Inter-Research Science Publisher

Marine Ecology Progress Series
Volume 753:19–35

Published January 30, 2025
https://doi.org/10.3354/meps14763

OPEN ACCESS

Detection of the *Diadema antillarum* scuticociliatosis *Philaster* clade on sympatric metazoa, plankton, and abiotic surfaces and assessment for its potential reemergence

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ABSTRACT: A ciliate belonging to the *Diadema antillarum* scuticociliatosis (DaSc)-associated *Philaster* clade (DaScPc) caused catastrophic long-spined urchin mass mortality in spring and summer of 2022. The ciliate can be grown in culture in both the presence and absence of *D. antillarum* tissues, suggesting that it may persist outside its host by consuming microorganisms or dissolved organic nutrients. We hypothesized that DaScPc was present outside its host during and after mass mortality and absent prior to 2022. We examined DaScPc in DNA extracted from 500 swabs of sympatric metazoa and abiotic surfaces, and plankton samples, collected at 35 sites in the Caribbean in 2022 and 2023. DaScPc was detected on corals, turf algae, and a sponge, predominantly at sites with active or prior DaSc. We examined whether it was present prior to 2022 by surveying extracted DNA from Caribbean corals and water collected near corals by PCR and by mining publicly available transcriptomes and metagenomes for DaScPc rRNAs. These efforts yielded no DaScPc genes. We further hypothesized that DaScPc may recruit to the specific corals detected in field surveys, and that these may then infect naïve hosts. A mesocosm experiment to test DaScPc recruitment suggested that, while it recruited to corals, it did so inconsistently between coral species. Incubation of corals that recruited DaScPc with naïve urchins yielded inconclusive results since urchins died without characteristic DaSc signs. Overall, our results suggest that DaScPc may occur outside its urchin host, and that it may have been absent in the region prior to 2022.

KEY WORDS: Scuticociliate · *Diadema* · *Philaster* · Organic matter · Caribbean

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1. INTRODUCTION

The long-spined sea urchin *Diadema antillarum*, a keystone species in Caribbean coral reef ecosystems affecting the balance between coral and algal benthic cover (Lessios 2016), experienced a mass mortality event beginning in January 2022 that led to up to a 99% reduction in densities at affected sites (Hylkema et al. 2023). The mass mortality event was first documented on the southern side of St. Thomas (US Virgin Islands), then affected *D. antillarum* widely across the Caribbean before dissipating by late summer. Affected *D. antillarum* exhibited abnormal behavior, including detachment from vertical surfaces, lack of response to stimuli, loss of tube foot control, use of spines for movement, stellate spine arrangement, loss of spines, and, ultimately, death (Hewson et al. 2023, Hylkema et al. 2023). The condition, known as *D. antillarum* scuticociliatosis (DaSc), was caused by a philasterine scuticociliate (Hewson et al. 2023), which forms a phylogenetic clade distinct from other *Philaster* spp., that we refer to here as the DaSc-*Philaster* clade (DaScPc) (B. Vilanova-Cuevas et al. preprint doi:10.1101/2023.09.11.557215). The identification of DaScPc as the causative agent prompted recognition of this ciliate as an emerging infectious threat to *D. antillarum* restocking efforts and remaining urchin populations which had previously experienced mass mortality in 1983–1984 (Lessios 2016).

More recently, urchin mass mortalities have been observed in the Mediterranean Sea (in *D. setosum*; Gokoglu et al. 2023, Zirler et al. 2023, Dinçtürk et al. 2024, Skouradakis et al. 2024), the Red Sea (*D. setosum* and *Echinothrix calamaris*; Ghallab et al. 2024, Roth et al. 2024), Oman (*D. setosum*; Ritchie et al. 2024), and the Western Indian Ocean (*E. calamaris* and *D. setosum*; Roth et al. 2024, Quod et al. 2025), where identical rRNA DaScPc sequences were recovered from affected urchins at most affected sites (Ritchie et al. 2024, Roth et al. 2024, Quod et al. 2025). These reports expand affected species to other urchins within the Diadematidae family and indicate that this pathogen has strong potential to invade new areas. While significant strides have been made in characterizing the pathogen, there is limited understanding of its environmental distribution and virulence beyond urchins. For instance, although DaScPc was detected in water and sediments adjacent to affected urchins (Hewson et al. 2023), it is unclear whether this pathogen is normally present in the coral reef habitat. Understanding its presence in sympatric habitats is vital to assess risks to remaining populations and restocking efforts of *D. antillarum*.

Ciliates play essential roles in pelagic food webs as components of the microbial loop. Most ciliates are bacterivores or grazers on phytoplankton and microzooplankton, and consequently aid in remineralizing organic nutrients and cycling trace metals (Sherr & Sherr 2002, Tuorto & Taghon 2014). Scuticociliates are a subclass (Scuticociliatia) of ciliates that have been linked to many marine disease outbreaks, often referred to as scuticociliatoses. These conditions manifest with clinical signs of epidermal or body wall lesions, hemorrhage, ulcers, and tissue necrosis. Scuticociliatoses are frequently severe and often fatal infections affecting metazoa, including teleosts, elasmobranchs, crustaceans, and possibly others (Saura et al. 2019). Scuticociliates that are histophagous (i.e. pathogens) may also be bacterivores or grazers. However, few studies have examined bacterivorous activity of pathogenic ciliates in nature (Kemp 1988, Xiong et al. 2015). DaScPc culture FWC2 (initially isolated from a DaSc-affected urchin from Florida as part of an earlier study to identify it as the causative agent of the condition) can be cultured in the absence of *D. antillarum* tissue homogenates, so it must be able to consume bacteria or dissolved organic compounds in culture media (Hewson et al. 2023).

Coral reefs are ecologically important habitats that harbor incredibly diverse microbial ecosystems (Rosenberg et al. 2007, Ainsworth et al. 2010, Barott et al. 2011, Glasl et al. 2019, Bonacolta et al. 2023). Scuticociliates within the genus *Philaster* are consistently associated with several coral diseases (Sweet & Séré 2016), where they may contribute to pathology through histophagy and consumption of zooxanthellae, leading to tissue damage, compromised immune response, and, in severe cases, coral mortality (Harikrishnan et al. 2010, 2012). The phylogenetic proximity of DaScPc to several of these coral-associated *Philaster* spp. brings into question whether DaScPc is a recent introduction to the Caribbean or if environmental conditions triggered DaScPc normally residing on corals to become pathogenic in *D. antillarum*.

The aim of this study was to examine the presence of DaScPc in nearby habitats at DaSc-affected sites. This study addressed 3 hypotheses: (1) DaScPc was present on sympatric metazoan surfaces and plankton during and after the onset of *D. antillarum* mass mortality; (2) DaScPc was absent in the Caribbean prior to *D. antillarum* mass mortality; and (3) DaScPc can recruit to metazoan surfaces and potentially reemerge to infect naïve host urchins. To address the first hypothesis, we surveyed DaScPc in metazoan-associated and plankton environments prior to, during, and after the DaSc outbreak at affected sites, as

well as reference sites (where DaSc never occurred). We haphazardly sampled the surfaces of common sympatric metazoa and plankton, along with several abiotic surfaces. Furthermore, we explored whether DaScPc in environmental samples was associated with specific bacterial taxa through 16S rRNA amplicon sequencing of specimens where DaScPc was detected. To address the second hypothesis, we mined publicly available survey sequence data and tested coral samples collected prior to the mass mortality event to examine whether DaScPc pre-dated the DaSc outbreak. To address the third hypothesis, we performed mesocosm experiments to examine recruitment of DaScPc to sympatric coral species identified in the field survey. Next, we examined the potential of DaScPc to infect naïve urchins after recruitment to corals in aquaria.

2. MATERIALS AND METHODS

2.1. Surface sample collection and processing

Surface swab specimens (n = 489) and plankton specimens (n = 11) were collected from 35 sites at 9 locations between August 2022 and November 2023 (Fig. 1; Table S1 in the Supplement at www.int-res.com/articles/suppl/m753p019_supp.pdf). Samples were collected from sites affected (i.e. sites which bore DaSc-affected urchins) at the time of sampling or those which were previously affected; sites that were never affected (i.e. reference sites); and ports/marinas. Swab specimens were collected by snorkelers at depths of ~1–4 m. Polyester swabs (Dry Transport Systems, Puritan Medical Products) were transported to the survey site sealed in an air-filled transport tube and withdrawn into the water immediately adjacent to the swabbed surface. The swab was gently rubbed for 10 s across a ~2 cm^2 surface before retrieval into the air-filled transport tube. Live swabbed surfaces, chosen haphazardly, were photographed to confirm the identity of invertebrate or plant species (seagrass and macroalgae). Small boat hulls were also swabbed following the same procedure. At some sites, identity of swabbed surfaces could not be determined (i.e. unidentifiable surface swabs; n = 59), and in some cases, the taxonomy of swabbed specimens was unclear (e.g. macroalgae; n = 11), so they were assigned to higher taxonomy. Swabs were transported at ambient temperature to shore, where the swab tips were cut off with clean scissors into cryovials containing RNAlater Solution (Invitrogen). Plankton samples, when collected, comprised 200–1000 ml seawater that was filtered immediately after collection through 0.22 µm polyethersulfone filters (VWR). The filters were folded and placed into cryovials containing RNAlater. All specimens were stored at −80 or −20°C before transport on dry ice to the laboratory at Cornell University.

DNA was extracted from swab tips and filters were extracted using the Quick-DNA Insect/Tissue kit (Zymo Research). The manufacturer's extraction protocol (v.2.21) was followed with these exceptions: we did not add betamercaptoethanol to the lysis buffer, bead bashing was shortened to 2 min, and DNA was eluted into nuclease-free sterile water instead of elution buffer. Extracted DNA was quantified by PicoGreen fluorescence (Invitrogen) and subjected to quantitative PCR.

2.2. Quantitative PCR of the *Philaster* 28S rRNA gene

Swab and plankton samples were initially assessed for DaScPc by quantitative PCR following established protocols (Hewson et al. 2023). Briefly, extracted DNA (1 µl) was subject to qPCR in 25 µl reactions containing 1× SSO Advanced Probes Supermix (Bio-Rad), 200 pmol each of primers Phil_28S_F (5'-TAG GGC AAG TCC TTG GAA TG-'3) and Phil_28S_R (5'-TGC CAC ATT TTA TCC ACA GC-3') and probe Phil_28S_Pr (FAM-5'-CAG CGT ACG CTG TTG CTG C-3'-TAMRA) in an ABI StepOne real-time thermal cycler. Specimens were run in duplicate against a synthetic oligonucleotide standard (5'-TAG GGC AAG TCC TTG GAA TGC AAA ACA GCG TAC GCT GTT GCT GCC AAA AGG GAA AGG GGT TAA TAT TCC TCT AGT CGG CTG TGG ATA AAA TGT GGC A-3') ranging from 2.41×10^7 to 2.41×10^0 copies per reaction and including 4 no-template controls (negative reactions) per run. Runs were performed in an ABI StepOne instrument, subject to an initial denaturation step of 50°C for 2 min and 94°C for 10 min, followed by 50 cycles of 94°C denaturation for 10 s and 56°C annealing for 60 s. Data were inspected for standard linearity ($R^2 > 0.98$). Swab specimens were considered a positive detection of DaScPc by qPCR (referred to as qDaScPc+) when both reactions were within 1 cycle threshold (Ct) value, and above a threshold of 10^2 copies per reaction, which is consistent with observations of copies per cell in cultured DaScPc (~10^2 $cell^{-1}$). qPCR inhibition was assessed in several (n = 20) swab specimens by amendment of extracted DNA with the synthetic oligonucleotide standard. However, Ct values of extracted DNA samples amended with a standard did not differ from the standard run without extracted DNA. Association

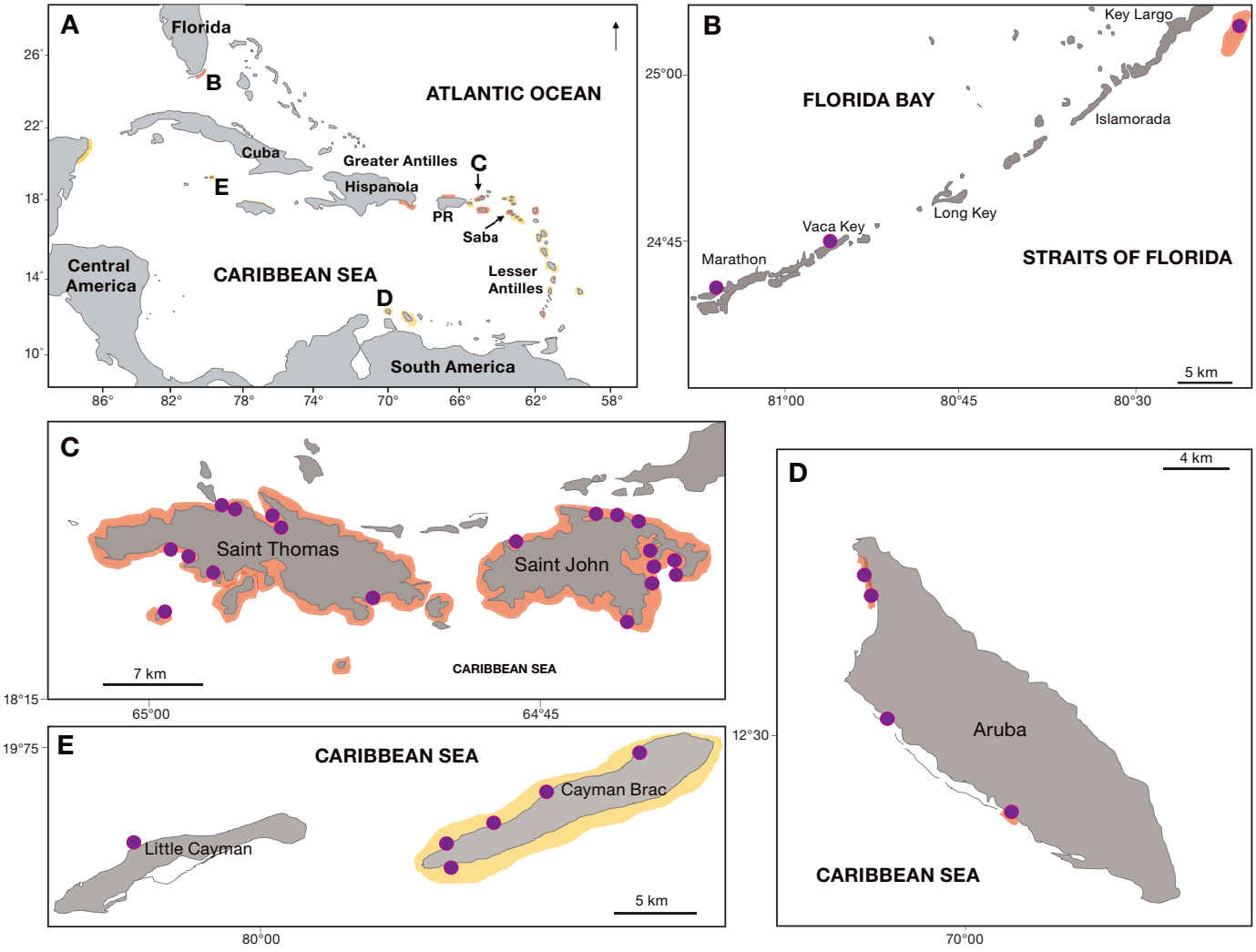

Fig. 1. Sampling locations in (A) the Caribbean Region, (B) Florida Keys, (C) United States Virgin Islands, (D) Aruba, and (E) Cayman Islands. Saba is omitted because it is very small (samples were taken from 1 site, Rocky Point). Yellow highlighted areas indicate approximate extent of *Diadema antillarum* scuticociliatosis (DaSc)-affected urchins, orange highlighted areas indicate where DaSc-associated *Philaster* clade (DaScPc) was detected in DaSc-affected urchins. Swab field survey sites are indicated by purple circles

between swab species, presence/absence of DaScPc, and site category (active or recent [less than 3 mo] DaSc signs) was examined by Fisher's exact test.

2.3. Screening of specimens by 18S rRNA gene PCR amplification

Specimens that were qDaScPc+ by qPCR (n = 37) were subject to further analysis to confirm DaScPc. The qPCR primer/probe set previously reported in the literature bore weak matches to related ciliate 28S rRNA. Moreover, the taxonomic coverage of the 28S rRNA gene is not as extensive as the 18S rRNA gene, which is used in most studies of voucher specimens and environmental/uncultured surveys (Hewson et al. 2023).

In an initial attempt to confirm the DaScPc sequence in qDaScPc+ specimens, the 37 qDaScPc+ samples were pooled in equal volume of extracted DNA separately by site and by species. Extracted DNA pools were then subject to PCR employing pan-*Ciliophora* 18S rRNA gene primers 384F (5'-YTB GAT GGT AGT GTA TTG GA-3') and 1147R (5'-GAC GGT ATC TRA TCG TCT TT-3') (Dopheide et al. 2008). PCR was performed in 50 µl reactions comprising 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (Promega PCR Nucleotide Mix), 200 pmol forward and reverse primers, 1 µl of 2 ng ml^{-1} BSA (Sigma), 5U *Taq* DNA polymerase (Invitrogen), and 2 µl of pooled DNA extracts. Thermal cycling was preceded by an initial

heating step for 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min in a BioRad MyCycler. The PCR amplicons (5 µl) were then visualized on a 1% agarose gel in 1× Tris-borate-EDTA after electrophoresis at 85 V for 45 min and staining with SYBR Gold. PCR products were cleaned up using the Zymo Clean & Concentrator-5 kit and subject to dye terminator (Sanger) sequencing at the Biotechnology Resource Center at Cornell University.

Initial assessment of Sanger sequence chromatograms identified 2 specimens (a site pool from Lindbergh Bay, St. Thomas, and a species pool from *Ircinia campana*) that had significant nucleotide overlap from PCR product sequencing. Hence, these specimens were cloned using the pGEM-T Easy Vector System (Promega) to resolve ciliate sequences. Five clones were randomly picked from each pool and prepared for sequencing using the Zyppy Plasmid Prep Kit (Zymo) before being Sanger sequenced.

2.4. Design of DaScPc primers and application to coral swab samples

A phylogenetic analysis performed previously (Hewson et al. 2023) and supplemented with sequences from abnormal urchins revealed a coherent clade of 18S rRNA sequences that were primarily associated with DaSc (B. Vilanova-Cuevas et al. preprint doi:10.1101/2023.09.11.557215). This clade separated from other *Philaster* spp., including *P. apodigitiformis* and coral-associated ciliates (Sweet & Séré 2016) with strong bootstrap support (Ritchie et al. 2024, Quod et al. 2025, B. Vilanova-Cuevas et al. preprint doi: 10.1101/2023.09.11.557215). We named this clade the DaSc-*Philaster* clade (DaScPc). Because this clade is distinct from the 18S rRNA sequences of *P. lucinda* and *P. guamensis* (both recovered from corals; Sweet & Bythell 2012, Sweet & Séré 2016, Sweet 2020) and *P. apodigitiformis* (originally identified from pufferfish eggs) (Miao et al. 2009), future reference to DaSc-associated ciliates should follow this clade designation until formal taxonomy is applied.

Sequences derived from swab samples using pan-*Ciliophora* PCR primers did not yield matches to DaScPc by BLASTn. Hence, we designed a second, nested approach to specifically target DaScPc previously reported (Ritchie et al. 2024, B. Vilanova-Cuevas et al. preprint doi:10.1101/2023.09.11.557215). We created an alignment of urchin-derived DaScPc sequences and representative 18S rRNA gene sequences spanning Ciliophora by BLASTn. We manually examined conserved nucleotide sequence regions and found a region within the amplicon obtained by the general ciliate primers (384F/1147R; Dopheide et al. 2008) on which to base the DaScPc primer. We then used the Primer3 program (Rozen & Skaletsky 1999) to identify a primer sequence (scutico-634F; 5′-TTG CAA TGA GAA CAA CGT AA-3′) that selectively amplified DaScPc sequences over related taxa. BLASTn analysis of the primer revealed that it shared 100% sequence identity with some metazoans but did not match other ciliate or protist 28S rRNAs in GenBank. We validated this new scutico-634F primer in combination with 1147R with the same PCR conditions described above except lowering the annealing temperature to 53°C and using only 1 µl of the product from the general ciliate PCR as template (i.e. nested PCR) (Ritchie et al. 2024, Quod et al. 2025, B. Vilanova-Cuevas et al. preprint doi:10.1101/2023.09.11.557215).

Amplicons from all 37 qDaScPc+ specimens were first PCR amplified with the pan-*Ciliophora* primers and then subject to a second round of PCR using the new DaScPc primer described above. Following gel electrophoresis, specimens yielding amplicons were Sanger sequenced. Because the pooled approach led to ambiguous identity of individual specimens within pools for a few specimens, the pan-*Ciliophora* and subsequent Scutico_634F-1147R PCR was repeated for individual specimens that yielded amplicons in site pools and processed for Sanger sequencing as above. DaScPc and other ciliate 18S rRNA sequences obtained in this study were deposited in GenBank under accessions PP851979—PP852041.

2.5. Publicly available database search

Publicly available metagenome and metatranscriptome databases at the Joint Genome Institute Integrated Microbial Genomes—Microbiome Expert Review (IMG-MER, https://img.jgi.doe.gov) (as of 27 February 2023) were examined for sequences that were similar to the 18S and 28S rRNA and internal transcribed spacer (ITS2) sequences of DaScPc culture FWC2 (Hewson et al. 2023) by BLASTn employing an e-value cutoff of 1×10^{-40}. Datasets were identified by the key word 'Invertebrates, All' and comprised 70 metagenomes and 90 metatranscriptomes. Matches with ≥95% nucleotide identity that were ≥200 nucleotides (nt) were considered in this analysis. Several sequences that were ≤200 nt (minimum length 179 nt) but had ≥96% identity were also considered in phylogenetic analyses. In addition, the

FWC2 18S and 28S rRNA sequences were aligned against the Transcriptome Sequence Assembly (TSA) database at NCBI (filtered for 'Animalia') by BLASTn.

2.6. PCR amplification from corals

Nested PCR amplification employing the scuticociliate primer 634F (B. Vilanova-Cuevas et al. preprint doi:10.1101/2023.09.11.557215) and general primer 1147R (Dopheide et al. 2008) was attempted on coral DNA extracts from the US Virgin Islands (n = 112), Belize (n = 23), and Florida (USA) (n = 17), representing a range of coral species collected between 2013 and 2021. Several specimens came from colonies affected by stony coral tissue loss disease (n = 43) or from colonies affected by black band disease (n = 24) (Table S2). These specimens had been collected as part of separate investigations (Meyer et al. 2017, 2019, Becker et al. 2022), which preceded the start of the *Diadema antillarum* mass mortality events.

2.7. Phylogenetic analysis of DaScPc sequences

Phylogenetic reconstructions were performed on overlapping portions of the 18S rRNA and 28S rRNA sequenced from PCR products or recovered from metagenomes/transcriptomes, including closest relatives to these in the nonredundant (nr) database at NCBI as identified through BLASTn. Alignments were performed using MUSCLE (Edgar 2004). Optimal models, substitution models, and site distributions were identified using the 'Find Best Model' function in MEGA X (Kumar et al. 2018) and applied to tree construction in the same platform. Phylogenetic reconstructions were supported by 1000 bootstrapping iterations.

2.8. Experimental assessment of coral specificity

The recruitment pattern of the DaScPc culture FWC2 (isolated from a DaSc-affected urchin and used in challenge experiments to establish pathogenicity as part of prior work; Hewson et al. 2023) to 4 coral species was assessed in experimental mesocosms in June 2023. Forty 10 cm^2 fragments of corals (10 each of *Siderastrea siderea, S. radians, Orbicella faveolata*, and *Montastraea cavernosa*) were obtained from the Florida Keys National Marine Sanctuary nursery (under National Marine Sanctuary Permit FKNMS-2023-058), wrapped in seawater-moistened bubble wrap, and transported in coolers to the laboratory at the University of South Florida (transit time ~6 h). There, corals were placed into 7.5 l glass aquaria (n = 25) filled with 7 l of 5 µm filtered seawater (collected from Marathon, Florida). Twenty aquaria held single individuals of each coral species, while 5 aquaria received a single individual from each of the 4 taxa in combination. Aquaria were subject to 50% water changes with fresh, 5 µm filtered seawater every 48 h with constant aeration via air stones. Aquaria were maintained at 26°C in a water bath, with a water pump and heater, under a 12:12 h light:dark cycle. Corals were fed 0.2 g of Ocean Nutrition Frozen Formula Foods Prime Reef blocks every 2 d.

Each aquarium was inoculated with ~100 FWC2 cells (enumerated by light microscopy) by pipetting onto the coral surface (or in the case of combined aquaria, in the space between all 4 coral fragments). Surface swabs of the corals were retrieved once a day and swabs were frozen at −80°C prior to DNA extraction. In addition, the aquarium glass surface was swabbed side-to-side in a ~2 cm^2 area after 24 and 48 h, and 200 ml water samples from each aquarium were retrieved and filtered through 5 µm polycarbonate filters, which were then frozen prior to DNA extraction.

DNA was extracted from swabs as described for the field survey. DNA from water filters and subsamples of the coral food were extracted using the Zymo Tissue and Insect kit and quantified by Pico Green fluorescence. The quantity of DaScPc was assessed by qPCR targeting the 28S rRNA gene of DaScPc culture FWC2 as previously described. The experiment ended after 96 h since real-time data on DaScPc abundance revealed the presence of the ciliate on all corals (with detection threshold of 2.41×10^3 copies). qPCR values of DaScPc 28S rRNA copy number were assessed for normality by a Shapiro-Wilk test. Differences between the proportion of qDaScPc+ corals across species as individuals and combined were assessed by χ^2 goodness of fit tests.

2.9. Experimental assessment of reemergence potential

We examined if DaScPc that recruited to corals (from the assessment of the coral specificity experiment) can infect *D. setosum.* This species was chosen because it is available through the aquarium trade (*D. antillarum* is not available) and was recently reported to be affected by DaSc in the Mediterranean and Red Sea (Zirler et al. 2023, Ritchie et al. 2024) and can be infected by DaScPc in experimental challenge

(Ritchie et al. 2024). Corals in the recruitment experiment described above were sorted into specimens which bore the greatest swab DaScPc 28S rRNA copy number, and those in which DaScPc was not detected in the experiment. *S. radians* was excluded from this experiment since all coral pieces recruited DaScPc in the recruitment experiment. *M. cavernosa* (3 qDaScPc+ and 3 DaScPc specimens that were not qDaScPc+, hereafter referred to as qDaScPc−), *S. siderea* (3 qDaScPc+ and 2 qDaScPc−), and *O. faveolata* (1 qDaScPc+ and 2 qDaScPc−) were placed into individual, cleaned aquaria which were refilled with 5 µm filtered Florida Keys water. A single *D. setosum* (obtained from Vivid Aquarium, Canoga Park, CA) was placed into each aquarium and monitored daily for spine loss and morbidity (lack of movement). *D. setosum* were screened for DaScPc by removing a single spine from each urchin using sterile forceps, and performing qPCR targeting the DaScPc 28S rRNA (see Section 2.2), which yielded no detections in any specimen. Aquarium husbandry and maintenance followed the assessment of coral specificity experiment and qPCR was used to detect DaScPc on the urchins throughout the experiment, with the addition of daily fecal pellet removal and feeding urchins with commercially available herbivore diet pellets (New Life Spectrum ALGAEMAX) every other day. The survival of specimens incubated with qDaScPc+ and qDaScPc− corals was compared by Wilcoxon signed-rank tests.

2.10. Microbial community amplification and analysis

Bacterial communities in environmental swab sample DNA extracts of qDaScPc+ samples that also yielded DaScPc sequences by conventional PCR were identified using dual-barcoded PCR amplification and sequencing of the V4 region of the 16S rRNA gene (Kozich et al. 2013, Parada et al. 2016, Walters et al. 2016). Each 40 µl PCR reaction comprised 1× PCR master mix (One-*Taq* Quick-Load 2× Master Mix with Standard; New England Biolabs), 0.125 µM of each barcoded primer (515f; 5′-GTG YCA GCM GCC GCG GTA A-3′ and 806r; 5′-GGA CTA CNV GGG TWT CTA AT-3′), and 2 µl of template (Apprill et al. 2015, Parada et al. 2016). The thermocycling conditions were as reported by Apprill et al. (2015). 16S rRNA amplicons were pooled at equimolar concentrations using a SequalPrep Normalization Plate kit (Invitrogen) and sequenced on 1 lane of Illumina MiSeq (v2 500 bp; 2 × 250 paired end) at the Cornell University Biotechnology Resource Center. 16S rRNA gene amplicon sequences were submitted to European Bioinformatics Institute (EBI accessions PRJEB76500 and ERP161028).

Data preprocessing was done using the online platform Qiita (Gonzalez et al. 2018), an interactive web-based platform that uses QIIME2 plugins to process microbiome data. The data and preprocessing information are available for visualization and export under Study ID 15474. Using Qiita, the samples were demultiplexed and quality control was applied to retain samples with a Phred Score above 30. Sequences were trimmed at 250 nt, Deblur (Amir et al. 2017) was used to denoise the sequences and produce an initial amplicon sequencing variant (ASV) table, and singleton removal was performed eliminating all features with frequency <10. The average and median of sequencing depth before preprocessing was 107 318 and 113 252, respectively. After preprocessing, the average and median of sequencing depth was 15 486 and 14 710, respectively. Prior to processing, the total library frequency was 5 795 150; after preprocessing, the total library frequency was 418 115. The Silva-138-99-515-806-nb-classifier (V.138) (Quast et al. 2013) was used to assign taxonomy, and chloroplast and mitochondrial sequences (which comprised 48 925 sequences or 9.29% of total sequences) were removed from the ASV table. Data were imported into R, where alpha diversity and taxonomic composition analyses were performed using the package 'Phyloseq' (v.1.46.0; McMurdie & Holmes 2013), and a graphical summary of the data was created using 'ggplot2' (v.3.5.0; Wickham 2016). For this analysis, quantitative PCR (qPCR)-determined DaScPc copies per ng of extracted DNA were categorized into 4 groups (negligible: 0−100; low: 100−800; medium: 800−5000; and high: >5000). Richness and diversity differences, across differences in DaScPc abundance, were assessed using Chao1 and Shannon indices, respectively (Chao 1984, Keylock 2005, McMurdie & Holmes 2013). Significant differences in the alpha diversity analysis were determined from a Kruskal Wallis test using 'ggpubr' (v.0.6.0; Kassambara 2023).

3. RESULTS

3.1. Survey of sympatric habitats

Of the 489 individual benthic swab specimens examined, 37 (5.8%) were qDaScPc+ (Fig. 2). These included specimens collected from corals, turf algae, sponges, and the hull of a small boat. In addition, we confirmed the presence of DaScPc on several surface specimens (metazoa could not be positively identified

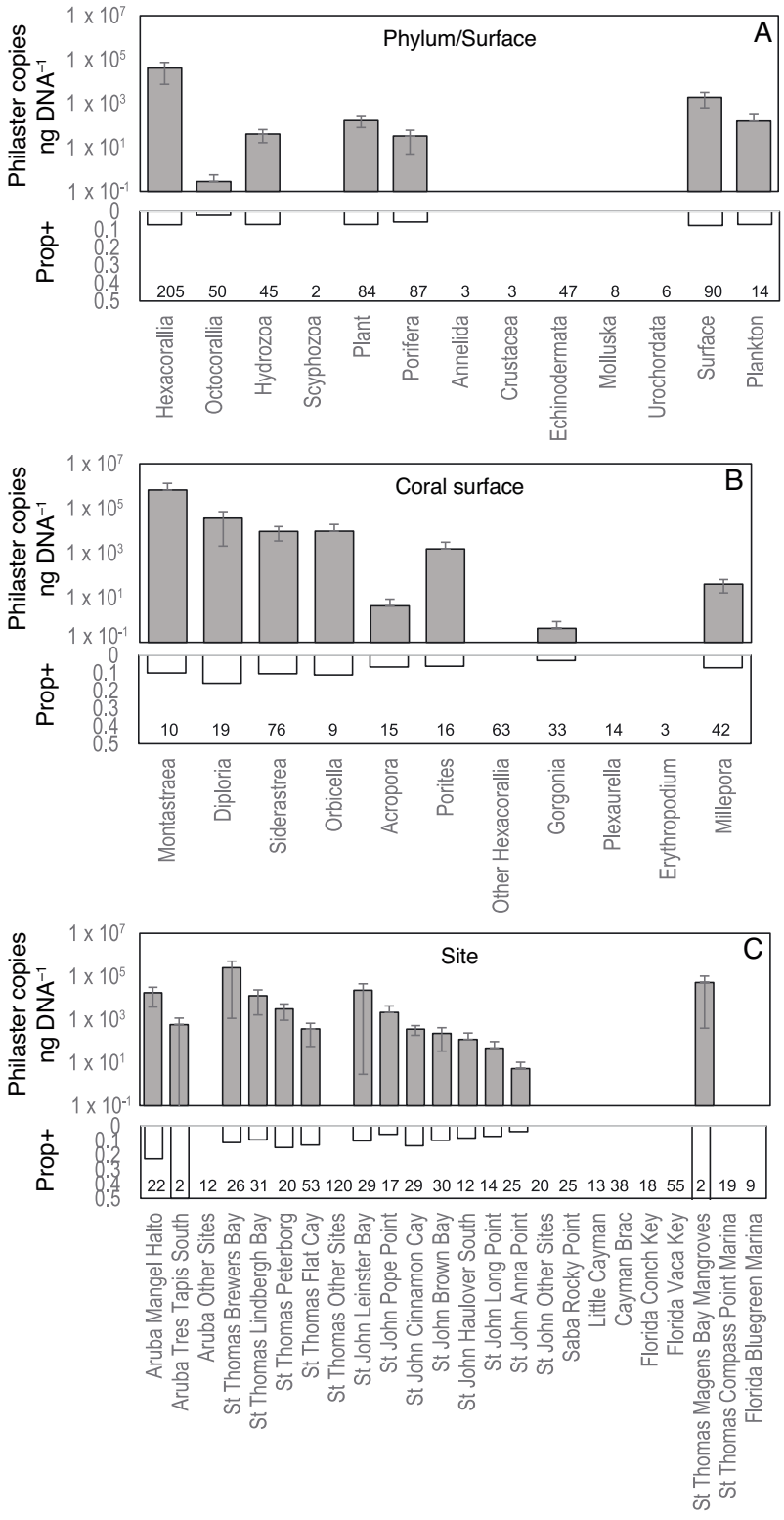

Fig. 2. Mean DaScPc 28S rRNA gene abundance (copies per ng of extracted DNA) and prevalence (Prop+; proportion that were positive by qPCR [qDaScPc+]) in swab DNA extracts. The data are presented (A) by surface type/phylum, (B) separately for coral genera, and (C) by site. The numbers below bars indicate total samples tested. Error bars = SE

in the field because of low water visibility) and in plankton samples at 1 site. Amongst corals from sites previously affected with DaSc, DaScPc was detected in only a single octocoral specimen, and detected in 14 and 4 (10 and 12%) of tested hexacorals and hydrozoa, respectively. Overall, DaScPc 28S rRNA copy numbers were greatest for hexacorals ([4.1 ± (SE) 3.3] × 10^4 copies ng DNA^{-1}) compared to other specimens ([4.3 ± 2.5] × 10^2 copies ng DNA^{-1}). Geographically, DaScPc was detected at most sites around St. Thomas, St. John, and Aruba, but were not detected near Saba, Florida, or the Cayman Islands. The proportion of qDaScPc+ of total specimens sampled was significantly associated with the coral *Siderastrea siderea* (p = 0.039, Fisher's exact test, df = 437) but not with other coral species.

3.2. 18S rRNA sequence confirmation of qDaScPc+ at survey sites and detection in coral tissue

The 37 qDaScPc+ swab samples were further investigated by nested PCR amplification using pan-*Ciliophora* primers (Dopheide et al. 2008) followed by the scutico-634F primer (Ritchie et al. 2024, B. Vilanova-Cuevas et al. preprint doi:10.1101/2023.09.11.557215) and 1147R primer (Dopheide et al. 2008). Initially by using a pooled approach (by site and by taxon/surface), 15 specimens yielded PCR amplicons using the pan-*Ciliophora* primers. These fell within taxonomic clades that included peri-, hypo-, and oligotrich ciliates, along with Suctoria and phyllopharyngid ciliates and a scuticociliate most closely related to *Paranophrys* (Fig. S1). Subsequent application of the scuticociliate primer scutico-634F on all qDaScPc+ specimens yielded amplicons of 18 pools (site + species). In addition, a further 22 amplicons were amplified directly from individual specimens. Amongst the scutico-634F amplified sequences, 28 fell within a distinct clade with DaScPc, 10

were more broadly classified within *Philaster* spp., and 2 did not match scuticociliates (1 most like *Trichodina* sp. and 1 of unclear taxonomy within *Peritrichia*) (Fig. S1).

3.3. Query for DaScPc on publicly available data sets preceding the 2022 mass mortality event

A total of 4 metagenome scaffolds yielded hits against the ITS2, 26 matched 18S rRNA, and 58 matched 28S rRNAs (Table S3). Four 18S rRNA sequences had sufficient overlap with other rRNAs retrieved from close relatives and those derived from direct PCR amplification to create a phylogenetic reconstruction (Fig. 3). All 4 sequences fell within the Philasteridae, but none within the DaScPc. Phylogenetic reconstruction of both ITS2 and 28S rRNAs revealed that retrieved sequences fell within *Philaster* spp., but there was insufficient resolution for these loci to distinguish between closely related *Philaster* and DaScPc (Fig. S3).

3.4. Bacterial+archaeal community analysis of samples that yielded DaScPc 18S rRNA sequences

The impetus for this investigation was to identify whether DaScPc was associated with specific bacterial taxa that might select for its recruitment onto environmental surfaces. We characterized the bacterial+archaeal community present on swab specimens that yielded DaScPc 18S rRNA sequences. Bacterial+archaeal community diversity was significantly greater in high (>5000 DaScPc copies per ng extracted DNA, n = 10) DaScPc 28S rRNA copy number specimens than in negligible DaScPc 28S rRNA copy number specimens (0–100 DaScPc copies per ng of extracted DNA, n = 5; diversity p = 0.028, Kruskal-Wallis tests; Fig. S2). Similarly, bacterial+archaeal communities were significantly richer in high DaScPc 28S rRNA copy number specimens than medium (800–5000, n = 5) DaScPc 28S rRNA copy number specimens (p = 0.04, Kruskal-Wallis test; Fig. S2). Linear discriminant analysis effect size (Lefse) failed to identify any correlation between DaScPc 28S rRNA copy number and specific bacterial taxa.

3.5. PCR amplification of coral samples preceding the 2022 mass mortality event

Although 29 coral DNA extracts yielded PCR amplicons via nested PCR targeting the 18S rRNA of DaScPc, only 15 yielded sufficient amplicon quantities for sequencing. Of these, 2 sequences most strongly matched Porifera (Ianthellidae), 4 matched ciliates other than the Philasteridae, and 9 matched Philasteridae. Alignment of these sequences yielded 7 sequences with sufficient overlap to create a phylogenetic reconstruction (Fig. 3). Of these, all 7 fell within sequences from the Philasteridae, but did not form part of the DaScPc.

3.6. Coral recruitment experiment

In the coral recruitment experiment, DaScPc culture FWC2 was detected on coral surfaces, aquarium glass, and in the water used for incubation. However, the overall DaScPc 28S rRNA copy number varied between the incubations and whether corals were incubated as individuals or in combination. DaScPc 28S rRNA was detected in 30 ± (SE) 13% of individual corals and 50 ± 13% of combined corals before inoculation occurred, but quantities were 831 ± 617 copies ng DNA^{-1} and therefore may have represented spurious hits to related ciliates (Fig. 4). The proportion of corals that were qDaScPc+ increased over the course of the experiment in all coral species, with 100% of *S. siderea* bearing DaScPc after 2 d incubation, and 100% of *Montastraea cavernosa* and *S. radians* bearing DaScPc after 4 d. We found that 40% of *Orbicella faveolata* were qDaScPc+ in both individual and combined incubations. DaScPc 28S rRNA copy number values were not normally distributed (Shapiro-Wilk test, $p < 0.0001$, df = 160). The overall pattern of qDaScPc+ as a proportion of total specimens tested over time was not significantly different between coral species (χ^2 goodness of fit test, p = 0.238, df = 3; Fig. 4). The DaScPc 28S rRNA copy number in corals was not different between coral species housed individually or in combined incubations, but increased over time in individual incubations (Kruskal-Wallis test, p = 0.007, df = 3) (Fig. 5). This was reflected in significantly higher abundances after 2 and 4 d in *M. cavernosa* (Wilcoxon signed rank test, p = 0.028, df = 10) and *S. radians* (Wilcoxon signed-rank test, p = 0.015, df = 10) compared to other species tested. DaScPc could be detected in both aquarium water and on aquarium glass surfaces, but only significantly increased over time on glass surfaces (Kruskal-Wallis tests, p = 0.029 for glass, p = 0.238 for water, df = 4).

3.7. Reemergence experiment

DaScPc was detected in *Diadema setosum* after being introduced to qDaScPc+ corals, from the pre-

Fig. 3. Phylogenetic reconstruction of *Ciliophora* sequences recovered from the swab survey, amplified directly from coral DNA extracts (prior to 2022), and recovered from the Joint Genome Institute Integrated Microbial Genomes–Microbiome Expert Review (JGI IMG-MER) by BLASTn. The reconstruction was performed on the 281–313 nucleotide portion of the 18S rRNA, including closest relatives in the nonredundant (nr) database at NCBI. The alignment was performed using MUSCLE (Edgar 2004). The tree was created with MEGA X (Kumar et al. 2018) using maximum likelihood and the Kimura-2 parameter model with gamma distributed sites and the nearest-neighbor interchange heuristic model. Bootstrap values represent 1000 iterations. Sequences in orange are those primed with the Scutico-634F and 1187R primers, while those in purple are primed with pan-*Ciliophora* 384F and 1187R. Blue sequences indicate those recovered from IMG-MER by BLASTn. The DaScPc clade, with 77% bootstrap value, is indicated

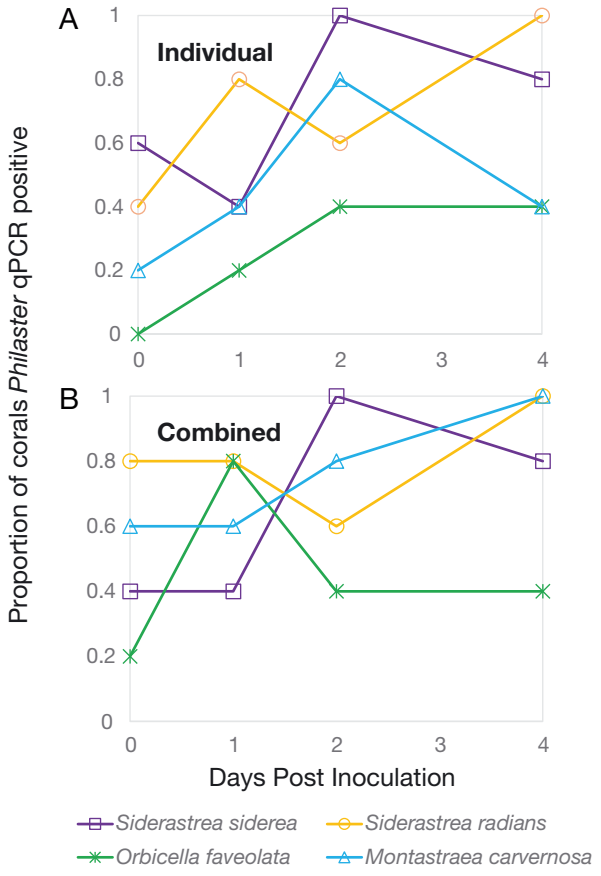

Fig. 4. Proportion of corals that were qDaScPc+ over the course of the experiment in (A) individual-species and (B) combined-species incubations. Proportions over time did not differ between corals (χ^2 goodness of fit test)

vious experiment. However, urchins died with signs inconsistent with the currently known clinical presentation of DaSc. No spine loss was observed and spines ceased moving. The pattern of mortality was unrelated to whether corals were qDaScPc+ or qDaScPc− (Fig. 6). Despite this, DaScPc was detected by qPCR in 5 out of 7 *D. setosum* introduced to qDaScPc+ corals, and to only 1 out of 7 *D. setosum* introduced to the qDaScPc− corals. Of qDaScPc+ urchins, Sanger sequencing revealed 4 that were within DaScPc, while the remaining 2 matched related *Philaster* spp. previously observed in corals. Amongst these 4 confirmed DaScPc urchins, 3 died within 96 h of incubation. Three of the 4 mortalities (1 incubated with *M. cavernosa* and 2 with *S. siderea*) were from qDaScPc+ corals, while 1 (incubated with *O. faveolata*) was qDaScPc−. DaScPc was also detected in coral tissue DNA extracts (by qPCR) in 1 of 7 corals identified as qDaScPc+ by swab at experiment initiation, while it was also detected in 2 of 7 corals identified as qDaScPc−.

4. DISCUSSION

4.1. Presence in environmental/sympatric habitats

The overall pattern of DaScPc detection following our approach suggests that the higher qPCR signal in the field survey, confirmed by 18S rRNA sequences of qDaScPc+ samples, was driven by the coral *Siderastrea siderea*, turf algae, and the sponge *Ircinia campana* (Fig. 7). The discrepancy between detection of DaScPc by qPCR, pan-*Ciliophora* primers in conventional PCR, and scutico-634F + 1147R primers in conventional PCR may relate to varying stringency of each primer set. The qPCR primer and probe were designed around a 28S rRNA gene transcript recovered from a DaSc-affected *Diadema antillarum* transcriptome (Hewson et al. 2023), which may be more conserved across ciliates within the Philasteridae than the scutico-634F primer. At the same time, DaScPc occurs as part of a diverse community of ciliates which may outnumber this taxon, hence it is not expected to dominate amplicons by using the pan-*Ciliophora* primers.

The surfaces of corals, turf algae, and sponges may be characterized by elevated dissolved organic matter (DOM) and inorganic nutrient conditions. Heterotrophic bacteria and archaea consume DOM and regenerate it into inorganic nutrients, and so high DOM concentrations support larger bacterial abundances (Ducklow 1983). Since DaScPc may also consume bacteria, they may therefore thrive in environments rich in DOM. The greatest DaScPc 28S rRNA copy numbers and proportion of qDaScPc+ samples of samples tested occurred at sites where DaSc was first observed in January 2022, but also at sites that had been previously affected by DaSc but sampled ~4 mo after the original outbreak.

Corals produce mucus on their tissue surfaces which supports microbial metabolism and plays a role in feeding and silt removal (Bhagwat et al. 2023). We observed differences in DaScPc detection between sampled phyla that may be related, at least in part, by their biology and ecology. Amongst hexacorals examined in the field survey, *Siderastrea* spp. was the most frequently detected and had the highest DaScPc quantities of any genus examined. *S. siderea* often forms mucus strings that are visible on its surface (Sekar et al. 2008), which fosters elevated microbial diversity and richness compared to other corals (Kellogg et al. 2014, Clark et al. 2021). Considering DaScPc may participate in bacterivory, we speculate that the elevated microbial activity surrounding the mucus on the coral surface could be a preferred sub-

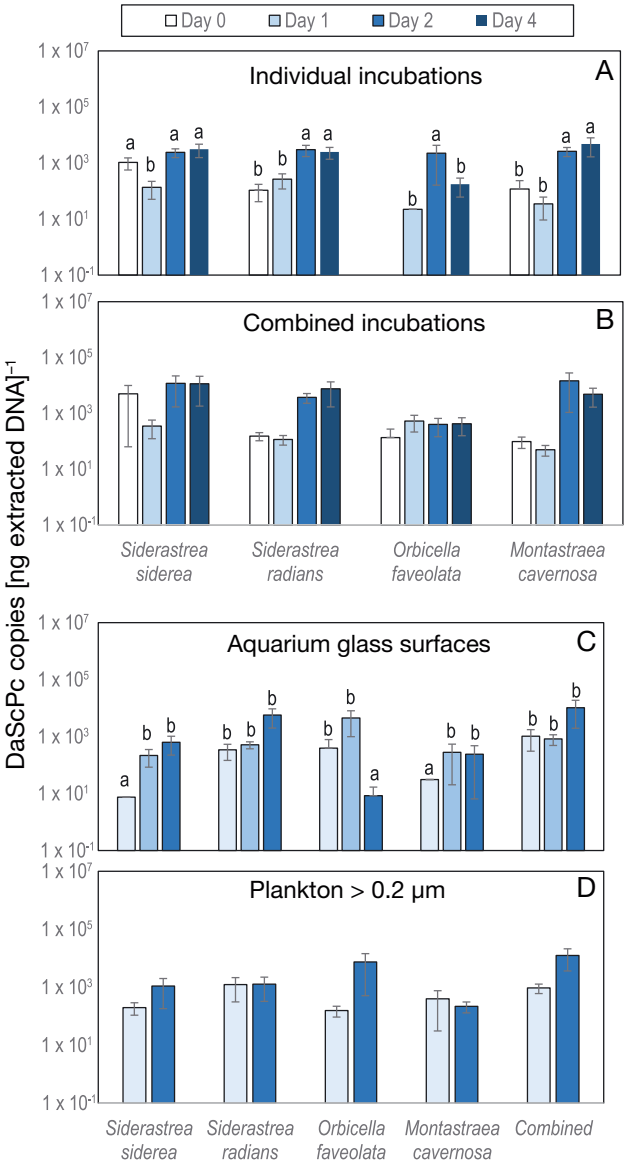

Fig. 5. Summary of DaScPc abundance in the coral recruitment experiment as determined by quantitative PCR of the DaScPc 28S rRNA gene (Hewson et al. 2023) in (A) individual incubations, (B) combined incubations, (C) aquarium glass surfaces, and (D) plankton. Lowercase letters above bars denote significant differences in the individual coral experiment (Kruskal-Wallis test, $p < 0.05$, df = 16) and on aquarium glass (Kruskal-Wallis test, $p < 0.05$, df = 20). Error bars = SE

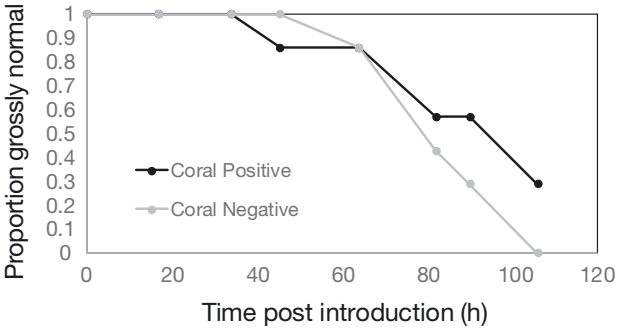

Fig. 6. Proportion of grossly normal *Diadema setosum* over time after introduction to corals identified as qDaScPc+ or qDaScPc−. There was no significant difference between proportions over time (Wilcoxon signed rank test)

strate for the ciliate. Moreover, the DOM exudate composition of *S. siderea* is distinct from other hard coral species and could in turn contribute to preferential recruitment of DaScPc to its surfaces (Weber et al. 2022). DaScPc was primarily detected amongst *S. siderea* from 2 sampling sites, one of which (St. Thomas, US Virgin Islands) was sampled 4 mo after *D. antillarum* mass mortality, and the other (Mango Halto, Aruba) that had dying *D. antillarum* at the time of sampling.

Similarly, macroalgae living on rock or biotic surfaces (turf algae) release DOM as a product of photosynthesis, supporting microbial activity on and adjacent to algal surfaces (Speare et al. 2020). Hence, we speculate that DaScPc may recruit to macroalgal surfaces from surrounding seawater to take up DOM or feed on microorganisms that thrive in these habitats.

Sponges produce DOM through release of wastes from consumed particulate matter filtered from seawater (Fiore et al. 2017). Previous metabolomic studies have demonstrated that *Ircinia campana* produces highly labile DOM, including nucleosides (Fiore et al. 2017). *I. campana* also hosts symbiotic algae in the family Symbiodiniaceae and has one of the highest tissue chlorophyll *a* concentrations of any known reef sponge (Southwell et al. 2008), suggesting that its contributions to DOM release may exceed those of other taxa. Similarly, *I. campana* releases large quantities of inorganic nitrogen into surrounding waters compared to co-occurring sponges (Southwell et al. 2008), which supports primary and secondary production on their surfaces and in adjacent waters. These metabolic traits could provide optimal conditions for DaScPc to persist after urchin disease events.

We hypothesized that DaScPc occurrence in the environment may be related to specific bacterial taxa inhabiting the habitats in which they are found. Bacterial+archaeal communities were more diverse in specimens that had higher DaScPc 28S rRNA copy numbers than those which had low DaScPc 28S rRNA gene copy numbers (Fig. S2). Taxonomic composition of bacteria on surfaces where DaScPc 18S rRNA sequences were recovered varied depending on surface type and location, but DaScPc 28S rRNA gene copy number was not correlated with any specific taxon. Biomass–richness relationships of aquatic microbial communities may follow positive, negative,

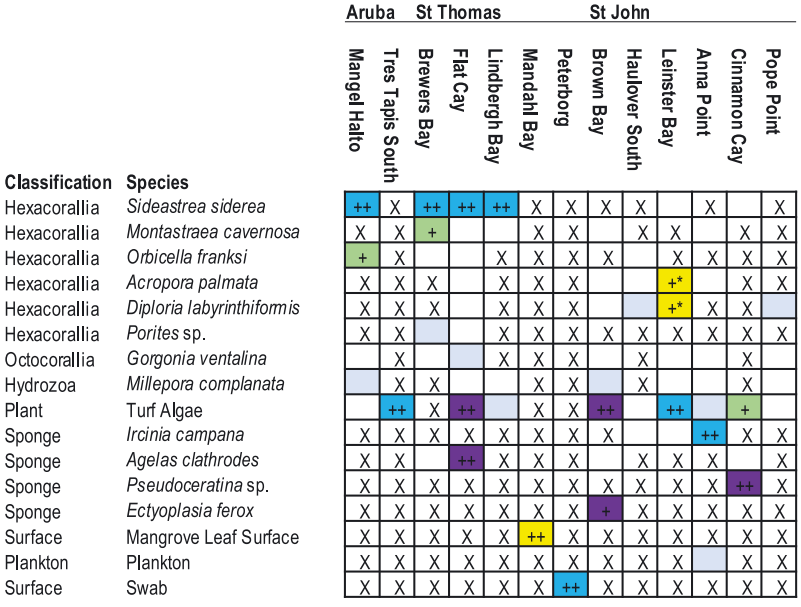

Fig. 7. Summary of DaScPc detection among sequences obtained using the pan-*Ciliophora* and scutico-634F nested PCR. Blue is DaScPc, green is *Philaster* spp., and yellow is unclear phylogeny. Purple = no amplicon from site or species pools but amplification in individual swabs; light blue = no amplification in site pool (and individual not attempted); white = no amplification from individual specimens. ++ = amplification in individual swabs and species pool; + = amplification of site pool and individual swab; +* = amplified in pool but not in individual swabs. Cells corresponding to locations that were not tested for a specific species are marked with an 'X'

hump-, and U-shaped distributions (reviewed by Smith 2007). We speculate that greater bacterial+archaeal diversity in specimens with higher DaScPc 28S rRNA copy numbers may reflect greater bacterial+archaeal abundance in these specimens, which in turn may support their bacterivorous feeding activity. However, the lack of association between specific taxa suggests that this feeding may be non-specific amongst target bacterial or archaeal taxa. Future work may explore how DaScPc responds to cell size, nutritional quality, and physiology of bacteria, since there may be redundancy in these factors between disparate bacterial taxa.

These results open a window on potential ecological drivers of DaScPc in habitats around urchins. The relationship between DOM concentration, bacterial abundance/production, diversity, and DaScPc growth should be validated in future work through empirical assessment.

Aside from their detection on animals and plants, DaScPc was confirmed through both qPCR targeting the 28S rRNA and through direct Sanger sequencing of the 18S rRNA gene amplified from swabs of small boat hulls from a previously affected site months after DaSc had occurred. Boat hulls, especially those with epiphytic or encrusting biofoulants, may serve as mobile environments on which ciliates, including DaScPc, may move between disparate locations (Schaerer et al. 2019). However, more study is needed to determine the longevity of DaScPc in these habitats, or how water movement (e.g. turbulence) affects its persistence. DaScPc has been recorded in urchins collected from Oman (Ritchie et al. 2024), the Red Sea (Roth et al. 2024), and the Western Indian Ocean (Quod et al. 2025), suggesting that it has moved into different regions through unidentified vectors. The locations at which DaSc was observed in the Caribbean (e.g. Grenada, Puerto Rico, and St. Thomas) experience widespread boat traffic and tourism. It is worth noting that stony coral tissue loss disease (SCTLD) was first reported in St. Thomas at the same site as DaSc (Brandt et al. 2021, Hewson et al. 2023). While the transmission vectors of both DaSc and SCLTD are currently unknown, it is plausible that similar mechanisms of transmission may occur for both conditions.

4.2. DaScPc was not detected in coral specimens prior to 2022

A key question in DaSc ecology is the provenance of this taxon. While diseases may manifest from dysbiotic events favoring parasitic infections by typically commensal organisms (Kelly et al. 1994, Egan & Gardiner 2016), diseases may also represent novel introductions to a region (e.g. *Haplosporidian nelsonii* MSX; Burreson et al. 2000). Our survey of 128 coral tissue samples and water samples around corals collected before 2022 did not yield DaScPc 18S rRNAs in any specimen. Furthermore, comparison of the 18S and 28S rRNAs of DaScPc against metagenomes at IMG-MER yielded no sequences within the DaScPc. Given that DaScPc was detected in this study in swabs of corals from regions actively or previously affected by DaSc, this result suggests DaScPc likely was not present in the Caribbean prior to the 2022 DaSc outbreak but may have recruited onto these surfaces after its initial introduction. However, this survey was based on a limited suite of available samples and public sequence data sets, so it may have been present in

other compartments or those not sampled at the time. Searching the Transcriptome Shotgun Assembly (TSA) archive at NCBI, 28S rRNA genes sharing 99.36% nucleotide identity to DaScPc were recovered from the bat star *Patiria miniata* in an aquarium study performed in southern California in 2016 (accession GHJN010795562.1) (Gildor et al. 2019). That study incubated sea stars in water from the Port of Long Beach, suggesting that ciliates within the genus *Philaster* were present in southern California at the time. Subsequent surveys of sea stars and urchins near San Diego did not detect DaScPc (Hewson et al. 2025). Because evolutionary rates of scuticociliate 28S rRNAs are not yet constrained, it is not possible to definitively identify these as DaScPc, since hits may have been to highly conserved loci within the large subunit operon.

4.3. Recruitment to corals

We further examined the ability of DaScPc to recruit to hexacoral surfaces in experimental mesocosms, testing the hypothesis that *S. siderea* would recruit more of the scuticociliate than other coral species. While *S. siderea* had the greatest DaScPc 28S rRNA copy number and prevalence amongst corals at experiment outset, over time these were no different from controls. Interestingly, we frequently observed DaScPc on aquarium glass surfaces and in plankton, with the highest abundances in aquaria containing all 4 coral species. This may suggest that DaScPc may grow on materials released from the corals in plankton, and since they are found on animal and plant surfaces, they may persist on abiotic surfaces in general.

Persistence of DaScPc on corals over time is also unclear from our data. While other philasterine ciliates have been associated with coral diseases, such as brown band disease (Randall et al. 2015, Ravindran et al. 2022), our work does not provide evidence to suggest that DaScPc is associated with any coral abnormalities. None of the corals in the experimental mesocosms that recruited DaScPc became abnormal over the course of the experiment. Likewise, although several corals that were swabbed in the field had diseases, including stony coral tissue loss disease, the detection and DaScPc 28S rRNA copy number were inconsistent with disease occurrence. Finally, bleached corals affected by the 2023 Caribbean heatwave did not yield DaScPc, suggesting that its occurrence is not tied directly to stressed/diseased corals.

4.4. Risk to surviving and reintroduced *Diadema*

A key question raised by detection of DaScPc in sympatric habitats is whether these ciliates may then reemerge to infect surviving or newly introduced *D. antillarum* at previously affected sites. Our data are inconclusive in this regard. While there was successful transmission of DaScPc from qDaScPc+ corals to *D. setosum* in aquaria, it is still unclear if their death was associated with the ciliate or coral response byproduct since their gross signs were mostly inconsistent with DaSc. Specimens did not lose spines over time, and in most specimens, the tube feet were intact at the time of their death, both characteristic signs of DaSc (Hylkema et al. 2023). Instead, it is likely that their death was caused either by antagonistic interactions with the corals (i.e. materials they exuded), or by unmeasured adverse water quality parameters in the aquariums. Several approaches are currently being developed or already used to restore *D. antillarum* populations (Hylkema et al. 2022, Williams 2022, de Breuyn et al. 2023, Wijers et al. 2023). These efforts could be in vain if DaSc reemerges. Due to the prevalence of DaScPc on marine surfaces after disease occurrence detected here, we suggest that testing for this pathogen should be included in measures taken to verify urchin health before restocking efforts are implemented. Furthermore, establishing the etiology of scuticociliatosis in wider Diadematidae species should be prioritized, since this has only been demonstrated in *D. antillarum* (Hewson et al. 2023) and *D. setosum* (Ritchie et al. 2024).

4.5. Limitation of methods

The study has limitations which may preclude definitive interpretation of results. The use of swabs in this survey, which were employed because of ease of collection and permitting requirements, may underestimate total DaScPc abundance, since swabs generally have poor retention of free-living microbial cells (Bruijns 2024). Furthermore, there may be differences in recovery between sample type (e.g. macroalga, coral, sponge, etc.) which may have influenced the relative detection of DaScPc between these surfaces. The use of qPCR for the detection of DaScPc inevitably can amplify related *Philaster* species, requiring further confirmation by conventional PCR and Sanger sequencing. While we considered detection of DaScPc through 2 loci, namely 28S rRNA for qPCR and 18S rRNA for conventional PCR, where sequences obtained from the latter were coherent

with the pathogenic culture FWC2, these may represent strains that vary in their ecology and pathogenicity within *D. antillarum*. Hence, future efforts should focus upon confirmation of their identity through other molecular markers, perhaps through multilocus sequence tagging, to confirm their presence. PCR and qPCR may also detect intact DNA in moribund or dying cells, hence detections may not represent live DaScPc cells. Uneven distribution of sample sizes between sites and surfaces led to differences in detection thresholds and can affect overall detection accuracy. The data collected are limited by the retrospective nature of the study, where samples are mostly limited to periods shortly after the 2022 DaSc outbreak began or were sampled opportunistically well after the DaSc outbreak had abated. These limitations highlight the need for cautious interpretation of the results and highlight areas for future work to address these methodological shortcomings and increase awareness of the ecology of DaScPc and its impact on coral reef health. Despite these limitations, our results demonstrate that DaScPc may persist in coral reef habitats for some time after DaSc occurrence and provide new information on the ecology of this pathogen.

It is also important to note that while we infer that DaScPc occurrence may be related to DOM-rich environments, directly measuring DOM was beyond the scope of this study. DOM is produced by an array of sources, including phototroph exudates, metazoan egestion and excretion and sloppy feeding, and viral lysis of (micro)organisms. While we report the DOM excretion characteristics of several metazoa on which DaScPc occurs, this phenomenon should be further investigated by directly testing these exudates, which can be chemically characterized, on DaScPc. Finally, our examination of DaScPc reemergence potential in naïve *D. setosum* was based solely on gross observations and qPCR detection. Employing histopathology approaches (Hewson et al. 2023) may have observed DaScPc within tissues prior to their mortality, which occurred in the absence of DaSc gross signs.

5. CONCLUSIONS

This study suggests that the *D. antillarum* pathogen DaScPc may occur on submerged surfaces at sites currently or previously affected by DaSc, especially around its putative locations of emergence in the Caribbean (St. Thomas and St. John), and that the ciliate can be recovered in surveys from hexacorals (especially *S. siderea*), sponges, and plants. We found no evidence for the presence of DaScPc in Caribbean coral samples recovered prior to 2022 despite similar *Philaster* spp. sequences recovered from previously published transcriptomes and through direct amplification of DNA extracts. Furthermore, DaScPc culture FWC2 recruits on at least 4 species of corals in the region and provides support that at least 1 coral species is less likely to harbor the cultured isolate. Moreover, DaScPc-FWC2 appears to recruit to metazoan surfaces, at least in aquarium settings. These data fortify that DaScPc may be a novel introduction to the Caribbean at the time of DaSc mass mortality, and that the taxon may persist in surrounding habitats for some time after affecting *D. antillarum*. Furthermore, our data suggest that DaScPc inhabiting corals may colonize naïve urchins. However, its ability to infect and cause mortality remains unclear. It is also unclear whether associations with metazoans other than *D. antillarum* are mutualistic, commensal, or parasitic. We propose that the DaScPc may have a life strategy which is partially derived from DOM or microbes thriving on exudates, and that it may become pathogenic in *D. antillarum* under as yet undefined conditions. We base this proposal on observation that DaScPc thrives in habitats previously described to have high DOM, bacterial richness, and diversity. However, there is still a need to understand how and why DaScPc infects urchins, which may be due to factors such as population density (which may affect transmission potential), physicochemical conditions, and animal stress, or material excreted from the corals under stress that may attract and support DaScPc.

Data archive. Nucleic acid sequence data associated with this work have been deposited in NCBI GenBank under accessions PP851979—PP852041. Data for 16S rRNA amplicons have been deposited at EBI under accessions PRJEB76 500 and ERP161028.

Acknowledgements. We are grateful to the Virgin Islands National Park, Jendahye Antione, Davis Stroebel, Kevin McCartney, Cayman Islands Department of Environment, Fundación Parke Nacional Aruba, Shen Jean Lim, Saba Conservation Foundation, Ricardo Bourdon, and Daniel Kasin, for assistance in fieldwork and sample processing. Specimens collected in the Florida Keys were taken under permit SAL-23-2564-SR from Florida Fish and Wildlife Conservation Commission and FKNMS-2023-058-A1 from the Florida Keys National Marine Sanctuary. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government. This work was supported by NSF OCE- 2049225 (I.H.), NSF OCE-2228940 (D.C.B), a David R. Atkinson Center for Sustainable

Futures rapid response grant (I.H.), and US Geological Survey Ecosystems Mission Area Biological Threats and Invasive Species Research Program (C.A.K.).

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Editorial responsibility: Scott C. Burgess, Tallahassee, Florida, USA
Reviewed by: E.R. Schmeltzer and 2 anonymous referees
Submitted: July 4, 2024; Accepted: November 26, 2024
Proofs received from author(s): January 24, 2025

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