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Sponging up diversity: Evaluating metabarcoding performance for a taxonomically challenging phylum within a complex cryptobenthic community

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Abstract

Despite their ecological importance, sponges are often avoided in biodiversity studies and monitoring programs because they are notoriously difficult to identify using morphological or molecular methods. Here, we investigate the metabarcoding performance of universal degenerate cytochrome c oxidase subunit I (COI) primers in detecting species from this challenging phylum in a cryptobenthic community. Twenty-two modified Autonomous Reef Monitoring Structures (ARMS) were deployed for 2 years in mesocosms receiving unfiltered seawater from an adjacent reef slope. Upon recovery, each unit was inspected by a marine sponge taxonomist who used a combination of taxonomy, imagery, and DNA barcoding (28S rRNA and COI) to identify sponges and generate a validated taxonomic richness value for each ARMS unit. A total of 69 unique sponge barcoded morphologies (BMs) were identified from the classes Calcarea, Demospongiae, and Homoscleromorpha. Metabarcoding identified 41 unique sponge molecular operational taxonomic units (MOTUs) from Demospongiae and Homoscleromorpha but the primers failed to amplify any species from the class Calcarea which comprised 22% of the BMs. Sponge richness did not differ between BMs and MOTUs assigned to the classes Demospongiae and Homoscleromorpha. However, assignments at the order and family level in Demospongiae underscore known limitations in sponge taxonomic resolution using the COI gene. The prevalence of false positives within the order Suberitida and the pervasiveness of false negatives within the order Haplosclerida highlighted both technical and biological constraints in the metabarcoding method. Overall, these results confirm the need for discretion in sponge MOTU assignments using universal degenerate barcoding primers that target a short fragment of the COI gene. However, they also demonstrate that COI metabarcoding is capable of capturing sponge richness from a complex natural community.

KEYWORDS
28S, COI, community metabarcoding, coral reefs, cryptobenthic, DNA barcoding, mesocosm, Porifera

1 | INTRODUCTION

DNA metabarcoding is redefining metazoan coral reef biodiversity surveys in the 21st century. Metabarcoding is progressively filling the extensive knowledge gap that exists in assessing the diversity and distribution of complex communities such as the cryptobenthos on coral reefs (Leray & Knowlton, 2016; Stat et al., 2017). Prior to the DNA revolution, coral reef biodiversity surveys were based on taxonomic identifications that predominantly targeted well-studied groups of organisms, such as fish and coral, that were conspicuous and had easily observed morphological features to distinguish species. In the field of marine biodiversity, body size is directly correlated with species knowledge (Fautin et al., 2010). The small and cryptic organisms that make up the vast majority of coral reef biodiversity are often ignored because they live deep within the reef matrix and require significant taxonomic expertise and time for accurate assessment (Fautin et al., 2010; Fisher et al., 2015). Even though metabarcoding has propelled the field of biodiversity into a new realm of biomonitoring, its successful application relies on extensive DNA barcode reference libraries and the power and efficiency of genetic markers and corresponding primers to detect species across multiple phyla (Deagle et al., 2014).

For coral reef cryptobenthic metazoans, a 313 bp segment of the mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (COI) gene is the marker most commonly targeted for biodiversity surveys because it has been proven effective in amplifying taxa across multiple phyla (Al-Rshaidat et al., 2016; Carvalho et al., 2019; Leray & Knowlton, 2015; Leray et al., 2013; Pearman et al., 2018; Ransome et al., 2017). However, evaluating the direct performance of these primers in estimating species richness from a complex cryptobenthic community in a natural environment is near-impossible given the paucity of marine invertebrate barcodes in reference libraries, the inevitable biases associated with PCR amplification (Elbrecht & Leese, 2015, 2017; Elbrecht et al., 2017; Gaspar & Thomas, 2013), sequencing (Bokulich et al., 2013; Huse et al., 2010; Kunin et al., 2010; Leray & Knowlton, 2017; Zhan et al., 2014), and the intrinsic challenges in species delimitations due to inconsistent rates of evolution within and across taxa (Brown et al., 2015; Meyer & Paulay, 2005). These biases can generate both false negatives (species being present in a sample but not detected) and false positives (species being detected but not present), thereby eroding the credibility of richness estimates in the absence of taxonomic validation.

One of the most diverse and functionally important groups within the cryptobenthos are sponges (phylum Porifera) (de Goeij et al., 2013; Richter & Wunsch, 1999; Richter et al., 2001; Rützler, 2004). Sponges play a pivotal role in sustaining coral reef biodiversity in nutrient-limited conditions (de Goeij et al., 2013). They absorb large quantities of dissolved organic matter released by seaweeds and corals and return that energy as particulate organic matter through the rapid proliferation and shedding of their cells. This released detritus is a major source of food that is transferred to higher trophic levels. Coined the sponge-loop, this process occurs predominantly within the cryptic habitats on coral reefs and helps to explain why coral reefs recycle nutrients effectively enough to sustain such high biodiversity in oligotrophic seas (de Goeij et al., 2008; Goeij et al., 2013; Rix et al., 2017, 2018). However, despite their diversity and crucial ecological importance, sponges have often been overlooked in biodiversity studies.

Sponges are among the most difficult metazoan groups to identify to species using both taxonomic and molecular methods. The simplicity and plasticity of their morphological characteristics combined with the lack of macroscopic hard structures in most species make them notoriously challenging to identify (Wörheide et al., 2007). Furthermore, sponges can harbor up to hundreds of in-faunal metazoans (Wendt et al., 1985) making them particularly challenging to DNA barcode because the DNA of its inhabitants can be co-extracted and either co-amplified or preferentially amplified in place of the sponge (Vargas et al., 2012). Moreover, compared to other metazoans, mitochondrial DNA (mtDNA) in the sponge class Demospongiae evolves slowly (Huang et al., 2008; Shearer et al., 2002) whereas mtDNA in the class Calcarea evolves rapidly (Lavrov et al., 2013). The high rate of evolution in Calcarea results in the failure of standard COI primers to amplify target mtDNA regions (Lavrov et al., 2013; Voigt et al., 2012). The slow rate of evolution in demosponges results in less frequent nucleotide substitutions in the COI gene, making standard barcode sequences essentially uninformative at the species level (Erpenbeck et al., 2006; Pöppe et al., 2010). Additionally, rampant polyphyly challenges species-level molecular resolution (Erpenbeck et al., 2007; Redmond et al., 2011). An extension to the standard 648 bp COI primers (Folmer et al., 1994) has provided greater resolution for some sponge species (Erpenbeck, Breeuwer, et al., 2006) but not others (Yang et al., 2017). Due to the unusual evolutionary patterns within the COI gene of many sponges, species delineations must be based on a combination of histological taxonomic and multi-locus phylogenetic approaches (Erpenbeck, Breeuwer, et al., 2006; Morrow & Cárdenas, 2015; Redmond et al., 2011; Yang et al., 2017). Even then, there are often mismatches between molecular markers in identifying species boundaries (Carella et al., 2016; DeBiasse & Hellberg, 2015; Heim et al., 2007). Due to these taxonomic and molecular idiosyncrasies, sponges have received less attention than other groups in empirical DNA metabarcoding studies, despite having been uncovered as the most prominent sessile phylum found in

many studies targeting the coral reef cryptobenthos (Al-Rshaidat et al., 2016; Carvalho et al., 2019; Nichols & Marko, 2019; Pearman et al., 2018; Ransome et al., 2017).

In spite of the known molecular challenges of marine sponges, given their diversity, abundance, and ecological importance in the cryptobenthos, we wanted to test the efficiency of the most commonly used universal COI primer for coral reef metazoans in capturing sponge diversity from a complex cryptobenthic community (Geller et al., 2013; Leray et al., 2013). To do this, we placed 22 Autonomous Reef Monitoring Structures (ARMS) in mesocosms receiving unfiltered raw seawater from an adjacent reef slope for 2 years. Upon recovery, each ARMS unit was carefully inspected and sponges were sorted into morphospecies groups and identified using a combination of taxonomy and DNA barcoding; thereby generating a validated taxonomic richness value for each unit. True richness values were then compared to results obtained from metabarcoding the homogenized cryptobenthic community that settled on each ARMS unit. We examined sponge composition between the assigned metabarcoding annotations and those identified through taxonomy and DNA barcoding to evaluate the similarities and differences in taxonomic classification. This study is the first of its kind to evaluate the direct performance of metabarcoding-derived observed richness estimates with a validated taxonomic richness value for an entire phylum present within a field-based experiment.

2 | MATERIALS AND METHODS

2.1 | Sampling

Twenty-two modified Autonomous Reef Monitoring Structure (ARMS) units composed of three gray type I PVC plates (23 cm × 23 cm) forming a two-tiered stack of one open and one semi-closed layer were placed in tanks located at the Hawai'i Institute of Marine Biology (HIMB) in Kāne'ohe Bay on the island of O'ahu (Figure S1). Tanks received unfiltered raw seawater over a 24-month period piped from an adjacent reef slope, which facilitated the settlement and colonization of marine taxa. Upon recovery, each ARMS unit was disassembled. The top and bottom of each plate were photographed in high resolution and examined by a sponge taxonomist. For each unit, identified sponge morphotypes were photographed, subsampled, and collected based on unique or uncertain morphology for subsequent genetic analyses. Subsampled sponges were placed in 95% ethanol for DNA barcoding and, if enough tissue was available, were also fixed for histological evaluation in two additional solutions; one containing 4% paraformaldehyde, and the other containing 4% glutaraldehyde, and 0.1 M sodium cacodylate with 0.35 M sucrose. When sponge subsampling by the taxonomist was complete, ARMS plates were scraped clear of all accumulated biomass. Scraped material from each ARMS unit was combined, immediately homogenized within a sterilized blender, and preserved in 95% ethanol for high-throughput sequencing of the cytochrome *c* oxidase I (COI) gene.

2.2 | Sponge DNA barcoding and identification

DNA was extracted from sponge specimens using the Promega E-Z 96 (R) Tissue DNA Kit (Promega Bio-Tek) following the manufacturer protocols. Polymerase chain reactions (PCR) were used to amplify partial fragments of the 28S rRNA and COI genes as these have been useful in sponge classification of underexplored sponge communities (Erpenbeck et al., 2016, 2020; Idan et al., 2018). Multiple primers were used hierarchically based on varying amplification success rates (Table S1). PCR reactions for both 28S rRNA and COI were carried out in 40 μl total volume including the following: 14.4 μl of H2O, 20 μl of BioMix Red (Bioline) PCR Mastermix, 0.8 μl of each primer (10 mM), 3.2 μl of bovine serum albumin (BSA) (100 mg/ml) and 0.8 μl of template DNA (1–30 ng/μl). The PCR program consisted of an initial denaturation of at 94°C for 3 min. Each of 34 cycles began with 30 s at 94°C, but annealing temperatures and time for each reaction varied according to the primer pair being used (Table S1). Annealing steps were followed by a 1 min extension at 72°C for each cycle. The PCR program ended with a final extension at 72°C varying between 5 and 10 min, according to the primer pair. PCR products were examined on a 1% agarose gel stained with GelRed and purified using ExoFAP (Exonuclease I and Fast Alkaline Phosphatase – Life Technologies). Products showing multiple bands were purified by gel excision prior to cleanup for sequencing. Sequencing reactions were performed in both directions using the Big Dye TM terminator v. 3.1, and sequencing was done on an ABI Prism 3730 XL automated sequencer at the University of Hawai'i Advanced Studies of Genomics, Proteomics and Bioinformatics sequencing facility.

Forward and reverse reads were trimmed (at an error probability limit of .05) before being assembled and edited by eye using Geneious 10 (Kearse et al., 2012). Inconsistent base calls were edited by selecting the highest confidence score from the two assembled chromatograms. All assembled chromatograms resulted in >90% high-quality base pair reads with a mean Phred quality score ≥40. Assembled sequences were saved and exported as a fasta file. Each fasta file from targeted gene sequences was checked for contamination using the BLAST (Altschul et al., 1990) function from GenBank. BLAST results that showed >85% sequence identity and a query cover of >60% to those belonging to Porifera were exported to Geneious 10 and aligned using the ClustalW algorithm with default parameters. Samples showing ≥2% sequence divergence combined with unique morphological features were classified as distinct barcoded morphologies (BMs) within three Classes (Demospongiae, Calcarea, and Homoscleromorpha), providing the true sponge richness values for each ARMS unit. BM classification to Order, Family, Genus and, if possible, species was based on morphological character comparisons to previous sponge collections in Hawai'i (Bergquist, 1967, 1977; De Laubenfels, 1950, 1951, 1954, 1957; Pons et al., 2017) while also integrating a >97% COI

and 28S rRNA sequence identity, independent of the metabarcoding. Neighbor-joining phylogenetic trees were generated from COI and 28S rRNA barcode data in Mega 10 (Kumar et al., 2018). Evolutionary distances were computed using the Tamura Nei model (Tamura & Nei, 1993) for COI with a total of 281 positions and Jukes-Cantor method for 28S rRNA (Jukes & Cantor, 1969) with 826 positions.

2.3 | DNA metabarcoding

Total genomic DNA was isolated using the DNeasy Powermax Soil Isolation Kit (Qiagen) following modifications to the manufacturer's protocol as per Ransome et al. (2017). Amplicons of the COI gene were generated via polymerase chain reaction (PCR) in triplicate 20 µl reaction volumes for each sample (ARMS unit), targeting a 313 bp fragment using the primers mlCOIintF and jgHCO2198 (Geller et al., 2013; Leray et al., 2013). Each 20 µl reaction included: 7.65 µl of nanopure H_2O, 10 µl of ImmoMix Red (2×; Bioline), 0.06 µl of each primer (10 µM), 0.15 µl BSA (10 mg/ml), and 1 µl template DNA (5–25 ng/µl). We used a touchdown PCR profile with 16 initial cycles: denaturation for 10 s at 95°C, annealing for 30 s at 62°C (−1°C per cycle), and extension for 60 s at 72°C, followed by 20 cycles at an annealing temperature of 46°C (Leray et al., 2013).

PCR products were quality assessed by gel electrophoresis in 2% agarose gel and amplification success was defined by the presence of a clear band of approximately 340 bp. PCR replications were then pooled, purified with AMPure XP beads (Beckman Coulter Life Sciences), and quality assessed again by gel electrophoresis. Illumina adapters were ligated to cleaned PCR products using the Kapa Hyper-Prep PCR-free Kit. Libraries were validated via qPCR using the KAPA library quantification kit and sized and checked for quality using an Agilent Technologies 2100 Bioanalyzer. Samples passing QC were shipped to the University of California, Riverside's Institute for Integrative Genome Biology for sequencing on an Illumina MiSeq platform using v3 chemistry (2 × 300 bp paired-ends).

We chose the user-friendly and computationally fast R modular package pipeline for metabarcoding bioinformatics—*Just Another Metabarcoding Pipeline* (JAMP—https://github.com/VascoElbrecht/JAMP - see rationale in supplementary materials)—which integrates Usearch v10.0.240 (Edgar, 2013), Vsearch v2.4.3 (Rognes et al., 2016), and Cutadapt 1.9 (Martin, 2011) to process samples. In brief, pre-processing of reads included sample demultiplexing, paired-end merging (Usearch, allowing for 25% mismatches in overlap), primer and adapter trimming (Cutadapt, allowing for 10% errors in primer matching), and sequence length filtration (Cutadapt: min/max 295/340 bp – see Figure S2 for rationale). Low-quality sequences were filtered and discarded using UPARSE fastq_filter with maxee = 0.25 and qmax at 60 (Edgar & Flyvbjerg, 2015), dereplicated (min. unique size = 2), and clustered with simultaneous chimera removal using Usearch (cluster_otus 97% identity). The pre-processed dereplicated reads of all samples (including singletons) were then matched against the respective clustered molecular operational taxonomic units (MOTUs) with a minimum match of 97% using usearch_global and strand plus within Usearch.

MOTUs were classified using three approaches to maximize and cross-check sponge assignments. We first ran a local BLASTn against 65 sponge COI DNA barcodes obtained from DNA barcoding efforts from this study and the MarineGEO Hawai'i biodiversity survey that occurred in Kāne'ohe Bay in 2017. We ran an additional BLASTn search against a curated reference database containing 16,679 COI sequences specific to coral reef fauna from the Mo`orea BIOCODE project Inventory (Meyer, 2017). Next, we classified sequences taxonomically using the ecotag algorithm (Boyer et al., 2016), which was based on a lowest common ancestor classification approach on representative sequences for each taxon in relation to a local reference COI database that contained 192,929 filtered COI sequences taken from GenBank and BOLD (*database COI Nov2018* – Wangensteen et al., 2018). Lastly, we assigned sequences using the R package, Informatic Sequence Classification Trees (INSECT), which takes a probabilistic approach (hidden Markov model) to assignment against a classification tree built from 396,413 sequences extracted from the MIDORI database and GenBank (Wilkinson et al., 2018). Due to the limited number of marine invertebrate barcodes within reference databases, BLASTn identifications were accepted at ≥85% identity, ≥85% coverage, and ≥200 alignment length following Ransome et al., 2017 who found that these cutoffs provided the greatest accuracy in maintaining the known phylum-level taxonomy across 16 tested phyla. Ecotag assignments were accepted if the "best identity" was ≥80% and INSECT assignments were set at a probability ≥0.90%.

All MOTUs identified as metazoans were translated into amino acids and aligned to the BIOCODE reference data set using Multiple Alignment of Coding Sequences (MACSE; Ranwez et al., 2011). MACSE detects interruptions in open reading frames from nucleotide substitutions that can result in stop codons which are likely to be pseudogenes. Any MOTUs that did not pass through MACSE were removed from the MOTU table and only MOTUs with a read abundance above 0.01% were considered in downstream analysis to reduce the number of false positives due to PCR and sequencing errors (Bokulich et al., 2013; Bista et al., 2017; Elbrecht et al., 2017a).

MOTUs identified as sponges were further examined in Geneious 10 and translated into amino acids to double check for stop codons and introns given that some members of Porifera are known to have introns within the targeted barcoding region (Schuster et al., 2017). Resulting annotated MOTUs were then examined across competing methods, and the final classification was based on the assignment with the greatest identity from the three methods. We accepted class, order, and family annotations if sequence identity was ≥90%, ≥92%, and ≥96%, respectively. We accepted classifications to the genus and species level if sequence identity was ≥98% and 100%, respectively (Yang et al., 2017). To cross-examine sponge annotations to class and order-level assignments, we aligned all sponge-identified sequences from the metabarcoding in Geneious with the successfully aligned COI DNA barcode sequences and generated a neighbor-joining phylogenetic tree in Mega10 (Kumar et al., 2018)

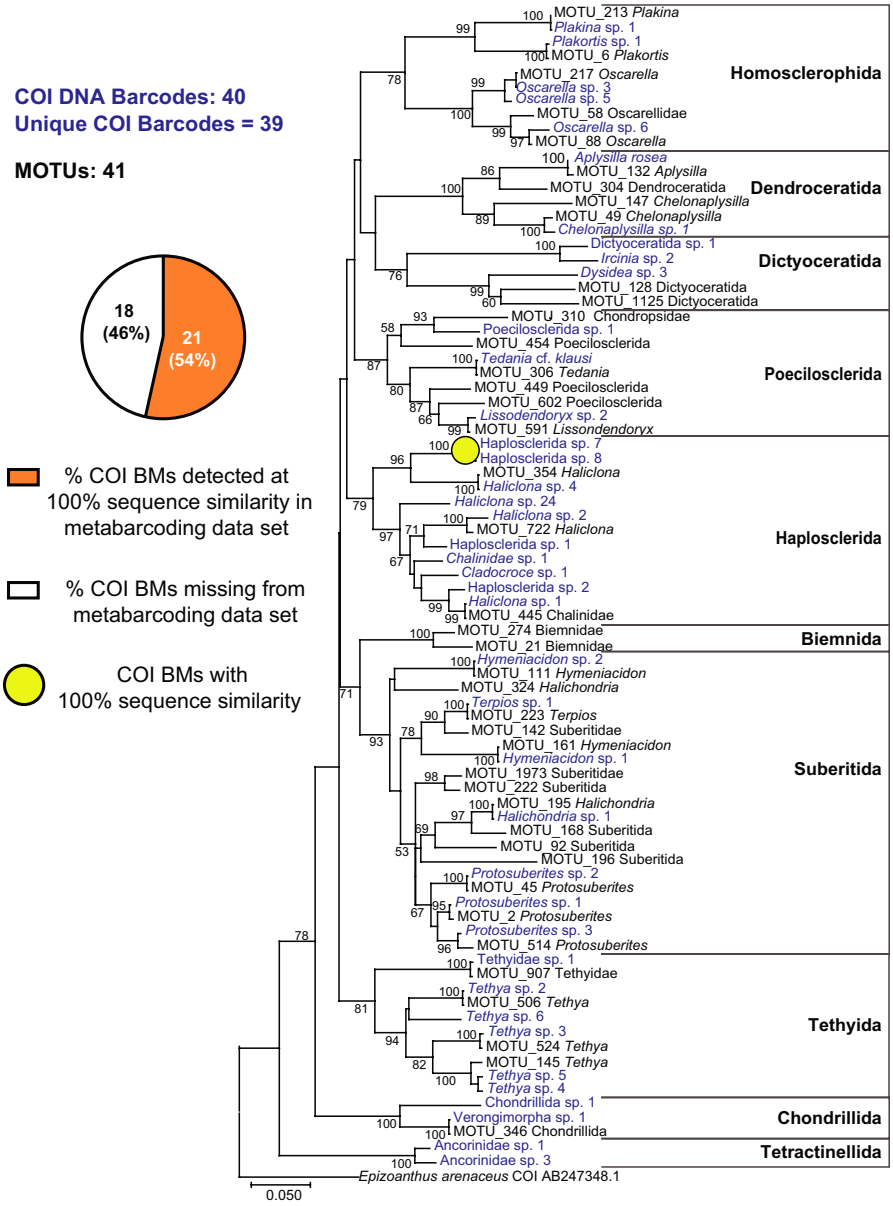

FIGURE 1 Neighbor-joining phylogenetic tree of sponge annotated molecular operational taxonomic units (MOTUs) (in black) and 40 COI barcoded morphology (BM) sequences (in blue) to validate class and order-level annotations. Tree was generated from partial sequences spanning 281 position of the COI gene. The yellow circle represents two distinct sponge species from the 28S rRNA barcodes that had identical COI DNA barcode sequences

using the Tamura Nei model with uniform rates among sites based on 1,000 bootstrap replicates (Tamura & Nei, 1993). A total of 270 positions were used to generate the tree (Figure 1). Sponge MOTUs that were identified only to the phylum level were assigned to a class and/or order level based on their position within the tree.

2.4 | Analyses

Data were analyzed using R version 3.5.2 (R Core Team, 2018). Richness was calculated using the specnumber function in *vegan* (Oksanen et al., 2019). We examined composition by class, order, and family based on the overall abundance of unique BMs (barcoded morphologies) and MOTUs (metabarcoding). Parametric and nonparametric (Wilcoxon signed-rank) paired *t* tests were used to examine differences between metabarcoding richness and the true richness with and without Calcarea from the total data set and based on assigned class and orders. Both types of paired *t* tests were conducted to cross-validate p-values due to small sample sizes and varying degrees of variances, ties, and zeros.

All MOTUs that matched at 100% sequence similarity to the specimen derived COI DNA barcodes were classified with the sponge name assigned to the barcode by the taxonomist (Figure 1). This enabled a direct comparison of the presence and/or absence of these particular sponges between methods across ARMS units. Any positive (metabarcoding) detection on an ARMS unit where the specimen of that species was absent was called a false positive and an absence of detection where the specimen was known to be present was called a false negative. A presence/absence heat map was produced using the Heatmap function in *ComplexHeatmap* (Gu et al., 2016) and compared to examine the efficacy of metabarcoding in species detection across replicates. To examine the overall contributions of the COI DNA barcoding effort in deriving species-level matches for MOTUs as well as in generating new GenBank database

species records, we compared BLAST results based on 100% sequence similarity with GenBank. All graphs were produced using *ggplot2* (Wickham, 2016).

3 | RESULTS

3.1 | DNA barcoding and morphology—capturing true sponge richness

A total of 240 sponges were subsampled from the 22 ARMS units. The sequencing success rate using the 28S rRNA primers was 100% resulting in a species list totaling 69 unique sponge BMs (6 homoscleromorphs, 15 calcareous, and 48 demosponges) (Table S2 and Figure S3). Primers targeting the standard COI barcoding gene had a lower overall success rate, identifying 39 unique sponge BMs (5 homoscleromorphs and 34 demosponges). As expected, no sequences from the class Calcarea were successfully amplified using the COI primers in this study. Demosponges identified as Haplosclerida sp. 7 and Haplosclerida sp. 8 from the 28S rRNA barcoding had the identical sequence from the COI barcodes (Figure 1). As a result of overall low COI amplification success rate and the reduced ability to delineate species using the COI gene, true sponge richness values across ARMS units were based on the combination of 28S rRNA barcodes and morphological comparisons with previous collections in Hawai'i (Figure S2). True sponge richness per ARMS unit ranged from 9 to 18 BMs with Calcarea and from 5 to 13 BMs without Calcarea (Table 1).

3.2 | Metabarcoding performance

A total of 41 unique sponge MOTUs were identified out of 295 annotated metazoan MOTUs (Table S3). Five MOTUs were assigned to the class Homoscleromorpha and 36 were assigned to the class Demospongiae. There were no MOTUs assigned to the class Calcarea. Sponge MOTU richness per ARMS unit ranged from 1 to 13 and 6 ARMS units had a greater MOTU richness than the true richness value when Calcarea were removed (Table 1).

There were significant differences in richness between methods ($p < .001$) but when Calcarea was removed from the true richness values, richness estimates did not differ overall ($p = .13$; Table 2, Figure 2a). At the class level, there were no richness differences between methods in either Homoscleromorpha ($p = .58$) or Demospongiae ($p = .15$) (Figure 2b, Table 2). At the order level, there were significant richness differences between BMs and MOTUs assigned to Haplosclerida, Poecilosclerida, and Suberitida ($p < .01$;

TABLE 1 ARMS unit metadata. ARMS units with a higher metabarcoding richness than the richness detected without Calcarea from the barcoded morphologies are in bold

ARMS unit	Raw sequence depth	Quality filtered sponge sequences	Sponge richness COI metabarcoding	Sponge richness 28S rRNA barcoding - no Calcarea	Sponge richness 28S rRNA barcoding -all sponges
1	99,151	6,134	3	8	13
2	79,252	2,120	5	8	13
3	41,939	3,712	9	10	14
4	133,359	5,613	8	10	18
5	188,537	724	10	13	18
6	147,690	1,827	1	6	13
7	**58,971**	**2,979**	**7**	6	10
8	23,080	3,362	9	11	17
9	136,750	3,130	7	10	15
10	87,137	1,265	7	7	11
11	76,079	397	5	9	16
12	**227,257**	**592**	**12**	10	16
13	**108,989**	**12,681**	**11**	6	9
14	329,916	865	10	10	14
15	**489,606**	**1,322**	**13**	9	16
16	217,388	855	10	11	17
17	86,486	3,943	5	8	15
18	69,353	410	8	10	18
19	**35,957**	**636**	**7**	5	12
20	**107,760**	**1,328**	**8**	6	9
21	71,096	1,178	3	5	9
22	176,210	433	7	7	11

TABLE 2 Parametric and nonparametric paired *t*-test results comparing metabarcoding sponge richness with the true sample richness from the barcoded morphologies with and without the class Calcarea, by class, and by order

Taxa group	Comparison	Mean difference	CI	CI	t	*p*-value	*p*-value Wilcoxon
	All Sponges	6.32	4.74	7.89	8.35	<.0001	<.0001
	No Calcarea	0.91	−0.29	2.11	1.56	.13	.13
Class	Demospongiae	0.86	−0.35	2.07	1.48	.15	.14
	Homoscleromorpha	0.04	−0.12	0.21	0.57	.58	.77
Order	Dendroceratida	−0.18	−0.48	0.11	−1.28	.21	.24
	Dictyoceratida	0.09	−0.18	0.36	0.69	.49	.57
	Haplosclerida	1.68	1.2	2.16	7.7	<.0001	<.001
	Poecilosclerida	0.72	0.29	1.16	3.46	<.01	.005
	Suberitida	−1.86	−2.56	−1.16	−5.52	<.0001	<.001
	Tethyida	0	−0.27	0.27	0	1	1
	Tetractinellida	0.32	−0.2	0.84	1.27	.22	.24
	Homosclerophorida	0.04	−0.12	0.21	0.57	.57	.77

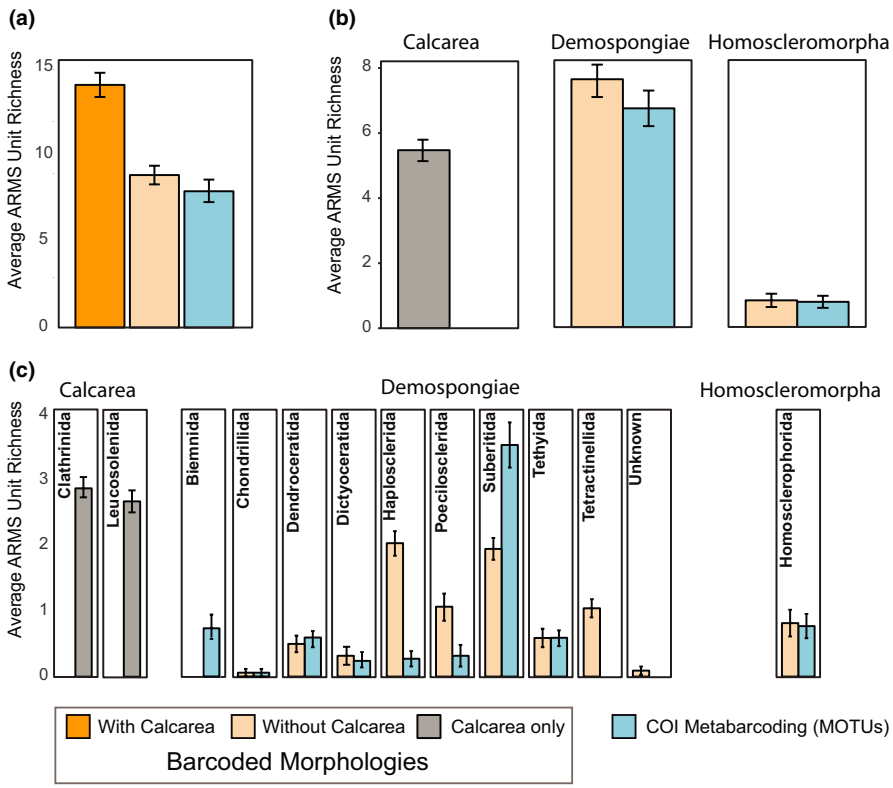

FIGURE 2 Average ARMS sponge richness for barcoded morphologies (28S rRNA and COI) and COI metabarcoding overall (a), by class (b), and by order (c) from 22 ARMS samples

Figures 2c and 3a, Table 2) with metabarcoding under-representing Haplosclerida and Poecilosclerida and overrepresenting Suberitida. There were no differences between methods within the orders Dendroceratida, Dictyoceratida, Homosclerophorida, or Tethyida. The order Tetractinellida was not found in the metabarcoding data set and the order Biemnida was present only in the metabarcoding data. Among families in orders represented by both methods, Irciniidae and Mycalidae were absent from the metabarcoding data and Chondropsidae was present only in the metabarcoding data (Figure 3b). The family Suberitidae was overrepresented in the metabarcoding data, and approximately 25% of both BMs and MOTUs could not be assigned to a family.

Of the 39 unique COI BMs, 21 (54%) were matched at 100% sequence similarity to metabarcoding MOTUs (Table S4). When examining the species occurrences of these 100% matches across all 22 ARMS units, 23% of the MOTUs were false positives, 24% were false negatives, and 53% matched the expected occurrence (Figure 4). The majority of the false positives (75%) occurred in MOTUs assigned to the order Suberitida and the majority of false negatives (79%) were associated with the order Haplosclerida. Only three ARMS units had

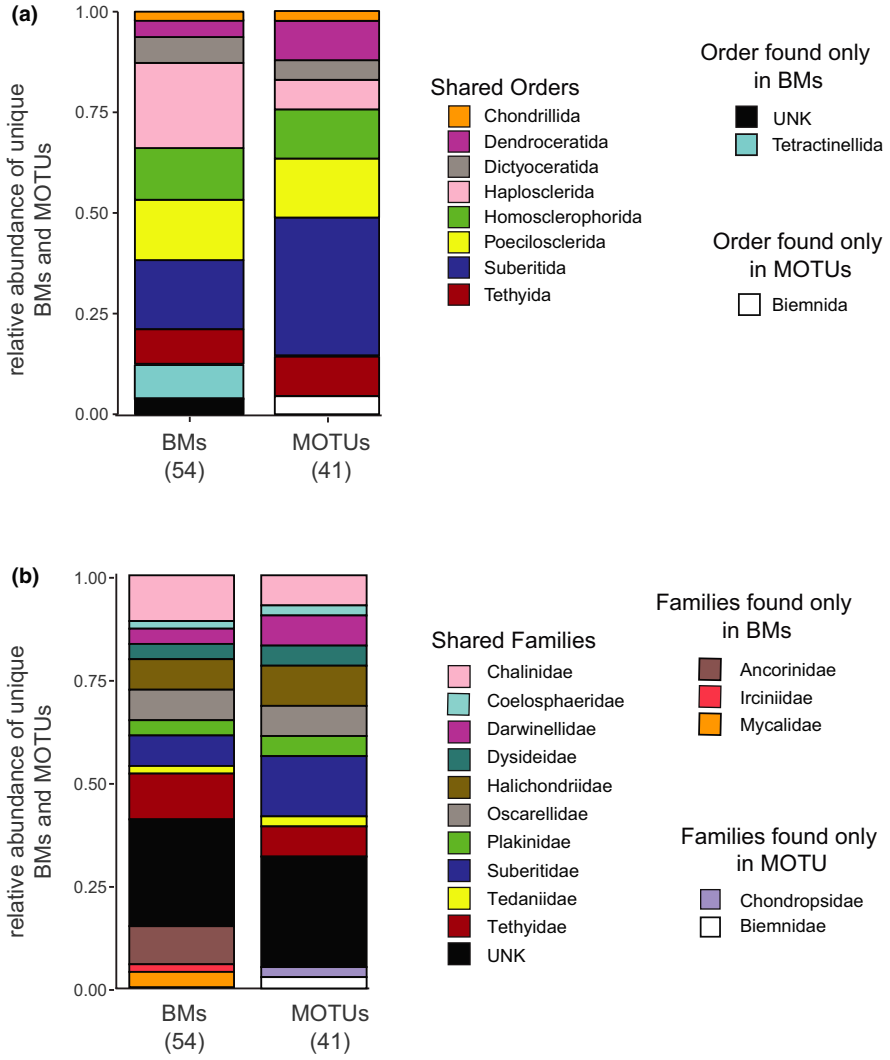

FIGURE 3 The relative abundance of unique barcoded morphologies (BMs) and molecular operational taxonomic units (MOTUs) within orders (a) and families (b). BMs are based on sponge identifications from 28S rRNA barcoding and morphology. MOTUs are based on sponge annotations from metabarcoding. The numbers in parenthesis equate to total sponge richness from 22 ARMS samples. No BMs from the Class Calcarea are included

MOTU detections matching the exact occurrences from the 28S rRNA (Figure 4).

Sponge COI DNA barcoding increased species-level MOTU identification by 37% (15 of the 41 total MOTUs) and overall, 30 out of the 39 successfully barcoded sponges (77%) were new species to GenBank based on comparisons using a 100% sequence identity (Table S4).

4 | DISCUSSION

Ecosystem function, sustainability, and resilience are all critically dependent on biodiversity, but the number of species in the sea and the spatial scale of their organization remains unknown (Palumbi et al., 2009). The capacity of the existing taxonomic workforce is insufficient to meet global demands for species descriptions and expert consultation on biodiversity surveys (Hopkins & Freckleton, 2002; Sangster & Luksenburg, 2015; Tancoigne & Dubois, 2013). As a result, DNA metabarcoding approaches, pioneered in microbial research, are being explored as a mechanism to provide high-throughput taxon identification through sequencing without a direct reliance on taxonomic expertise (Coissac et al., 2012; Taberlet et al., 2012). Metabarcoding approaches have shown great promise in some systems to monitor alpha- and beta-diversity of well-studied taxa (Ji et al., 2013; Nichols & Marko, 2019; Stat et al., 2017). But metabarcoding performance on lesser known taxonomic groups in the natural environment is less certain and would be more arduous to substantiate without taxonomic validation due to the patchiness of DNA barcodes in reference libraries (Ransome et al., 2017).

Despite the concerted efforts of the "Sponge DNA Barcoding Project" (Wörheide et al., 2007), sponges arguably remain the most difficult group among the lesser known metazoans to identify. The promise of DNA barcoding approaches to facilitate identification has yet to be realized due to the genetic peculiarities of this problematic group (Solé-Cava & Wörheide, 2007; Wörheide et al., 2007). Evaluation of genetic approaches is typically conducted against well-characterized or mock community samples in which the exact composition is known (e.g., Elbrecht & Leese, 2017; Ji et al., 2013; Nichols & Marko, 2019; Zhang et al., 2018). The performance of metabarcoding approaches for sponges has yet to be evaluated, likely due to an expectation that metabarcoding would perform poorly, but also because of how difficult this group is to validate against

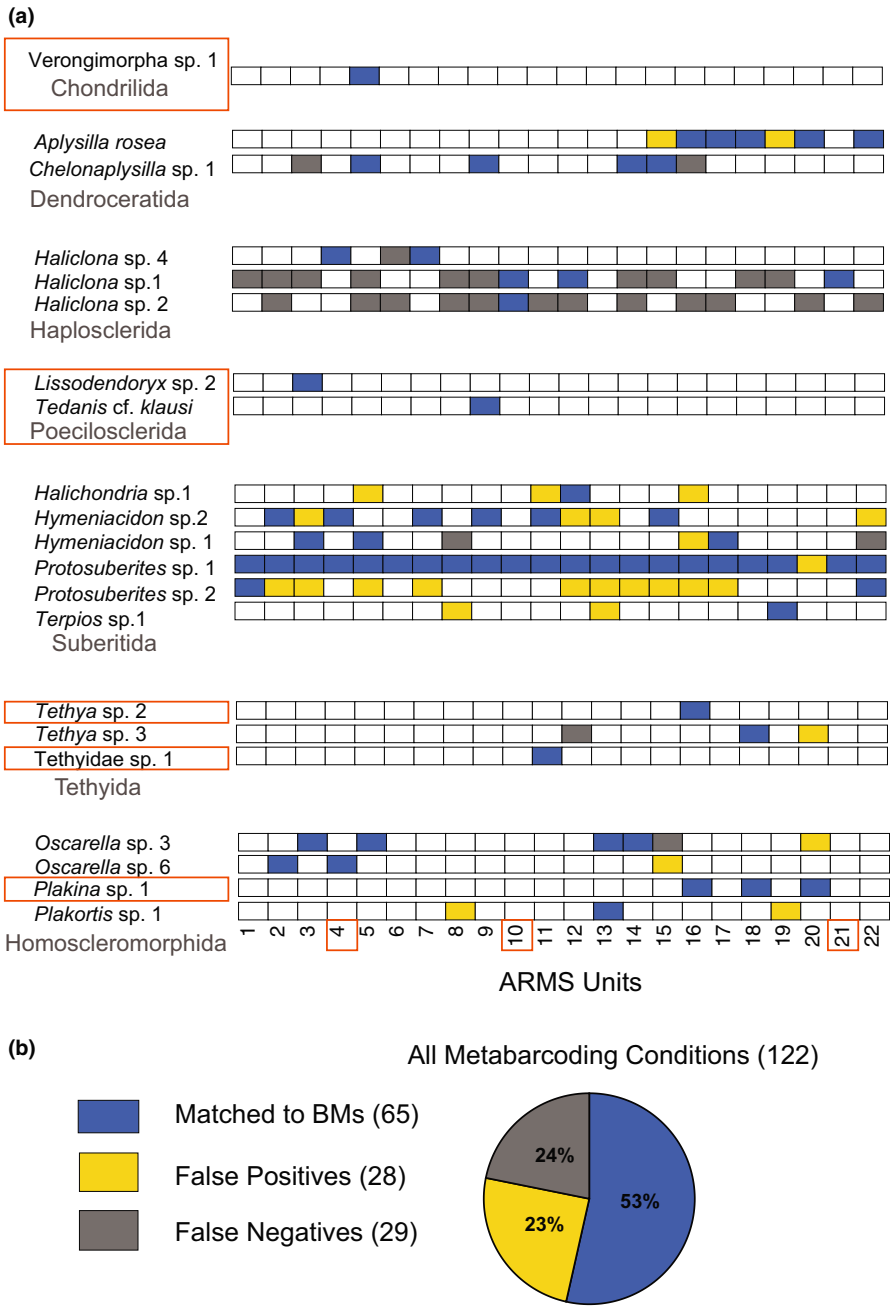

FIGURE 4 (a) Presence/absence occurrence comparisons of 21 species across ARMS units. Selected species were based on subsampled sponges that were successfully barcoded with both COI and 28S rRNA genes and had >100% sequence similarity match between COI barcodes and molecular operational taxonomic units (MOTUs) from COI metabarcoding (Figure 1). Each box represents an individual ARMS unit (22 total). The orange boxes highlight species that were found in the same units by both methods as well as the ARMS units that had exact matches across all 21 species. (b) The pie chart represents the percentage of matched, false positives, and false negatives considering all conditions found within the metabarcoding data

an established community. Our results confirm some expectations about the shortcomings of a COI approach for sponge diversity, such as preferential amplification and sequencing, and limited species to family-level classifications. But despite these limitations, this study was still able to generate accurate richness estimates of sponges overall (in the absence of class Calcarea) and at the class level using universal primers on a complex natural community.

A common concern with DNA metabarcoding is whether results reflect the true community (Deagle et al., 2014; Ficetola et al., 2016; Ji et al., 2013; Taberlet et al., 2012). When applying this technique to complex communities of multiple understudied phyla, such as the coral reef cryptobenthos, the question intensifies. Challenges include the inevitable PCR amplification and sequencing biases associated with using universal primers that amplify widely divergent taxa, and the variable rates of evolution within and among phyla that affect sequence clustering with a fixed divergence threshold (Bokulich et al., 2013; Brown et al., 2015; Elbrecht & Leese, 2015; Elbrecht, Peinert, et al., 2017; Elbrecht et al., 2017; Fonseca et al., 2010; Kunin et al., 2010). However, if abundance and identity of species present within a community is not known a priori, it is difficult to know which taxa are biased in metabarcoding data as a result of technical and/or biological constraints. Our findings begin to address these unknown biases within Porifera.

Our DNA metabarcoding approach allocated 41 MOTUs to the phylum Porifera, 28 MOTUs less than the 69 verified BMs. This shortage resulted largely from the known failure of standard COI primers to amplify members of the class Calcarea, which resulted in a loss of 15 species, or 22% of the total sponge diversity

(Figure 2a). The remaining 13 missing species included 12 fewer MOTUs within the class Demospongiae and 1 within the class Homoscleromorpha. Missing sponge MOTUs (false negatives) may be a result from differing sponge biomass biasing PCR amplification (Elbrecht, Peinert, et al., 2017; Elbrecht, Vamos, et al., 2017), primer-specific species detection limitations (Elbrecht & Leese, 2015), the stringency of sequence quality-filtering used in the bioinformatic process, the use of a fixed 97% sequence similarity for clustering (Brown et al., 2015

), and/or various challenges inherent in amplifying this gene in sponges (Vargas et al., 2012).

In this study, we intentionally avoided attempting to find the "sweet spot" of sponge sequence divergence thresholds for MOTU clustering using COI. The differing rates of evolution that can exist among members of the same families or genera (Redmond et al., 2011), compounded with the known discrepancies in family- and even order-level resolution, make such thresholds arbitrary (Erpenbeck et al., 2007; Redmond et al., 2011; Wang & Lavrov, 2008; Yang et al., 2017

). With many metazoans showing on average <2% intraspecific variation but more than 4% interspecific divergence from their nearest neighbor, a standard of 3% sequence divergence (97% sequence similarity) has been proposed for species delineation in many COI barcoding efforts (Hebert et al., 2004; Hebert et al., 2003). Applying this standard showed congruence in richness between BMs and metabarcoding approaches when the Calcarea were removed from the analysis (41 of 54 species). Richness did not differ significantly between methods for classes Demospongiae and Homoscleromorpha (Figure 2a,b, Table 2) and several order-level richness comparisons (Table 2). However, the orders Haplosclerida, Poecilosclerida, and Suberitida were the exception, with metabarcoding overestimating Suberitida richness (creating false positives) and underestimating Haplosclerida and Poecilosclerida richness (creating false negatives) (Figures 2c and 3a).

One possible explanation for the significant over- and underestimations of MOTUs within these orders is intraspecific variation (Brown et al., 2015; Meyer & Paulay, 2005). If intraspecific variation within Suberitida exceeds the 3% divergence threshold used to cluster sequences, this could explain MOTU overestimation within this order. Likewise, if intraspecific variation within Haplosclerida and Poecilosclerida is less than 3% divergence this could explain MOTU underestimation within these orders. The overestimation of MOTUs within the order Suberitida could also result in part from nuclear mitochondrial-like sequences (NUMTS), also known as pseudogenes (Bensasson et al., 2001). These nonfunctional copies of mtDNA sequences integrate into the nucleus and can easily be co-amplified when targeting a conserved fragment of COI using degenerate primers. When present, pseudogenes lead to an overestimation of the number of unique MOTUs (Deagle et al., 2014; Song et al., 2008). Relative to other sponges, members of the families Suberitidae and Halichondriidae within the order Suberitida have been found to have high amplification and sequencing success rates (Vargas et al., 2012). Accordingly, we found a high proportion of sequences assigned to Suberitidae (Figure 2b). As with previous studies, we attempted to minimize the inclusion of pseudogenes using the MACSE pipeline, which detects and quantifies interruptions in open reading frames, flagging and removing sequences that have nucleotide substitutions resulting in frameshifts and stop codons as likely pseudogenes (Ji et al., 2013; Leray & Knowlton, 2015, 2017; Yu et al., 2012). In addition, we visually analyzed MOTU sequences in Geneious to search for introns and stop codons. Despite these measures, given the high degeneracy of our universal primers and the high sequencing success rates for family Suberitidae, we cannot rule out the possibility that some of the excess MOTUs result from pseudogenes; although this does not explain the large number of false positives found within the subset of Suberitida MOTUs we examined (Figure 4).

We found 35 false positives when we examined the presence of 21 MOTUs across the 22 ARMS units and 21 of these (75%) occurred within the order Suberitida (Figure 4). Some of these false positives may be the result of small sponges or recently settled sponge larvae that were overlooked by the taxonomist but happened to be detected by metabarcoding (particularly given the high amplification and sequencing success rates within Suberitida) (Vargas et al., 2012). False positives within Suberitida could also be a result of morphospecies misidentification by the taxonomist. All morphospecies identified on the plates were subsampled for DNA barcoding; however, all species within the order Suberitida express a yellow color or will turn yellow when stressed (Figure S4). Thus, it is possible that some of the visually censused Suberitida specimens were catalogued inaccurately and some false positives may in fact be true detections. Similarly, sponge species within the order Biemnida, also frequently yellow, may have been misidentified as they occurred in the metabarcoding dataset but not the BM dataset. A yellow biemnid, *Biemna fistulosa*, is known to occur within Kāneʻohe Bay.

False positives may also be a result of tag jumping (Schnell et al., 2015). Although this is challenging to detect, we believe it may explain the two false positives for *Plakortis* sp. 1 found in ARMS Units 8 and 21 (Figure 4). This species was one of the only BMs detected within a single ARMS unit for which there were false positives in the metabarcoding data. *Plakortis* sp. 1 was dominant in Unit 13 both in terms of plate coverage (Figure S5) and metabarcoding read abundance (Table S3). Thus, it seems probable that the 10 reads of *Plakortis* sp. 1 found in Unit 8 and the 5 reads found in Unit 21 (indicated as false positives) were the result of tag jumping during sequencing (Schnell et al., 2015). Similarly, the false positive detected in Unit 20 for the species *Protosuberites* sp. 1 could be a result of tag jumping given that this sponge species (MOTU) had the highest number of reads overall and was detected visually in all ARMS units except Unit 20.

Despite the prevalence of both false positives and false negatives (primarily from the orders Suberitida and Haplosclerida, respectively), our metabarcoding approach was effective at detecting rare BMs (those found in a single ARMS unit and/or at very low biomass). Such taxa include Verongiomorpha sp. 1, *Lissodendoryx* sp. 2, *Tedania* cf. *klausi*, *Tethya* sp. 2, and Tethyidae sp. 1), (Figure 4; Figure S6 – Verongiomorpha sp. 1 example).

Discrepancies in family-level classifications (Figure 3b) could be a result of metabarcoding failing to amplify particular families (Ircinidae within Dictyoceratida and Ancorinidae within Tetractinellida – Figure S2) (Vargas et al., 2012), difficulties in resolving family-level taxonomy using COI, and/or the well-known conflicts in sponge classification between the 28S rRNA and COI genes (Carella et al., 2016; DeBiasse & Hellberg, 2015; Erpenbeck, Breeuwer, et al., 2006; Erpenbeck, Hooper, et al., 2006; Erpenbeck et al., 2007; Heim et al., 2007; Morrow & Cárdenas, 2015; Redmond et al., 2011; Yang et al., 2017). Such difficulties are not necessarily a failing of the metabarcoding approach, but rather known limitations of the short amplicon fragment lengths as well as taxonomic challenges associated with sponges. Nearly 25% of species were unclassified by either method, indicating considerable future challenges associated with species discrimination in sponges regardless of the approach. It will take dedicated taxonomic study and population of reference databases with reliably identified and vouchered individuals to overcome these challenges if sponge biodiversity is to be better understood in coral reef cryptobenthic environments. Our results highlight this pressing need as 30 of the 39 COI sequenced BM sponge vouchers from this effort are new species additions to GenBank and from these, 15 out of the 41 sponge MOTUs were identified to the species level, increasing metabarcoding sponge annotations by 37%.

5 | CONCLUSIONS

Here, we compare a community metabarcoding approach to careful taxonomic evaluation of a naturally occurring sponge community. We used a user-friendly and computationally fast metabarcoding R modular package bioinformatic pipeline on a complex coral reef cryptobenthic community. After taking into consideration the inability of the primers used to amplify sponge species from the class Calcarea, we found that metabarcoding adequately captured richness overall and at the class level for demosponges and homoscleromorphs. We uncovered technical and biological constraints for the orders Suberitida, Haplosclerida, and Poecilosclerida that complicated richness comparisons, but found congruence with other orders. We successfully detected rare sponges with low biomass, and most likely detected real species on some ARMS units that were missed by the taxonomist due to morphotype similarities. Although there are unavoidable challenges when metabarcoding a complex community composed of multiple phyla, this study imparts a greater level of confidence in richness values obtained for sponges overall and at the class level when using a 97% sequence similarity threshold for MOTU clustering.

There has been much debate in the literature regarding the advantages and disadvantages of MOTU clustering, differing clustering algorithms, exact sequencing variants, and strong opinions can be found on all sides (Brown et al., 2015; Callahan et al., 2017; Flynn et al., 2015; Glassman & Martiny, 2018; Nearing et al., 2018; Prodan et al., 2020; Schmidt et al., 2015). This active debate can confuse and discourage researchers from entering the otherwise promising field of DNA metabarcoding. Debate occurs because there are methodological trade-offs and no perfect solution currently exists. Choosing an appropriate clustering method (or not) should be based on the system being studied and the question of interest. The field will undoubtedly advance as more empirical performance tests and validations such as this are examined across differing phyla. However, metabarcoding approaches will still be confounded when applied on a global scale if method standardized if not upheld (Ransome et al., 2017), metabarcoding performance is not tested across all phyla, and if each taxon does not have multiple vouchered reference sequences from across their geographic ranges (Meyer & Paulay, 2005). This complexity is why the importance of regional reference databases cannot be overemphasized; any metabarcoding approach will be further confounded without DNA barcodes attached to regional voucher specimens. DNA barcoding in this study alone found 77% of the sponge vouchers to be new species to the GenBank database. With the continued decline in training and funding for taxonomists, regionally vouchered databases are a distant goal, and without them the true promise of eDNA and DNA metabarcoding approaches to biodiversity characterization and monitoring of complex communities may never be reached. But, if we collectively make a concerted effort to include and fund taxonomists in empirical field-based metabarcoding performance tests, interpretability will substantially improve and the path toward reliable biomonitoring of complex communities using genetic approaches in our changing environment could become a reality.

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CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

CJ involved in Mesocosm set-up and maintenance. MT and JV involved in conceptualization. MT, JV, and MW involved in field sampling and laboratory work. JV involved in taxonomic identification. MT and JV involved in formal analysis. MT involved in visualization. MT involved in writing—original draft preparation. MT and RJT: involved in funding acquisition and project administration. All authors contributed to review and editing of manuscript and gave final approval for this publication.

DATA AVAILABILITY STATEMENT

All data files, R code, and materials used in the analysis will be made available in some form to any researcher for purposes of reproducing

or extending the analysis. Sequence barcodes that support the findings of this study have been deposited in GenBank (see Table S2) and submitted to the Barcode of Life Data System. Raw illumina sequences are available in NCBI SRA (Sequence Read Archive, Accession: SRS7105074-SRS7105095).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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