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# Evaluation of octocoral-specific primers for environmental DNA (eDNA) metabarcoding of the coral reefs of Okinawa, Japan

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## Abstract

Along with traditional surveys, environmental DNA (eDNA) offers a promising non-invasive, rapid, and cost-effective tool for monitoring coral reef communities and tracking changes driven by anthropogenic pressures and climate change. Most anthozoan eDNA studies have focused on class Hexacorallia and remain limited regarding class Octocorallia, with existing primers specifically targeting deep-sea groups, octocorals in general (both mtMutS), or anthozoans in general (28S rDNA). In this study, we evaluated three octocoral-specific primer sets (original mtMutS, new mtMutS, and 28S rDNA), targeting mitochondrial and nuclear genes for eDNA metabarcoding by comparing their performance *in silico*, *in vitro*, on mock communities, and on environmental samples. Mock communities were prepared from tissue samples of Octocorallia (20 families) collected from coral reefs of Okinawa and the northwestern Pacific Ocean. In the Okinawa field, photo-transect surveys were conducted, and seawater samples were collected at shallow coral reefs. The mock and field samples were amplified using the three primer sets. Only the new mtMutS primer set successfully detected all selected families within the mock community. We observed three genera from the family Sarcophytidae and one genus from Cladiellidae in visual surveys, but only the new mtMutS primer set confirmed their presence in eDNA samples. This study highlights the potential of the new mtMutS primer set as an effective tool for detecting octocoral biodiversity, and we recommend its use as a preferred marker for future assessments. Monitoring efforts are critical for understanding how octocoral communities will continue to change and for considering management strategies to ensure their future.


## Introduction

Octocorals (class Octocorallia, subphylum Anthozoa), such as soft corals, blue corals, sea fans, sea pens, and sea whips, are considered significant and ecologically conspicuous components of benthic communities and can be found from shallow waters to the deep sea worldwide (Fabricius and Alderslade 2001; Pérez et al. 2016; Lau et al. 2020). At some subtropical and tropical coral reefs, octocorals are a dominant benthic component (McFadden et al. 2025), and in some cases this dominance has been linked to phase shifts, especially in areas with declining water quality (Baum et al. 2016), higher levels of anthropogenic pressure (Lalas et al. 2024), coral bleaching (Stobart et al. 2005), and reef degradation (Rodriguez et al. 2020). It has been speculated that shifts to octocoral dominance may be due to many octocoral species' resilience to contemporary stressors (Ruzicka et al. 2013) and their capacity to recruit at comparatively higher densities and maintain rapid initial vertical growth rates (Lasker et al. 2020). Shallow tropical and subtropical coral reef monitoring has often been based on scleractinian coral datasets (Gardner et al. 2003; Bruno and Selig 2007; De'ath et al. 2012), although there are some notable exceptions (e.g., Lasker et al. 2020; Liberman et al. 2025). As a consequence, octocorals are often not surveyed or their diversity is underestimated when evaluating long-term marine ecosystem conditions.

Several methods for benthic community monitoring are well established, from *in situ* surveys to remote sensing-based methods (English et al. 1997; Hill and Wilkinson 2004; Teague et al. 2022). Each method has its own limitations, but the most commonly used method is *in situ* visual surveys recording directly or using a camera with simple image capture along transects. These SCUBA diving-based methods often focus on a subset of large and well-studied taxonomic groups as surrogates (Plaisance et al. 2011) and come with several limitations, such as inherent risks, high amounts of logistical effort, and limitations to accessible water depths (Reimer and Gösser 2023). However, taxonomic expertise and species detection abilities limit which taxa are recorded, and cryptic groups may be overlooked (Edgar et al. 2004). In addition, visual surveys are time and resource-intensive; the geographic area covered tends to be limited, resulting in patchy data (Muenzel et al. 2024). Thus, biologist need non-invasive, rapid, and cost-effective approaches to robustly monitor aquatic species underwater across wider scales, and environmental DNA (eDNA) approaches can help fulfill this need (Adams et al. 2019; Huang et al. 2022).

Environmental DNA (eDNA) applications, therefore, are ideal complements to traditional survey methods to increase detection rates and robustness of biodiversity monitoring (Reimer and Gösser 2023). With metabarcoding technology, this approach can rapidly and simultaneously identify species in the environment by amplifying specific DNA marker regions

(DiBattista et al. 2020). eDNA-based methods generally amplify DNA from various environmental samples, including water, soil, or air (Clare et al. 2021) under the assumption that there is free genetic material released from the skin, mucus, urine, saliva, feces, secretions, eggs, sperm, blood, etc. (Taberlet et al. 2012). Environmental DNA (eDNA) has recently been widely applied to reveal freshwater and marine aquatic organisms' diversities, abundances, distributions, and habitats (Stoeckle et al. 2017; Li et al. 2018). Thus, eDNA is a promising tool for monitoring the species presence in their habitats (Bohmann et al. 2014) and for tracking changes due to anthropogenic pressures (DiBattista et al. 2020).

Thus far, eDNA studies related to anthozoans have largely focused on the order Scleractinia within the Hexacorallia (Nichols and Marko 2019; Alexander et al. 2020; Shinzato et al. 2021; Dugal et al. 2022; Gösser et al. 2022; West et al. 2022; Nishitsuji et al. 2023; Açıkbaş et al. 2024; Nishitsuji et al. 2024; Hisata et al. 2025). Only a few studies have targeted anthozoans more broadly (DiBattista et al. 2020; McCartin et al. 2024), while studies directly targeting octocorals are scarce (Everett and Park 2018; Satoh et al. 2026). In studies targeting octocorals, an original mitochondrial MutS (mtMutS) eDNA primer set has been developed for deep-sea species (Everett and Park 2018), and a nuclear 28S rDNA primer set has been designed to examine anthozoans in general (McCartin et al. 2024).

Since metabarcoding primers targeting conserved rRNA or rDNA genes can amplify sequences from many non-target taxa (Marquina et al. 2019), we recently developed a new mtMutS primer set to amplify all octocorals (Hakim et al. 2025), as this marker has the highest substitution rate among mitochondrial genes (France and Hoover 2002), and is widely used for taxonomic work (McFadden et al. 2011; Quattrini et al. 2022). Octocorals are the only animal group with the mtMutS gene in their mitochondrial genomes (Muthye et al. 2022), and primers targeting this gene may enable more efficient and sensitive detection of octocoral diversity, while reducing the proportion of non-target taxa reads. Since the use of multiple markers is recommended in metabarcoding analyses for robust biodiversity monitoring (Liu and Zhang 2021), and octocoral-specific metabarcoding primers from the nuclear 28S rDNA are also available, evaluating the mitochondrial MutS (mtMutS) primer set with the 28S rDNA primer set offers a complementary strategy. In this study, we therefore evaluated three octocoral-specific primer sets targeting mtMutS and nuclear 28S rDNA genes for environmental DNA (eDNA) metabarcoding by comparing their performance *in silico* and *in vitro*, as well as with mock communities and eDNA samples from coral reefs in Okinawa Island, Japan.

## Materials and methods

# In silico evaluation

In our recent study, a new mtMutS primer set (AAH-MutS_F01 and AAH-MutS_R250; Hakim et al. 2025) was designed to amplify all octocorals via examinations of 2,126 sequences covering 92% (81/88) of all octocoral families (WoRMS 2025; Supplementary Table S1). Sequences were collected from GenBank using the criteria and validation data procedure described in Hakim et al. (2026). The new mtMutS primer set was developed by considering the conserved regions of the primer nucleotide sequence with a target length of 209–251 bp (Table 1). The new and original mtMutS primer sets overlap partially and are located within the common full-length barcode region of ND42599F and Mut-3458R primers from France and Hoover (2002) and Sánchez et al. (2003) (Fig. 1). *In silico* evaluation was performed for the original (Everett and Park 2018; hereafter “original primer set”, etc.), and our new mtMutS primer sets (Hakim et al. 2025; hereafter “new primer set”, etc.) by calculating primer-binding site conservation and visualizing the results using the PrimerMiner package (Elbrecht and Leese 2017), and by estimating primer mismatches in R (R Core Team 2025). However, some sequences used to design a new mtMutS primer set did not cover the target region of the original mtMutS primer set, reducing the dataset for *in silico* analysis of the original mtMutS to 2,004 sequences. Mismatches of the 28S rDNA primer were described in McCartin et al. (2024). The three octocoral-specific primer sets used in this *in silico* analysis and in subsequent analyses are presented in Table 1.

Table 1
Three metabarcoding primer sets targeting octocorals (Class Octocorallia) using mitochondrial mtMutS and nuclear 28S rDNA genes examined in this study.

| Primer names | Number of sequences in original analyses | Targeted gene | Sequences (5′ to 3′) | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| Original mtMutS primer | | | | | |
| Octo_eDNA_Mut2F | 78 (26 families) | mtMutS | GCTTATCCAGATTGGTAAGTTCTATGAA | 285–345 | Everett and Park 2018 |
| Octo_eDNA_Mut2R | | | ACAGTCCTCTAAATTACAACTAGGAGAATA | | |
| New mtMutS primer | | | | | |
| AAH-MutS_F01 | 2,216 (81 families) | mtMutS | YTRCTTCAAATGGGRTTTCC | 209–251 | This study |
| AAH-MutS_R250 | | | ACTATGYCCAYTCATGGCWG | | |
| 28S rDNA primer | | | | | |
| Anth-28S-eDNA-F | 483 (64 families) | 28S rDNA | CGTGAAACCGYTRRAAGGG | ~ 400 | McCartin et al. 2024 |
| Anth-28S-eDNA-R | | | TTGGTCCGTGTTTCAAGACG | | |

## In vitro evaluation and mock community analysis

*In vitro* evaluations were performed using tissue-derived DNA from shallow reef octocorals from Okinawa and deep-sea octocorals from Kita-Koho Seamount, Kyushu-Palau Ridge (northwestern Pacific Ocean) including 32 genera representative of 20 families (Supplementary Table S2) currently deposited at the Molecular Invertebrate Systematics and Ecology lab (University of the Ryukyus, Japan). Additionally, five museum specimens were obtained from the Okinawa Churaumi Aquarium Zoological Collection. Total genomic DNA was extracted from preserved tissue (70–99% ethanol) using a DNeasy Blood & Tissue kit (Qiagen, Germany) with an elution volume of 200 µL (100 µL for museum specimens). DNA concentrations were measured using a Nanodrop (Thermo Fisher Scientific, USA), and DNA extractions were diluted if necessary to obtain DNA concentrations for all samples ranging from 2 to 20 $ng.L^{-1}$ for further analyses.

*In vitro* polymerase chain reactions (PCR) of 32 octocoral tissue-derived genomic DNA samples were conducted using the three octocoral metabarcoding primer sets. PCR amplification conditions of the original mtMutS and 28S rDNA primer sets followed McCartin et al. (2024) with modifications of removing the touchdown step and using 35 cycles. The new mtMutS metabarcoding primer set PCRs were conducted following Hakim et al. (2025). Amplified samples from all primers were purified using a standard ExoSAP protocol and were sent to Fasmac (Kanagawa Prefecture, Japan) for sequencing. All sequences were used as data references for metabarcoding data analyses.

Mock community samples were prepared by pooling 10 µL of genomic DNA from each sample, in a 1.5 mL tube. Using all octocoral metabarcoding primer sets with the sequence adapter (forward overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence] 3' and reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence] 3'), amplifications were performed in three replicates per reaction, with a total volume of 25 µL: 12.5 µL KAPA HiFi, 9 µL RNase-free water, 0.75 µL of each primer, and 2 µL of mock community samples. Negative PCR controls were prepared using 2 µL RNase-free water. Amplification conditions using original mtMutS and 28S rDNA primer sets were performed following McCartin et al. (2024), while a new mtMutS primer set was amplified using touchdown PCR with cycling conditions as follows: initial denaturation at 98°C for 30 s; then 14 cycles of denaturation at 98°C for 10 s, the touchdown annealing step from 65°C to 52°C for 10 s, and

extension at 72°C for 15 s; followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 51°C for 10 s, and extension at 72°C for 15 s; and a final extension at 72°C for 15 min.

PCR products were cleaned, and amplicon sequencing libraries were prepared using a KAPA Hyper Prep Kit without fragmentation (Açıkbaş et al. 2024). Sequencing was performed on a NextSeq using a NextSeq 1000 P1 600-cycles cartridge, with paired-end 2 x 300 bp sequencing for all products at the Okinawa Institute of Science and Technology (OIST). The performance of mock community analyses was evaluated by assessing each primer set's ability to detect 32 octocoral genera in mock community samples.

## Visual survey, seawater collection, and filtration

Visual observations by scuba diving were conducted on August 25, 2023, in Makiminato (26°16'30.4" N, 127°42'52.0" E) and on August 28, 2023, in Sunabe (26°19'33.1" N, 127°44'37.1" E), Okinawa, Japan. Three replicates of 20 meter transect were laid with 5 m distance between each transect, following the contour of the reef slope in shallow waters (5–6 meters depth). Photographs of quadrats (1 x 1 m) laid next to the transect were taken every meter. Photographs were analyzed by recording the presence of octocorals and identifying them to the genus level only, as identification to species level would require morphological and genetic analysis of coral tissue samples

In the middle of each transect, 2 liters of seawater were sampled 1 meter above the coral reef using a Mighty Pack (Maruemu) for a total of three seawater samples per site. A 2 L water bottle was brought along to the sampling site, opened for 5 minutes, and contents were then transferred to a Mighty Pack placed in a cooler box with ice with the rest of the water samples to act as a field contamination control. Samples were then transported to the laboratory for filtration on the same day (within four hours from collection). Before filtering, another 2 L water bottle was opened at the place of filtration and transferred to a Mighty Pack and filtered along the other samples to act as a filtration control. Water was filtered using a Sterivex cartridge with a pore size of 0.45 µm (Merck Millipore), following Oka et al. (2021) with modifications following Açıkbaş et al. (2024). Filters were preserved with RNALater (Thermo Fisher Scientific, United States) and stored at − 80°C until the extractions.

## eDNA extraction, amplification, library preparation, and MiSeq sequencing

Environmental DNA was extracted, and 150 µL of eDNA was eluted following the protocol described by Açıkbaş et al. (2024). Extracted eDNA was amplified using the three different octocoral metabarcoding primer sets following the same protocols as described in the mock community analysis, except for the new mtMutS primer set, where only 1 µL of eDNA sample was used. Library preparation was conducted with the same protocol for mock community analysis. Sequencing was performed on the MiSeq using a MiSeq v3 500-cycle cartridge, with paired-end 2 x 300 bp sequencing for all products at OIST.

## Bioinformatic analyses

Raw Illumina reads from the mock community and eDNA analyses were demultiplexed and sequencing adapters and primers were trimmed using Cutadapt (Martin 2011). Quality of the sequences was filtered using DADA2 (Callahan et al. 2016) in RStudio (R Core Team 2025). For quality filtering, we trimmed all sequences to 175 bp on both the forward and reverse for the original mtMutS and to 150 bp for the new mtMutS. Maximum expected error rate was 2 per read, no ambiguous bases allowed, and a minimum length of 100 bp for both mtMutS primer sets. Learning error rates, generating and denoising amplicon sequence variants (ASVs), merging forward and reverse reads, generating count table, and performing chimera removal were conducted using the default settings in DADA2. For 28S rDNA, sequences were also processed with DADA2 as described by McCartin et al. (2024).

The reference database for taxonomic assignment was created by downloading sequences from the NCBI (National Center for Biotechnology Information) using Creating Reference databases for Amplicon-Based Sequencing (CRABS) v1.7.7 (Jeunen et al. 2023). We selected all cnidarian sequences for 28S rDNA and all octocoral sequences for mtMutS, then imported the sequences, including taxonomy information provided by NCBI. Sequences were extracted using *in silico* PCR and selected by pairwise global alignment with a minimum percent identity and coverage of 90%. Sequences in the reference database were dereplicated, filtered (by sequence length within the range of each primer set; 300 – 450 bp for 28S rDNA and 200 – 350 bp for original and new mtMutS), and exported in a BLAST-compatible format.

ASV sequences from each primer set were aligned to each reference database using blastn (Chen et al. 2015), with a minimum percent identity and query coverage of 50%. Blast results were parsed using Ancestor Lineage EXplorer (ALEX) v0.1.0 to identify the most recent common ancestor (Jeunen 2025). ASVs with similarities of 90–95% were assigned to family, and between 85% and 90% were assigned to order (Elbrecht et al. 2017). All data were curated using *ampvis2* (Andersen et al. 2018), Biostrings (Pagès et al. 2024), *decontam* (Davis et al. 2018), and *Phyloseq* packages (McMurdie and Holmes 2013). The *decontam* package was used to identify and remove potential contaminants using all negative control samples, including field, laboratory, and PCR. Furthermore, ASV sequences were filtered based on amplicon length to maintain target length of 290 – 320 bp for original mtMutS, 205 – 255 bp for new mtMutS, and 380 – 415 bp for 28S rDNA. Singletons were removed to reduce spurious sequences, but a minimum abundance cutoff was not applied to avoid excluding rare taxa.

## Results

# Primer-binding site conservations and mismatches

The average primer-binding site conservation of the original mtMutS was 96.05% for the forward primer and 94.83% for the reverse primer. At the same time, the new mtMutS primer showed a higher value of 99.59% conservation for the forward and 98.13% for the reverse (Fig. 2). The zero (0) mismatch percentages for the forward and reverse were 35.94% and 22.78% for the original mtMutS primer set and 92.47% and 68.58% for the new mtMutS primer set, respectively (Table 2). In addition, mismatches of five or more bases in the original mtMutS primer set were observed in the forward primer for families Acanthogorgiidae, Briareidae, Euplexauridae, Nidaliidae, Subergorgiidae, and Veretillidae, and in the reverse primer for Acanthogorgiidae, Coralliidae, Ifalukellidae, Sarcophytidae, and Veretillidae. In contrast, only family Cornulariidae had mismatches of five or more bases in the reverse primer of the new mtMutS primer set. The position of the mismatch bases varied across families, with mismatches exceeding three bases in the original mtMutS primer set; only Ifalukellidae (*Plumigorgia* and *Trichogorgia*) had one base located near the 3' end of the reverse primer (Supplementary Table S3). In the new mtMutS primer set, only *Cornularia* (Cornulariidae) had two bases located near the 3' end of the reverse primer (Supplementary Table S4).

Table 2

Percentages of the mismatch from each octocoral-specific primer set (the original mtMutS primer set, Everett and Park 2018; the new mtMutS primer set, this study; and the 28S rDNA primer set, McCartin et al. 2024) at the family level. The families listed below each mismatch category represent taxa contributing more than three base mismatches.

| **Number of different bases** | **Original mtMutS (2,004 sequences)** | | **New mtMutS (2,126 sequences)** | | **28S rDNA (483 sequences; McCartin et al. 2024)** | |
|---|---|---|---|---|---|---|
| | **Octo_eDNA_Mut2F** | **Octo_eDNA_Mut2R** | **AAH-MutS_F01** | **AAH-MutS_R250** | **Anth-28S-eDNA-F** | **Anth-28S-eDNA-R** |
| 0 | 35.94 | 22.78 | 92.47 | 68.58 | 91.90 | 99.25 |
| 1 | 35.99 | 33.10 | 6.91 | 27.70 | 6.97 | 3.77 |
| 2 | 15.30 | 20.79 | 0.61 | 3.33 | 1.13 | 3.77 |
| 3 | 7.67 | 6.48 | 0.00 | 0.85 | 0.00 | 0.00 |
| 4 | 4.43 | 13.31 | 0.00 | 0.14 | 0.00 | 0.00 |
| | Capnellidae, Cladiellidae, Gorgoniidae, Nidaliidae, Subergorgiidae, Tubiporidae | Coralliidae, Clavulariidae, Chrysogorgiidae, Ifalukellidae, Primnoidae, Sarcophytidae, Umbellulidae, Virgulariidae | - | Scleralcyonacea *incertae sedis* | - | - |
| 5 | 0.65 | 3.39 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Acanthogorgiidae, Euplexauridae, Nidaliidae, Subergorgiidae, Veretillidae | Acanthogorgiidae, Coralliidae, Ifalukellidae, Sarcophytidae, Veretillidae | - | - | - | - |
| 6 | 0.00 | 0.10 | 0.00 | 0.09 | 0.00 | 0.00 |
| | - | Ifalukellidae | - | Cornulariidae | - | - |
| 7 | 0.00 | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 |
| | - | Briareidae | - | - | - | - |

## Performance of octocoral-specific primer sets on single sample and mock community

In *in vitro* PCR using single-tissue-derived DNA, all primer sets (original mtMutS, new mtMutS, and 28S rDNA) successfully amplified all samples from 32 genera of 20 families, except for the 28S rDNA primer set, which failed to amplify *Heteroxenia* (Xeniidae) (Supplementary Table S2). Of 20 families in the mock community, Coralliidae and Subergorgiidae were not detected by the original mtMutS primer set, and Chrysogorgiidae was not detected by the 28S rDNA primer set. In contrast, the new mtMutS primer set successfully detected all families in the mock community sample (Fig. 3). At the genus level, several genera were not detected, including seven genera (*Hanabira, Hemicorallium, Klyxum, Muriceides*, *Paramuricea*, *Sarcophyton*, and *Subergorgia*) by the original mtMutS primer set, two genera (*Klyxum* and *Sarcophyton*) by the new mtMutS primer set, and four genera (*Chrysogorgia, Heteroxenia, Muriceides*, and *Nanipora*) by the 28S rDNA primer set. Besides the octocoral ASVs obtained from the mock community, the 28S rDNA primer set also revealed ASVs from

other taxa, including the class Hydrozoa (with three families) and 22% (52 of 239 ASVs) of unassigned ASVs, while the original and new mtMutS primer sets did not detect any non-target taxa.

## Visual survey records and eDNA metabarcoding in Sunabe and Makiminato, Okinawa

In the visual surveys of two shallow water coral reefs (Sunabe and Makiminato) in Okinawa Island (Fig. 4a-c), we observed four genera of soft corals (Octocorallia), including three genera from the family Sarcophytidae (*Lobophytum*, *Sarcophyton*, and *Sclerophytum*) and one genus from Cladiellidae (*Klyxum*) (Fig. 4d-g). Via detection of octocoral ASVs from seawater, the 28S rDNA primer set obtained six families from two orders, the original mtMutS primer set revealed 11 families from one order, and the new mtMutS primer set identified 10 families from two orders (Fig. 5a). All three primer sets detected Acrossotidae, Nephtheidae, Sarcophytidae, Sinulariidae, and Xeniidae. Furthermore, original and new mtMutS primer sets obtained ASVs from Acanthogorgiidae, Cladiellidae, Clavulariidae, and Isididae, while these four families were not detected by the 28S rDNA primer set. Furthermore, Arulidae and Carijoidae were detected only by the original mtMutS primer set, and Helioporidae were detected only with the new mtMutS primer set. Since four ASVs of 28S rDNA were identified and close to *Parasphaerasclera rotifera* (order Scleralcyonacea: family Parasphaerascleridae) with similarities of 86.86–88.28%, we decided to keep the assignment at the order level. In addition, only the new mtMutS primer set successfully detected all genera (*Klyxum*, *Lobophytum*, *Sarcophyton*, and *Sclerophytum*) observed in the visual survey (Fig. 5b). Based on the total number of genera, the original mtMutS primer set detected the highest number of genera with 17 genera, while the new mtMutS primer set detected 14 genera, and the 28S rDNA primer set identified 10 genera.

Based on the number of ASVs detected in the eDNA samples (Fig. 6a-c), the original mtMutS primer set obtained 158 (100%) octocoral ASVs, and the new mtMutS primer set 159 (68.24%) octocoral ASVs, with 74 (31.76%) unassigned ASVs (Fig. 6a, b). In contrast, the 28S rDNA primer set identified 316 (47.23%) octocoral ASVs, 251 (37.52%) unassigned ASVs, and 102 (15.25%) ASVs from other classes of Cnidaria, including Hexacorallia, Hydrozoa, and Scyphozoa (Fig. 6c). Sarcophytidae was the dominant ASV for all primer sets, with a total number of ASVs for original mtMutS, new mtMutS, and 28S rDNA primer sets of 130, 131, and 305, respectively.

The relative abundances of octocoral read counts from all primer sets also showed Sarcophytidae as the dominant family with more than 90% of reads belonging to this family in both locations (Fig. 6d-f). However, the three metabarcoding primer sets differed in the relative abundance of reads in the second-most dominant family in Makiminato: Isididae for the original mtMutS primer set, Cladiellidae for the new mtMutS primer set, and Acrossotidae for the 28S rDNA primer set. Nephtheidae was the second-most dominant octocoral family in Sunabe for the original and new mtMutS primer sets, while Scleralcyonacea was second-most dominant for the 28S rDNA primer set.

Octocoral ASV richness varied by genus across all three primer sets at the two shallow-water sites in Okinawa (Fig. 7). Overall, Sunabe had a higher number of detected genera than Makiminato across all primer sets. *Calcigorgia*, *Dendronephthya*, *Scleronephthya*, *Stereonephthya*, Sarcophytidae sp., Helioporidae sp., and Scleralcyonacea sp. were only found in Sunabe, while Arulidae sp., *Carijoa*, and *Xenia* were only found in Makiminato. Although Sarcophytidae was the family with the most dominant ASVs, the composition of the detected genera differed in the three primer sets. New mtMutS and 28S rDNA primer sets obtained *Sclerophytum* as the dominant ASV, while the original mtMutS primer set found *Sarcophyton* as the dominant ASV.

## Discussion

## Primer compatibility and detection on tissue-derived DNA

Octocoral-specific primers for eDNA metabarcoding are essential and needed, as most eDNA coral studies so far have focused on scleractinian corals (Hexacorallia) and have been comparatively limited for octocorals (Octocorallia). The use of specific markers aims to increase amplification success and obtain a better resolution for the group of interest (Ficetola et al. 2019). The mtMutS primer sets were designed to amplify only the class Octocorallia (octocorals), while the 28S rDNA primer set also targets the order Antipatharia (black coral). The utility of the mtMutS gene as an octocoral metabarcoding marker has distinct advantages over other markers. The MutS gene is unique to octocorals within animals and has been found in the mitochondrial genomes of all octocorals (Muthye and Lavrov 2021). Furthermore, the mtMutS region has been reported as the most variable region of mitochondrial octocoral DNA (France and Hoover 2002; McFadden et al. 2011), providing the necessary sequence divergence, making it useful for studying octocoral taxonomy and biodiversity.

In metabarcoding studies, primer-template mismatches can result in significantly underestimating the number of amplified sequences, and thus lower species richness estimates and lower overall biodiversity assessments (Bru et al. 2008; Piñol et al. 2015). The new mtMutS primer set performed better than the original mtMutS primer set with higher primer binding site conservation and fewer primer-template mismatches. A higher zero (0) mismatch percentage can result in a higher amplification success rate for a target group (Shaffer et al. 2025). The original Everett and Park (2018) mtMutS primer set had low levels of complete sequence matches; only 35.94% of octocoral taxa for the forward and 22.78% for the reverse primer (N = 2,004 sequences; 81 families). These low levels of primer match may be due to Everett and Park (2018)'s dataset being focused on deep-sea octocorals from the Northeast Pacific. In contrast, our new mtMutS primer set had higher levels of complete matches than the original mtMutS primers; 92.47% for forward and 68.58% for reverse primers (N = 2,126 sequences; 80 families).

However, amplification efficiency is not only affected by the quantity of mismatches but also by the position of primer-template mismatches (Bru et al. 2008; Clarke et al. 2017; Rejali et al. 2018), with mismatches within 5 bp of the 3' end of the primer significantly reducing PCR efficacy (Lefever et al. 2013; Wu et al. 2009). With mismatches of five or more bases, the original mtMutS primer set may not be functional for the nine families Acanthogorgiidae, Briareidae, Coralliidae, Euplexauridae, Ifalukellidae, Nidaliidae, Sarcophytidae, Subergorgiidae, and Veretillidae, with Ifalukellidae (*Plumigorgia* and *Trichogorgia*) from the original mtMutS primer having one or two base pair differences near the 3' end for each reverse primer. Consistent with these predicted mismatches, several genera detected *in vitro* with the original mtMutS primer set were absent or underrepresented in mock community analyses. These false negatives, which refer to the failure to detect a taxon in the mock community or in eDNA samples where it is known to be present (Burian et al. 2021), may occur due to primer-template mismatches, quality of tissue extractions, or quantity of DNA released into the environment (especially in eDNA samples). With the original mtMutS primer set, based on the *in silico* analysis, some taxa were likely not amplified in the mock community, reflecting the high number of mismatches, such as *Hanabira*, *Hemicorallium*, *Klyxum*, and *Subergorgia*. Other false negative results by the original mtMutS primer set occurred with *Muriceides*, *Paramuricea*, and *Sarcophyton* with one to three mismatches from both primers (forward and reverse) (Supplementary Table S3). The two genera undetected by the new mtMutS primer set, *Klyxum* and *Sarcophyton*, had only zero to one mismatch in both primers (Supplementary Table S4). This is likely because an organism's total genome DNA (gDNA) is not equivalent to the amplifiable template DNA, with an assay targeting only a small fragment of the total DNA present (Shaffer et al. 2025), even when the DNA concentrations used for each species in the mock community have been specified.

The 28S rDNA primer set did not amplify *Heteroxenia*, and this primer set might not be functional for detecting this genus at all. Amplification conditions can affect primer performance, and the conditions may vary between single-taxa PCR (*in vitro*) and mock community metabarcoding PCR (Marinchel et al. 2023). Even when primers have perfect sequence matches to target taxa, PCR bias can still occur, with approximately 60% of the bias coming from PCR and sequencing, while other factors that can influence amplification bias are more challenging to quantify and predict (Shaffer et al. 2025). However, mock community methods are very useful to assess the reliability of metabarcoding results from both qualitative and quantitative point of view (Iwaszkiewicz-Eggebrecht et al. 2023), including the identity and relative

abundance estimates of known species (Porazinska et al. 2009; Egge et al. 2013; Pochon et al. 2013). In addition, such analyses can facilitate the development of more efficient and standardized protocols, as well as validate eDNA studies or highlight their limitations (Marinchel et al. 2023).

In our mock community sample, only octocoral genera were included, but the 28S rDNA primer set identified Hydrozoa (Cnidaria), and even generated some unassigned ASVs that were outside of Cnidaria. It is likely that our tissue samples were not necessarily clean and free of other organisms. Some Hydrozoa may live near or directly on other cnidarians (Puce et al. 2008; Bo et al. 2011), and there is always the possibility of endosymbionts, ectosymbionts, and other bacteria also being present (Knowlton and Rohwer 2003; van de Water et al. 2018). eDNA extracted from environmental samples can capture non-target DNA by genetic material from both living and dead organisms (Veilleux et al. 2021). Therefore, negative controls were incorporated in the PCR process, identified and potential contaminants were removed during data analyses, thus minimizing any laboratory-introduced contamination. By combining primer-template mismatch assessment, single-tissue-derived DNA PCR, and mock community analysis as in this study, we can improve and recommend primer selection in eDNA metabarcoding, thereby enhancing the detection of octocoral diversity in coral reef communities. Furthermore, standardizing primer selection can improve comparability and reproducibility between studies (Ren et al. 2025).

## The presence of octocoral in the shallow waters of Makiminato and Sunabe, Okinawa

Using eDNA samples collected in the shallow waters of Okinawa Island, only the new mtMutS primer set successfully detected all four genera observed visually (*Lobophytum, Sarcophyton, Sclerophytum*, and *Klyxum*), while the original mtMutS and 28S rDNA primer sets did not detect *Klyxum*. Overall, by applying the three octocoral-specific primer sets, we detected more genera, including 20 genera of 13 families, most of which could not be captured using traditional visual observations. This highlights eDNA metabarcoding as a promising method to supplement traditional survey methods (Reimer and Gösser 2023) by confirming octocoral genera observed visually and detecting more families and genera in this study. In addition, eDNA can be especially advantageous for detecting rare and cryptic organisms that may be overlooked in divers operated surveys (Nichols and Marko 2019; Alexander et al. 2020).

Small octocorals are challenging to recognize and detect visually, and sometimes colonies growing in crevices and under other structures are overlooked when analyzing photographs. Based on records of Okinawan octocorals, *Cladiella* and *Clavularia* have been reported from the shallow waters of Makiminato and Sunabe, Okinawa Island (Lalas et al. 2024). In addition, other octocoral taxa (e.g., Nephtheidae) have been observed in our study sites, such as in deeper areas than where the transects were established (AAH, personal observation). This could explain detection of taxa that were not observed in the photo quadrats. Future studies involving specimen collections could also verify the results of this study.

Comparing mtMutS with 28S rDNA, recorded octocorals had a significant overlap, while 10 genera belonging to seven families could not be detected by 28S rDNA (Fig. 5). In addition, the original mtMutS primer set revealed more families than the new mtMutS primer set. The new mtMutS primer set could not detect Arulidae and Carijoidae, while the original mtMutS could not detect Helioporidae. Arulidae and Carijoidae were not part of the mock community, so we do not know if the lack of amplification here means that the primers could not detect them at all. At the genus level, *Sclerophytum* was dominant for the new mtMutS and original 28S rDNA primer sets, while *Sarcophyton* was dominant for the original mtMutS set (Fig. 7). These differences in results indicate potential biases in the primers and underscore the value of combining multiple markers to detect a more complete octocoral biodiversity. The shallow coral reef of Makiminato and Sunabe are dominated by *Sclerophytum* (Lalas et al. 2024), and our eDNA results using the new mtMutS and 28S rDNA primer sets were not inconsistent with previous findings.

It is worth noting that for the new mtMutS primer set we used less eDNA template (half volume compared to the other primers) for amplification, producing clear PCR bands, but using smaller eDNA template volumes may allow fewer taxa to

be detected. eDNA detection is also affected by PCR reaction conditions (Takahara et al. 2015), and rare ASVs are more easily detected in treatments with larger template volumes (Ushio et al. 2022). Three technical PCR replicates were performed; however, additional replicates could have improved detection of low-abundance taxa. Highly abundant species or groups dominate PCR amplification, and subsequent sequencing results might prevent and overshadow rare taxa (Skelton et al. 2023). In our case, Sarcophytidae was highly abundant with over 90% of ASVs, which may have caused difficulties in detecting rarer octocoral species. Future work should evaluate the use of blocking primers to reduce amplification of dominant taxa (Vestheim and Jarman 2008; Boessenkool et al. 2012) and thereby enhancing sensitivity for detecting rare octocoral ASVs. Emerging approaches such as “clustered regularly interspaced short palindromic repeat” (CRISPR)-based depletion of abundant sequences may also offer future potential (Song and Xie 2020), although these methods are not yet widely applied in metabarcoding workflows (Owens et al. 2024).

## Conclusions

Environmental DNA (eDNA) metabarcoding approaches using octocoral-specific primer sets complemented and confirmed octocoral biodiversity observed by traditional survey methods (visual surveys) and detected overall more families and genera through eDNA samples than visual surveys. A new mtMutS primer set is promising and has potential as an effective marker for detecting and monitoring octocoral biodiversity based on *in silico*, *in vitro*, mock community analyses, and eDNA sample evaluation. Therefore, we recommend its use as a preferred marker for future octocoral biodiversity assessment and octocoral community monitoring. Such biodiversity studies testing the performance of eDNA metabarcoding primer sets are important to confirm the best approaches (involving multiplexing of different marker regions) for octocoral biodiversity monitoring. The collection of such information is critical for understanding octocoral biodiversity, how it will continue to change, and for considering management strategies to ensure their future.

## Declarations

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Ethical approval**

The authors followed all applicable international, national, and/or institutional guidelines for animal testing, animal care, and use of animals.

**Sampling and field studies**

No permits are needed for coral observation and seawater collection in Okinawa, Japan. However, the authors have obtained all necessary permits for sample collection for *in vitro* evaluation.

**Data availability statement**

Data and materials that support the findings of this research are available from the corresponding author upon reasonable request.

**Author contributions**

AAH conceived and designed the research, consulting with JDR and TR. AAH, JAAL, and AA conducted the field observation and seawater collection. AAH, JAAL, EAJ, GMC, KBBB, and JJ conducted molecular analyses. YF and MN provided specimens. JDR, TK, and TR provided supervision and materials. All authors analyzed the data. AAH wrote the manuscript. All authors read, edited, and approved the manuscript.

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## Figures

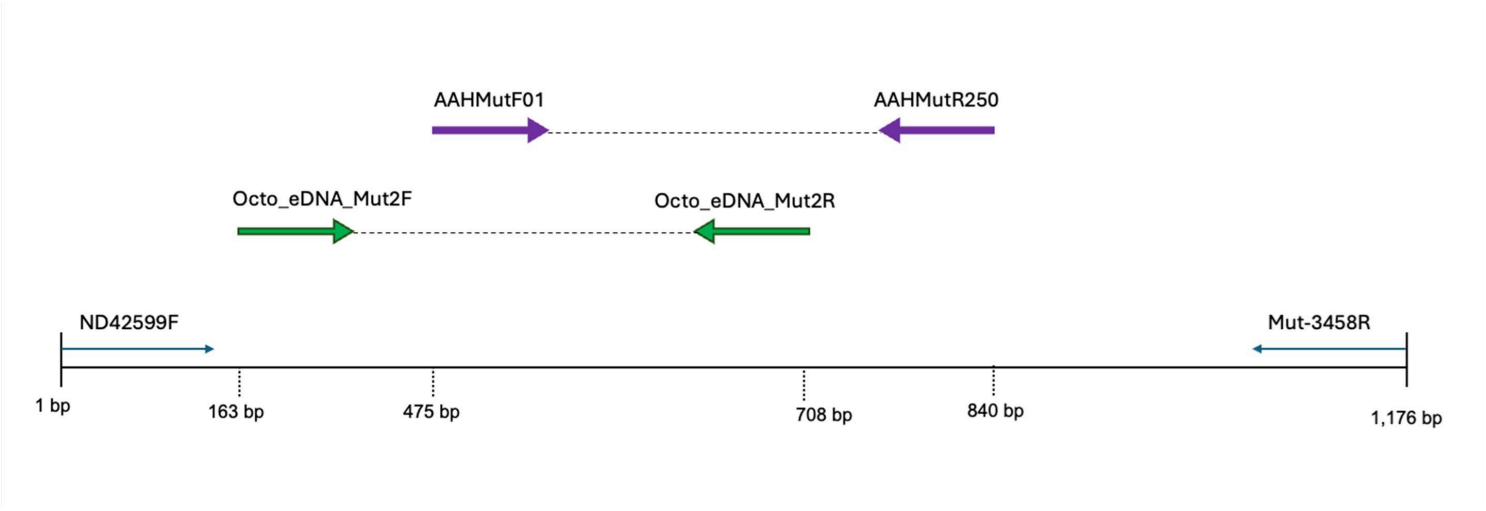



**Figure 1**

Position of new (purple, AAH-MutS_F01 and AAH-MutS_R250, this study) and original (green, Octo_eDNA_Mut2F and Octo_eDNA_Mut2R, Everett and Park 2018) primer sets of mtMutS compared to DNA barcoding region using the common primer pair (gray, ND42599F and Mut-3458R, France and Hoover 2002; Sánchez et al. 2003).

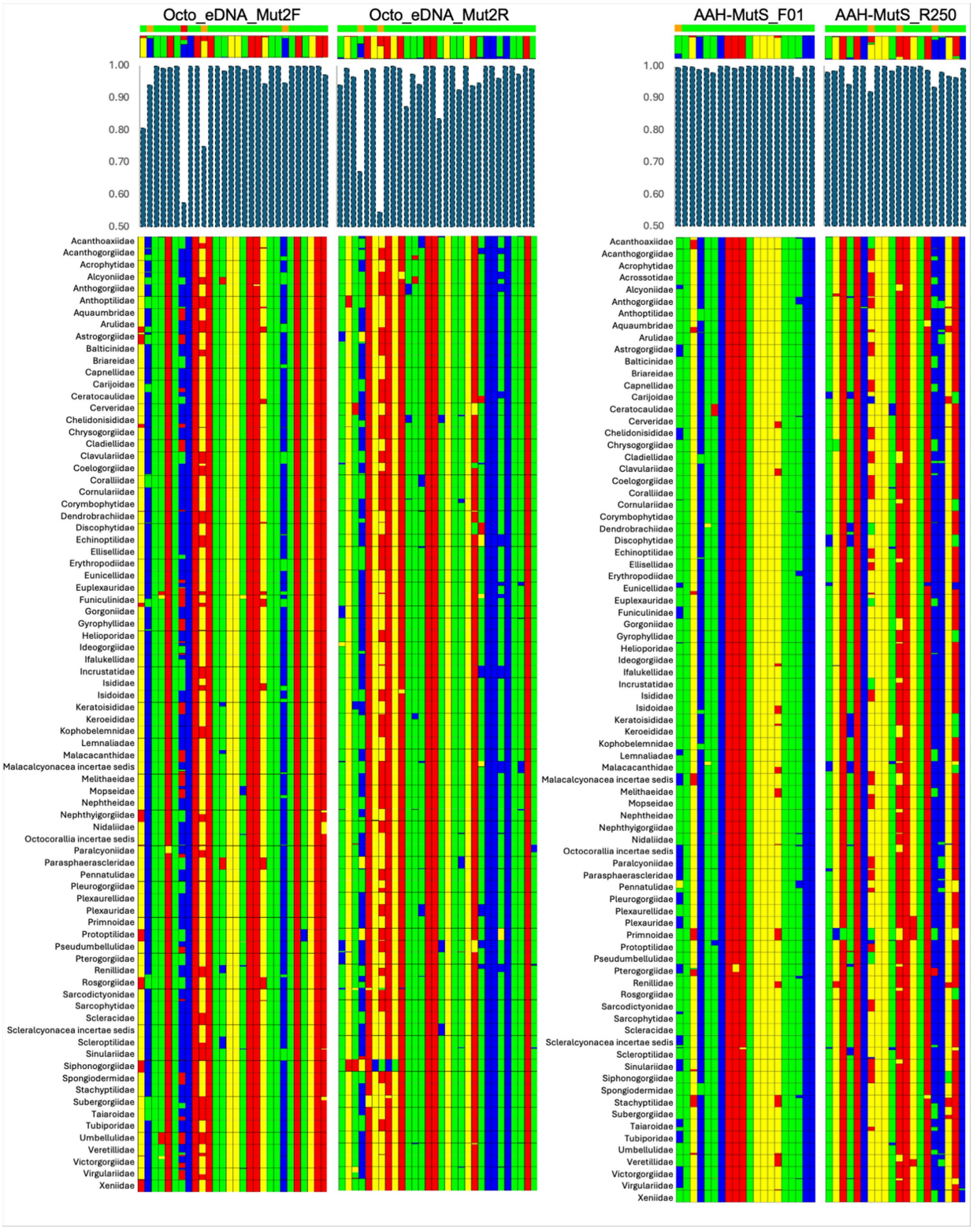



**Figure 2**

Primer-binding site conservation of original mtMutS primer set (Octo_eDNA_Mut2F and Octo_eDNA_Mut2R, Everett and Park 2018) using 2,004 sequences from 80 families and new mtMutS primer set (AAH-MutS_F01 and AAH-MutS_R250,

this study) using 2,126 sequences from 81 families. Each different color represents a different base, red: adenine, yellow: guanine, blue: cytosine, green: thymine.

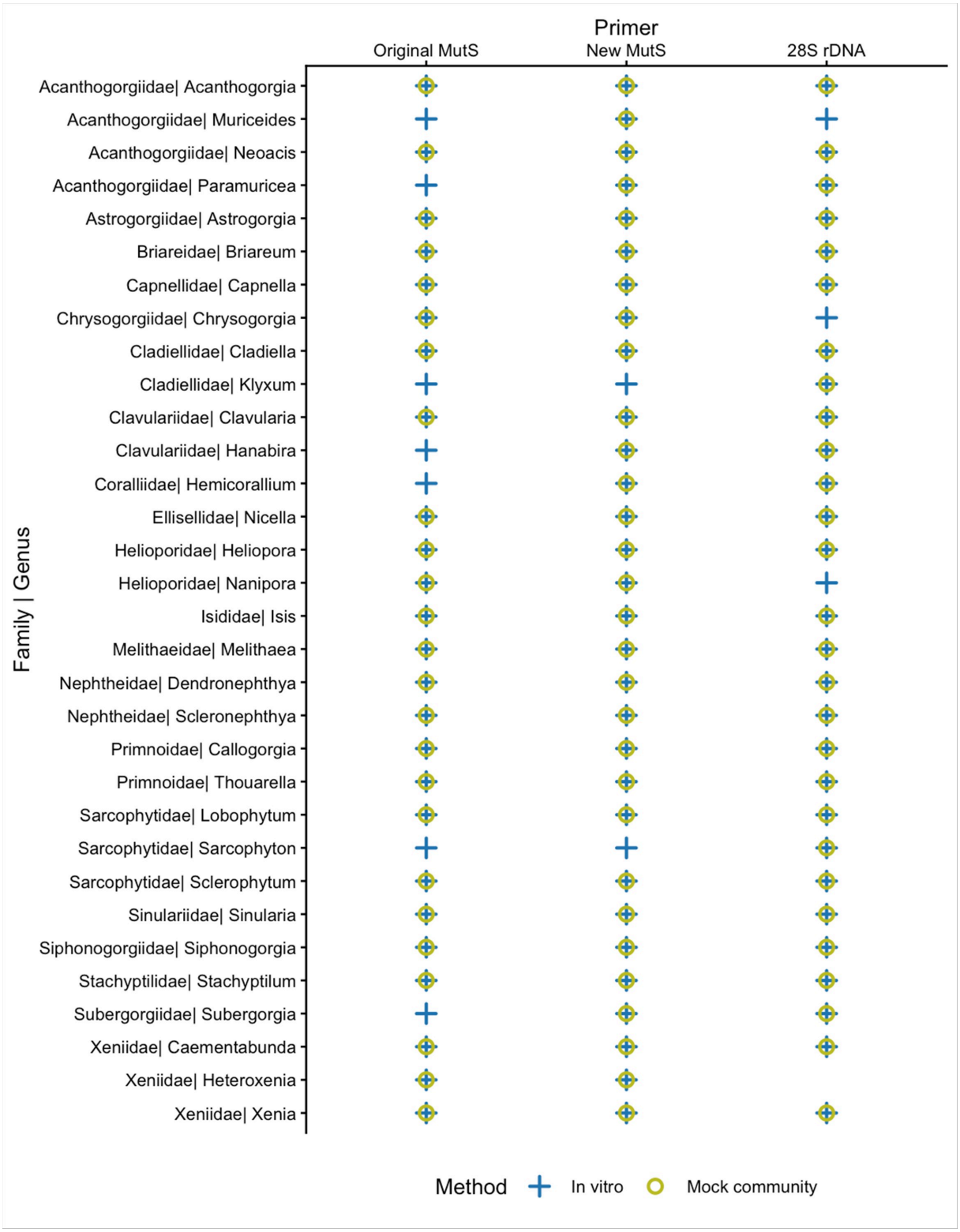



**Figure 3**

Evaluation of octocoral-specific primer sets (the original mtMutS primer set, Everett and Park 2018; the new mtMutS primer set, this study; and the 28S rDNA primer set, McCartin et al. 2024) by detecting DNA derived from single tissues and mock communities of 32 genera from 20 families.

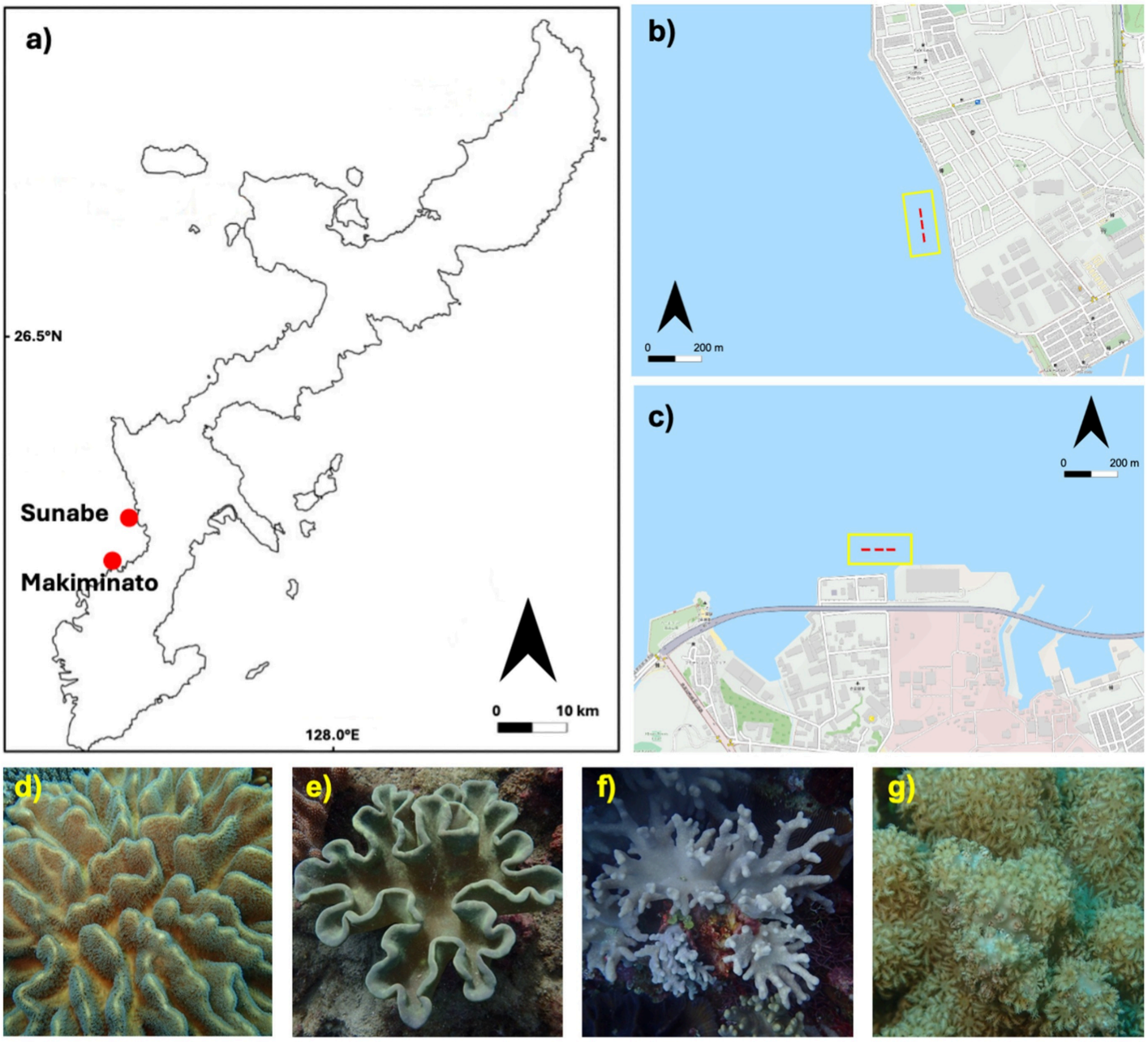



**Figure 4**

Location of visual surveys and eDNA sample collections in the shallow waters of Okinawa Island, Japan. **a)** Okinawa Island; **b)** Sunabe; and **c)** Makiminato, the red lines indicate the positions of the 20 m transects laid out parallel to the shoreline (© OpenStreetMap). Soft corals (Octocorallia) observed at the two sites of shallow waters in Okinawa Island based on visual surveys. **d)** *Lobophytum*; **e)** *Sarcophyton*; **f)** *Sclerophytum*; and **g)** *Klyxum*.

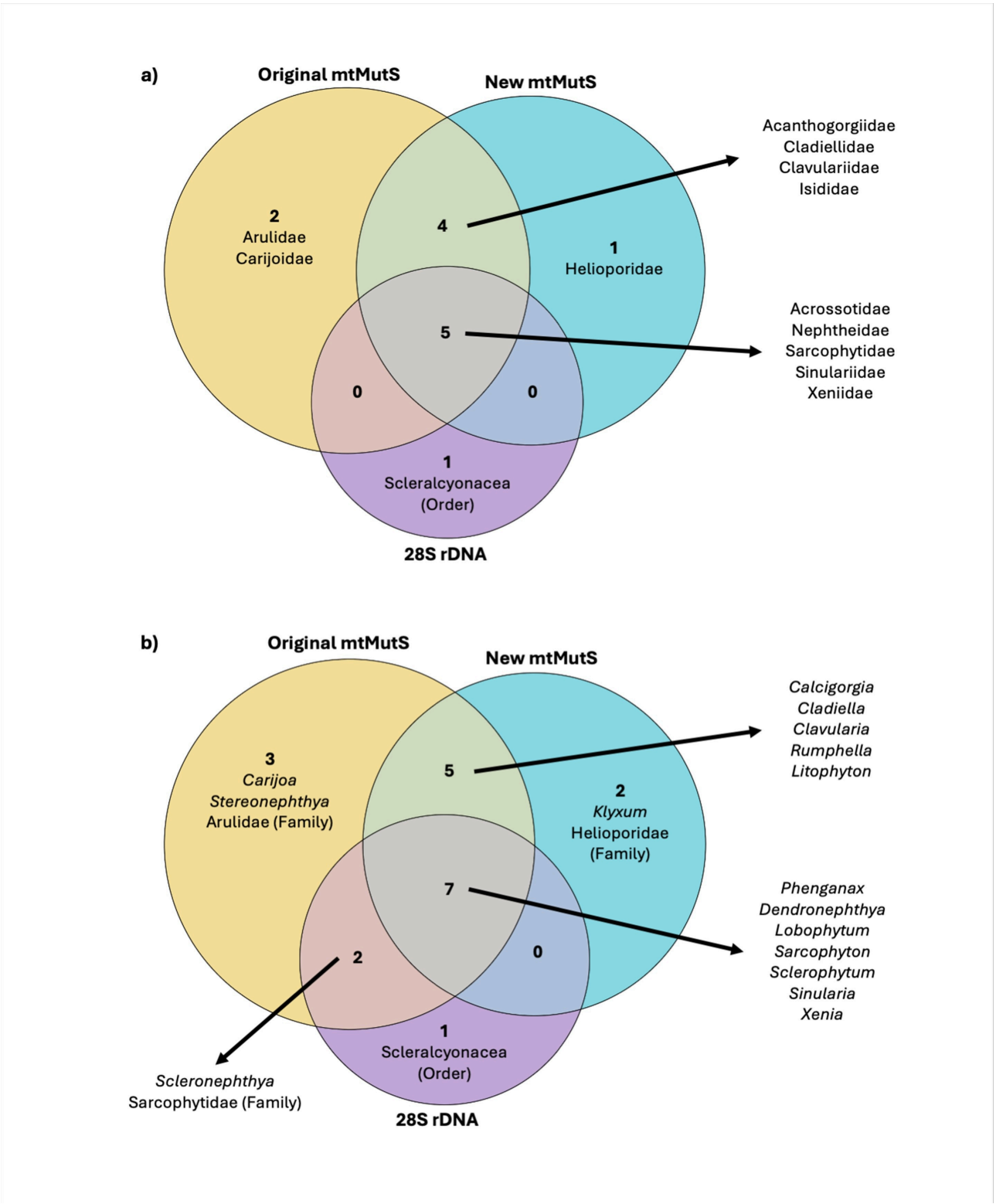



**Figure 5**

Effectiveness of three metabarcoding primers in detecting octocorals in environmental DNA (eDNA) from the two sites of shallow waters in Okinawa Island samples. **a)** family level and **b)** genus level. Note that not all ASVs could be confidently assigned to the family or genus level; some were retained at higher taxonomic ranks (e.g., order).

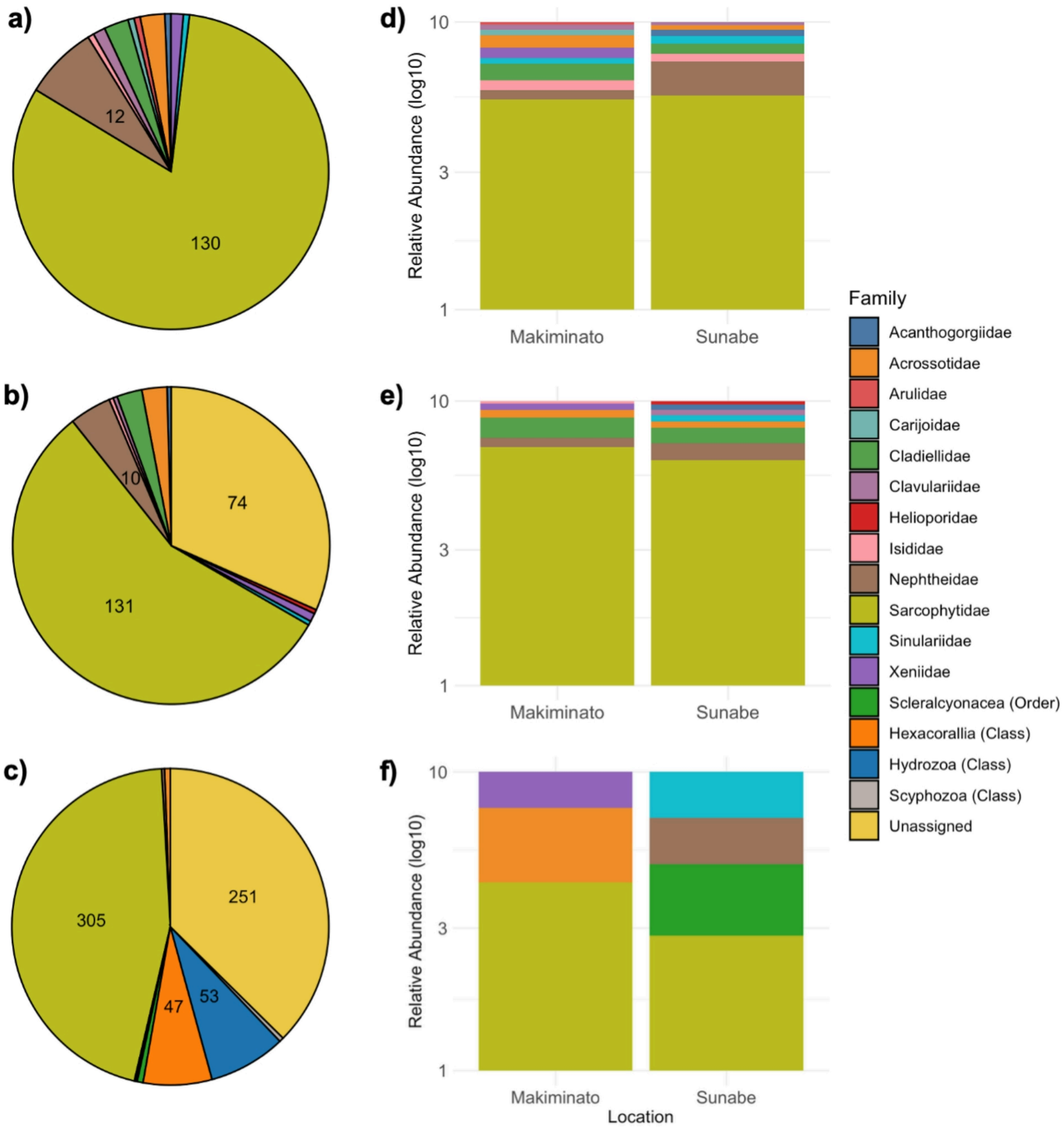



**Figure 6**

The number of ASVs detected in the eDNA samples for **a)**original mtMutS; **b)** new mtMutS; and **c)** 28S rDNA. The relative abundance of octocoral read counts for **d)** original mtMutS; **e)** new mtMutS; and **f)** 28S rDNA.

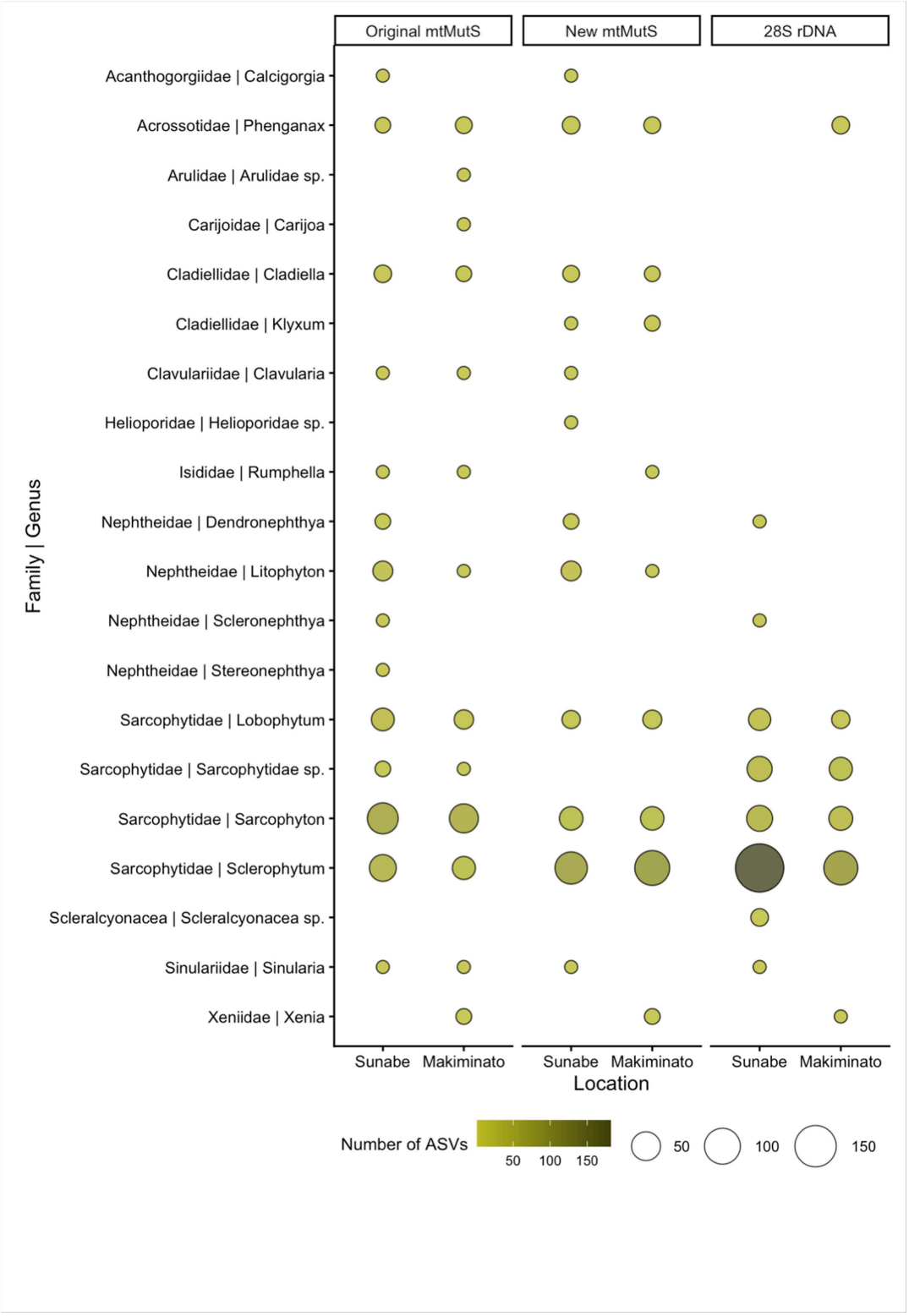



**Figure 7**

The number of ASVs from each genus in the shallow water of Okinawa Island detected by original mtMutS, new mtMutS, and 28S rDNA metabarcoding.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SubmitSupplementaryTable.xlsx