reference database for metazoan mitochondrial DNA (Machida et al., 2017) and to our own reference database (results were given preference) in GALAXY using the tool Blastn based on the expected value for a ≥ 97% identity match. Sequences matching in both percent and bitscore to multiple scleractinian genera were discarded, as taxonomy could not be confirmed. All taxonomic nomenclature was checked against the WoRMS database.

2.4 | Statistical analyses

Since different numbers of libraries per collection site were generated during library preparation (Table S2), and libraries differ in the number of eDNA reads obtained, statistical evaluations and comparisons were conducted with standardized values for the eDNA reads per sampling site and time point. For this purpose, eDNA reads from each of the sampling sites were pooled together and divided by the number of libraries. For qualitative comparison and visualization of genera found in transects as well as in the eDNA reads balloon plots were created using RStudio and the ggplot2 (tidyverse) package for data visualization (Wickham, 2016).

We created a bray-curtis distance matrix for relative abundance of coral genera per visual transect and abundance of eDNA reads per sample, as well as a jaccard distance matrix with the R package "vegan" (Oksanen et al., 2020) and visually analyzed the community composition based on this matrix with non-metric multidimensional scaling (NMDS; Figure S3). We then performed PERMANOVA to statistically analyze the effects of sample location (Location), sampling time (Day vs. night: eDNA) and method (eDNA vs Transect), on the recovered community composition, using the "adonis" function from the "vegan" package, with 5000 permutations (Table S3).

We used a generalized linear model (GLM) with a negative binomial distribution and mixed effects with location as an offset (1|Location), which also included a zero-inflation term to account for coral genera with no occurrence at sites. We checked for overdispersion and patterns in the model residuals. We tested the significance of the variables "sampling time", and "library preparation method" (explanatory variables) as a predictor of the number of eDNA reads (response variable) by calculating the likelihood ratio test using the "drop1" function with a chi-squared distribution (Table S4). Linear regressions were used to test for log-linear relationship between obtained coral reads per genus and percentage coral cover based on transect data in RStudio (Nichols & Marko, 2019).

3 | RESULTS

3.1 | Visual surveys

In total, 26 scleractinian genera (mean of 19 ± 2.67 per site) from 14 families were identified from all sites during the visual survey. Data from visual surveys showed significant differences (GLM: $\chi^2 = 9.1804$, $p < 0.05$) among total coral cover ranging from 26.37% at Haad Khom up to 41.88% at Haad Salad. Marine flora (turf, coralline and macro-algae), other invertebrates (soft corals and sponges) and abiotic substrate (sand, rubble, and dead coral) were responsible for the remaining benthic cover. At Haad Yao 23 genera were found, followed by Haad Salad with 18 and Haad Khom with 16 genera. Overall, *Porites* was the dominant genus at Haad Salad and Haad Yao with a relative coral cover of 63.39% and 48.61% respectively. Haad Khom showed the highest percentages of *Acropora* (37.00%), which were low at Haad Salad (2.6%) and Haad Yao (3.1%). Abundances of genera belonging to the families Merulinidae, Fungiidae and Pocilloporidae varied widely across locations (Figure 2). Coral communities based on relative abundance of detected coral genera showed significant differences among the three sampling sites (PERMANOVA: df = 2, *F*-Model = 5.589, $p < 0.01$).

3.2 | Reference database

The total of 138 samples taken on coral colonies could be assigned to 34 different coral genera across 15 families of Scleractinia. We could amplify and sequence successfully 89 of these samples that could be assigned to 23 genera based on taxonomical classification, resulting in 23 unique consensus sequences in our reference database. Other tissue samples from Fungiidae, Diploastraeidae and Lobophylliidae were not amplifiable with the chosen primer. The marker chosen worked well in the delimitation of genera within most families.

3.3 | Comparison of library preparation approaches and visual survey

The total number of eDNA reads obtained from the NEB library preparation was 9,506,458 (mean of 194,009 ± 80,881 per sample). Total reads per site ranged from 2,619,512 in Haad Yao up to 3,789,170 in Haad Khom. Total quality filtered reads were 1,097,112 (mean of 22,390 ± 12.021 per sample) leading to 401 ± 195 OTUs per sample after post-clustering and de-noising. The total number of eDNA reads obtained from fusion primer library preparation was 14,863,188 (mean of 594,528 ± 662,493 per sample). Total reads per site ranged from 3,624,044 in Haad Khom up to 6,400,764 in Haad Salad. Total quality filtered reads were 2,763,084 (mean of

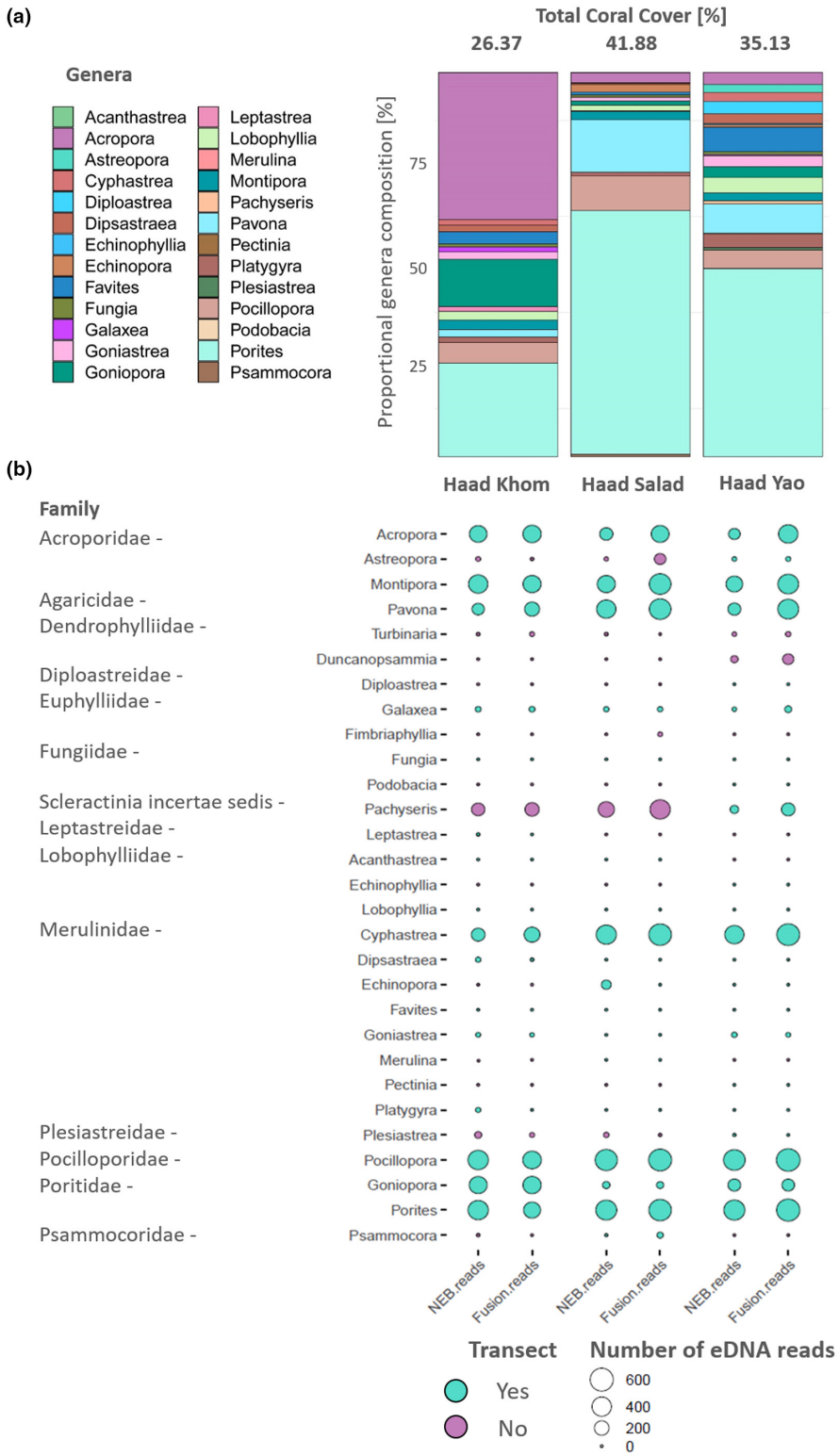

FIGURE 2 (a) Total coral cover of the three reef sites where transects and water samples were taken. The bar chart shows the relative composition of coral cover with respect to individual genera of stony corals identified during the visual transects. (b) Balloon plot of the number of eDNA reads for coral genera at the three reef sites as well as if there were visually identified during transects. Size of the circle indicates eDNA read number. Color indicates presence in transect (violet = no; light blue = yes).

110,423 ± 155,484 per sample) leading to 269 ± 118 OTUs per sample after post-clustering and denoising. Number of eDNA reads differed significantly between the two methods (GLM: $\chi 2 = 5.7662$, $p < 0.05$). Tap water controls and extraction controls did not amplify. For a detailed overview please see Table S2.

In the NEB library prepared dataset 96.33% of all OTUs and in fusion primer library prepared dataset 99.08% of all OTUs were assigned to metazoans (Figure 3). Cnidaria were dominant with approximately 91% of assigned OTUs belonging to this phylum in both library approaches. Within the Cnidaria the groups of Hydrozoa (<0.05%), Scyphozoa (<0.05%) and Anthozoa (over 90%) were detectable with no significant differences among the library approaches (Figure 3). The remaining metazoans were predominantly assigned to Porifera with approximately 4%, followed by Mammalia

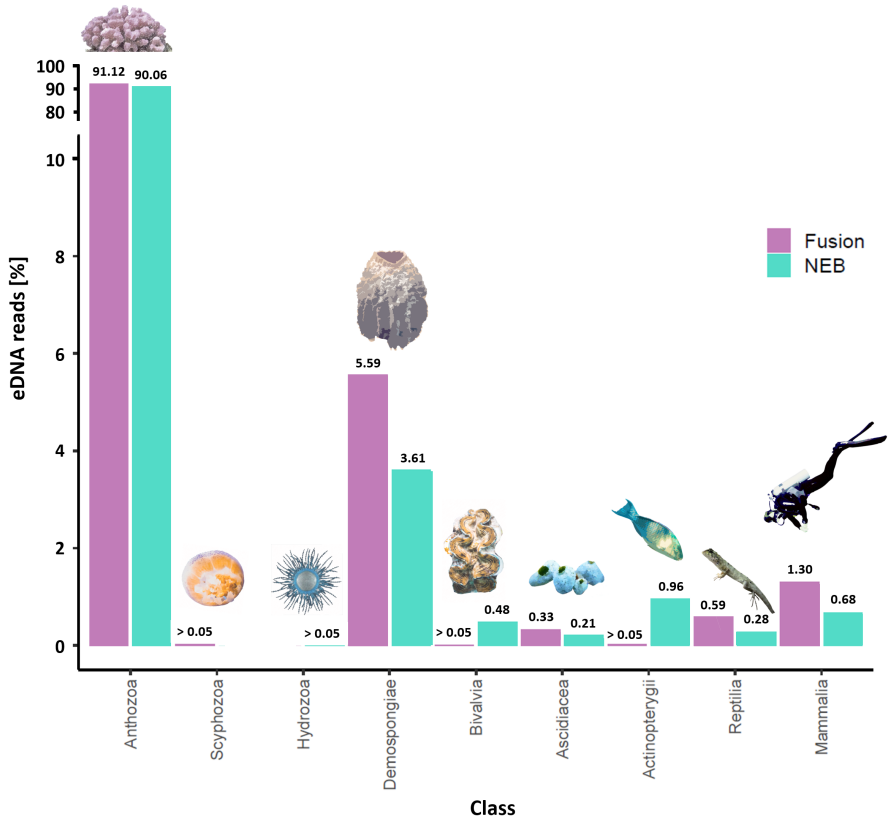

FIGURE 3 Percentage of total assigned eDNA OTUs of selected classes of metazoans, for a section of cytochrome oxidase-1 (~400 bp), using two different library preparation methods, a commercial kit (NEB) and custom-made fusion primers (fusion) that already contained the sequencing primer and all necessary Illumina-adaptors and inline barcodes. PCR amplification and next-generation sequencing of the amplicon resulted in a high percentage of sequenced reads from the Anthozoa, with other taxa represented to a lesser extent. Sequences that could not be assigned or involved taxa outside of the metazoans were removed.

with approximately 1%. After reblasting all data with our own database overall OTUs belonging to 19 scleractinian genera (mean genera per site: 13.3 ±1.9) were detectable in the commercial library preparation dataset. The fusion primer dataset consisted of 17 scleractinian genera (mean genera per site: 13±0.0) (Figure 2). Combining both library approaches led to the detection of 20 scleractinian genera from 14 families. At Haad Khom 17 genera of scleractinia were found, followed by Haad Salad with 15 and Haad Yao with 14 genera. There were significant differences between assemblages obtained by the two library preparation methods (PERMANOVA: df = 1, *F*-Model = 5.069, $p < 0.01$). Most discrepancies between the two library approaches were caused by the selection and number of seawater samples, as only a subset was used for the fusion primer, and by low sequence resolution of members of the Merulinidae. Overall, reads belonging to the OTU *Porites* and the OTU *Pocillopora* were dominant in the dataset with ~15–23% of all reads. Additionally, all three sites showed high percentages of reads belonging to *Acropora* and *Montipora* with ~10% and ~15% respectively (Figure 2).

The number of scleractinian-related reads differed significantly among samples taken during the day and night (GLM: $\chi^2 = 6.8196$, $p < 0.01$). More scleractinian genera were found in the night-time samples than the daytime samples independently of the chosen library preparation (NEB night 12.66±1.52; NEB day 10 ±1 and Fusion night 11.7 ±1.5; Fusion day 10.33±0.6) (ANOVA: df = 2, *F*-Model 8.1, $p < 0.05$). Community composition also differed significantly between day and night-time samples (PERMANOVA: df = 1, *F*-Model = 4.448, $p < 0.01$). Similar observations were also observed for the mean number of reads with the NEB library approach, with 1407±681 and 1820±658 reads in day and night datasets respectively, and also with the fusion primer, with 2217±911 and 2529±898 reads in day and night datasets, respectively (Figure 4).

Visual census data detected 26 scleractinian genera whereas the two library approaches used for eDNA detection together yielded 20 scleractinian genera (Figure 5). *Duncanopsammia*, *Turbinaria* (both Dendrophylliidae) and *Fimbriaphyllia* (Euphylliidae) were not found in the visual surveys. Conversely, *Fungia*, *Podobacia* (both Fungiidae), *Acanthastrea*, *Echinophyllia* and *Lobophyllia* (all three Lobophylliidae) and *Diploastrea* (Diploastreidae) were not found within the eDNA OTUs, most likely due to non-amplification. There were significant differences in community composition between the eDNA approach and visual transect data (PERMANOVA: df = 1, *F*-Model = 51.6009, $p < 0.01$).

The number of DNA reads found in the dataset explained 32% of the variation in percentage of coral cover between sites ($R^2 = 0.32$) and suggest a predictive relationship between eDNA read abundance found in the water samples and the coral cover of the tested sites. Reads from the NEB preparations showed slightly higher explanations of the variation on percentage coral cover ($R^2 = 0.34$) as reads from the fusion library ($R^2 = 0.26$) or both applications pooled together ($R^2 = 0.32$) (Figure 6).

4 | DISCUSSION

Our study successfully demonstrates eDNA as an efficient and complementary method to visual census data for monitoring scleractinian

FIGURE 4 Balloon plot showing coral genera found in the eDNA samples during the day (light blue) and night (violet) in comparison to both library preparation methods used, commercial kit (NEB) and fusion primers (fusion). Size of the circle indicates eDNA read number.

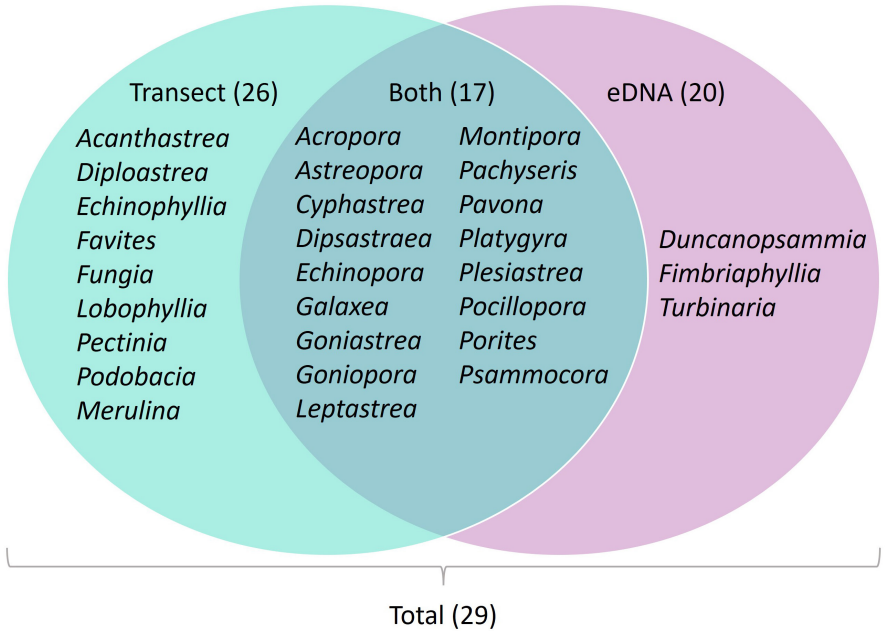

FIGURE 5 Shown are coral genera found only in the visual transects (light blue), both assays (center) and only in eDNA reads (pink).

coral diversity. Further, our data suggest a correlation between abundance of eDNA reads and visually determined percentage coral cover in the analyzed reef sites. This highlights the potential of eDNA metabarcoding approaches for marine biodiversity detection and the possible integration into existing monitoring efforts, especially in scleractinian corals (Alexander et al., 2020; Dugal et al., 2022;

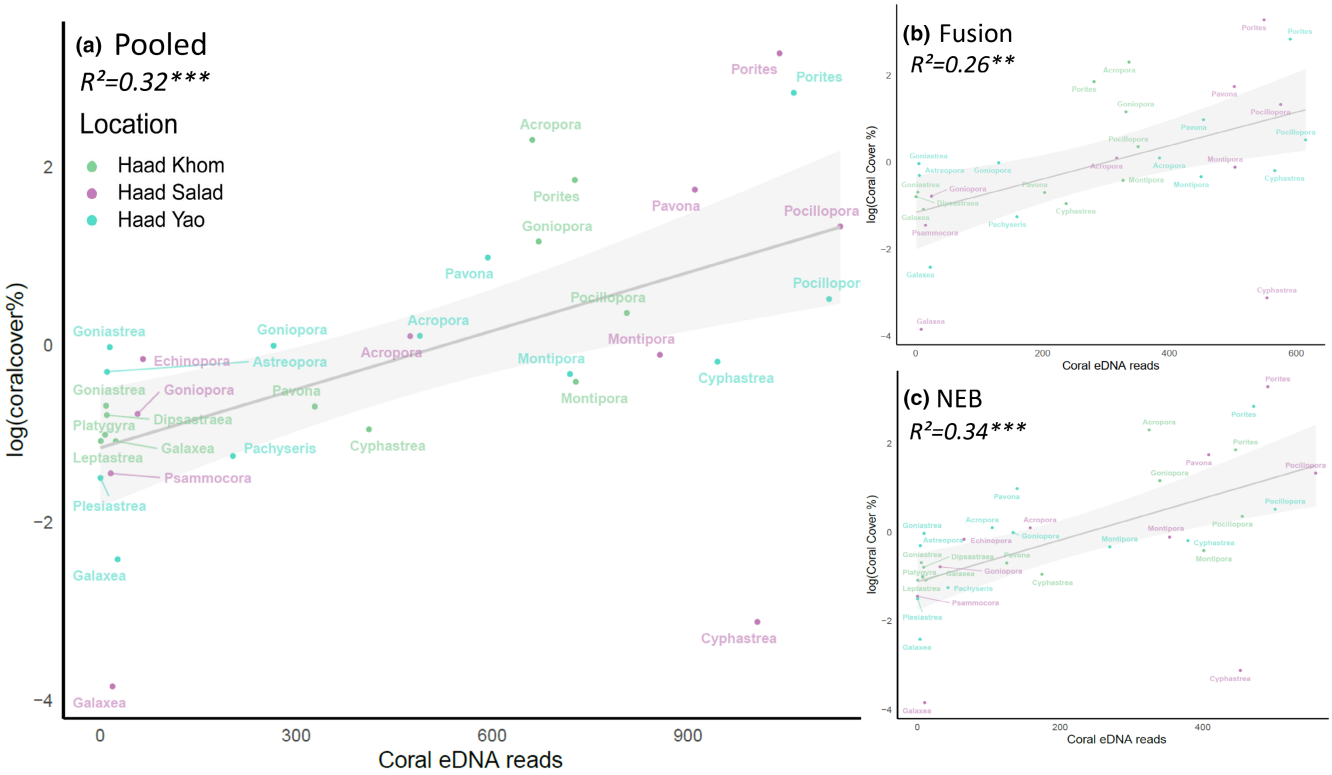

FIGURE 6 Scatterplots of standardized coral eDNA reads versus log percentage cover (%) of stony coral genera. A linear regression and 95% prediction intervals are plotted, showing a significant log-linear relationship for COI. R2 is given for pooled eDNA reads as well as individually for both library preparation methods.

Nichols & Marko, 2019; West et al., 2021). However, our results also revealed, besides the obvious need for suitable laboratory infrastructure, methodical and technical shortcomings that need to be addressed in future studies.

4.1 | Overall comparison of approaches

Twenty different coral families and 75 genera have been described for the Gulf of Thailand (Veron et al., 2021: http://www.coralsoftheworld.org; WoRMS (2022): https://www.marinespecies.org). Our study retrieved Scleractinia from 14 families assigned to 29 different genera by combining eDNA metabarcoding and visual census data (Figure 5). The genera found in this study reflect 38.7% (Transect: 34.7%; eDNA: 26.7%) of the known coral genera (in this region) and suggest a high overall coral biodiversity for the island of Koh Pha-ngan, especially considering that only three reef sites were included in our study. Hence, our data obtained is in line with earlier published studies that found similar diversity patterns in the Samui archipelago (Monchanin et al., 2021; Sutthacheep et al., 2013; Veron et al., 2015). Of course, it must be kept in mind that the actual diversity could be much higher than described here. It is conceivable that our collection sites do not harbor all species of respective genera. For example, we did not observe representatives of Plerogyridae at the sites studied for eDNA samples and transects but they do occur elsewhere on Koh Pha-ngnan (*Physogyra liechtensteini*; Table S1). Furthermore, there is always the possibility that rare species have simply been overlooked or that species have been identified incorrectly.

Our study detected a large overlap of 17 genera between the visual surveys and the eDNA reads (Figure 5), but also 12 genera that were only detected using either the eDNA method or visual monitoring, emphasizing the advantage of combining both approaches in biodiversity surveys (Everett & Park, 2018; Kelly et al., 2016; Stat et al., 2019). In our eDNA dataset, we found two genera of Denrophyilliidae, *Turbinaria* and *Duncanopsammia*, that did not appear in the transects. This is because cryptic and rare species are often missed in traditional visual surveys (Mumby et al., 1997; Pearman et al., 2016). Often visual surveys are guided by protocols from government agencies (Walsh et al., 2010) and, hence, resources and time of investigations is limited. More intensive visual sampling, although expensive and time-consuming, would likely yield the missing taxa, as the collection for our reference database shows. Here an advantage of the eDNA method becomes apparent, where theoretically more comprehensive samples of the entire community in a given area can be taken with minimal sampling effort (Nichols & Marko, 2019). However, the opposite case, some visually detected species (Lobophylliidae, Diploastreidae and Fungiidae) were absent from our eDNA dataset (Figure 5). This recommends the integration of eDNA metabarcoding as a complementary tool to traditional survey methods for species detection rather than a replacement (Alexander et al., 2020; West et al., 2021) and highlights that; eDNA

holds promise for detecting rare species that would be missed in purely visual data. Another problem with visual surveys of coral diversity, is the high level of expertise required to identify corals to compile reliable biodiversity data sets (West et al., 2021).

4.2 | Quantification of coral cover

To date, few studies have used eDNA metabarcoding to quantify biodiversity (Bakker et al., 2017; Kutti et al., 2020; Nichols & Marko, 2019; Rourke et al., 2022; Thomsen et al., 2016) and very few in corals. For coral communities where the quantitative use of eDNA abundance data was statistically tested, divergent results emerged in two different ecosystems with different levels of diversity. While eDNA metabarcoding appeared promising for quantifying the coral community in Hawai'i (Nichols & Marko, 2019), the method did not provide a valid correlation between the number of eDNA reads and coral cover for more diverse coral communities (West et al., 2021).

Our data reveals that eDNA metabarcoding could also be a promising methodology in regions with higher biodiversity, as in Nichols and Marko (2019). Further, there is a significant correlation between number of eDNA reads and log-transformed percentage coral cover of associated scleractinian corals at the genus level. However, the same analysis showed a higher correlation at the family level (Figure S4), suggesting resolution biases of the marker at the genus level, especially within the Merulinidae. Furthermore, the limited number of reef locations ($n = 3$) should be kept in mind here. Hence, further investigation is needed to determine whether eDNA is a valid method for quantifying coral genera. Exemplarily, varying eDNA shedding rates may also play a role, as they can vary among taxonomic groups or even among individuals of a given species (Holman et al., 2022; Jo et al., 2019; Klymus et al., 2015; Wood et al., 2020), but there is a lack of information on specific eDNA abundance rates of corals, especially among different species (Nichols et al., 2022). Although one does not necessarily expect diurnal variation in abundance of eDNA for sessile species, the results showed significant differences in the species compositions and the abundance of reads between day and night samples. This affects the detection and quantification and is likely related to the activity phases of corals in general or that of specific species. Higher abundances of coral eDNA reads in general were seen at night including some genera that were not seen during the day. Many corals are thought to feed primarily at night, when zooplankton density on the reef is highest (Heidelberg et al., 2004; Sebens & DeRiemer, 1977; Yahel et al., 2005), while other species extend their tentacles both during the day and at night (Johannes & Tepley, 1974; Sorokin, 2013). For example, nocturnal tentacle extension has been described for *Platygyra* spp. (Yahel et al., 2005), which we found only in the nocturnal eDNA samples, whereas *Porites* spp., which produced tenfold the reads in both daytime and night-time samples, are described to extend their tentacles for feeding during the day and at night (Johannes & Tepley, 1974). Another example of a species found only in the night-time samples is *Duncanopsammia sp.*, which may also indicate nocturnal activity of this species, but this would need to be shown in future studies. Therefore, future studies should also focus more on the sampling time point so that it coincides with the activity patterns of the target species or even extend sampling points throughout the day to achieve broader species detection and to validate quantification. However, besides those obvious limitations that urgently need to be addressed, our results are in line with the study of Nichols and Marko (2019), suggesting that eDNA could be a promising method to identify individual coral genera and quantify coral cover.

4.3 | Limitations of eDNA approach

4.3.1 | Overall limitations

External circumstances, such as sample location, sampling time and sample composition, can each affect the DNA composition of a given eDNA sample and may mask certain DNA purely by chance. A potential limiting factor in our case is the number and quantity of samples. It has already been shown in studies with similar sample numbers, that increasing the total number of samples per site would have likely led to higher species detection rates (West et al., 2020) and accumulation curves indicate that more samples than were taken in our individual sampling efforts are needed to fully assess community composition at a site (Figure S5). How eDNA analyses are limited by sampling timepoints (days, weeks, or months), location (large scale versus small scale) and other abiotic factors that may affect eDNA longevity as temperature or water movement is a subject of ongoing research (Deiner & Altermatt, 2014; Jeunen et al., 2019, 2020; Koziol et al., 2019). Information about eDNA longevity and movement in seawater varies widely, and estimates range from hours to days (Andruszkiewicz et al., 2021; Holman et al., 2022; Paul et al., 1989). However, results from our samples, taken one month apart, indicated the reliability of the approach as they did not differ in detected species composition and abundance. This supports earlier published studies (DiBattista et al., 2020; Nichols & Marko, 2019). It is possible that environmental and ecological conditions influence detection rates and area (Jeunen et al., 2019). However, the spatial discrimination of eDNA signals from reefs in this study suggests that DNA dispersal between reefs is unlikely, as agrees with results reported in other studies (Alexander et al., 2020; Cole et al., 2022; Stat et al., 2019).

Different protocols during laboratory work (e.g., the use of half filter or whole filters) may also be potential sources of error (Bohman et al., 2021). In our case, the data obtained show that both library preparation approaches, provide comparable data in the final evaluation. Discrepancies in the results can be explained by our choice of samples. However, it should also be noted that read depth and number of OTUS can vary depending on the library preparation approach. Thus, methodological differences (e.g., increased number of cycles/different PCR conditions may bias read depth up to chosen bioinformatic pipelines) should be considered when comparing different methods in future studies.

4.3.2 | Specific methodical limitations

Finding a single metabarcoding gene marker for stony corals is difficult (Shearer & Coffroth, 2008). The use of mitochondrial (mt) DNA has several advantages; for example, mt-DNA fragments are likely to be more abundant in environmental samples than nuclear DNA fragments due to their high copy number per cell and are presumably less susceptible to degradation in the environment (Bylemans et al., 2018). Mt-DNA is regularly used for metazoan barcoding (Hebert et al., 2003) covering a broad range of different genera and mt genes have also been used extensively for molecular phylogenetic analyses of Scleractinia (Benzoni et al., 2011; Budd et al., 2010; Fukami, 2008; Huang et al., 2009; Kitahara et al., 2010). In addition, many mt nucleotide sequences are publicly available in databases such as NCBI or Midori (Machida et al., 2017), which can improve the accuracy of analyses. Therefore, mt markers are regularly used in eDNA studies, including corals (Nichols & Marko, 2019; Shinzato et al., 2021). However, genomic variation in mt genes is highly conserved among different coral species (Shearer et al., 2002; Shearer & Coffroth, 2008), making species-level discrimination of stony corals with this barcode region nearly impossible and challenging even at the genus level (Shinzato et al., 2021).

Thus, representatives of the Merulinidae, could not be reliably resolved to the genus level in our eDNA metabarcoding approach. Although reference sequences could be collected for many representatives of this family (Table S2), they mostly differed by only 1 or 2 base pairs and had a within-family genetic distance of only 2% (Table S5), resulting in low genus-level resolution in the eDNA analyses. Therefore, the assumed threshold of 97% sequence similarity in OTU clustering may also be partially responsible for not accurately resolving genus differences. Most eDNA reads were assigned to *Cyphastrea sp.* although visual data indicated that other genera such as *Favites sp.* or *Dipsastraea sp.* were more abundant. However, a higher assignment threshold could result in fewer hits and bias the results rather than lead to a more accurate analysis (Alexander et al., 2020; Brown et al., 2015; Mysara et al., 2017).

The primer pair used in our study was adopted from an eDNA metabarcoding study of stony corals in Hawai'i, where it reliably recognized the predominant coral diversity (Nichols & Marko, 2019). However, the primer pair was not optimized to compensate for the higher diversity of scleractinian corals in the Gulf of Thailand (Monchanin et al., 2021; Sutthacheep et al., 2013). DNA from representatives of Lobophyllidae, Diploastreidae, and Fungiidae could not be amplified to the quality required for our database. Assuming that the non-amplification is due to primer problems such as mutations, insertions, or deletions in primer binding sites; affected specimens are not amplified and therefore not detected with eDNA approaches. This is quite possible, as several alterations in the COI gene have been described, especially in coral groups that were not amplified in our assay (Fukami et al., 2007; Huang et al., 2009). Therefore, our results reflect the need for optimization or a new primer design for COI, as is already being pursued for stony corals (Shinzato et al., 2021).

Another option would be to use nuclear markers such as ITS rRNA to improve taxonomic assignments and circumvent the limitations of mt databases (Deiner et al., 2017). Particularly in scleractinian corals, ITS markers are commonly used for species differentiation and have also led to robust species assignments in eDNA studies (Alexander et al., 2020; Chen et al., 2004; Dugal et al., 2022; West et al., 2021). However, in a recent study species resolution could not be guaranteed for all coral groups (Ip et al., 2022). In addition, the high mutation rate can also lead to high intragenomic variation, which can affect the robust taxonomic assignment of ITS sequences with multiple copies to a reference sequence. As a result, unknown sequences at such loci may be discarded if they do not have an exact database match, and the dataset is reduced to known and sequenced species diversity (Deiner et al., 2017; West et al., 2021).

Therefore, in addition to designing new primer pairs for the taxon under study, combining different primer pairs in a multi-assay approach seems to be a promising strategy to achieve a better resolution of the actual species diversity. In this context, combinations of different mt marker genes (COI, 16 S: Nichols & Marko, 2019; COI, 12 S: Shinzato et al., 2021) and nuclear genes (ITS: Dugal et al., 2022; West et al., 2021) and the combination of mt and nuclear markers (16 S, ITS: Alexander et al., 2020) have already been tested in coral eDNA studies.

Nevertheless, our study and others (Nichols & Marko, 2019; Shinzato et al., 2021) show that genus-level monitoring of stony corals using mt genes is quite feasible and useful. However, future studies should favor multi-assay approaches that include nuclear markers to combine the advantages of marker systems and obtain the best possible combination of species detection and assignment.

4.3.3 | The importance of a reference database

The ability to interpret eDNA data is also limited by the quality and quantity of reference sequences available, which in other studies resulted in the failure to determine a large proportion of OTUs found and hence in a higher portion of false-negative results (DiBattista et al., 2020; Koziol et al., 2019). In some cases, databases are not up to date with the latest taxonomic status or unintentionally contain incorrect information. These circumstances can therefore lead to incorrect taxonomic information being assigned to an eDNA read. Those problems may arise frequently and often limit the integration of visual and eDNA data. We addressed this problem by the construction of a COI custom-made reference database based on corals collected directly on field sites. As previously reported, the reliability of a metabarcoding approach can be improved by selecting higher quality sequence reference databases (Dugal et al., 2022; Nichols & Marko, 2019;

Pompanon et al., 2012). It has already been shown for ITS2 that a local reference database improved robustness of species detection (Dugal et al., 2022; West et al., 2021). Using our custom reference database provided better results (less false negatives as well as outdated taxonomic classification) than querying against NCBI GenBank, the world's largest freely accessible annotated collection of nucleotide sequences.

5 | CONCLUSION

Coral reefs are under severe stress due to anthropogenic activities and climate change (Hughes et al., 2017; Knowlton et al., 2021). Better understanding of their resilience to changing environmental conditions and to correctly assess ecosystem health information on diversity indices such as species richness and abundance is of great importance (Margules & Pressey, 2000; Richards, 2013). Therefore, more efficient and robust survey methods are needed. While the monitoring method used will also depend on the primary biodiversity question at hand, it has been shown, not only in this study, that combining visual monitoring with eDNA metabarcoding provides much greater taxonomic richness than a single method alone (Everett & Park, 2018; Kelly et al., 2016; Stat et al., 2019). Furthermore, eDNA metabarcoding also shows potential for tracking changes in coral cover as well as general shifts in community composition under oceanographic conditions or highly variable anthropogenic pressures (DiBattista et al., 2020, 2022; Kelly et al., 2016; Nichols & Marko, 2019). Similarly, identifying cryptic taxa or invasive species in coral reefs at risk of being missed by traditional visual survey methods demonstrates the integration of multiple survey methods provides the most comprehensive snapshot of diversity, especially at hard-to-reach or remote sites (Ip et al., 2021). Building on the rapid developments in eDNA metabarcoding, the establishment of local databases as well as the expansion of existing public sequence databases, the eDNA method continues to improve and will soon be integrated into best practices for marine resource management, if not already (Dugal et al., 2022; Shinzato et al., 2021).

AUTHOR CONTRIBUTIONS

F.G. conceived the idea for the study, participated in the planning of the study, collected tissue samples, collected water samples and conducted transects, performed statistical analysis, and wrote the manuscript; M.S. conceived the idea for the study, participated in the planning of the study, collected tissue samples, collected water samples and conducted transects, performed the laboratory work, performed statistical analysis, and wrote the manuscript; P.M. collected tissue and water samples, conducted transects, and helped with the laboratory work; E. S. helped conduct and organize the field work and participated in the collection of tissue and water samples; R.T. participated in the planning of the study, wrote the manuscript, and critically revised it; all authors gave their final approval for publication and agree to be responsible for the work done here.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

Visual survey data, photographs of coral colonies collected for the reference database and materials used in the analysis are available for download on Dryad Digital Repository (https://doi.org/10.5061/dryad.3n5tb2rmm). Sequence data (demultiplexed, unfiltered reads) that support the findings of this study have been deposited in the NCBI Sequence Read Archive (BioProject ID: PRJNA894349).

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SUPPORTING INFORMATION

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