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REPORT

Using underwater mini-ROV for coral eDNA survey: a case study in Okinawan mesophotic ecosystems

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Abstract Mesophotic coral ecosystems (MCEs) are light-dependent communities occurring at depths of 30–150 m. They have been suggested to serve as refuge against thermal stress during heat waves for some coral species. Recent studies on MCEs have revealed a high diversity of communities, some unique, and that these ecosystems are far from being immune to anthropogenic threats. However, the depths at which these ecosystems are found make their exploration and study challenging. Consequently, most suitable environments for MCEs remain unexplored. To facilitate the detection and characterization of MCEs, we improved the methodology for mesophotic scleractinian survey by environmental DNA (eDNA) metabarcoding analysis using seawater collected by underwater mini-Remote Operated Vehicle (mini-ROV). We tested this improved approach at upper mesophotic sites in Okinawa, Japan, with different corals dominating the communities (i.e., *Alveopora*-dominated, *Seriatopora*-dominated, and *Acropora*-dominated communities). Despite the proximity of the different sites, our eDNA metabarcoding analyses detected the dominant coral genera specific to each site. In addition, this study detected numerous other genera present at these sites, including *Acropora*, *Pachyseris, Galaxea, Lobophyllia, Montipora, Pocillopora, Porites,* and others. Therefore, this study might support a new technical gate for comprehensive survey of MCEs using eDNA samples collected by underwater mini-ROV, although further technical improvement is required for quantitative estimation.

Keywords Scleractinians · Upper mesophotic reefs · Ryukyu Islands · Broader survey · Aquatic robotics · Genera-level detection · Biodiversity

Introduction

Coral reefs extend across tropical and subtropical seas and are the richest source of ocean biodiversity with approximately 30% of marine species living there (Knowlton et al. 2010). Reef-building corals, mainly scleractinians, are mostly known from shallow waters (3–20 m in depth). However, photosymbiotic corals can grow at depths below 30 m and down to 100 m or more where the light intensity is very limited, in ecosystems called mesophotic coral ecosystems (MCEs) (Puglise et al. 2009; Hinderstein et al. 2010; Muir and Pichon 2019; Pyle and Copus 2019). Some reef-building corals thrive only in shallow waters, while others are found in relatively deep waters (25–60 m) and even deeper than 170 m, depending on the species biological property and location (e.g., Rouze et al. 2021). These trends are closely

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related to location and physical parameters (water clarity, temperature regimes, stratification, etc.) (Kahng et al. 2019).

Recent technological advances have made it possible to survey MCEs at depths of 100 m and more. In parallel with technical diving, underwear robotics became more accessible, and it is now possible to survey MCEs at 100–200 m using reasonably priced underwater robots (Armstrong et al. 2019). Taking advantage of advanced underwater robotic techniques, MCEs in many areas have been explored to show that at deeper depths, reef-building corals are decreasing, and a transition occurs toward deep ecosystems dominated by gorgonians, black corals, and sponges (Stefanoudis et al. 2019). However, there is still much that remains unknown about the present status of MCEs. In many locations, including the Ryukyu Islands, their occurrence remains mostly unknown in the absence of extensive surveys; for the MCEs that are known, often the coral diversity assessments are preliminary, and little to no information is available on the coral community dynamics and health.

Environmental DNA (eDNA) comprises the total DNA extracted from an environmental sample, including DNA from organisms, or fragments of organisms as well as extracellular DNA deriving from cellular degradation (Taberlet et al. 2012). Furthermore, eDNA terminology can be refined by referring to the sources (e.g., water eDNA) or the targeted taxa (e.g., coral eDNA) (Pawlowski et al. 2020). High-throughput sequencing of eDNA samples allowed eDNA metabarcoding studies to monitor a variety of terrestrial and aquatic ecosystems and to target any taxa, from plants and animals to bacteria (Deiner et al. 2017). Recently, several eDNA metabarcoding studies have been conducted in coral reefs (e.g., Shinzato et al. 2018; Nichols and Marko 2019; Alexander et al. 2020; Gösser et al. 2023; Nishitsuji et al. 2023; Hoban et al. 2023; Ip et al. 2023). After a pilot study of Shinzato et al. (2018) in aquaria using the putative mitochondrial control region of *Acropora*, Nichols and Marko (2019) were the first to use an eDNA approach in the field to assess corals in the relatively low coral diversity environment of Hawaii based on mitochondrial partial cytochrome C oxidase subunit I (COI) and 12S ribosomal RNA genes (12S rDNA). Following these pioneering works, Alexander et al. (2020) and Dugal et al. (2021) addressed the issue of field monitoring of coral eDNA in high-diversity locations in Australia using primers for both internal transcribed spacer 2 (ITS2) region of rDNA and 12S rDNA (only in Alexander et al. 2020) targeting not only corals but also other marine metazoans. These studies identified up to 25 and 37 coral genera, respectively, and highlighted the importance of an extensive custom reference database obtained from collected specimens. Gösser et al. (2023) compared coral eDNA based on COI and visual census data in Thailand and recovered 20 genera with eDNA and 26 with the visual census approach, with 17 genera overlapping both approaches. Illustrating the versatility of eDNA metabarcoding, Ip et al. (2023) targeted the coral spawning period to detect coral and fish DNA in seawater samples to monitor coral spawning activity and related shifts in fish trophic structure. In Japan, Shinzato et al. (2021) designed a set of primers for mitochondrial 12S rDNA to theoretically distinguish between 36 genera at that time. This approach was tested on Okinawan shallow reefs and confirmed that this method could distinguish and/or cover most of the directly observed coral genera at approximately 80% of monitored locations (Nishitsuji et al. 2023).

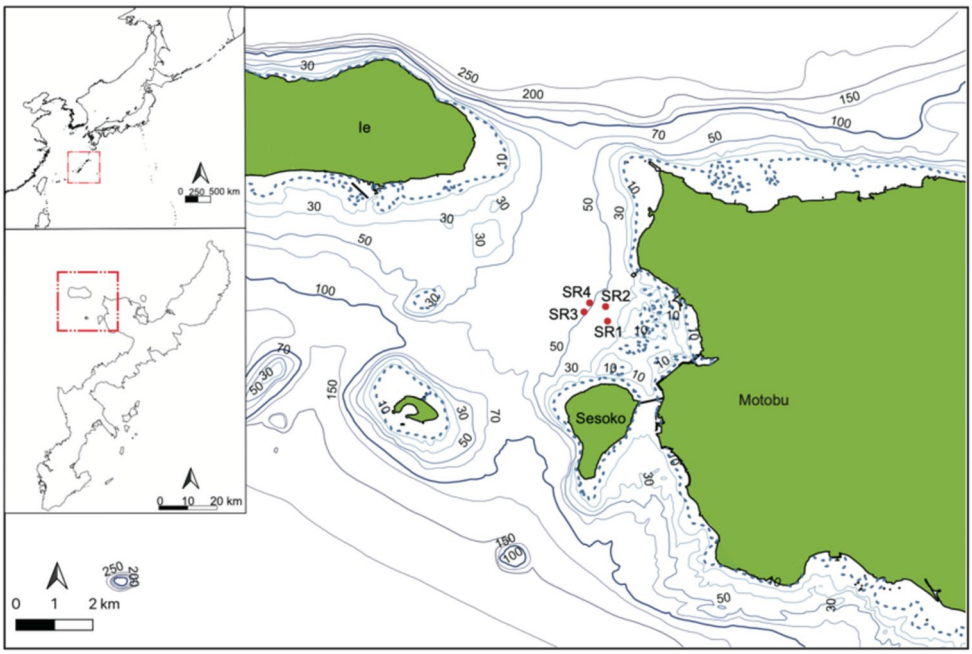
In parallel to these molecular approaches, the use of underwater robotics, including Remotely Operated Vehicles (ROVs) and Autonomous Underwater Vehicles (AUVs) has been suggested for surveys of coral reefs, especially for environments difficult to access, such as MCEs (Madin et al. 2019; Armstrong et al. 2019). For example, ROVs have been used to expand knowledge on various aspects of mesophotic diversity and ecology (e.g., Hollarsmith et al. 2020; Strader et al. 2021), and AUVs allowed to obtain imaging data on mesophotic communities over wide areas (e.g., Osuka et al. 2021; Noguchi et al. 2022). Combining eDNA and robotics, an ROV equipped with a 500 ml water sampler allowed the detection of several coral genera in water sampled between 60 and 80 m depth in Okinawa (Nishitsuji et al. 2024). However, the limitation of one sample per dive, limited battery capacity, and lack of baseline data limited the interpretation of the data obtained. Here, we aim to address these limitations by using an upgraded ROV model with two water samplers and replaceable batteries in the field on several mesophotic sites with known coral communities (Sinniger et al. 2022). In studies that targeted 12S rDNA, eDNA metabarcoding identified more scleractinian genera than directly observation (e.g., Nishitsuji et al. 2023). This study aimed to examine whether eDNA metabarcoding of samples collected from sub-mesophotic fields by ROV might provide a novel tool to broader survey of MCEs.

Materials and methods

Monitoring locations, mini-ROV, and water collection

Four monitoring sites north of Sesoko Island and west of Motobu Peninsula, Okinawa, Japan (Fig. 1a), were surveyed on April 25, 2023. Monitoring locations with latitude and longitude information, depth, volume of collected seawater, and others are shown in Fig. 1b. Shigeo Reef (SR) sites, SR1 and SR2, are approximately 37–45 m in depth, and deeper Shigeo Reef sites, SR3 and SR4, approximately 54–59 m in depth (Fig. 1b). According to surveys done by Sinniger et al. (2013, 2019, 2022), a relatively high scleractinian generic diversity with a dominance of *Acropora tenella* is found at SR1, and *Seriatopora hystrix* is most frequent at SR2. In

(a)



(b)

Site	Location	Latitude	Longitude	Depth (m)	Water volume (mL)		Fig.	Video
					Left	Right		
SR1	Shigeo Reef	26°40.2332' N	127°51.9562' E	37~40	420	400	Fig. 2b	S1
SR2	Shigeo Reef	26°40.4624' N	127°51.9257' E	45	400	300	Fig. 2c	S2
SR3	Deep Shigeo Reef	26°40.3790' N	127°51.5872' E	57~59	400	350	Fig. 2d	S3
SR4	Deep Shigeo Reef	26°40.5209' N	127°51.6755' E	54~57	400	300	Fig. 2e	S4

Fig. 1 A scleractinian coral eDNA survey at four mesophotic sites using an underwater mini-ROV. **a** Four monitoring locations (SR1-SR4) at the Shigeo Reef near Motobu Peninsula. The location of Okinawa Island, Japan, and the survey area are shown upper-left squares. **b** Information about sampling locations, latitude, longitude, approximate depth, figures, and videos

addition, SR3 has the lowest coral coverage and is dominated by solitary corals such as *Cynarina*, and *Alveopora* is a dominant species at SR4 (Sinniger et al. 2019).

Mini-ROV used in this study was a FIFISH W6 Plus (https://www.qysea.com/jp /products/fifish-v6/) (Fig. 2a) with 300 m cable between the ROV and its controller. Two water samplers (FIFISH model No. QY-WS-500) with a capacity of 500 mL were attached to the bottom of the FIFISH W6 Plus (Fig. 2a), although the actual amount of water sampled is 350–400 mL in most cases (Fig. 1b), due to incomplete regulation of ROV position from the boat to get full volume of water samples. The W6 Plus could be operated with replaceable batteries so that nearly 6 h of operation was possible on a one-day cruise (Supplementary Videos S1–S4). Three persons were needed for the ROV operations, piloting, guiding, deployment/recovery, and management. Seawater samples were collected approximately 0.5–1 m above the reef bottom without damaging corals. Upon recovery of the ROV, seawater collected by each of the two samplers was immediately filtered through separate 0.45-µm Sterivex filters (Merck), followed by the addition of 1 mL of RNAlater (Qiagen) to the filtrate to prevent DNA degradation (Shinzato et al. 2021). Filters were maintained at 4℃ before transfer to a − 20 °C freezer in the laboratory for eDNA metabarcoding analysis.

PCR primers for eDNA metabarcoding analyses

We used a set of primers for scleractinian-specific eDNA metabarcoding method, which targeted mitochondrial 12S rDNA, Scle_12S_Fw (5′-CCAGCMGACGCGGTRANACTTA-3′) and Scle_12S_Rv (5′-AAWTTGACGACG GCCATGC-3′) (Shinzato et al. 2021). This primer set was able to identify 36 scleractinian coral genera as of 2022 (Nishitsuji et al. 2023). An increasing number of scleractinian mitogenome information was deposited to NCBI on 10th October 2023, from which we could download publicly available mitogenomes of 114 scleractinian species.

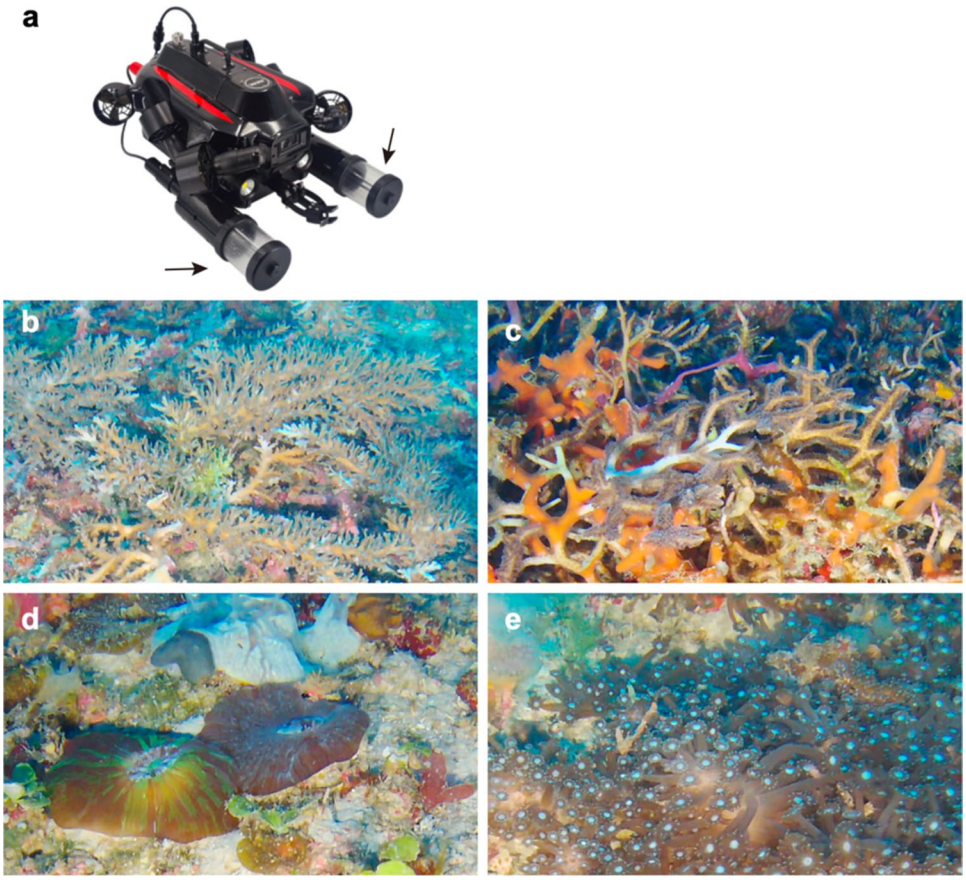


Fig. 2 **a** W6-type mini-ROV with two seawater samplers (arrows). **b** Shigeo Reef site SR1. View from mini-ROV camera. See Supplementary Video S1 for more image. **c** Shigeo Reef site SR2 with dominancy of *Seriatopora hystrix.* See Video S2 for more image. **d** Shigeo Reef site SR3. See Video S3 for more image. **e** Shigeo Reef siteSR4 with dominancy of *Alveopora catalai.* See Video S4 for more image

In the present study, the entire mitochondrial genomes of *Acropora tenella*, *Alveopora catalai*, and *Leptoseris papyracea* were sequenced (see below), and were added to the data set to facilitate this eDNA metabarcoding analysis. We confirmed that the primer set can be utilized for 115 species and resulted in amplicons with lengths of 396–465 bp (Supplementary Figs. S1 and S2).

eDNA metabarcoding analyses

Duplicated eDNA samples each were processed independently by DNA extraction, PCR amplification, cDNA preparation, sequencing, and metabarcoding analyses until the final step of combination of them to get their averages. eDNA in Sterivex filters was extracted following instructions in the Environmental DNA Sampling and Experiment Manual v. 2.1 (Minamoto et al. 2021; Shinzato et al. 2021; Nishitsuji et al. 2023). PCR amplification was performed in a final volume of 25 µL using 2 µL eluted eDNA sample. The amplification mixture contained 0.3 U of Tks Gflex DNA Polymerase (Takara), 12.5 µL of 2 × Gflex PCR Buffer (Takara), and 0.5 µM of each primers. The mixture was denatured at 94 °C for 1 min, followed by 35 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 30 s, with a final extension of 68 °C for 5 min. Control experiment for PCR amplification was carried out sing DNA of *Acropora tenuis* as positive control and using surface seawater from the coast with more than 30 m depth and distilled water as negative controls (Supplementary Fig. S3).

PCR products were extracted and cleaned with a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd.). Amplicon sequencing libraries of cleaned PCR products were prepared using a KAPA Hyper Prep Kit (Roche) without fragmentation. Libraries were multiplexed, and 300-bp paired-end reads were sequenced on a MiSeq platform (Illumina) using a MiSeq Reagent kit v. 3 (600 cycles). Raw-sequence data (DRA accession: DRA017764) was deposited in DDBJ under BioProject ID PRJDB12132. The number of sequence reads, total base-pair length, and average and maximum length of reads of each sample are shown in Supplementary Table S1.

Low-quality sequences (–quality-cutoff = 20 and minimum-length = 200) and Illumina sequence adaptors were trimmed with CUTADAPT v4.3 (Martin 2011). The ZOTU (Zero-radius Operational Taxonomic Units) method was adapted to identify scleractinian genera with minor modifications to the method described by Shinzato et al. (2021). In this study, all ZOTU analyses using USEARCH v11.0.677 (Edgar 2010) were performed sample-by-sample. To reduce missing of scleractinian sequences, ZOTUs with a BLAST

e-value ≤ 1e-20, percent identity ≥ 90%, and query coverage ≥ 95% against 12S rRNA sequences of scleractinians were first used. Obtained data were further analyzed, and numbers of mapped sequences for each ZOTU were counted using the USEARCH "otutab" command with a percent identity of 100% (−id 1.00).

NCBI nt contains numerous unverified sequences, increasing the uncertainty of taxonomic identification in the metabarcoding analysis. To increase the accuracy of the analysis, we included only sequences that were verified by NCBI into our database, i.e., sequences tagged "UNVERIFIED" were removed. Firstly, complete mitogenomes of scleractinians deposited in NCBI were downloaded on 10th October 2023. We aligned the sequence region amplified by the primer set on a genus-by-genus basis by using MAFFT v7 (Katoh and Standley 2013). In most cases, the sequence region was identical within a genus. In such cases, we clustered them into one sequence to reduce file size and increase computational efficiency. If different sequences were found within a genus, we retained all sequences in the custom database. In addition, species of which mitogenome has not been reported but with reported 12S rDNA region to which the primer set can bind were included in the custom database. In the case of *Acropora*, three types of sequences were identified: sequences shared by most *Acropora*, sequences possessed by only one *Acropora* spp. (Accession ID: MW773218) and sequences shared by Caribbean *Acropora* (*Ac. cevicornis* and *Ac. prolifera*). Since Caribbean *Acropora* do not exist around Japan and due to the uncertainty of the sequence of *Acropora* spp., we only retained sequences that most *Acropora* possessed in the database. An alignment of the sequences used in the custom database is provided in Supplementary Figure S2. Using the custom database, taxonomic assignment of ZOTUs was performed with Assign-Taxonomy-with-BLAST (Li and Godzik 2006) and the best estimated genus was selected.

Several genera shared identical amplicon sequences (Supplementary Figs. S1 and S2), preventing exact genus identification. In these situations, the sequences were categorized as "multiple" (Supplementary Tables S3). This study focused on scleractinian-specific ZOTUs, and the other ZOTUs will be analyzed in a future study (Satoh et al., unpublished).

Sequencing of the mitochondrial genome of three species

The entire mitochondrial genome of *Acropora tenella*, *Alveopora catalai*, and *Leptoseris papyracea* were sequenced to facilitate this eDNA metabarcoding analysis, because having local reference sequences helps to accurately match the eDNA data to scleractinian genera. In addition, with the increase of coral metabarcoding studies using various markers, sequencing whole mitochondrial genomes will provide flexibility in future metabarcoding studies. Moreover, the raw sequencing data will likely also include other nuclear markers such as ribosomal genes. Sampling of these species was carried out from Shigeo Reef by SCUBA diving. Sampling information and the result of DNA sequencing are summarized in Supplementary Table S2.

Genomic DNA was isolated from fragments of corals by a Maxwell RSC Blood DNA Kit (Promega). 1 µg of DNA from each sample was used for PCR-free library preparation with KAPA Hyper Prep Kits (Roche). After the cleanup of the sequence library with AMPure XP beads, a size selection of 620–820 bp was performed with BluePippin (Sage science), and sequencing was carried out on an Illumina MiSeq platform (600 cycles). Raw-sequence data (DRA accession: DRA017588) was deposited in DNA Databank of Japan (DDBJ) under BioProject ID PRJDB17204. Low-quality sequences (quality-cutoff = 20 and minimum-length = 200) and Illumina sequence adaptors were trimmed with CUTADAPT v4.3 (Martin 2011). Clean reads were de novo assembled with GetOrganelle v1.7.7.0 (Jin et al. 2020) with the option "animal_mt." Genes in mitochondrion were annotated using MITOS2 webserver (Bernt et al. 2013) with the options "Reference: RefSeq 63 Metazoa" and "Genetic Code: 4 Mold." We obtained the assembly of the three coral species' circular mitochondrial genomes (mitogenomes). The complete mitogenomes of *A. tenella*, *A. catalai*, and *L. papryacea* consisted of 18,252 bp, 18,145, and 18,345 bp, with 13 protein-coding genes (Supplementary Table S1). The sequence information was incorporated into the bioinformatic pipeline for ZOTU analyses mentioned above.

Results

An average of 1,101,983 reads were obtained from the samples collected (ranging from 953,990 to 1,193,975 reads per sample), and an average of 252,429 reads per sample was successfully merged (Supplementary Table S1). After chimera filtering and sequence error correction, 1,512 ZOTUs per sample were detected (Supplementary Table S1). Although the primer set was designed to amplify the mitochondrial 12S rDNA of scleractinians, approximately 47% of the merged reads were mapped to the scleractinian-specific ZOTUs (Supplementary Table S1). Non-scleractinian ZOTUs corresponded to other groups of Anthozoa, and results will be reported separately. Among the scleractinian ZOTUs, 98% could be assigned to a unique genus (96–100% per site), while the remaining ZOTUs were assigned to groups of multiple genera (Supplementary Table S3).

The ROV used here was not set for quantitative visual surveys and only allowed visual estimation of the coral diversity and frequency at the study sites. The visual information obtained were used to confirm that the sites sampled

corresponded to the sites previously surveyed (Fig. 2). The ROV observation confirmed the distribution of four scleractinian corals: *Acropora, Alveopora, Pachyseris,* and *Seriatopora*. The abundant genera were *Acropora* (mostly *A. tenella*), *Seriatopora*, and *Pachyseris* (mostly *P. speciosa*), together with together with some *Cycloseris* and *Galaxea* at SR1 (Fig. 2b; Supplementary Video S1). The dominance of *Seriatopora* with some *Galaxea* was noticed at site SR2 (Fig. 2c; Supplemental Video S2). As expected from previous studies, *Alveopora* was abundant at SR4 (Fig. 2e; Supplemental Video S4), together with *Stylophora*, *Echinophyllia*, *Merulinids*, *Pavona*, *Pachyseris*, Lobophyllia, and *Galaxea*. SR3 showed the lowest coral coverage and diversity, despite being located close to SR4, with sparse Lobophyllia and *Cycloseris* (Fig. 2d; Supplemental Video S3). These observations confirmed the suitability of the sites selected to test the eDNA metabarcoding approach for mesophotic corals.

eDNA of scleractinians at four sites

Occurrence of Alveopora and Seriatopora

Alveopora and *Seriatopora* are corals particularly abundant at SR4 and SR2, respectively. We examined first whether *Alveopora* are able to be detected by eDNA at SR4, since SR4 was known to host *Alveopora* and the ROV images confirmed the abundance of this genus at the exact dive point (Fig. 2e; Supplemental Video S4). As expected, a large number of ZOTUs that correspond to *Alveopora* appeared at SR4 but not at the other sites (Table 1). Next, we examined whether *Seriatopora* are able to be detected by eDNA at SR2 (Fig. 2c; Supplemental Video S2), since the site SR2 has been characterized by a local dominancy of *Seriatopora hystrix* (Sinniger et al. 2019, 2022). *Seriatopora*-corresponding ZOTUs were counted at SR2 (Table 1). However, the number of *Seriatopora*-corresponding ZOTUs at SR2 was unexpectedly small (667), and the ZOTUs were also detected at SR1 (the number was 206) (Table 1). Sites SR2 and SR1 are of shallower Shigeo Reef and in close proximity. Deeper Shigeo Reef S3 and S4 did not show *Seriatopora*-corresponding ZOTUs (Table 1). Therefore, these results indicate that scleractinian-specific eDNA metabarcoding analysis might detect a specific coral genus, dominancy of which was known at a given site.

Table 1 ZOTU numbers mapped to four scleractinian genera at four sites of Shigeo Reef

	SR1	SR2	SR3	SR4
Acropora	259,260	34,124	10,361	6,716
Alveopora	**0**	**0**	**0**	**22,480**
Pachyseris	12,621	6,767	5,970	8,727
Seriatopora	**206**	**667**	**0**	**0**

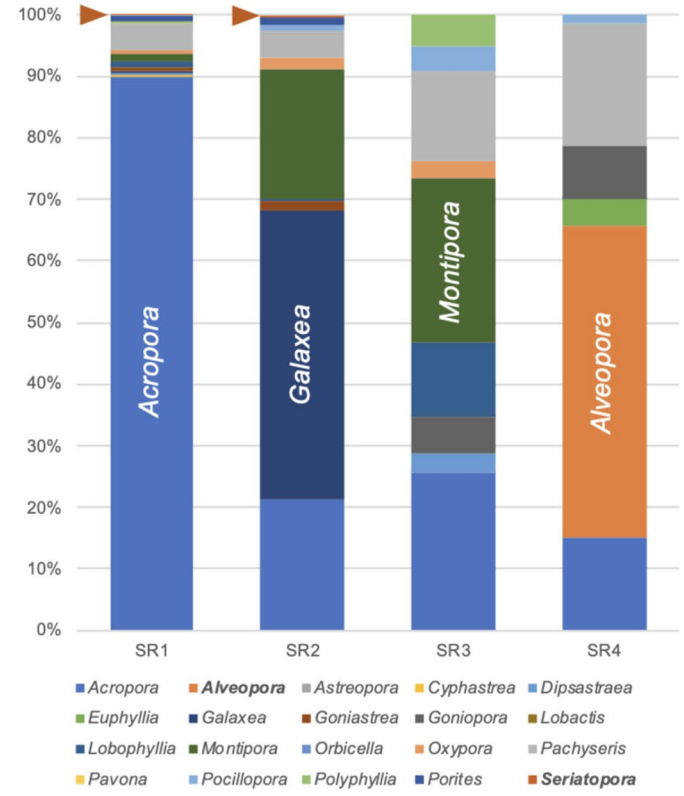
Features at each site

Site SR1 (37–40 m depth): the eDNA metabarcoding analysis showed the presence of scleractinian corals of at least 20 genera from this site (Fig. 3; Supplementary Tables S4 and S5), with additional ZOTUs that could be assigned to multiple genera (Supplementary Table S6). Of the single genus identifications, nearly 90% of reads corresponded to ZOTUs assigned to *Acropora* (Fig. 3; Supplementary Table S5), supporting the observed dominancy of *Acropora* species at site SR1. ZOTUs were called against the amplicon sequence of *A. tenella* and *A. tenuis.* However, the nucleotide sequences of the two species were completely identical to the other 24 *Acropora* species. Therefore, this analysis could not always mention that *A. tenella* was the dominant species of these genera. The next genera with high ZOTU scores were *Pachyseris* then *Montipora* and *Porites* (Fig. 3; Supplementary Table S5). Numerous reads formed ZOTUs assigned to *Lobophyllia*, *Oxypora*, *Galaxea*, and *Goniastrea,* and a few reads were assigned to various other genera, including *Seriatopora* (Supplementary Table S5). Among the other scleractinian ZOTUs assigned to a multiple genera category, most reads could be identified either to *Dipsastraea* or *Mussa*, the second most abundant category groups identified to *Merulina* or *Mycedium* (Supplementary Table S6). Other groups include *Agaricidae*, various combinations of Acroporidae, a few *Merulinidae*, and some combinations of genera from unrelated families (Supplementary Table S6).

Site SR2 (45 m in depth): Slightly less coral diversity was detected from this site with scleractinian corals of 13 genera (Fig. 3; Supplementary Tables S4 and S5). Nearly half of the reads were identified as *Galaxea* (45.4%) (Fig. 3; Supplementary Table S5), followed by *Montipora* and *Acropora*, with 20 and 30% reads, respectively. Other groups include *Pachyseris* and *Seriatopora* and a few other genera with less than 5% reads (Fig. 3). Despite *Seriatopora* representing a minor proportion of the reads obtained from the site with 0.24% of the reads, SR2 was the site with the largest amount of *Seriatopora* sequences. Among the ZOTUs that could not be assigned to a single genus, *Gardineroseris*/*Leptoseris*/*Pavona* comprised a significant number of reads (98% of the total scleractinian reads for multiple at this site; Supplementary Table S6). Despite a relatively high coral cover compared to other sites, this site resulted in the lowest number of reads. The reason for this low yield was obscure and could simply be an artifact of sequencing.

Site SR3 (57–59 m in depth): among the 13 genera identified from this site, *Montipora* and *Acropora* counted for 15% and 12%, respectively (Fig. 3, Supplementary Tables S4 and S5). *Pachyseris* and *Lobophyllia* were next with 15.1% and

(a)



(b)

	SR1	SR2	SR3	SR4
Acropora	87.87%	20.29%	25.53%	14.92%
Alveopora	0.00%	0.00%	0.00%	49.95%
Astreopora	0.10%	0.05%	0.00%	0.00%
Cyphastrea	0.42%	0.00%	0.00%	0.00%
Dipsastraea	0.11%	0.00%	3.24%	0.00%
Euphyllia	0.00%	0.00%	0.00%	4.10%
Galaxea	0.43%	44.93%	0.00%	0.00%
Goniastrea	0.39%	1.33%	0.00%	0.00%
Goniopora	0.00%	0.00%	5.75%	8.75%
Lobactis	0.01%	0.00%	0.13%	0.00%
Lobophyllia	0.99%	0.38%	12.02%	0.00%
Montipora	1.13%	20.33%	26.80%	0.00%
Orbicella	0.00%	0.00%	0.00%	0.00%
Oxypora	0.66%	1.70%	2.65%	0.00%
Pachyseris	4.28%	4.02%	14.71%	19.39%
Pavona	0.12%	0.00%	0.00%	0.00%
Pocillopora	0.00%	1.05%	4.15%	1.48%
Polyphyllia	0.12%	0.00%	4.99%	0.00%
Porites	1.11%	1.20%	0.00%	0.00%
Seriatopora	0.07%	0.40%	0.00%	0.00%

Fig. 3 **a** Bar graph showing the distribution and approximate proportions of scleractinian corals at four monitoring sites (SR1-SR4) of mesophotic Shigeo Reef. Names of scleractinian coral genera are shown in different colors at the bottom. Brown arrowheads indicate *Seriatopora*. **b** Percentages of sequence reads mapped to coral genera in each eDNA sample. Percentages are colored in the heatmap. This analysis used ZOTUs mapped to single genera category

11.5% of the reads, respectively, followed by various genera, including *Goniopora*, *Dipsastraea*, and others (Fig. 3, Supplementary Tables S4 and S5). Almost all the ZOTUs could be assigned to a single genus (Supplementary Table S6).

Site SR4 (54–57 m in depth):

Over half of the scleractinian reads could be assigned to the genus *Alveopora*, followed by *Pachyseris*, *Acropora*, *Goniopora*, and *Euphyllia* (19.7%, 14.6%, 8.2%, and 4.1%, respectively) with a minor proportion of *Pocillopora* and *Stylophora* (Fig. 3, Supplementary Tables S4 and S5). Additional reads were assigned to the *Merulina/Mycedium* cluster (10%) (Supplementary Table S6).

Discussion

Diversity of mesophotic coral genera at Shigeo Reef

The depths at which MCEs occur challenge biodiversity studies, especially when combined with the environmental plasticity exhibited by scleractinian corals (Sinniger et al. 2016; Muir and Pichon 2019). Previous studies have shown that Japanese MCEs exhibit some of the highest diversity of scleractinian corals in the world (Sinniger and Harii 2018; Sinniger et al. 2019). Yet, they are also affected by increasing anthropogenic impact and natural events such as typhoons (Fujita et al. 2012; White et al. 2017). In the context of the deep reef refugia hypothesis (Bongaerts and Smith 2019), recent studies in Okinawa suggested that the abundant *Seriatopora* corals found at MCEs support the role of MCEs as a refuge for this coral (Sinniger et al. 2013, 2017), although mesophotic larvae that were exposed to light conditions corresponding to shallow depths (5 and 10 m) ex situ showed bleaching and a significant reduction in settlement rates (Prasetia et al. 2022). To properly assess the threats and potentials of MCEs, it is essential to improve the efficiency of coral biodiversity surveys at mesophotic depths in this region.

Efficiency of the eDNA metabarcoding method for mesophotic coral survey

The major aim of this study was to examine the reliability of eDNA metabarcoding method using seawater samples collected from semi-mesophotic reefs (35–80 m in depth) by mini-ROV. A first attempt to survey mesophotic coral eDNA revealed several technical issues such as sampler number, battery, cable, and others (Nishitsuji et al. 2024). The mini-ROV FiFiSH W6 Plus used in the current study answered these issues. Nishitsuji et al. (2024) also found a lack of baseline data to compare with the eDNA results. Here, we addressed this issue by surveying four sites of Shigeo Reef, Okinawa, Japan, for which we knew the existence of distinct communities. The ratio of scleractinian sequences recovered from the eDNA (65%) was surprisingly low compared to the more than 90% recovered from surface water with the same primer set previously (Shinzato et al. 2021). However, several findings on this eDNA survey hint at a validation of the eDNA approach. SR1 is a site mostly dominated by *Acropora tenella* (Sinniger et al. 2013), and the eDNA recovered an unambiguous majority of *Acropora*-assigned reads at this site (nearly 220,000 reads, or 89% of the sequences obtained at this site, versus less than 30,000 and less than 26% of the reads at other sites). The site SR2 has been characterized by a local dominancy of *Seriatopora hystrix* (Sinniger et al. 2019, 2022). While *Seriatopora* sequences were rare in the eDNA dataset, SR2 was the site with the highest proportion of *Seriatopora* sequences. The paucity of *Seriatopora* sequences could result from primer bias or more likely from biological sources with this genus shedding less DNA in the environment than other corals. At SR2, most reads were assigned to *Galaxea*; this genus is present at the site and could be observed on the ROV. SR4 is one of the best examples of supporting the eDNA approach, as this site was known to host *Alveopora*, and the ROV images confirmed the abundance of this genus at the exact dive point. In addition, this site is currently the only location in Japan where *Euphyllia paradivisa* was recorded (Eyal et al. 2016), and this could explain the 4% of sequences assigned to this genus and its occurrence only at this site. SR3, on the other hand, was one of the most poorly explored sites, and the ROV footage tends to confirm the first observations made by divers (Sinniger pers. comm.) that this site has a rather low coral cover dominated by solitary Lobophylliid corals. However, the eDNA recovered at SR3 suggests a slightly different diversity and this may be due to the proximity to other coral communities. However, SR3 is also the site with the most sequences assigned to *Lobophyllia* (Fig. 3). While the decrease of *Acropora* sequences from S1 to S4 could also suggest a carryover of the massively abundant *Acropora* DNA between samples, this is unlikely to have happened at the sampling stage as SR4 was the first sampled site, followed by SR1, SR3, and finally SR2. However, the carryover problem should always be in mind in eDNA metabarcoding analysis. Also, improvement of the mini-ROV to allow simultaneous eDNA and quantitative visual survey could be obtained by installing a downward facing camera and laser scale, combined with a geopositioning system to estimate the distance traveled.

In a low coral diversity region such as Hawaii, eDNA metabarcoding was suggested to be usable to infer coral coverage based on the high correlation between reads numbers and percent cover from visual surveys (Nichols and Marko 2019). The same approach was conducted in Thailand and suggested both a correlation between eDNA and coral cover and a primer bias at the genus level (Gösser et al. 2023). However, this potential was not confirmed in a higher biodiversity environment like NW Australia (West et al. 2022). While recovering some interesting signals, our data question the relevance of the quantitative approach as exemplified by the amounts of reads overall or the dominance of solitary Lobophylliids in SR3 that is not reflected by the eDNA that instead recovered a larger proportion of *Montipora*, *Acropora*, and *Pachyseris*. A bias in amplification due to the primers is always a possibility in metabarcoding studies (Fonseca 2018); however, in our case, the stability of the 12S rDNA region within scleractinians, makes it less likely to result in a strong primer-induced bias. The usage of different methods by different laboratory may be another factor that causes a bias to compare results of different studies.

Depending on the dispersal vector, different corals' DNA may also disperse in the environment at different rates. However, our results do not support a strong eDNA dispersal potential of coral eDNA, considering the differences observed at each site, despite the sites being located within 300–700 m from each other. This is coherent with the theory that eDNA decays faster in marine environments and at higher temperatures such as 30 °C and more (Lamb et al. 2022), both conditions being fulfilled at our sites. A preferred hypothesis relates to the fact that all corals may not contribute to the eDNA pool equally at a given time due to intrinsic variability in their physiology. Different DNA shedding rates have been demonstrated in several marine organisms (Sassoubre et al. 2016; Allan et al. 2020), and our data support the hypothesis that such differences in shedding rates occur within scleractinian corals.

Beyond the quantitative aspect, our study identified 29 single genera in the eDNA from Shigeo reef and up to a few additional genera with the ZOTUs that could not be unambiguously assigned to a single genus. In the shallow reefs of the Cocos Keeling Island (Indian Ocean), 25 genera were recovered (Alexander et al. 2020), while in the highly diverse Rowley Shoals in Western Australia, 37 genera were recovered (Dugal et al. 2021). Considering that both of these studies were mostly based on ITS2, which is much more

variable, the 29 genera identified with the 12S data presented here support the idea of Okinawa as a particularly diverse location for mesophotic corals.

The apparent depth-related decrease in genera detected from 20 at SR1 (37–40 m in depth) to 8 at SR4 (54–57 m) (Table 1), reflects different types of coral assemblages rather than suggesting an impoverishment of the diversity with depth. While the decrease in generic diversity with depth is known (e.g., Bridge et al. 2012; Diaz et al. 2023), in Okinawa, this decrease occurs at deeper depths than those investigated here (Sinniger et al. 2022). Future surveys over a larger depth range are needed to compare this decrease with the eDNA data.

Application of eDNA metabarcoding methods for coral survey

The ITS2 region was used in previous studies and showed great potential to identify corals from eDNA (Alexander et al. 2020; Dugal et al. 2021). However, the highly variable, multicopy nature of the ITS2 region requires an accurate and complete reference database for proper interpretation of the results. At mesophotic depths, the current lack of knowledge on coral biodiversity prevents the creation of such a reference database. Therefore, the phylogenetic information of the mitochondrial 12S ribosomal RNA allows better assignment to higher taxonomic levels (family level) when exact matches are not found in the reference database. The sequences of interest can then motivate further specimen-based investigations to discover and describe this unknown diversity. However, with the rapidly increasing number of mesophotic specimens and sequences available to researchers, a shift to more variable markers supported by extensive voucher-based reference databases is the most likely path for future coral metabarcoding studies.

Consistent with the findings of the previous studies on eDNA metabarcoding, several limitations remain when it comes to coral eDNA metabarcoding. The main challenges are the limited taxonomic resolution, the difficulty in detecting all the coral diversity and quantifying the results, although this is a general limitation of eDNA metabarcoding, not just coral eDNA. The limited taxonomic resolution is also a caveat for visual surveys, especially for encrusting species with small corallites, as exact coral identification often requires a detailed examination of skeleton vouchers. With improvements in coral molecular taxonomy, improving baseline data and exploring various markers may help understand coral diversity at lower taxonomic resolution. Increased detection of less abundant species may be achieved by improvement of the sampling methods (larger volumes, in situ filters, etc.) and experimental experimentation to understand how corals contribute to the eDNA pool and how this contribution varies among taxa will allow a better interpretation of the eDNA metabarcoding data.

Conclusion

Even acknowledging the current limitations of coral eDNA metabarcoding, the combination of eDNA and underwater robotics, as illustrated here, may greatly improve the screening and monitoring of difficult-to-access coral assemblages. With a proper reference database and protocols, technicians or researchers with limited taxonomic backgrounds could conduct this approach on a large scale in a standardized manner. This first screening approach will allow dedicating resources and manpower of expert coral taxonomists and ecologists to in-depth studies of the most relevant sites only.

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Author contributions NS conceived the experiment. FS and SH led to the sampling locations. NO, YS, and SN operated mini-ROV. NS and HN collected seawater samples. YY developed the primer set. HN and YY carried out sequencing. KH and YY performed ZOTU analysis. NS, FS, and SH interpreted the results and prepared the draft manuscript. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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Data availability Electric supplementary material is available online.

Declarations

Conflict of interest We declare no competing interests.

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