



REPORT

Unveiling hidden sponge biodiversity within the Hawaiian reef cryptofauna

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Abstract Our perception of reef diversity is dominated by corals, fish, and a few other groups that visibly dominate the reef surface. However, the bulk of reef biodiversity resides within the reef framework, and this cryptobiota is fundamentally important for the surface community. Sponges are abundant and conspicuous on the reef surface in productive, continental reefs, but largely vanish from surveys of the oligotrophic reefs of Oceania. However, their diversity in the cryptobiota remains poorly characterized. Here, we explore the contribution of cryptobenthic sponges to overall sponge diversity on 1750 m² of reef habitat in Kāneʻohe Bay and Waimanalo in the island of Oʻahu, Hawaiʻi. We also assessed cryptic sponges using 15 m² of autonomous reef monitoring structures (ARMS) deployed in this same area. We used integrative taxonomy combining morphology, COI and 28S barcoding to

delineate and track species, most of which are poorly known or undescribed. We documented 186 OTUs, 150 of which are new records for the Hawaiian Islands, increasing the known sponge fauna of Kāneʻohe Bay by 3.5-fold, and that of the Hawaiian Islands by 2.5-fold. More than of the sponge OTUs were cryptobenthic. Reef sampling provided access to 31% (44 OTUs), whereas 52% (75 OTUs) were retrieved exclusively from ARMS. These results illustrate that the interstices of ARMS units provide suitable habitat for settlement of cryptobenthic sponges that would otherwise be impossible to access through traditional field surveys. Tracking species with provisional names, using integrative species delineation anchored to vouchers, images, and DNA barcodes provides a powerful approach for working with such a poorly understood fauna.

Keywords ARMS · Porifera · Cryptobenthic coral reefs · COI · 28S rRNA barcoding

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Introduction

Sponges (Phylum Porifera) are foundational components of coral reef ecosystems both as surface dwellers and within the complex matrix of the reef framework. Structurally, they contribute to the topological complexity of reefs and provide habitat for other organisms (Pawlik 2011). Among holobiomes, sponges hold the largest diversity of microorganisms (Thomas et al. 2016; Moitinho-Silva et al. 2017). They have remarkable water filtering capacities (Gili and Coma 1998) and, when coupled with their microbial symbionts, can perform key ecological functions such as changing the nitrogen composition of the surrounding seawater (Corredor et al. 1988; Southwell et al. 2008; Gantt et al. 2019) which can facilitate growth of

neighboring corals and seaweed (Slattery et al. 2013; Easson et al. 2014). Sponges in cryptobenthic spaces are the only metazoans able to transform biologically unavailable forms of dissolved organic matter (DOM) into nutritional, particulate organic carbon (POC), by continuously producing and shedding of sponge cells (De Goeij et al. 2013). Acting as POC bioreactors surrounded by an endless supply of coral-derived DOM, sponges feed detritivores at lower trophic levels and in turn sustain coral reef biodiversity under oligotrophic conditions (Rix et al. 2018).

One of the most striking differences among reefs in different regions of the tropical ocean is in the abundance and diversity of large sponges. Sponges are major space occupiers on Caribbean reefs in particular and are also abundant in the “continental” western Pacific, but become rare and inconspicuous around the islands of Oceania (Birkeland 1987; Wilkinson 1987). These differences are driven in part by productivity and further reflected in the increasing relative abundance of photosymbiotic sponges on more oligotrophic reefs (Wilkinson 1987). This gradient in abundance is apparent in the documented diversity of reef sponges, with hundreds of species recorded from continental reefs compared with tens of species from oceanic islands (Van Soest et al. 2012).

While the diversity, abundance, and ecology of macro-sponges on the reef surface have received substantial attention, the smaller, less conspicuous, and hidden sponges of the reef interior have been much less studied. Most of the diversity on reefs is in the subsurface spaces that permeate the reef matrix, and sponges are a dominant space occupier of these cryptobenthic habitats (Richter et al. 2001). Cryptobenthic sponges have received limited attention, although their ecological and nutritional importance to the ecosystem has long been realized (Wulff 1984; De Goeij et al. 2013). Despite their ecological importance, cryptobenthic sponges, particularly throughout Oceania, have been poorly studied (Van Soest et al. 2012) and likely add to the estimated 64% of sponge species (25,000) that remain to be discovered (Appeltans et al. 2012). Sponge collections have been conducted for over two hundred yrs within temperate systems such as the Northeast Atlantic and Mediterranean, whereas remote locations in the Pacific have been comparatively underexplored (Van Soest et al. 2012). The geographic isolation of remote Pacific reefs combined with the taxonomic challenges of positively identifying sponges and the pervasiveness of undescribed and undiscovered sponges hidden within reef crevices has challenged diversity estimates throughout the marine tropics (Richter et al. 2001). Uncovering these sponges across isolated oceanic islands is difficult and collecting cryptobenthic sponges without destroying the surrounding reef is nearly impossible.

One tool that has proven efficient in the last decade for collecting cryptobenthic fauna is the autonomous reef monitoring structures (ARMS: Zimmerman and Martin 2004; Brainard et al. 2009; Knowlton et al. 2010). The sessile community of invertebrates that settle on ARMS after 2–3 yrs is usually scraped, homogenized and metabarcoded to examine the entire homogenized sessile community via universal primers that amplify a short fragment of the mitochondrial (mtDNA) cytochrome c oxidase subunit I (COI) gene (Geller et al. 2013; Leray et al. 2013). Although these primers amplify sponges, the ability to identify those sponges to lower taxonomic levels is severely limited by both: (1) the availability of a well curated local sponge database with individual barcodes associated with voucher specimens and (2) the notoriously poor performance of COI barcodes for identifying sponge species (Vargas et al. 2012). Despite successful deployment and recovery of these structures, funding for taxonomic analysis of the settling fauna is limited to a handful of regional surveys (Leray & Knowlton 2015; Al-Rshaidat et al. 2016; Ransome et al. 2017; Carvalho et al. 2019). Even among these regional surveys, little systematic attention has been given to the phylum Porifera. Sponges have been overlooked in such studies due in part to the taxonomic challenge of identifying these soft-bodied organisms, and in part to the reliance on metabarcoding techniques that perform poorly for this phylum (Timmers et al. 2020).

As a result of such challenges, sponge diversity, despite representing a substantial proportion of reads in metabarcoding studies to date, is typically included at only the level of phylum (Leray & Knowlton 2015; Stat et al. 2017; Nichols & Marko 2019). But continued poor resolution of such an integral phylum to coral reef communities is detrimental to our understanding of the evolutionary and ecological processes governing biodiversity. Given the critical ecological role that sponges play in reef ecosystems and the increased vulnerability of coral reefs as a result of global human impacts (Carpenter et al. 2008; Jackson 2008; Knowlton & Jackson 2008), a baseline of this functionally important phylum in cryptobenthic communities is crucial.

Given the pressing need to establish a baseline of the overlooked cryptobenthic sponge diversity of tropical Pacific reefs, we set out to monitor sponge species richness over a 2-yr period on ARMS deployed in Kāneʻohe Bay, Oʻahu. The diversity of the cryptobenthic sponge fauna from ARMS units was compared against the diversity of surface-dwelling and cryptobenthic sponges observed from visual surveys within Kāneʻohe Bay. Previous sponge biodiversity surveys in the bay (De Laubenfels 1950; Bergquist 1967; Pons et al. 2017; Vicente et al. 2020) collectively documented sponge richness at 51 species to

date (Table S1). However, these surveys focused on the exposed reef, rather than within the cryptobenthic environments of the reef interior. The reef surface in Kāneʻohe Bay is dominated by corals, which account for 40–60% of benthic cover (Bahr et al. 2015), while sponge cover, with the exception of the invasive *Mycale grandis* (Coles et al. 2007), accounts for <1% (JVs personal observations-Fig. 1a). The opposite is true for the cryptobenthic reef, where sponges are a rich and dominant phylum in Kāneʻohe Bay (Fig. 1b) (Timmers et al. 2020), as typical of oceanic island reefs. Timmers et al. (2020) identified 69 cryptobenthic sponge species which exceed sponge richness previously reported for Kāneʻohe Bay. The remarkable increase in sponge richness attributed to sponges that recruited onto ARMS in Kāneʻohe Bay motivated us to compare ARMS sponge richness to sponge richness found on patch reefs, including cryptobenthic reef spaces. Here we take a multi-locus sequencing approach of properly vouchered sponge specimens collected from both visual surveys and ARMS platforms to examine the extent of cryptobenthic sponge biodiversity within one of the most isolated archipelagos on the planet.

Materials and method

Sponge collection from autonomous reef monitoring structures and field surveys

Autonomous reef monitoring structures (ARMS) are standardized sampling devices that mimic reef interstices, attracting cryptobiota colonization (Brainard et al. 2009; Knowlton et al. 2010). Standard ARMS units are

comprised of an eight-tiered stack of gray Type I PVC plates (22.9×22.9 cm), arranged in four open and four semi-closed layers (Figure S1a; Leray and Knowlton 2015), while modified ARMS units are composed of a two-tiered stack of one open and one semi-closed layer (Figure S1b; Timmers et al. 2020). Sponges were sampled from six standard ARMS deployed along the reef slope of Moku o Loʻe (Coconut Island) and from six modified ARMS (Figure S1c) hovering in the water column attached to a Moku o Loʻe intake pipe, along an adjacent reef slope. An additional 24 modified units were placed within mesocosm tanks on Moku o Loʻe that received unfiltered seawater from the same intake pipe but were exposed to future climate conditions as described in Bahr et al. (2020) (Figure S1a). The ARMS in mesocosm tanks and those at the intake pipe were retrieved for sponge subsampling every two months for two yrs and sponges from the full ARMS on the reef slope were collected once upon recovery, in July 2018. The ARMS units in ensemble provided a total combined sampling surface area of 15 m^2 at each period of collection. At each collection period, ARMS units were disassembled for high-resolution plate imagery and carefully examined for newly settled sponge recruits. Sponges showing unique morphological features on each plate were individually photographed, carefully subsampled, and fixed in 95% ethanol for DNA extraction. If enough tissue was available, sponges were additionally fixed in two solutions, one containing 4% paraformaldehyde in seawater, and the other containing 4% glutaraldehyde in 0.1 M sodium cacodylate with 0.35 M sucrose for future histological evaluation. A total of 439 sponge samples were collected from the ARMS units.

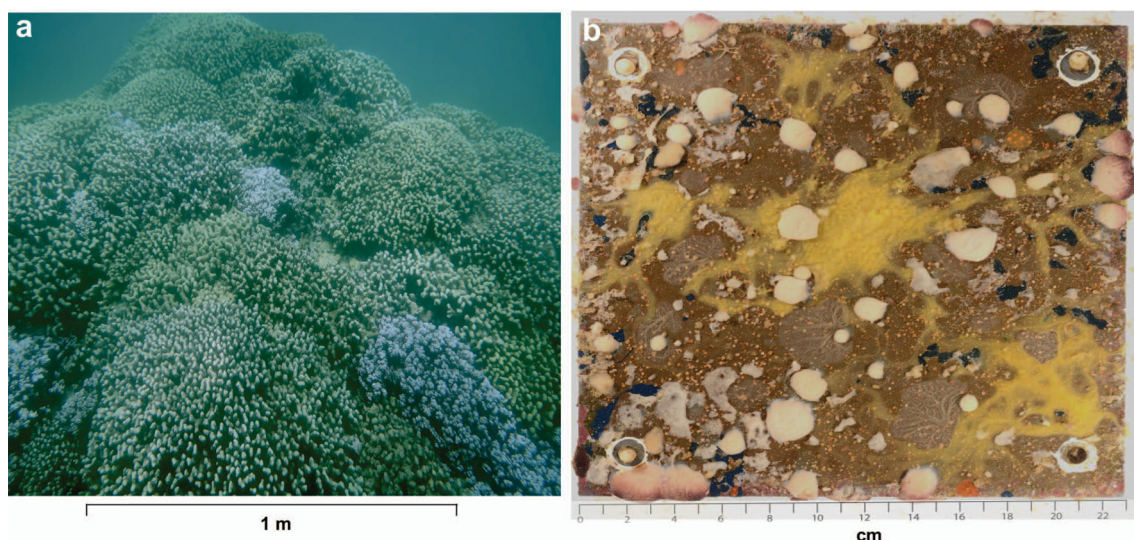


Fig. 1 Coral-dominated reef of Kāneʻohe Bay (a) and sponge dominated cryptobenthic community by 12 sponge species from an ARMS plate (b)

Sponges were also collected on reef substrates along a 50-m transect line from 34 sites in Kāneʻohe Bay and one site on the Makai Pier in Waimanalo (see Table S2 for GPS coordinates and collection depth) throughout the 2-yr mesocosm experimental period. Collection on Kāneʻohe Bay reef sites included samples taken by global taxonomic experts during the Smithsonian-led MarineGEO biodiversity surveys in 2017. A total of 163 marine sponges showing unique morphologies were haphazardly collected in the photic zone of the reef at a depth range of 1–16 m from within crevices, beneath coral rubble, fouling upon structures and under overhangs. Presence/absence of sponge OTUs was recorded at each surveyed site (Table S3), was photographed, and fixed in 95% ethanol for DNA extraction. Additional sponge metadata pertaining to specimen morphology, such as color, consistency, surface, oscules, exudates, and odors, was also recorded.

All samples were vouchered with the Florida Museum of Natural History at the University of Florida (UF Porifera) and the Hawaiʻi Institute of Marine Biology (“KB” or “KBOA”) (Table S4). Images and associated metadata of each sponge sample are publicly available at <https://www.invertebase.org/portal/> and <http://specifyportal.flmnh.ufl.edu/iz/>. All samples were collected under special activities collection permit (SAP) nos. 2018–03 and 2019–16 (covering the period of January 13, 2017, through April 10, 2019) issued by the State of Hawaiʻi Division of Aquatic Resources.

Sponge DNA extraction and sequencing

Vouchered specimens were subsampled for DNA extraction using the E-Z 96 Tissue DNA Kit (Promega Bio-Tek, Norcross, GA, USA) following the manufacturer protocols. Care was taken to subsample only sponge material free of other organisms which would contaminate the sponge DNA extract. Multiple primers were used in a stepwise fashion to successfully amplify partial fragments of both 28S rRNA and COI genes using polymerase chain reaction (PCR) (Table S1 in Timmers et al. 2020). Fragments of the COI were initially attempted with primer pairs LCO1490/COXR1 (1400 bp fragment) (Folmer et al. 1994), followed by primers jgLCO1490/jgHCO2198 (Geller et al. 2013) (648 bp fragment) within the previous PCR fragment region and a final attempt with subsequent internal primers mlCOIint/jgHCO2198 (313 bp) (Leray et al. 2013). Similar to the approach used for the COI, amplification of 28S rRNA fragments was first attempted with primers F63mod/1072RV (1050 bp) (Medina et al. 2001), followed by internal primers (28S-C2-fwd/28S-D2-rev) (450 bp) (Chombard et al. 1998) within the previous fragment, and a final attempt with 28SMycF/1072RV (1000 bp). PCRs were carried out in a total volume of 40 µL including the

following: 14.4 µL of H₂O, 20 µL of BioMix Red (Bioline, Taunton, MA, USA) 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). PCR products were examined on a 1% agarose gel stained with GelRed and purified using ExoFAP (Exonuclease I and FastAP–Life Technologies, Carlsbad, CA) prior to sequencing. When products showed multiple bands above the 100-bp ladder mark, products were purified using gel excision by loading 40 µL of the PCR product onto a 2% agarose gel made with 1× modified (no EDTA) TAE running them at 50 mV. After 90-min bands were excised with a sterile scalpel, loaded onto a column filter fitted inside a 1.5-ml centrifuge tube, and centrifuged for 10 min at 5000 rpm. 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 Hawaiʻi Advanced Studies of Genomics, Proteomics and Bioinformatics sequencing facility.

Forward and reverse reads were trimmed and edited by eye using Geneious 10 (Kearse et al. 2012). Assembled and edited sequences were exported as *fasta* files and checked for contamination by using the BLAST (Altschul et al. 1990) function in GenBank. Sequences showing >85% sequence identity to those belonging to Porifera were kept and used for further analysis. 28S rRNA sequences for 592 samples were produced, but only 340 sequences were deposited in GenBank as many were repetitive sequences with 100% identity. When available, up to three replicate sequences per OTU were deposited and assigned accession numbers MW016037–MW016376. A total of 98 COI sequences were deposited in GenBank and assigned accession numbers MW059039–MW059109; MW144969–MW144988; MW143251–MW143256; MW349624 (Table S4).

Phylogenetic analysis and taxonomic assignments

Sequences were aligned with the closest sequence relatives in the GenBank database using the ClustalW algorithm with default parameters in Geneious. Sequence KJ483037.1 *Parazoanthus puertoricense* was used as an outgroup for all phylogenetic topologies of partial 28S rRNA sequences and AB247348.1 *Epizoanthus arenaceus* was used as an outgroup for the phylogenetic topology of partial COI sequences. Bayesian inference (BI) using MrBayes version 3.2.1 (Huelsenbeck & Ronquist 2001) and a maximum likelihood (ML) framework using RaxML (Stamatakis 2006) were added to the phylogenetic analysis. The GTR substitution model and GTRGAMMA nucleotide model with 1000 bootstrap replicates were implemented in the BI and ML analyses respectively. The BI was run using

5 million generations sampled every 200 generations. The analysis was stopped when the standard deviation (SD) of split frequencies fell below 0.01.

Most sponge OTUs were delineated using a combination of $\geq 1\%$ COI and 28S rRNA sequence divergence combined with unique morphological features and classified as distinct operational taxonomic units (OTUs). A handful of OTUs that were morphologically clearly differentiable, but had $\leq 1\%$ sequence divergence were also recognized as distinct OTUs (Table S6). This conserved threshold was chosen based on the different rates of evolution that can exist within poriferan families and even genera which make the selection of an accurate threshold for delineating sponge OTUs arbitrary (Erpenbeck et al. 2007; Wang & Lavrov 2008; Redmond et al. 2011; Voigt & Wörheide 2016; Yang et al. 2017).

Preliminary assessments of morphological characters (i. e., color, consistency, surface, oscules, and skeleton composition) were made mostly from OTUs that matched previous vouchered sponge collections in Kāneʻohe Bay (De Laubenfels 1950; Bergquist 1967, 1977; Pons et al. 2017) (Table S5). We assigned OTUs to taxonomic levels based on the placement of each barcode into the lowest clade (Bayesian posterior probability of ≥ 50) in the COI and 28S rRNA tree topologies. On average, taxonomic identities followed these barcode sequence identity percentages: Order ($>90\%$), Family ($>95\%$), Genus ($>98\%$) and for the species above (100%). Phylogenetic topologies were first generated with only full-length amplicons for COI and 28S rRNA and then repeated with shorter sequences to maximize the inclusion of reference sequences from GenBank. Matches and identification at the species level (17 OTUs) were based on sequences and a preliminary analysis of skeleton and spicule composition which matched sequences from vouchers in GenBank linked to a publication with a rigorous morphological assessment and description of the voucher. The remaining OTUs (including GenBank accession matches without taxonomic support) were identified as “sp.” since further morphological analysis is needed for accurate classification. In addition, species identification is impossible using molecular methods for polyphyletic groups (such as sub-orders, families and genera within Haplosclerida) without a complete morphological assessment of OTUs. However, the objective here is to determine species richness mostly based on molecular OTUs rather than a full species description of OTUs.

Diversity assessment

R v.3.6.3 (R Core Team 2020) was used to visualize and analyze the molecular diversity assessments of sponges recruited on ARMS and reef substrates. Phylogenetic

analyses of COI and 28S rRNA sequence data were used to prepare a taxonomy table (Table S5) for OTU classification (OTU) to the lowest level possible. An OTU distribution table (Table S2) specifying OTU presence/absence on either ARMS or reef substrate at each of the 35 sites was used to map sponge OTU richness using the ggmap v.3.0.0.901 package (Kahle & Wickham 2013). We used the *specaccum* function from the vegan v.2.5–6 (Oksanen et al. 2013) package to generate OTU richness rarefaction curves for comparison between the two substrates across the most speciose sponge groups according to sponge class and order. Number of OTUs as a function of sites was used to generate rarefaction curves for reef substrate sponges, and number of OTUs as a function of time points was used for ARMS as these were only present at one site. Venn diagrams were generated using the limma v.3.42.2 (Ritchie et al. 2015) package to determine the number of shared OTUs between the survey method types. Calculation of new OTU records was based on species comparisons to previous studies focused on Kāneʻohe Bay sponge collections.

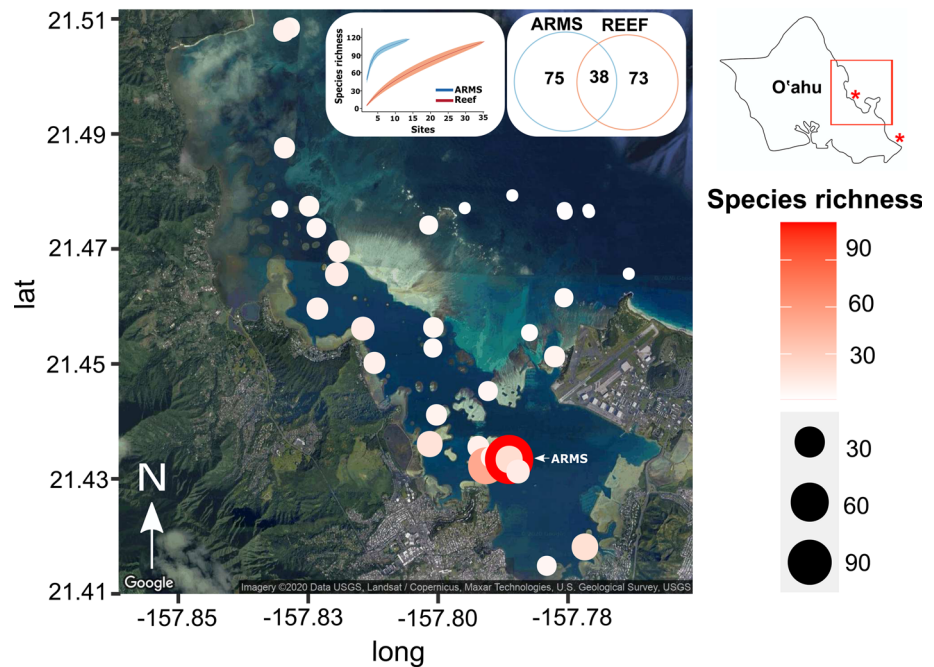
Results

Sponge community richness between reef sites and ARMS

186 sponge OTUs were delineated from 616 voucher specimens (Fig. 2, Table S2). A total of 183 OTUs were provided with sequences from either COI, 28S rRNA, or both loci as follows: 28S rRNA sequences were provided for 178 OTUs (97% of OTUs), COI sequences were provided for 88 OTUs (48%), and 28S rRNA+COI sequences were provided for 81 OTUs (44%) (Table S4). No successful sequences were obtained from three species (*Calcarea* sp. 2 UF 3782, *Poecilosclerida* sp. 15 UF 3721, *Demospongiae* sp. 42 KBOA061118432), but we included these OTUs because they were clearly distinguishable morphologically from other species in our collection (see images <https://www.invertebase.org/portal/>). Eight other species that were clearly morphologically distinct but had $>99\%$ sequence similarity to other OTUs were also recognized (Table S6).

Sponge OTUs from reef environments (111 OTUs from 1750 m² surveyed area) and ARMS units (113 OTUs from 15.0 m² ARMS surfaces) were numerically similar, but only 38 OTUs were shared between collection methods (Fig. 2-inset Venn diagram). Sponge diversity varied between 1 and 51 OTUs across the 35 surveyed reef sites. A total of 142 OTUs were confined to cryptobenthic reef spaces provided by ARMS (98 OTUs) and reef environments (67 OTUs) (Table S5), and 23 OTUs were shared

Fig. 2 Distribution of 186 sponge OTUs collected from ARMS monitored throughout a 2-yr period (pointed by arrow), 34 sites in Kāneʻohe Bay and the Makai pier site (indicated by red asterisk outside of red box). Rarefaction curve of species richness is compared between 12 sampling periods on ARMS (treated as sites) vs. the 35 reef sites. Inset Venn diagram shows 38 OTUs were shared between all reef sites and ARMS. GPS coordinates and OTU distribution table are found in Table S2 and Table S3 respectively



between habitats. From the 38 shared OTUs, 15 were found on both cryptobenthic and open reef habitats.

The class Demospongiae dominated the fauna with 142 OTUs, followed by Calcarea (31 OTUs) and Homoscleromorpha (12 OTUs). 28S rRNA was successfully amplified across all 18 sampled orders of sponges, but COI primers in all combinations failed for all Calcarea (2 orders), and for three orders of demosponges (Bubarida, Verongiida and Chondrosiida) (Fig. 3; Table S8). Failure of COI amplification was expected for Calcarea as mtDNA within this class evolves rapidly with high substitution rates (Voigt et al. 2012; Lavrov et al. 2013). However, amplification within Bubarida, Verongiida, and Chondrosiida is likely incidental. High success rate and 28S rRNA sequence length of all sponge classes allowed for a more detailed phylogenetic analysis within the different sponge classes and orders. Phylogenetic assignment of 28S rRNA sequences to the order level had a Bayesian posterior probability of >0.84 and RaXML bootstrap >50 for all orders except the Scopalinida which had short reference sequences that could not be included in the alignments (Table S7).

Richness of demospongiae

The 142 demosponge OTUs were dominated by the orders Poecilosclerida (31 OTUs), Haplosclerida (30 OTUs), Suberitida (23 OTUs), Dictyoceratida (14 OTUs), Tethyida (8 OTUs), Tetractinellida (8 OTUs), and Dendroceratida (8 OTUs). The orders Clionaida (4 OTUs), Axinellida (3 OTUs), Bubarida (2 OTUs), Biemnida (1 OTU),

Scopalinida (1 OTU), Chondrillida (2 OTUs), Chondrosiida (1 OTUs), and Verongiida (1 OTU) were less diverse. OTUs that were less abundant and were not assigned to specific orders within demosponges included Heteroscleromorpha (2 OTUs), Keratosa (2 OTUs), and Verongimorpha (1 OTU). Within Poecilosclerida, 15 OTUs had COI or 28S rRNA sequences that matched GenBank reference sequences at $>95\%$. Phylogenetic analysis and comparison of external morphological characters allowed further classification of these poecilosclerids into seven *Mycale* spp. including *M. parishii*, two *Tedania* spp. including *T. cf. klausi*, three *Lissodendoryx* spp. including *L. hawaiiiana*, two *Iotrochota* spp. including *I. protea*, and *Monanchora clathrata* (Fig. 4). Eight poecilosclerid OTUs were documented in this survey from among the 11 previously reported in Kāneʻohe Bay (Table S1), and 22 are new records (Fig. 4). More species of poecilosclerids were found on reef sites (21 OTUs) than ARMS (18 OTUs), and 8 OTUs were shared between these habitats (Fig. 4).

Haplosclerida diversity was greater on ARMS (21 OTUs) than on reef sites (14 OTUs), with six OTUs shared (Fig. 4). Morphological characters and $>95\%$ sequence matches with GenBank reference sequences allowed 16 OTUs to be further classified into two Chalinidae spp., two *Callyspongia* spp., nine *Haliclona* spp., *Cladocroce* sp. 1, *Haliclona caerulea*, and *Gelliodes wilsoni*. These collections added an additional 25 haplosclerid OTUs to the previous 8 recorded in Kāneʻohe Bay (Table S1 Fig. 4).

Similar to haplosclerids, Suberitida richness was greater in ARMS (16 OTUs) than reef sites (13 OTUs), with six OTUs shared (Fig. 5). Sequences with $>95\%$ identity to

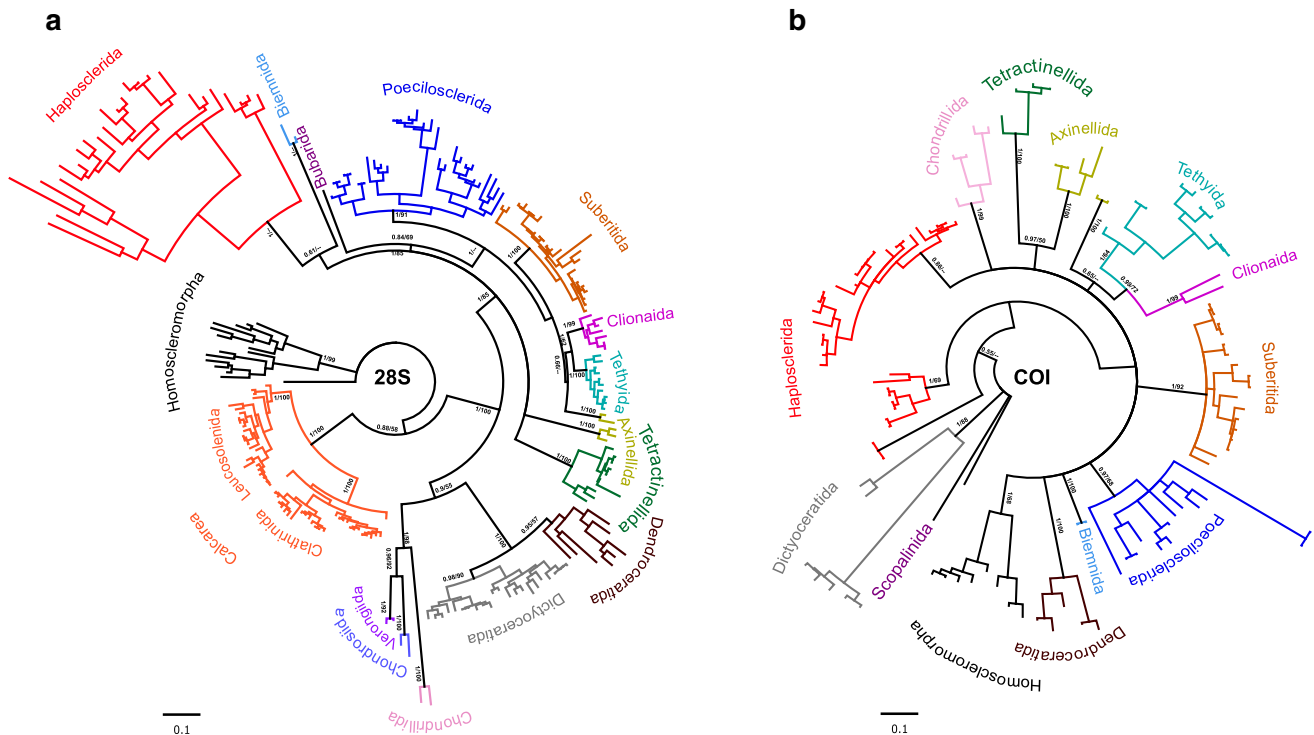


Fig. 3 Bayesian and maximum likelihood topology from partial 28S rRNA sequences (a) and Folmer (5') region of the cox1 gene (b). A total of 1484 positions were used to generate the 28S rRNA tree and 577 positions were used to generate the COI tree. Numerical values at

nodes show Bayesian posterior probabilities followed by RAxML bootstrap values (also available in Table S6). Nodes with “—” refer to bootstrap values of <50 generated by RAxML. Colors denote different sponge groups either by order or class

GenBank reference sequences and morphological analysis further classified 19 OTUs into six Suberitidae spp., four *Hymeniacidon* spp., five *Halichondria* spp. including *Halichondria* cf. *coerulea*, two *Suberites* spp. including *Suberites* cf. *aurantiacus*, and one species each of *Terpios*, and *Amorphinopsis*. A total of 18 OTUs are new records, while six were shared with seven previously recorded Suberitida from Kāneʻohe Bay (Table S1).

Tethyida and Tetractinellida also contributed to the greater richness of ARMS than reef sponges (Figs. 4 and 5). Among eight OTUs classified as Tethyida, seven were >95% identical in COI or 28S rRNA sequences to *Tethya* spp.. Four *Tethya* spp. were closely related phylogenetically (>98% sequence identity) and were shared between ARMS and reef habitats. Unlike Tethyida, Tetractinellida species were largely confined to ARMS (6 OTUs) with only two OTUs found on reef sites (Fig. 5). Of the 16 Tetractinellida and Tethyida species encountered, *Tethya* sp. 1 was recently reported by Vicente et al. (2020) and *Tethya* sp. 3 matched the morphology of *Tethya* cf. *diplo-derma* (a Caribbean species) reported by De Laubenfels (1950), while 14 are new species records for Kāneʻohe Bay (Table S1).

Dictyoceratida and Dendroceratida (=Keratosa) were more diverse on reef sites (18 OTUs) than ARMS (11

OTUs), with five OTUs shared (Fig. 6). Sequences matching COI and 28S rRNA reference sequences at >95% identity allowed the further classification of Dendroceratida OTUs into four *Chelonaplysilla* spp. including *Chelonaplysilla erecta*, and to *Aplysilla rosea*. Similarly, dictyoceratid OTUs could be further classified into six *Dysidea* spp., including *D.* cf. *arenaria*, *D.* cf. *pallidus*, and to *Lamellodysidea* cf. *chlorea* and an *Ircinia* sp.. Dendroceratida have not been previously reported from Kāneʻohe Bay; all 10 species records are new. Three dictyoceratid OTUs matched previous records, while nine are new. New species records were also attributed to orders Clionaida (2), Axinellida (2), Bubarida (2), Scopalinida (1), Chondrillida (1), Chondrosiida (1), and subclasses Verongimorpha (1) and Heteroscleromorpha (2).

Richness of calcarea and homoscleromorpha

The 31 OTUs of Calcarea encountered include 13 *Calcinea* and 17 *Calcaronea* (Fig. 7). Low Bayesian posterior probabilities (<0.5) and RAxML bootstrap values (<50) allowed only limited assignment of some OTUs to the orders Leucosolenida within *Calcaronea* and *Clathrinida* within *Calcinea*. Calcarea richness in ARMS (20 OTUs) exceeded that of the reef (14 OTUs), and only 3 OTUs

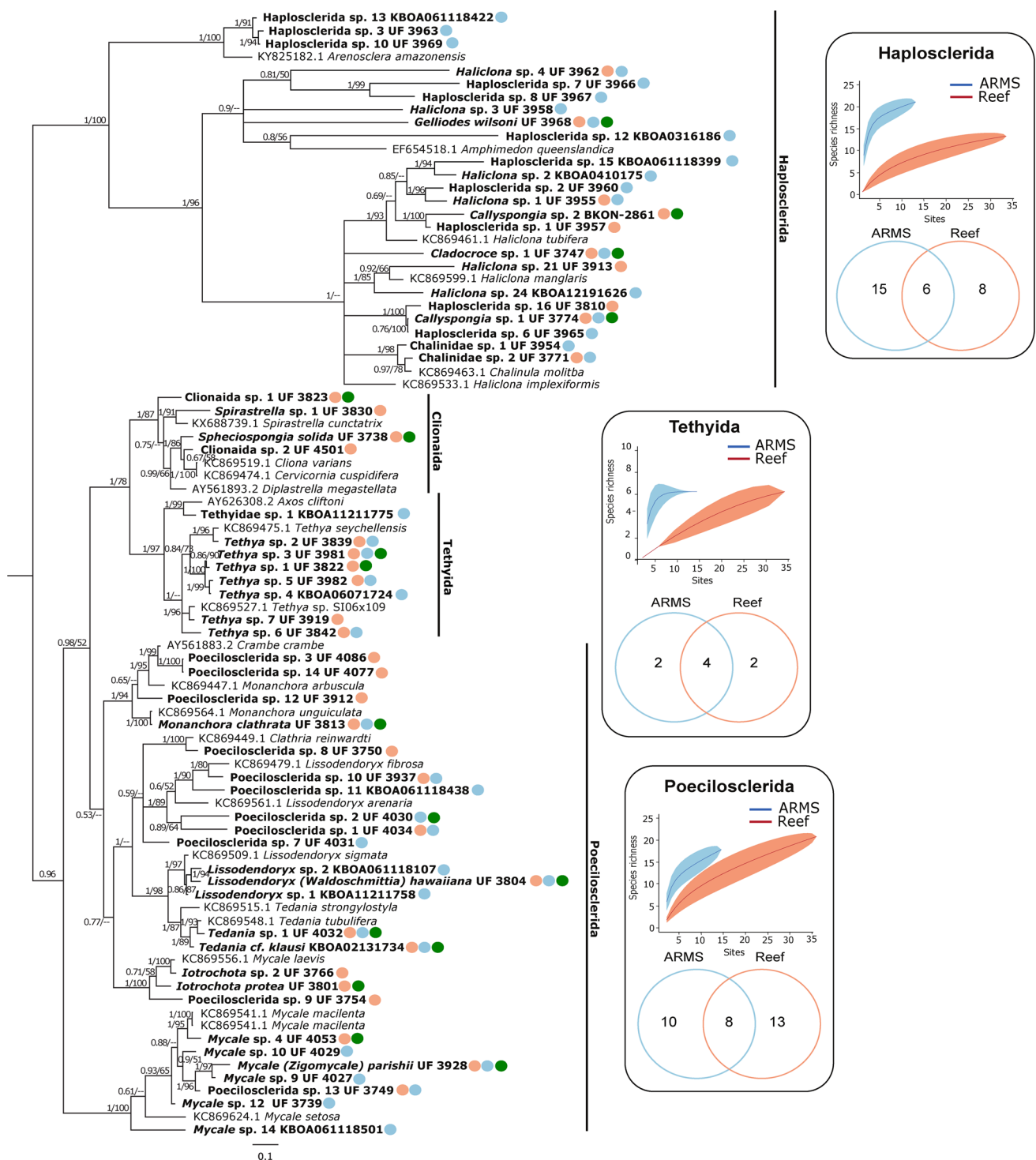


Fig. 4 Bayesian and maximum likelihood topology from 702 bp positions of partial 28S rRNA sequences for OTUs belonging to orders Haplosclerida, Clionaida, Tethyida, and Poecilosclerida. Sequences in bold were generated from taxa in this study. Color of circles adjacent to sequence names indicate whether sponges were collected from ARMS (blue), reef (pink), and whether they matched previous species collected from Kāne'ohe Bay (green), listed in

Table S1. Numerical values at nodes show Bayesian posterior probabilities followed by RAXML bootstrap values. Nodes with “–” refer to bootstarp values <50 generated by RAXML. Short sequences from eight haplosclerid, and six poecilosclerid OTUs were omitted from the alignment. Subplots (right) show species richness rarefaction curves and Venn diagrams indicating distribution of OTUs of each sponge class between habitat types

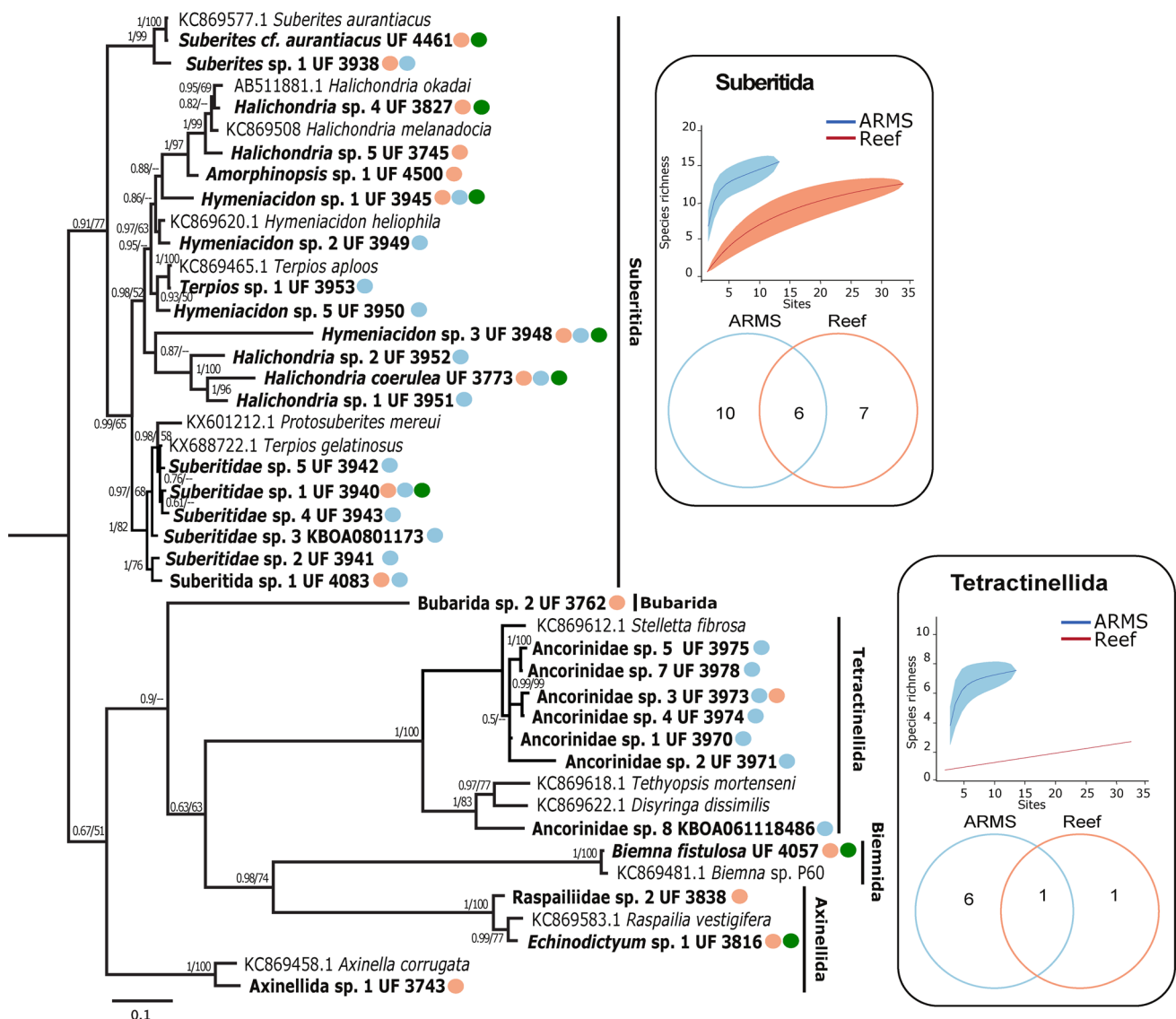


Fig. 5 Bayesian and maximum likelihood topology from 847 bp positions of partial 28S rRNA sequences for OTUs belonging to orders Suberitida, Tetractinellida, Biemnida, and Axinellida. Sequences in bold were generated from taxa in this study. Color of circles adjacent to sequence names indicate whether sponges were collected from ARMS (blue), reef (pink), and whether they matched previous species collected from Kāneʻohe Bay (green), listed in Table S1. Clades highlighted in blue denote cryptic speciation of

OTUs. Numerical values at nodes show Bayesian posterior probabilities followed by RAxML bootstrap values. Nodes with “—” refer to bootstrap values of <50 generated by RAxML. Short sequences from four Suberitida, one Tetractinellida, and two Axinellida OTUs were omitted from the alignment. Subplots (right) show species richness rarefaction curves and Venn diagrams indicating distribution of OTUs of each sponge class between habitat types

were shared between habitats. Survey of the calcareous sponges added 29 new sponge species records for Kāneʻohe Bay with only two species (*Leucettidae* sp. 3 UF 4024 and *Leucosolenida* sp. 14) matching previously reported species as *Leucetta solida* (a Mediterranean species) (de Laubenfels 1950), and *Leucosolenida* sp. (Pons et al. 2017) (Table S1). The phylogenetic analysis showed that seven species sampled were >99% identical in 28S sequences to *Calcarea* species reported from other areas of the Indo-west Pacific: *Clathrina luteoculcitella* (Van Soest

& De Voogd 2015) from Indonesia, *Ernstia variabilis* from French Polynesia (Klautau et al. 2020), *Leucetta primigenia* from the Great Barrier Reef (Thacker et al. 2013), *Anamixilla torressi* and *A. singaporensis* from Indonesia (Van Soest & De Voogd 2015, 2018), *Leucandra nicolae* from the Coral Sea, and *Sycettusa hastifera* specimens from the Red Sea (Voigt et al. 2012). The remaining 23 OTUs showed <99% sequence identity to other available *Calcarea* sequences.

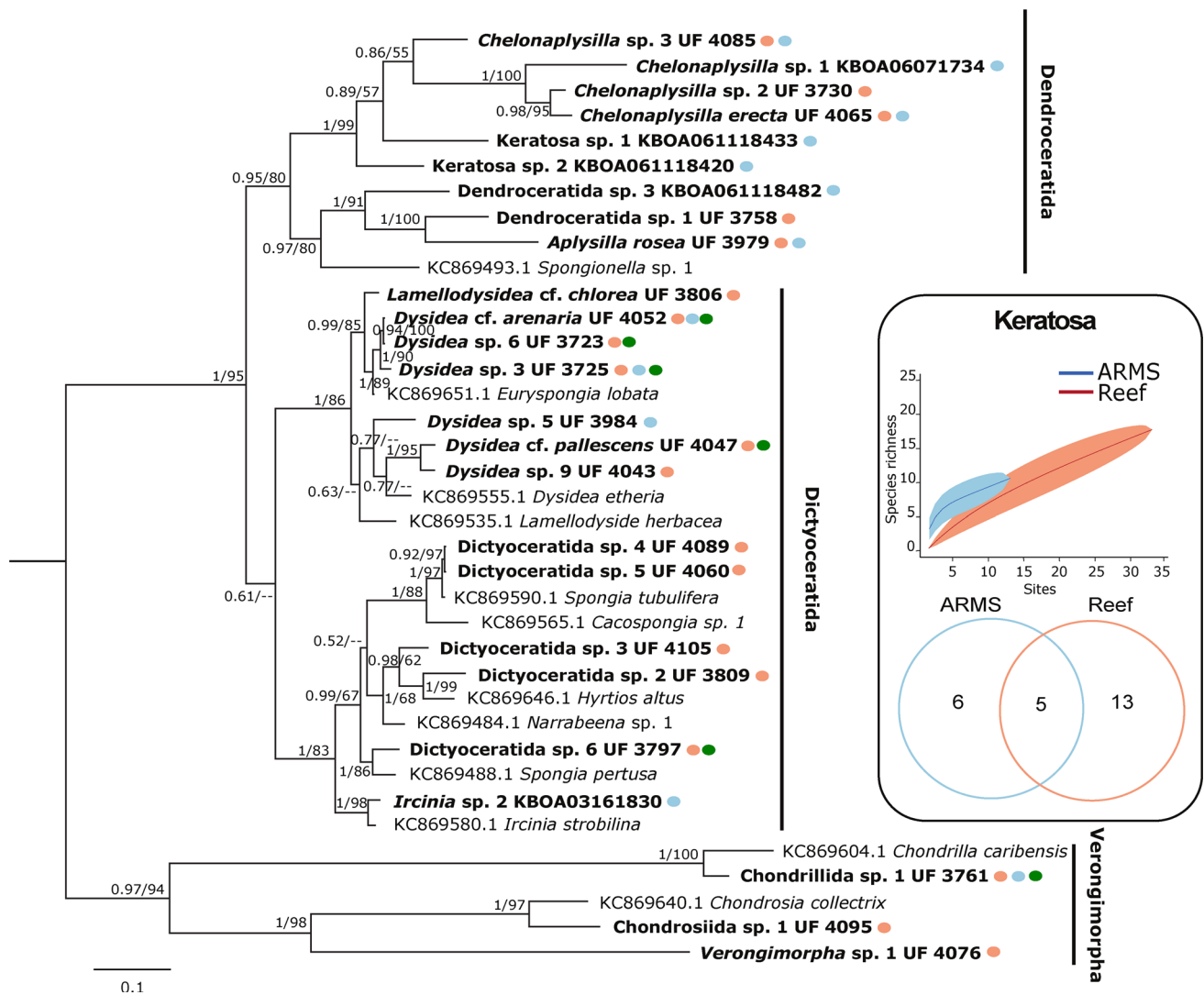


Fig. 6 Bayesian and maximum likelihood topology from 709 bp positions of partial 28S rRNA sequences for OTUs belonging to subclass Keratosa (Dendroceratida and Dictyoceratida), Chondrillida, Chondrosiida and Verongimorpha. Sequences in bold were generated from taxa in this study. Color of circles adjacent to sequence names indicate whether sponges were collected from ARMS (blue), reef (pink), and whether they matched previous species collected from Kāneʻohe Bay (green), listed in Table S1. Numerical values at nodes

show Bayesian posterior probabilities followed by RAxML bootstrap values. Nodes with “–” refer to bootstrap values of <50 generated by RAxML. Short sequences from Dictyoceratida sp. 2, Dysideidae sp. 1, Chondrillida sp. 3 and *Pseudoceratina* sp. 1 were omitted from the alignment. Subplots (right) show species richness rarefaction curves and Venn diagrams indicating distribution of OTUs of each sponge class between habitat types

Of 12 Homoscleromorpha OTUs, eight were collected from ARMS, seven from reef sites, and three were shared between habitats. *Oscarella* spp. were the most speciose, with seven OTUs matching COI sequences at 95–96% of *O. microlobata* and *O. lobularis* from the Mediterranean (Gazave et al. 2010) (Table S4). *Oscarella* spp. were found almost exclusively in ARMS. Other species collected matched 28S and COI sequences at >95% belonging to *Plakina* (2), *Plakinastrella* (1), *Plakortis* (1), and *Corticium* (1). All homoscleromorphs are new species records for Kāneʻohe Bay except for *Plakortis* sp. 1 UF 3472 which matches the description of *Plakortis simplex* by De

Laubenfels (1950). Nevertheless, pairwise comparisons of COI and 28S rRNA sequences between *P. simplex* and *Plakortis* sp. 1 were 94% and 87% identical respectively, indicating that De Laubenfels’ assignment of Kāneʻohe Bay samples to that species, described from the Mediterranean, was in error.

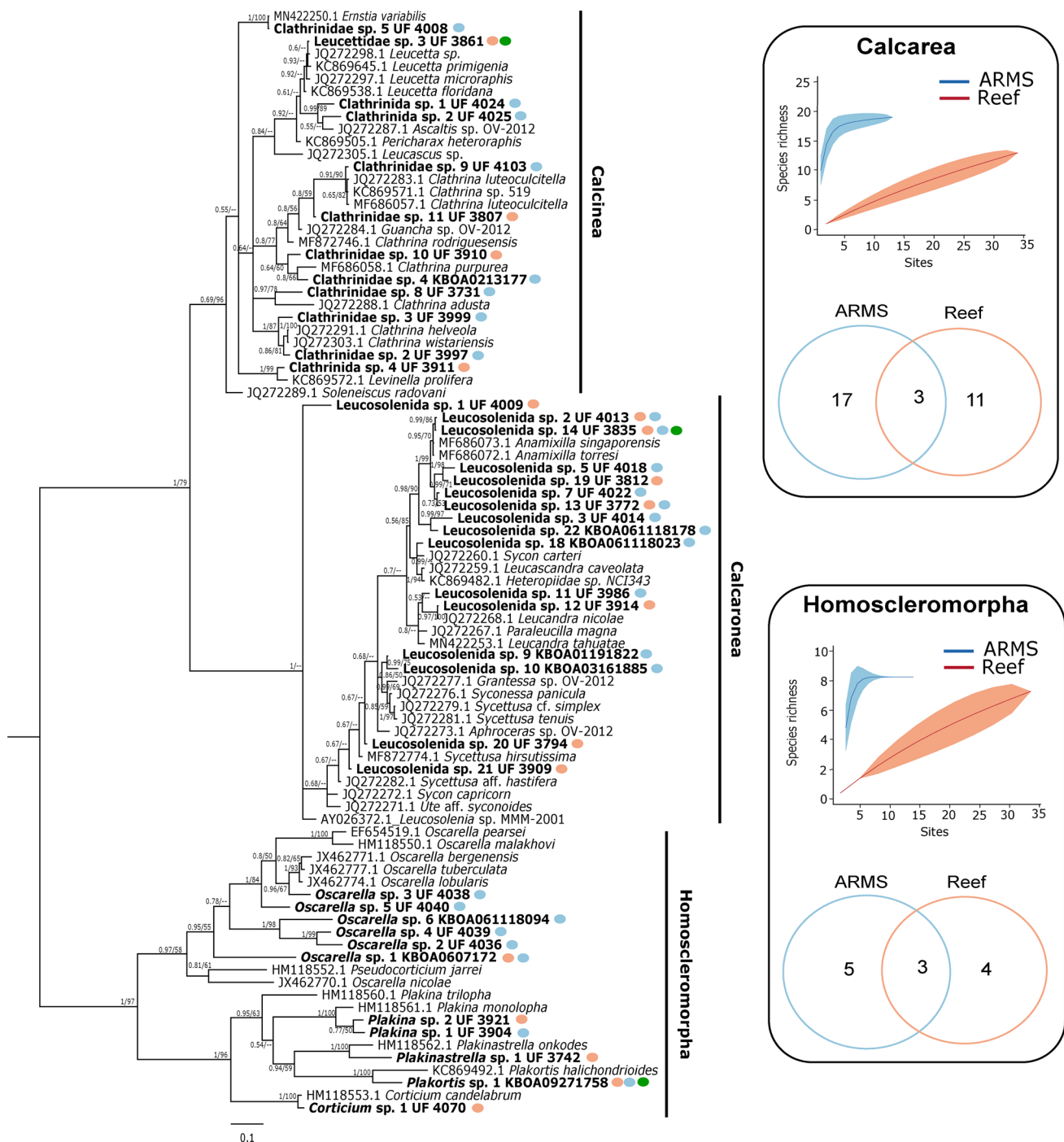


Fig. 7 Bayesian and maximum likelihood topology from 466 bp positions of partial 28S rRNA sequences for OTUs belonging to classes Calcarea and Homoscleromorpha. Sequences in bold were generated from taxa in this study. Color of circles adjacent to sequence names indicate whether sponges were collected from ARMS (blue), reef (pink), and whether they matched previous species collected from Kāneʻohe Bay (green), listed in Table S1. Numerical

values at nodes show Bayesian posterior probabilities followed by RAxML bootstrap values. Nodes with “—” refer to the bootstrap values of <50 generated by RAxML. Short sequences from three calcareous OTUs and *Oscarella* sp. 7 were omitted from the alignment. Subplots (right) show species richness rarefaction curves and Venn diagrams indicating distribution of OTUs of each sponge class between habitat types

Discussion

We documented 186 species of sponges from Kāneʻohe Bay, thus increasing the known fauna of this major reef system more than 3.5-fold. For comparison, 51 species have been previously recorded in the bay (Table S1), while the last review of the entire Hawaiian fauna lists 67 species (Bergquist 1977), albeit other species have been reported since (e.g., Van Soest et al. 2012; Calcinaï et al. 2013). Most of the new species records for Kāneʻohe Bay are likely also new records for the Hawaiian Islands, greatly increasing the known diversity of the archipelago. These results underscore how poorly known tropical sponge faunas are in even places subjected to substantial past studies (De Laubenfels 1951, 1954, 1957; Bergquist 1977; Pons et al. 2017; Vicente et al. 2020).

Sponges are generally not considered to be as diverse or abundant in the oligotrophic waters of the insular central Pacific (Kelly-Borges & Valentine 1995) compared to other ecoregions of the tropical Pacific such as the coral triangle (Van Soest et al. 2012). Within Oceania, macro-sponges on the reef surface are largely confined to more productive lagoonal waters and rare on the outer reef. For example, previous sponge surveys in Moʻorea, French Polynesia, focused mostly on macrosponges, report eight species with <1% cover (Freeman & Easson 2016). In Palmyra Atoll, 24 species of macro sponges, with 27% cover, are only confined to the lagoon habitat of the atoll (Knapp et al. 2013). Similar patterns are observed in the Mariana Islands, where many of the 124 species documented are known only from the deep lagoon of Apra Harbor on Guam (Paulay et al. 1997; Kelly et al. 2003). While low abundance and diversity may be true for surface-dwelling sponges, the great diversity of sponges documented here are within the confines of cryptobenthic spaces, demonstrating the absolute dominance of cryptobenthic sponges in Hawaiʻi. This suggests that the cryptobenthic community in the insular central Pacific is far more diverse than previously realized.

The utilization of ARMS units greatly enhanced our collection and identification of cryptobenthic sponges, exceeding the diversity of sponges from the reef itself (113 ARMS species vs 111 reef species) despite sampling two orders of magnitude less area (Fig. 2). These results confirm previous observations from other comparative diversity surveys between reef and cryptobenthic fauna from ARMS in the Red Sea, where sponges from ARMS surveys were among the top three phyla to increase in richness and abundance when compared to the surrounding reef surface (Pearman et al. 2016). Nonetheless, in this study we also compared the sponge diversity of ARMS to sponge diversity confined to the cryptobenthic community

which was responsible for 76% (142 OTUs) of all species. Sampling from the cryptobenthic reef provided exclusive access to 31% (44 OTUs) of species, while sampling from ARMS provided exclusive access to 52% (75 OTUs) of the cryptobenthic specific diversity. These results suggest that the interstices of ARMS units provide suitable habitat for settlement of cryptobenthic sponges that would be impossible to access through traditional field surveys.

Sampling of ARMS and reef sponges revealed 142 new species records for Kāneʻohe Bay, including 108 demosponges, 24 Calcarea and 10 Homoscleromorpha. Diversity estimates for sponges belonging to orders Clathrinida, Leucosolenida, Homosclerophorida, Tetractinellida, Suberitida, Tethyida, and Haplosclerida from ARMS all exceeded the number of species from these groups found on the reef (see rarefaction curves in Figs. 4, 5 and 7). Keratose sponges, however, were more diverse on the open reef than cryptobenthic spaces, perhaps revealing a sensitivity of keratose diversity to low light environments. For the purpose of this study, we compared molecular OTU diversity with species previously reported for Kāneʻohe Bay including those previously provided with sequence data (Pons et al. 2017). However, sponge diversity from just the ARMS in our study almost doubles the diversity previously reported for the entire Hawaiian archipelago (De Laubenfels 1951, 1954, 1957; Bergquist 1977; Van Soest et al. 2012; Calcinaï et al. 2013), suggesting that sampling of surface-dwelling sponges through traditional survey techniques severely underestimate richness of this phylum in coral reef ecosystems.

Regionally, our cryptobenthic diversity surveys in Kāneʻohe Bay also shed light on the magnitude of Hawaiian cryptobenthic diversity of Calcareous sponges when compared to other biodiversity rich ecoregions of the world. For example, sampling efforts from 15 ecoregions of the Western Indian Ocean identified 45 calcareous species from 140 specimens (Van Soest & De Voogd 2018); sampling in eight ecoregions of the coral triangle identified 37 species from 155 samples collected (Van Soest & De Voogd 2015); and nine species identified from over 100 samples spanning the entire French Polynesian archipelago (Klautau et al. 2020). In our study, 20 calcareous species were recovered from ARMS and an additional 11 species from reef habitats from just a single island location of the Hawaiian archipelago. These results illustrate the usefulness of ARMS as a tool to recover diversity of cryptobenthic sponges such as the Calcarea from throughout this understudied region.

Our results demonstrate that cryptobenthic sponge biodiversity in remote Pacific island archipelagos is largely overlooked and that integrative taxonomy combining morphological assessments with multi-loci 28S rRNA and COI barcoding greatly improves detection of cryptic

species and richness estimates for sponges (Erpenbeck et al. 2016b, 2020). Integrating molecular and morphological methods is important because classical taxonomic approaches based on conserved morphological characters can sometimes lead to incorrect classification of species and underestimate actual species richness, particularly among challenging groups such as sponges (Muricy et al. 1996; Xavier et al. 2010; Uriz & Turon 2012; Vicente et al. 2019). Likewise, classification solely based on molecular techniques can also limit taxonomic accuracy (Rubinoff & Holland 2005; Neigel et al. 2007). Continuous application of an integrative, detailed morphological assessment to our collection will likely increase diversity estimate as we discover more sponge species with slowly evolving COI and 28S rRNA sequences.

Moreover, integrating museum vouchers and species descriptions of sponges with COI and 28S rRNA barcoding will greatly improve taxonomic accuracy of classification in future metabarcoding studies using next generation sequencing (NGS) technology. Biodiversity surveys using NGS are advancing at a faster pace than taxonomists can complete accurate taxonomic assessments of species discovered from cryptobenthic communities such as ARMS. As a result, GenBank databases provide only limited identification for metabarcoding studies. For example, in a recent metabarcoding dataset, up to 95% of metazoan OTUs encountered were not identifiable to species (Nguyen et al. 2020). The ability to assign a species name to a sequence is particularly problematic among sponges for which molecular databases are exceedingly sparse and less than 1% of holotype specimens in museum collections have been barcoded to date (Erpenbeck et al. 2016a). Furthermore, in some groups, including the cryptobenthic sponges studied here, the majority of species are undescribed and thus not identifiable in the conventional sense. Nevertheless, once rigorously delineated by integrative study, species can be tracked using provisional names that are tied to vouchers, and documented with images as well as sequence data, thus creating a reference for tracking species in such a poorly known fauna. Such studies are needed to support the annotation of next generation sequencing data that rely on the DNA databases to support biodiversity assessment and monitoring. Our database for Kāneʻohe Bay sponges has already improved sponge classification in a local COI targeted metabarcoding effort by increasing species-level OTU identifications by 37% (Timmers et al. 2020).

A primary goal for state managers is to establish a baseline of species present in Hawaiʻi, to identify and protect native species, detect future sponge introductions, and monitor changes in the sponge community in response to human impacts. Our surveys of the cryptobenthic fauna show that this community remains highly undersampled.

Future taxonomic efforts will provide accurate species identification integrating morphological assessments with sequence data provided in this study. However, in order to identify species as endemic, native, or introduced, sampling and vouchers of the cryptobenthic sponge community from ARMS must be standardized and compared from other sites throughout the world. Taxonomic efforts should be coupled with sequencing initiatives such as the sponge barcoding project (Wörheide et al. 2007) which continue to enrich the GenBank Porifera database. Particular emphasis should be given to barcoding approaches using multi-loci (COI and 28S rRNA) as these are more effective at discerning related species to aid future metabarcoding studies in resolving the poriferan community of cryptobenthic reefs.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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