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TITLE

Unlocking the phylogenetic diversity, primary habitats, and abundances of free-living Symbiodiniaceae on a coral reef

RUNNING TITLE

The habitats of diverse free-living Symbiodiniaceae

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ABSTRACT

Dinoflagellates of the family Symbiodiniaceae form mutualistic symbioses with marine invertebrates such as reef-building corals, but also inhabit reef environments as free-living cells. Most coral species acquire Symbiodiniaceae horizontally from the surrounding environment during the larval and/or recruitment phase, however the phylogenetic diversity and ecology of free-living Symbiodiniaceae on coral reefs is largely unknown. We coupled environmental DNA sequencing and genus-specific qPCR to resolve the community structure and cell abundances of free-living Symbiodiniaceae in the water column, sediment, and macroalgae and compared these to coral symbionts. Sampling was conducted at two time points, one of which coincided with the annual coral spawning event when recombination between hosts and free-living Symbiodiniaceae is assumed to be critical. Amplicons of the internal transcribed spacer (ITS2) region were assigned

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to 12 of the 15 Symbiodiniaceae genera or genera-equivalent lineages. Community compositions were separated by habitat, with water samples containing a high proportion of sequences corresponding to coral symbionts of the genus *Cladocopium*, potentially as a result of cell expulsion from *in hospite* populations. Sediment-associated Symbiodiniaceae communities were distinct, potentially due to the presence of exclusively free-living species. Intriguingly, macroalgal surfaces displayed the highest cell abundances of Symbiodiniaceae, suggesting a key role for macroalgae in ensuring the ecological success of corals through maintenance of a continuum between environmental and symbiotic populations of Symbiodiniaceae.

KEYWORDS

Symbiodiniaceae, free-living, ITS2, qPCR, habitat, benthic, epiphytic

INTRODUCTION

Dinoflagellates of the family Symbiodiniaceae are key primary producers in marine ecosystems (LaJeunesse et al., 2018). The Symbiodiniaceae evolved to live in symbiosis with numerous marine organisms from foraminifera to scleractinian corals (Carlos, Baillie, Kawachi, & Maruyama, 1999; LaJeunesse, 2002; Pochon, Montoya-Burgos, Stadelmann, & Pawlowski, 2006; Rowan & Powers, 1991), but also can maintain a “free-living” lifestyle as part of (i.e. “transiently” free-living) or their entire (i.e. “exclusively” free-living) life history (Cunning, Yost, Guarinello, Putnam, & Gates, 2015; Granados-Cifuentes, Neigel, Leberg, & Rodriguez-Lanetty, 2015; LaJeunesse, 2002; Takabayashi, Adams, Pochon, & Gates, 2012; Yamashita & Koike, 2013). The family Symbiodiniaceae was recently divided into 15 lineages of which nine are formally described as genera (LaJeunesse et al., 2018; Nitschke et al., 2020), comprising probably hundreds of species (LaJeunesse et al., 2014; Thornhill, Lewis, Wham, & LaJeunesse, 2014). To date, most

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of the major lineages within the Symbiodiniaceae have been detected in reef environments (Cunning et al., 2015; Quigley, Bay, & Willis, 2017), although how this community interacts with and influences host-symbiont associations on reefs is poorly understood (Nitschke, Davy, & Ward, 2016; Quigley et al., 2017; Yamashita, Suzuki, Kai, Hayashibara, & Koike, 2014).

Free-living Symbiodiniaceae are essential for the 80-85% of coral species (Baird, Guest, & Willis, 2009; Harrison, 2011) that establish endosymbiotic relationships anew each generation (Coffroth, Lewis, Santos, & Weaver, 2006; Nitschke et al., 2016), a process referred to as horizontal transmission. During coral dispersal, horizontal transmission may aid the acquisition of regionally-adapted symbiont species or genotypes suited to the local environment (Ali et al., 2019; Baird, Cumbo, Leggat, & Rodriguez-Lanetty, 2007; Cumbo, Baird, & Van Oppen, 2013; Howells et al., 2012; Lajeunesse et al., 2004; Schwarz, Krupp, & Weis, 1999). Indeed, symbiont identity can modify the substrate preference of settling coral larvae (Winkler, Pandolfi, & Sampayo, 2015), and exposure of coral recruits to benthic sediment enhances the establishment of symbioses (Adams, Cumbo, & Takabayashi, 2009; Ali et al., 2019; Cumbo et al., 2013; Nitschke et al., 2016; Quigley et al., 2017) and can alter the photobiology of coral recruits (Quigley et al., 2017). Such recombination may also occur in adult corals during the rise of opportunistic species following perturbation (Berkelmans & Van Oppen, 2006; Grottoli et al., 2014; LaJeunesse, Smith, Finney, & Oxenford, 2009), yet whether such observations represent a rearrangement of community compositions already present within the coral (Jones, Berkelmans, van Oppen, Mieog, & Sinclair, 2008), the acquisition of exogenous symbionts from the free-living community (Boulotte et al., 2016), or some combination of the two, is an open question. As such, it is clear that uncovering the ecological roles of free-living Symbiodiniaceae is essential to understand the functioning of coral reefs.

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Whilst *in hospite* Symbiodiniaceae diversity has been examined extensively, there are comparatively few studies characterising *ex hospite*, habitat-associated Symbiodiniaceae. Culturing techniques and "symbiont samplers" (i.e. symbiont-free larvae that are at the stage of symbiont acquisition) have been used to capture free-living Symbiodiniaceae from the water column above reefs (Coffroth et al., 2006; Coffroth, Santos, & Goulet, 2001; Gómez-Cabrera, Ortiz, Loh, Ward, & Hoegh-Guldberg, 2008; Thornhill, Michael, LaJeunesse, Schmidt, & Fitt, 2006) and from sediments (Carlos et al., 1999; Hirose, Reimer, Hidaka, & Suda, 2008; Nitschke et al., 2020), with a range of molecular techniques (e.g. Restriction Fragment Length Polymorphism [RFLP], Denaturing-Gradient Gel Electrophoresis [DGGE]) used to characterise genetic diversity. However, most Symbiodiniaceae are recalcitrant to culturing (Coffroth, 2005; Krueger & Gates, 2012; Santos, Taylor, & Coffroth, 2001), and not all free-living Symbiodiniaceae can initiate symbiosis (Coffroth et al., 2006; LaJeunesse, 2002; Yamashita et al., 2014), therefore these early studies likely underestimated diversity. Next generation sequencing (NGS) has revolutionised community-level assessments of marine dinoflagellates, uncovering extensive cryptic biodiversity (Hume, Mejia-Restrepo, Voolstra, & Berumen, 2020; Kohli, Neilan, Brown, Hoppenrath, & Murray, 2014; Stern et al., 2010) and is increasingly utilised to profile *in hospite* Symbiodiniaceae community structure (Boulotte et al., 2016; Camp et al., 2020; Hume et al., 2020; Thomas, Kendrick, Kennington, Richards, & Stat, 2014; Ziegler et al., 2017). Few studies have leveraged NGS for free-living Symbiodiniaceae characterisation. Notably, Cunning et al. (2015) retrieved Symbiodiniaceae sequences from *Symbiodinium*, *Cladocopium*, *Durusdinium*, *Fugacium* and *Gerakladium* with 1,186 Operational Taxonomic Units (OTUs) across *in hospite* and free-living habitats (water and sediment samples) in American Samoa. Quigley et al. (2017) reported 1,562 phylogenetically diverse OTUs on the Great Barrier Reef (Australia), but only 10.6% of

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sediment-associated Symbiodiniaceae partnered with coral juveniles. As the identity of endosymbiotic Symbiodiniaceae can change across the coral ontogeny (Abrego, Van Oppen, & Willis, 2009), we require detailed information of the temporal dynamics in Symbiodiniaceae community structure, and from a wider range of reef habitats that may serve as potential reservoirs of symbionts.

We applied NGS to samples from coral reef habitats of Heron Island in the southern region of the Great Barrier Reef (GBR), where *in hospite* Symbiodiniaceae diversity has been extensively characterised (Fisher, Malme, & Dove, 2012; Gardner et al., 2017; LaJeunesse et al., 2003; Sampayo, Dove, & LaJeunesse, 2009; Stat, Loh, Hoegh-Guldberg, & Carter, 2008; Stat, Loh, LaJeunesse, Hoegh-Guldberg, & Carter, 2009). Two previous studies of free-living Symbiodiniaceae at Heron Island reported *Cladocopium* on macroalgal surfaces (Venera-Ponton, Diaz-Pulido, Rodriguez-Lanetty, & Hoegh-Guldberg, 2010) and *Symbiodinium* and *Cladocopium* in reef water and sediment (Sweet, 2014). Our sampling focused on two key time periods; (i) during coral spawning where host-symbiont recombination events are likely to be common, and (ii) in summer, when water temperatures are at their peak. We paired Symbiodiniaceae diversity assessments with quantitative PCR (qPCR) estimates of cell abundances using genus-specific rDNA (28S) assays (Yamashita, Suzuki, Hayashibara, & Koike, 2011; Yamashita, Suzuki, Hayashibara, & Koike, 2013) for the first quantitative molecular ecology study of free-living Symbiodiniaceae.

MATERIALS AND METHODS

Collection of coral and environmental samples

Sampling was conducted on the reef-flat of Heron Island (HI), southern GBR, Australia (23.26°S,

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151.54°E). Three sites (located 5-20 m from shore, depth ca. 1 m; SI.1, Figure S1) were selected and each comprised of a small (1-2 m diameter) mono-specific cluster of coral colonies so as to isolate local, host-specific overlap with the free-living Symbiodiniaceae community: Sites 1 - *Acropora aspera*; 2 - *Montipora digitata*; and 3 - *Pocillopora damicornis*. These three species are common on the GBR (Madin et al., 2016), particularly widespread on the shallow reef flat of HI (Nitschke et al., 2018), and typically form symbioses with three significant evolutionary radiations of *Cladocopium* [C3, C15, and C1/C42, respectively, sensu LaJeunesse (2005)]. Sampling was repeated over two time points (referred to as seasons hereafter). First, during late October 2015 approaching a period of coral spawning [5th November 2015; Gissi, Stauber, Reichelt-Brushett, Harrison, and Jolley (2017)], referred to hereafter as "spawning". Second, during mid-March 2016 in late summer, referred to hereafter as "summer". Sea-surface temperatures (SST) and photosynthetically available radiation (PAR) contrasting these seasons are available as Supplementary Information (SI.1, Figure S2). To characterise *in hospite* symbiont diversity, reference host samples were collected from each site (*n* = 4 per species per season) as small fragments (~2 cm), which were rinsed with 0.2 µm filtered seawater (FSW) and stored at -80°C until analysis.

Seawater, benthic sediment, and macroalgae samples (*n* = 4 per sample type per site and per season) were collected for free-living Symbiodiniaceae profiling. Seawater (10 L) was collected ca. 1 m from individual coral colonies at each site. Water samples were pre-filtered through a 20 µm nylon filter (NITEX®, SEFAR, Heiden, Switzerland) to remove large particles and cells were concentrated using gentle vacuum onto a 3 µm polycarbonate filter (Isopore™, EMD Millipore, MA, USA). Cells were resuspended 10 times with FSW to collect all filtrate, and microscopy was used to confirm that no microalgae-like organisms remained on the filter. The final suspension

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was concentrated at 2000 *g* for 5 min and the pellet stored at -80°C until analysis. A wet volume of 25 mL of the surface layer (~1 cm) of sediment near to a coral colony (ca. 1 m) at each site was collected using a sterile 50 mL centrifuge tube, with the excess seawater decanted. To account for disturbance during sediment scooping, 25 mL of interstitial sediment water was collected by inserting a custom sediment corer (3 cm diameter) capped with a modified syringe 2 cm deep into the sediment. The interstitial water was drawn through the isolated sediment core into the syringe and combined with the sediment sample for a total volume of 50 mL. Finally, ~50 g of the macroalgae *Padina* sp. was collected from benthic substrates adjacent to corals into a sterile sample bag. *Padina* was selected as it was present across all sites and seasons, and free-living Symbiodiniaceae have previously been detected as associated with this alga at HI (Venera-Ponton et al., 2010). Both sediment and macroalgae samples were re-suspended in 1 L of FSW and shaken vigorously 50 times to dislodge surface-attached Symbiodiniaceae. Each suspension was passed in a series through 250, 125, 63 μm stacked sieves, a 20 μm nylon filter, and then a 3 μm polycarbonate filter to capture particles containing Symbiodiniaceae cells. Samples were immediately processed and stored as above.

DNA extraction and next generation sequencing

Total DNA was extracted from both host and environmental samples using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) on an automated QIAcube (QIAGEN) instrument, according to the manufacturer instructions with modifications; specifically, (i) 2 × Buffer ATL and Proteinase K, (ii) digestion at 56°C overnight for host samples and 3 hrs for environmental samples, (iii) and the DNA eluted twice to a final volume of 100 μL with Buffer AE. Extraction negative controls (consisting of no sample) were processed at the same time as the samples.

A master mix for fusion tag primers, consisting of Illumina adaptor and sequencing primers,

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barcode indexes unique to this study, and the template-specific primers, were prepared on an automated QIAgility (QIAGEN) instrument in an ultra-clean facility at Curtin University. The ITS2 primer set, ITSD [5′-GTGAATTGCAGAACTCCGTG-3′; Pochon, Pawlowski, Zaninetti, and Rowan (2001)] and ITS2rev2 [5′-CCTCCGCTTACTTATATGCTT-3′; Stat, Pochon, Cowie, and Gates (2009)] that targets the partial 5.8S, entire ITS2, and partial 28S region of nuclear ribosomal DNA of Symbiodiniaceae, was used for amplicon sequencing. PCR reagents consisted of 1 × GeneAmp® PCR Gold Buffer (Life Technologies, CA, USA), 2 mM $MgCl_2$, 0.25 µM dNTPs, 10 µg BSA, 5 pmol of each primer, 0.12 × SYBR® Green (Life Technologies), 1 Unit AmpliTaq Gold® DNA polymerase (Life Technologies), 2 µL of DNA, and Ultrapure™ Distilled Water (Life Technologies) made up to 25 µL. Amplification of target DNA was performed in a single round of polymerase chain reaction (PCR) on duplicates of each sample on an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA) under the following conditions: initial denaturing at 95°C for 5 min, followed by 35-38 cycles (for endosymbiont samples) or 45 cycles (for environmental samples which were assumed to be substantially less abundant than endosymbiont samples) of 30 sec at 95°C, 30 sec at 52°C, and 45 sec at 72°C, and a final extension at 72°C for 10 min. Duplicates originating from each sample were combined prior to amplicon pooling and library preparation.

The amplicon library was prepared by pooling PCR products into equimolar ratios based on qPCR C_T values and ΔRn values, as well as quantification using a Labchip® GX Touch HT (Perkin Elmer, MA, USA). PCR negative controls were performed on the blank DNA extraction controls and included in the final library. Amplicons were size-selected using a Pippin Prep (Sage Science, MA, USA) and purified using the QIAquick PCR Purification Kit (QIAGEN). The volume of purified library added for sequencing was determined using a Labchip® GX Touch HT, and

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sequenced using a 500 cycle MiSeq® v2 Reagent Kit and standard flow cell (2 × 250 paired end) on an Illumina MiSeq platform (Illumina, CA, USA) at Curtin University.

ITS2 sequences of Symbiodiniaceae cultures

Total genomic DNA was extracted from 14 culture strains of Symbiodiniaceae (Table 1) from the genera *Fugacium*, *Symbiodinium*, and *Durusdinium* using the Wizard DNA prep protocol (Promega, WI, USA). Extracted DNA was submitted to MRDNA (Molecular Research LP, TX, USA) and amplicon sequencing (bTEFAP®) was performed using the same primer set as above, on the Illumina Miseq platform (Illumina, CA, USA) with 2 × 300 bp chemistry.

Bioinformatic analysis

Libraries were demultiplexed and primers were trimmed from raw sequences using cutadapt (Martin, 2011). DADA2 (Callahan et al., 2016) was used to denoise sequences and generate amplicon sequencing variants (ASVs) using the recommended ITS2 workflow. Each batch of samples (e.g. environmental samples from each season, cultures) were run through DADA2 separately, and then merged using the *mergeSequenceTables* function of DADA2. The effect of sequencing depth on the generation of ASVs was investigated with rarefaction curves. ASVs were assigned a taxonomic identity using the RDP classifier (Wang, Garrity, Tiedje, & Cole, 2007) against a custom reference database. To aid in removal of non-Symbiodiniaceae sequences (e.g. non-target dinoflagellates), a subset (one sequence per genus) of a previously published general ITS2 database (Ankenbrand, Keller, Wolf, Schultz, & Förster, 2015) was combined with a Symbiodiniaceae-specific database enriched with additional Dinophyceae sequences (see github.com/nitschkematthew/Symbiodatabaceae to reproduce this reference file). The default RDP bootstrap value of 50 was used to assign taxonomic ranks to an ASV; below this threshold the rank was set to 'unassigned'. Non-Symbiodiniaceae sequences were at this point removed prior to

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any further analyses. Importantly, the reference Symbiodiniaceae sequences assigned to study Symbiodiniaceae ASVs reported below are indicative of closest matches only (e.g. ASV5|C15_AY239369).

Symbiodiniaceae diversity estimates from ITS2 sequences are confounded by intragenomic variation [IGV, sensu Thornhill, Lajeunesse, and Santos (2007)]. However, repeat observations of co-occurring sets of IGVs (analogous to DGGE bands) can be used to define operational units, i.e. 'defining intra-genomic variant profiles' [DIV profiles, sensu Hume et al. (2019)]. To detect overlap between Symbiodiniaceae in coral hosts and free-living Symbiodiniaceae in reef environments at a level that incorporates IGV, we submitted the raw ITS2 sequences retrieved from coral samples to Symportal (Hume et al., 2019), a dynamically generated database of cooccurring Symbiodiniaceae DIVs from previous studies (Camp et al., 2020; Gardner et al., 2019; Hume et al., 2020). To search for complete sets of DIV sequences (as defined by DIV profiles) in our environmental samples, we reprocessed the raw sequences following the Symportal quality control documentation [using the Mothur bioinformatic pipeline (Schloss et al., 2009)]. Briefly, paired ends were joined and sequences containing ambiguous bases or homopolymers runs (exceeding 5 bases) were removed. Singleton and doublet sequences were also removed and sequence length filtering (to a minimum of 184 and maximum of 310 bases) applied. As above, we used the RDP classifier to identify and remove non-Symbiodiniaceae sequences, and then used exact sequence matching of Symportal DIV sequences to the study sequences. Symportal performs an *in silico* PCR using the SymVar primer pair (Hume et al., 2018). As such, Symportal DIV sequences were shorter than the study sequences, which were generated with the ITSD and ITS2rev2 primers (see above). Exact matching produced sequence super-sets that contained polymorphisms in regions flanking the SymVar DIVs, the abundances of

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which were summed towards the total abundance of each DIV.

For *Fugacium*, *Durusdinium*, and *Symbiodinium*, the top three DADA2 ASVs by abundance from each sequenced culture were defined as the culture-specific DIVs. Finally, ASVs not present in coral hosts or cultures were visualised as sequence-similarity networks (SSN), with inter-ASV and ASV-reference sequence similarity computed using a k-mer (k = 7) approach using the R package kmer (https://github.com/shaunpwilkinson/kmer). Sequence similarities are reported as the k-similarity (%), or in terms of total base pair mismatches following sequence alignment [using the R package DECIPHER, *AlignSeqs*, Wright (2016)].

Quantitative analysis of free-living Symbiodiniaceae cell abundance

The abundance of free-living *Cladocopium*, *Durusdinium*, *Breviolum*, *Fugacium*, and *Symbiodinium* was quantified in each habitat using qPCR with genus-specific primer sets targeting 28S rDNA (Yamashita et al., 2011). Other genera were not quantified either because they were absent from NGS data or material to produce standard curves was not available. Each primer set was first tested against monoclonal culture strains (SI.2). An aliquot of 10 mL from each strain (*n* = 3 cultures per strain) was collected during exponential growth and DNA was extracted with the same protocols as described above, but these were performed manually. Cell abundances used for rDNA copy-number normalisation were calculated using a haemocytometer and light microscope (ECLIPSE Ni-E, Nikon, Tokyo, Japan) with an automated capturing system (NIS-Elements Advanced Research, version 4.30, Nikon, Tokyo, Japan) as previously reported (Fujise et al., 2018; Suggett et al., 2015).

To check the efficiency and detection limit of each primer set, serial dilutions of culture DNA were prepared for five concentrations (approx. 0.1, 1, 10, 100, and 1,000 cells per reaction; exact

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cell numbers in each standard are shown in SI.2) and distributed to 384 well plates (HSP3805, BIO-RAD, CA, USA) on an automated epMotion® 5075i (Eppendorf, Hamburg, Germany) along with master mix for each primer set. qPCR reagents included 2.5 µL of iTaq™ Universal SYBR® Green SuperMix (BIO-RAD), 0.4 µM of each forward and reverse primer, 2 µL of DNA, and 0.1 µL of Ultrapure™ Distilled Water (Life Technologies). All qPCR assays were performed in triplicate on a CFX384™ Real-Time System (BIO-RAD). To check the specificity of each primer set, approx. 1,000 cells per reaction derived from other non-target genera were also subjected to qPCR. According to the specificity and efficiency tests, genera-specific qPCR assays were produced (see SI.1 for all assay conditions).

DNA extraction efficiency and PCR inhibition were also tested across water, sediment and macroalgae samples to ensure comparability in qPCR-retrieved cell numbers. A suspension of a known cell number of *Effrenium* were added to samples collected from three different habitats and total DNA was extracted as outlined above. A qPCR assay was performed using *Effrenium*-specific primers with undiluted, 0.25 and 1.25 ng μL^{-1} of DNA. The C_T values as compared to a standard of DNA extracted from *Effrenium* culture confirmed the absence of PCR inhibition at concentrations of 0.25 ng μL^{-1} of DNA for all substrates. At 1.25 ng μL^{-1} of DNA, the absence of PCR inhibition was confirmed for water and macroalgae samples. Similar DNA extraction efficiencies ranging between 94-100 % across three substrates were obtained.

The final qPCR assay to quantify free-living Symbiodiniaceae cells consisted of either 0.25 or 1.25 ng μL^{-1} template DNA and master mix for each primer set, with each sample run as a 5 µL reaction in triplicate in 384 well plates as above. Serial dilutions of DNA (as above) from congeneric cultures (one per genus) were used as quantification standards (see SI.2). According to

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the specificity and efficiency assays, minimum quantification ranges (MQR) were determined (see SI.2). Cell abundances occurring below the MQR were excluded from further analysis.

Cell concentrations were first normalised against sample volume (cells mL^{-1}) for water and sediment (Littman, van Oppen, & Willis, 2008) and against sample weight (cells g^{-1} wet weight) for macroalgae (Aligizaki & Nikolaidis, 2006; Kim, Yih, Kim, Myung, & Jeong, 2011). For cross-habitat comparison, macroalgae sample normalisation required conversion from wet weight (g) to volume (cm^3) using the relationship between wet weight (g) per unit of surface area (m^2) at 66.4 g m^{-2} for *Padina pavonia* (Mercado, Gordillo, Figueroa, & Niell, 1998). A boundary layer thickness of 350 µm was then assumed [data for *Padina australis* from Hofmann, Fink, Bischof, and de Beer (2015)] as a proxy for habitat volume for epiphytic Symbiodiniaceae, although it is currently unknown how tightly associated Symbiodiniaceae cells are to macroalgal thalli (Porto, Granados, Restrepo, & Sanchez, 2008).

Statistical analysis

To examine Symbiodiniaceae community structure across free-living habitats we used a permutational multivariate analysis of variance (PERMANOVA) approach. PERMANOVA [vegan, *adonis*, Oksanen et al. (2007)] was performed on weighted UniFrac (Lozupone & Knight, 2005) distance matrices. The weighted UniFrac distance incorporates information on both abundance and phylogenetic relatedness between members of a community. The UniFrac phylogenetic tree was produced by first dividing ITS2 ASV sequences into intra-genus subsets and aligning them [DECIPHER, *AlignSeqs*, Wright (2016)], and then alignments were converted to pairwise distances [seqinr, *dist.alignment*, Charif and Lobry (2007)]. As inter-genus ITS2 Symbiodiniaceae sequences are typically too variable for alignment, we substituted the mean 28S inter-genus distances from Nitschke et al. (2020) into the matrix [similar to Claar, Tietjen, Cox,

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Gates, and Baum (2020)], and from this a phylogenetic tree was generated [phangorn, *upgma*, Schliep (2011)]. ASV abundances were Hellinger transformed and the weighted UniFrac distances computed in phyloseq (McMurdie & Holmes, 2013). PERMANOVA was performed on UniFrac distances with variance partitioned across habitat (water, sediment, macroalgae), site (sites 1-3), and sampling period (spawning, summer), defined in the model in this order. As not all samples were sequenced to sufficient depth for analysis (any samples with less than 250 Symbiodiniaceae reads were removed from the dataset) the design was unbalanced which reduces the power of PERMANOVA to separate treatment effects from dispersion around group centroids. Variable dispersion between levels of each factor were tested [vegan, *betadisper*], and where a factor was significant in PERMANOVA yet also had significant differences in dispersion, a bootstrapping analysis was conducted to assess the impact of design imbalance (Claar et al., 2020). We randomly subsampled each group to match the sample size of the smallest group, and re-ran the PERMANOVA. This was repeated 100 times, and factors only interpreted if significance was maintained across all random subsets. Differential abundance between habitats and seasons were tested at the genus level using DeSeq2 (Love, Huber, & Anders, 2014) with the Wald test and a parametric fit of dispersion around group means.

qPCR-based cell abundances for *Cladocopium*, *Durusdinium* and *Symbiodinium* were analysed using ANOVA, whereas *Breviolum* and *Fugacium* were either absent from the sample or below the MQR, and as such were excluded. Normality of residuals were visually inspected in a QQ-plot. However, due to these data failing to meet assumptions of homoscedasticity of variance in a Levene's test, separate Welch's one-way tests of the effects of habitat and season on Symbiodiniaceae cell abundances were performed. Games Howell pairwise comparisons tests, which are suitable in the absence of homoscedasticity, were used to evaluate within-group

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differences. Symbiodiniaceae genus was not examined as a factor and all tests were performed on within-genus cell abundance data. These within-genera comparisons make the assumption that the intra-genus copy number is consistent across species and comparable to the cultures selected to produce the assay DNA standards. All of the bioinformatic and statistical analyses were conducted in R (version 3.6.1) and code to reproduce all output is available at Github (github.com/nitschkematthew/Free_living_Symbiodiniaceae_HI).

RESULTS

Symbiodiniaceae ASV community structure

ITS2 sequencing yielded ca. three million Symbiodiniaceae sequences across culture, host, and environmental samples after demultiplexing, quality filtering (there were no Symbiodiniaceae sequences present in the negative controls), and removal of non-Symbiodiniaceae sequences (which accounted for 54% of the library). From HI samples (excluding the cultures), a total of 533 Symbiodiniaceae ASVs were assigned to 12 genera [or genera-equivalent clades (LaJeunesse et al., 2018)]: *Cladocopium*; *Symbiodinium*; *Durusdinium*; *Gerakladium*; *Freudenthalidium*; *Fugacium*; Foraminifera-specific Clade D (referred to as Foraminifera D hereafter); Clade I, *Breviolum*; *Halluxium*; Clade Fr4; and Clade Fr2, in order of greatest to least ASV abundance (Figure 1). Of the ASVs from the 12 sediment samples from Summer, despite significant sequencing depth, 79.68 to 99.46% were assigned to the family Prorocentraceae, and only four of these 12 samples were included in further analyses of ASVs. No single Symbiodiniaceae ASV was found in common across all environmental samples, however ASV5 (assigned to *Cladocopium* C15_AY239369), was always observed in water samples. During spawning, ASV13, ASV33, and ASV17 (assigned to *Cladocopium* C3*_HM031101, *Cladocopium* C1_AF195144, and *Durusdinium* D1_DQ838545, respectively) were also present in every water sample.

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PERMANOVA analysis of environmental samples identified habitat and season as significant factors (Table 2), whereas site was not significant. Of the two-factor and three-factor interactions, only habitat × season was significant (Table 2). As significant differences in group dispersion were detected across habitats (betadisper F = 7.47, P = 0.001), with sediment containing greater dispersion due to separation by season (Figure 1), boot-strapping was conducted to determine if the habitat and season effects were robust to randomised subsampling. Habitat was significant in 100%, season in 42%, and their interaction in 95% of boot-strapped PERMANOVAs. Several genus-level differences in abundance were evident (adjusted P < 0.05, SI.3); Foraminifera D, *Fugacium*, and Clade I were elevated in the sediment compared to the water, and *Freudenthalidium* and Foraminifera D were elevated in the sediment compared to macroalgae. *Cladocopium* and *Durusdinium* however were elevated in the water and macroalgae relative to the sediment. Seasonal differences were also evident, with *Durusdinium*, *Fugacium*, *Freudenthalidium*, and *Halluxium* all more abundant during spawning compared to summer.

***Cladocopium* DIVs across symbiotic and free-living habitats**

To investigate diversity at a resolution that incorporates ITS2 intragenomic variation, we used Symportal to generate DIV profiles of Symbiodiniaceae within coral hosts. Symportal generated five DIV profiles comprising 16 total DIV sequences from the genus *Cladocopium* across *Acropora aspera*, *Montipora digitata,* and *Pocillopora damicornis*; no other Symbiodiniaceae genera were observed within these samples (full Symportal output is available as Supplementary Material [SI.4]). The DIV profiles of *A. aspera* (which, according to Symportal, comprised 81-92% of the total sequences in each sample) were C50b/C50p-C3-C50f-C3bm-C3dt (two replicates) or C50b-C50p-C3 (two replicates) during spawning, however during summer only the

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C50b/C50p-C3-C50f-C3bm-C3dt profile was detected across all replicates. One DIV profile, C15-C15dq-C15dr-C15ch, was produced from *M. digitata* across both seasons comprising 89-93% of the sequences in each sample. For *P. damicornis*, two profiles were produced; C42a/C1-C42.2-C1b-C1j-C1au (55-83% of the sequences in each sample) was dominant in all samples during spawning, however an additional profile of C42.2/C1-C1b-C1au (67% of the sample) was produced from a single sample in summer.

Complete sets of *Cladocopium* DIV sequences (as defined by Symportal generated DIV profiles) were retrieved from environmental samples (Figure 2). For example, all DIVs of the C50b/C50p-C3-C50f-C3bm-C3dt profile were recovered from water samples during spawning, with C50b and C50p in greater abundances than the minor DIVs, consistent with what is found in *A. aspera*. In summer however, the least abundant DIV C3dt was absent from all but two water samples. In all sediment samples the C50b/C50p-C3-C50f-C3bm-C3dt profile was incomplete. In 10 out of 24 macroalgae samples all DIVs of this profile were recovered, however the C3 DIV was always highest in abundance in contrast to what is found in *A. aspera* (Figure 2). The C15-C15dq-C15dr-C15ch profile was complete in all water samples across both seasons, and in 10 out of 12 and 5 out of 12 macroalgae samples in Spawning and Summer, respectively. The C15 DIV sequence was always in greatest abundance as expected based on sequences recovered from *M. digitata*. A full set of DIVs from the C42a/C1-C42.2-C1b-C1j-C1au profile was not retrieved from any single environmental sample (Figure 2). Notably, the C42a DIV, which is co-dominant with the C1 DIV in *P. damicornis*, was absent in 60 out of 64 environmental samples, whereas the C1, C42.2, and C1b DIV sequences were typically recovered (e.g. 100% of water samples during Spawning). Across all sediment samples, while multiple *Cladocopium* DIV sequences were retrieved, there were no completed DIV profiles.

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Fugacium, Symbiodinium, and Durusdinium DIVs across cultures and free-living habitats

Each culture of *Fugacium* and *Symbiodinium* was dominated by a single DADA2 generated 'DIV' that made up 74.2% to 95.6% of the sample total (Figure 3), with the exception of Isolate_28 (an uncharacterised *Symbiodinium* sp) which had co-dominant DIVs (Figure 3). For *Durusdinium* however, all three cultures had comparatively balanced DIV profiles, with the first and second DIVs comprising 64.5% and 19.8%, 61.6% and 24.7%, and 43.2% and 33.4% of the relative abundances in CCMP2556, RB7, and RD03, respectively. In CCMP2556 and RB7, ASV17 and ASV36 are exact matches to the D1 and D4 sequences *sensu* LaJeunesse et al. (2014). All cultures contained rare DIVs in the range of ca. 1% relative abundance. DIVs from five of the 14 Symbiodiniaceae cultures were detected in the free-living habitats (Figure 3): CCMP2434 (*Fugacium* sp), CCMP2592 (a *Symbiodinium* sp with the ITS2 A5 majority sequence), Isolate_29 (similar to *Symbiodinium microadriaticum* with an ITS2 A1 majority sequence), and CCMP2556 (similar profile to RB7, *Durusdinium trenchii*).

From *Fugacium*, the numerically dominant DIV of CCMP2434 (ASV15|F_AF333516) was detected in two water samples during spawning, and in two sediment samples; one during spawning and one during summer. In all instances ASV15|F_AF333516 was at background abundances. The second CCMP2434 DIV (ASV25|F_AF333516) was also detected alone in a single macroalgae sample during summer, however this was exceptionally rare, totalling 14 reads. From *Symbiodinium*, the numerically dominant DIV (ASV28|A5_AF333508) of CCMP2592 was detected in water and sediment samples during spawning, but only in macroalgae samples during summer (Figure 3). Neither of the minor CCMP2592 DIVs (ASV472|A5_AF333508 or ASV668|A5_AF333508) were detected in free-living habitats. The numerically dominant DIV of

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Isolate_29 (ASV29|A1_AB097463) was common in water and macroalgae samples in both spawning and summer, forming up to 33.1% of individual samples (Figure 3). As for CCMP2592, neither of the minor DIVs from Isolate_29 were detected. The top two numerically abundant DIVs from *D. trenchii* (ASV17 and ASV36) were detected together in 41% of the water samples during spawning. In other habitats and across both seasons, ASV17 occurred alone and without the other *D. trenchii* DIVs.

Intra-genus sequence similarities and abundances of Symbiodiniaceae ASVs

Symbiodiniaceae ASVs not present in corals or cultures were visualised in a SSN with reference sequences assigned by the RDP classifier (Figure 4). The relative abundance values reported below represent the community compositions pooled across habitats in each season after removal of the host-specific sequences and the culture DIVs described above. A further 21 ASVs not present in any of the three corals were assigned to *Cladocopium*, with 18 of these forming a densely-linked cluster with a high percentage of sequence similarity amongst ASVs and reference sequences (Figure 4; e.g. ASV613 which is an exact match for C66_AY589771). The most abundant of these were assigned to reference sequences such as C_AY624604 (previously reported from *Acropora aspera* at HI), C_EF428340, and C_AF195153, (*Cladocopium* sequences that do not have alphanumeric ITS2 designators), but also to reference sequences such as C35a_FJ529584, which was previously reported for another common HI coral, *Stylophora pistillata*.

ASV1352 was assigned to ITS2 sequence H7 of the recently described genus and type species *Halluxium pauxillum*, and was linked to a second ASV (ASV5214) at 99.2% k-similarity (1 bp difference). *Halluxium* were primarily associated with sediment samples (Figure 4). ASVs

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assigned to Clade Fr2 were also abundant in sediments, however all had a k-similarity < 90 % amongst ASV-reference comparisons (the closest match was 10 bp different to F2b_AJ29152). Eight ASVs were assigned to *Freudenthalidium*. *Freudenthalidium heronense* (F3.7) and *Freudenthalidium endolithicum* (F3.8) formed distinct networks made up of three and two high-similarity ASVs (>98% similarity to reference sequences or 1-3 bp difference), respectively. *Freudenthalidium* were found in all habitats across both seasons, and comprised 15.8% of the sediment community during spawning. A cluster of eight out of the 12 total Clade Fr4 sequences were assigned to F4.6_AM748586, however in all instances sequence similarities between ASVs and reference sequences were low (82-88% similarity, 23-27 bp difference) and were rare in abundance across the study (up to 3% relative abundance of macroalgae communities in summer). 20 ASVs were assigned to *Fugacium* and while they were also rare across the study (< 5% relative abundance), they were diverse and formed 4-5 clusters with high levels of sequence similarity to reference sequences (e.g. ASV3816 is an exact match to F5.1a_AM748591). *Breviolum* was exceptionally rare across the study (Figure 4) with only two ASVs assigned to this genus (both assigned to B1_DQ838547, with ASV210 an exact match and ASV4606 at 98.2% similarity or 2 bp difference), and all but four reads were detected in macroalgae samples. Clade I was also rare (< 3% relative abundance), and all 10 ASVs (which formed two clusters in the sequence similarity network) were up to 95% similar (7 bp difference) to reference sequences such as I2_FN561560 and I4_FN561562.

Foraminifera D reference sequences (D1.1_AM748564 and D1.2_AM748617) were assigned to six ASVs, however, sequence similarities were amongst the lowest in the study (< 65%). The D1.1 and D1.2 reference sequences are longer than typical ITS2 sequences [e.g. D1.1_AM748564 is 382 bp in length (including the 5.8S and 28S motifs)], and our study sequences have four regions

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indicative of deletions (among other mismatches) when aligned to this reference. These ASVs formed a single cluster in the network (Figure 4) and were typically rare, comprising up to 6.3% of the sediment community during spawning. All five of the remaining *Durusdinium* ASVs from free-living habitats formed a single cluster in the network with high sequence similarity to the D1_DQ838545 reference sequence (e.g. ASV496 at 98.9% similarity or 2 bp difference); these formed 10.6% of the water column community during spawning. ASVs assigned to *Gerakladium* were the most consistent members of the study, present at 9.6% to 14.6% relative abundance across all habitats and seasons sampled. *Gerakladium* formed four clusters in the network with high similarity to reference sequences reported from the soft coral *Stereonephthea* (e.g. ASV600 with 96.3% similarity or 3 bp difference to AB253788) and benthic soritid foraminifera (e.g ASV394 with 97.5% similarity or 3 bp difference to G3_AM74860). Finally, 18 ASVs were assigned to the genus *Symbiodinium*, forming seven clusters made up of diverse reference sequences (e.g. A1_AB097463, A3_DQ838546 and A_AF184949) and ASVs. Three of these clusters made up the majority of the community (e.g. ASV207, ASV217, and ASV518) and dominated sediment samples in summer, comprising up to 85.8% of the community (Figure 4).

Quantifying cell abundances of free-living Symbiodiniaceae

Symbiodinium, *Cladocopium* and *Durusdinium* were detected above the MQR, however, *Breviolum* and *Fugacium* were below the MQR, consistent with the low relative abundance of these genera in the ITS2 community compositions. Pooling data across seasons, all three genera exhibited the same pattern of increasing cell abundances from water, to sediment, and to macroalgae (Figure 5). For *Cladocopium*, habitat was a significant factor ($F = 29.55$, $P < 0.001$) with cell abundances at 3.79 ± 1.48 cells/cm^3 in the water, which was significantly lower than those in sediments and macroalgae at 248.28 ± 208.43 and 1079.27 ± 685.85 cells/cm^3,

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respectively (SI.5, Figure 5). There was no effect of season on the abundance of *Cladocopium*, however only one out of 12 sediment samples in summer was within the MQR. For *Durusdinium*, habitat was also a significant factor (F = 26.40, P < 0.0001) with cell abundances at 0.46 ± 0.32, 48.65 ± 51.86 and 89.02 ± 57.54 cells/cm^3 in the water, sediment, and macroalgae respectively with significant pairwise comparisons observed between water and macroalgae (SI.5). A significant effect of season was detectable for *Durusdinium* cell abundance in macroalgae, (F = 15.58, P = 0.002), with decreases from 116.81 ± 59.46 to 47.34 ± 11.02 cells/cm^3 from spawning to summer, respectively. As for *Cladocopium*, only a single sample from the sediment during summer contained *Durusdinium* at abundances within the MQR. The genus *Symbiodinium* was consistent in prevalence compared to *Cladocopium* and *Durusdinium* and was detected in all samples. Habitat was also a significant factor for *Symbiodinium* (F = 132.79, P < 0.0001) with cell abundance at 0.43 ± 0.34, 72.38 ± 30.83 and 173.09 ± 69.76 cells/cm^3 in the water, sediment, and macroalgae respectively (Figure 5), with all pairwise comparisons significantly different (SI.5). *Symbiodinium* cell abundance on macroalgal surfaces significantly fell from 233.88 ± 42.46 to 117.37 ± 31.82 cells/cm^3 from spawning to summer (F = 54.67, P < 0.0001).

DISCUSSION

Free-living Symbiodiniaceae are essential for coral species that establish symbiotic relationships through horizontal transmission (Coffroth et al., 2006; Nitschke et al., 2016). Despite the role free-living Symbiodiniaceae may play in reef formation and function, their diversity and distribution on reefs remain unknown. We provide four key insights into Symbiodiniaceae ecology: first, there is overlap of ITS2 sequences between coral-symbiotic and free-living Symbiodiniaceae communities; second, free-living Symbiodiniaceae communities encompass greater genetic disparity at the genus level compared to scleractinian coral symbionts; third,

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sediment communities are distinct from other habitats, potentially due to exclusively free-living species or links to non-coral hosts; and fourth, macroalgal surfaces harbour abundant Symbiodiniaceae communities, suggesting a previously unknown role in maintaining an exchange between environmental and symbiotic Symbiodiniaceae populations.

Overlap between free-living and symbiotic Symbiodiniaceae communities

The repeatable DIV profiles retrieved from *A. aspera*, *M. digitata* and *P. damicornis* likely correspond to undescribed species of *Cladocopium* 'C3', 'C15', and Pocilloporid-specific 'C1/C42', each of which are members of three separate radiations that have occurred within the genus (LaJeunesse, 2005). These observations align with previous records from the southern GBR (Fisher et al., 2012; LaJeunesse et al., 2003; Sampayo et al., 2009; Stat, Loh, et al., 2009). For example, Stat et al. (2008) used DGGE to produce C42 (and related variants) and C15 ITS2 profiles from *P. damicornis* and *M. digitata*, respectively. Symportal generated two C3 profiles across colonies of *A. aspera*. *Acropora* in the southern GBR are often reported to harbour a 'generalist' C3 (LaJeunesse et al., 2003). Our two C3 DIV profiles could reflect strain-level diversity consistent with two C3 genotypes of chloroplast $psbA^{ncr}$ previously retrieved from *A. aspera* colonies at Heron reef (Hillyer, Tumanov, Villas-Bôas, & Davy, 2016). This *Cladocopium* diversity requires further investigation with additional markers to taxonomically resolve (Hume et al., 2015; Lee, Jeong, & Lajeunesse, 2020).

Utilising Symportal DIV profiles to search for Symbiodiniaceae community overlap across symbiotic and free-living habitats presents some challenges. For example, complete C15-C15dq-C15dr-C15ch profiles were always recovered from water samples, but were never completed in sediments, where instead ITS2 sequences from multiple other Symbiodiniaceae

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genera were abundant (discussed below). It is not possible to know if the minor DIVs of the C15 profile (e.g. C15dr) were absent from sediment samples due to insufficient sequencing depth (i.e. non-DIV templates outcompeted minor DIVs in the PCR reaction), or if they were truly absent from the sample and the partial DIV profile corresponds to a related, but different C15 species or genotype. As other coral species (e.g. *Porites cylindrica* and *Porites lutea*) at HI also harbour *Cladocopium* of the C15 radiation (Fisher et al., 2012; LaJeunesse et al., 2003), and multiple other C15 DIV profiles have been generated on the GBR (Camp et al., 2020), reconciling Symbiodiniaceae identities in symbiotic and free-living habitats ultimately requires complete ITS2 profiles and/or additional genetic markers. To increase the likelihood of recovering complete ITS2 DIV profiles, future studies may look to weight the molar concentrations of sample types according to the expected differences in Symbiodiniaceae species richness during library preparation.

This consideration aside, that complete sets of DIV sequences from the C3 and C15 *Cladocopium* profiles were detected in free-living habitats is strong support for coral-symbiotic/free-living community overlap. The implications of this overlap and that the distribution of hosts may influence free-living Symbiodiniaceae community structure are significant when considering symbiont transmission modes. *Acropora* juveniles often acquire novel Symbiodiniaceae not commonly observed in adults (Abrego et al., 2009; Adams et al., 2009; Cumbo et al., 2013; Gómez-Cabrera et al., 2008; Quigley et al., 2017; Yamashita et al., 2013), and the lag time in reaching the mature (i.e. homologous) symbiosis may be dependent on supply from nearby conspecifics via distribution across free-living habitats (Nitschke et al., 2016). In contrast to *A. aspera*, *M. digitata* transmit symbionts vertically (Stat et al., 2008), and in these cases the symbionts are recalcitrant to culturing [e.g. Krueger and Gates (2012)] and potentially do not

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persist for long outside of hosts (Wang, Meng, Sampayo, Tang, & Chen, 2011). In the present study, the ubiquitous and complete C15 DIV profiles in water samples suggests ample opportunity for cryptic horizontal transmission (Byler, Carmi-Veal, Fine, & Goulet, 2013) of transiently free-living Symbiodiniaceae between species that are otherwise hypothesised to be 'closed' symbioses, which could ultimately contribute to the homogenisation of symbiont genotypes across large spatial scales (Pettay & LaJeunesse, 2013). Strain-level markers (e.g. microsatellites) will be required to track and detect such mixed-mode transmission (Quigley, Warner, Bay, & Willis, 2018) across coral conspecifics.

For *P. damicornis*, 7 out of 8 DIV profiles were defined by codominant C42a/C1 DIV sequences, yet the C42a sequence was almost never recovered from the environment. Instead, the recovered DIVs closely resembled the C42.2/C1-C1b-C1au profile found in a single *P. damicornis* colony. If *P. damicornis* colonies are assumed to be the sources of these free-living Symbiodiniaceae sequences (e.g. through symbiont cell expulsion), it is likely that the C42.2/C1-C1b-C1au profile is more prevalent at HI than expected based on its occurrence in a single host sample in the present study. Sampayo, Franceschinis, Hoegh - Guldberg, and Dove (2007) suggested that site-level differences (at the scale of 2-7 km) are evident in the cooccurrence patterns of C42 sequence variants within *P. damicornis* at HI. As indicated by the lack of C42a in the environment, it is plausible that the near-shore C42a/C1 dominated *P. damicornis* of this study may represent a rare combination of sequence variants compared to what occurs at the reef scale.

The phylogenetic diversity of free-living Symbiodiniaceae

In addition to *Cladocopium*, the Symbiodiniaceae sequences retrieved from free-living habitats traverses the majority of the family with high genetic disparity at the genus level compared to GBR scleractinian coral symbionts. *Symbiodinium* are occasional coral-symbionts in the southern

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GBR (Gómez-Cabrera et al., 2008; Ulstrup, Hill, Van Oppen, Larkum, & Ralph, 2008), and have been previously reported as free-living at HI (Sweet, 2014). DIVs from CCMP2592, an undescribed *Symbiodinium* species isolated more than 20 years ago in the southern GBR from the coral *Heliofungia actiniformis* (Moore, 2006); a culture with A1-like ITS2 sequences similar to *S. microadriaticum*; and a range of additional *Symbiodinium* ASVs were retrieved from free-living habitats. The unusual symbiosis between *Acropora longicyathus* and A1-like *Symbiodinium* in the southern GBR (LaJeunesse, Loh, & Trench, 2009) aligns with our observations of free-living A1-like ASVs at HI. *Durusdinium*, which are also uncommon as coral symbionts in the southern GBR (LaJeunesse et al., 2003; Stat et al., 2008) are reported for the first time as free-living at HI, with multiple DIVs from cultures of *Durusdinium trenchii* detected across all habitats. However, the remaining genetic diversity can only be contextualised when examining the wider community of symbiotic reef organisms. For example, an undescribed species of *Gerakladium* forms symbioses with the sponge *Cliona orientalis* at HI (Ramsby et al., 2017), and here *Gerakladium* were consistently detected in the free-living community. *Breviolum*, which is generally rare in the tropical Pacific and was also rare in the present study, associates with the soft coral *Nephthea* at HI (LaJeunesse et al., 2003).

Other genus-level assignments were to Symbiodiniaceae usually considered as symbionts of large-benthic foraminifera [e.g. *Amphisorus* (Pochon et al., 2001)], which have not been studied extensively in the southern GBR. Of the three *Fugacium* cultures sequenced, only CCMP2434, which was also originally isolated from the southern GBR (Moore, 2006), was detected in free-living habitats. The genera *Halluxium* and *Freudenthalidium* (formerly sub-clade Fr3 and clade H) were recently described from single cell isolates from HI sediments (Nitschke et al., 2020) and are here confirmed as persistent in a benthic habitat. '*Symbiodinium*' subclades Fr2,

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Fr4, '*Symbiodinium*' clade I and '*Symbiodinium*' foraminifera-specific clade D typically had low bootstrap support to reference sequences, suggesting insufficient reference material and a high degree of genetic novelty for these lineages in this location. These potentially represent sequences from free-living sister lineages of those described in symbioses with foraminifera, as recently found for free-living *Halluxium* versus symbiotic *Halluxium* (Nitschke et al., 2020). Altogether, the immense phylogenetic diversity reported here spans tens of millions of years of radiation (LaJeunesse et al., 2018) of which a small fraction of the community are likely to be viable symbionts of corals (Quigley et al., 2017).

Habitat compartmentalisation and temporal shifts

Multivariate community analysis with the ITS2 marker is challenging because unique sequences are not equivalent to species units. We opted for a phylogenetically-informed distance metric (the UniFrac distance) to capture the total between-sample sequence diversity, which hypothetically reflects adaptation to the study environments (Lozupone & Knight, 2005). With this approach variance was significantly partitioned by habitat with water and macroalgae communities exhibiting *Cladocopium* and *Durusdinium* driven overlap, whereas sediment communities were distinct due to greater relative abundances of *Symbiodinium*, *Freudenthalidium*, foraminifera-specific clade D, and *Fugacium*. Symbiodiniaceae are benthic dinoflagellates (Adams et al., 2009; Coffroth et al., 2006; Granados-Cifuentes et al., 2015; Hirose et al., 2008; Nitschke et al., 2020; Quigley et al., 2017; Takabayashi et al., 2012) and sediment appears to be a favourable environment for growth [as seen *in vitro* (Nitschke, Davy, Cribb, & Ward, 2015)]. Such environmental compartmentalisation (Cunning et al., 2015) suggests that Symbiodiniaceae diversification has been stimulated by adaptation to free-living habitats, in addition to their entry into endosymbiotic environments. As sediment has been suggested as the ancestral habitat of the Symbiodiniaceae (Granados-Cifuentes et al., 2015), and multiple Symbiodiniaceae species may be

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exclusively free-living (Granados-Cifuentes et al., 2015; Jeong et al., 2014; Nitschke et al., 2015; Yamashita & Koike, 2013), future studies should seek to identify the traits and genomic hallmarks required for switching between free-living versus symbiotic lifestyles across ecologically and evolutionary relevant timescales.

Our effort to detect temporal changes (spawning versus summer) in the free-living Symbiodiniaceae communities was impacted by non-target amplification of Porocentrales (among other dinoflagellates) in the sediment during summer. From our ITS2 sequencing data alone it is not possible to deduce if Symbiodiniaceae in the sediment were reduced (or absent) in summer, or if a bloom of Porocentrales exhausted sequencing depth, outcompeting Symbiodiniaceae templates to background abundances. However, as *Symbiodinium* and *Durusdinium* were detectable with qPCR primers (discussed below) during summer, the latter scenario seems likely. Regardless, from ITS2 ASVs retrieved from water and macroalgae samples, temporal changes were limited compared to habitat compartmentalisation, which aligns with the only other study to explicitly investigate temporal changes in free-living Symbiodiniaceae (Granados-Cifuentes et al., 2015). Granados-Cifuentes et al. (2015) hypothesised that peak Symbiodiniaceae abundances will co-occur with coral reproduction events. *Symbiodinium* and *Durusdinium* frequently dominate the early symbiont communities of coral juveniles (Abrego et al., 2009; Gómez-Cabrera et al., 2008; Quigley et al., 2017; Yamashita et al., 2014), and *Durusdinium trenchii* is often referred to as a sub-optimal or opportunistic symbiont in regions where it has been introduced or in symbiotic systems where it is considered heterologous (LaJeunesse, Smith, et al., 2009; Matthews et al., 2017). In the present study, *Durusdinium* ASVs significantly comprised a higher proportion of the community during spawning compared to summer, and this was supported by qPCR-generated cell abundances in the macroalgal habitat. Exposure to heterologous symbionts such as *D. trenchii*

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during recruitment may in part explain why up to 3.5 years are required for GBR *Acropora* juveniles to reach a stable symbiosis with homologous symbionts of mature conspecifics (Abrego et al., 2009). Ultimately, a higher temporal resolution of sampling [e.g. monthly (Lee et al., 2016)] across annual cycles in concert with the sampling of corals in various stages of development will be necessary to resolve complex patterns in free-living Symbiodiniaceae abundances, compositions, and interactions with coral hosts.

Macroalgal surfaces harbour an abundant community of Symbiodiniaceae

Cell abundances of free-living *Symbiodinium, Cladocopium*, and *Durusdinium* were obtained using genus-specific primer pairs and found to be the highest for macroalgae samples compared to sediments, and lowest overall in the water column. There are few studies of free-living Symbiodiniaceae abundances and the range of methods employed limit direct comparisons. Littman et al. (2008) utilised microscopy and FlowCAM to contrast sediments and the water column; Yamashita et al. (2013) quantified planktonic Symbiodiniaceae using the same primer pairs used in this study; and Lin, Chen, and Chen (2017) evaluated the abundance of Symbiodiniaceae in aquaria using qPCR. Our results fall within the intermediate range of these observations, but in general align with abundant communities in sediments compared to the water column (Littman et al., 2008). Our approach relies on the assumption that rDNA copy numbers are consistent within Symbiodiniaceae genera. Recent efforts to quantify *in hospite*, genus-specific cell abundances with rDNA primers have also made this assumption (Saad et al., 2020), however this remains to be empirically tested across representative collections of Symbiodiniaceae species.

The highest abundance of free-living Symbiodiniaceae cells was found on the surface of our target macroalgae (*Padina* sp.), in particular those belonging to *Cladocopium*. Macroalgae are thought to be important habitats for dinoflagellates (Aligizaki & Nikolaidis, 2006; Kim et al., 2011; Kohli et

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al., 2014) including Symbiodiniaceae (Porto et al., 2008; Venera-Ponton et al., 2010), with epiphytic microbes typically residing within the algal thalli boundary layer (Noisette & Hurd, 2018; Schaffelke, 1999). Macroalgae provide inorganic nutrients (Wada et al., 2007), a large surface area for attachment (Parsons & Preskitt, 2007), and light attenuation (Porto et al., 2008), and it appears likely that macroalgae play an important, and as of yet untested role as a reservoir of symbionts alongside benthic sediment (Adams et al., 2009; Nitschke et al., 2016). Furthermore, Porto et al. (2008) suggested herbivorous fishes may disperse epiphytic free-living Symbiodiniaceae, and viable Symbiodiniaceae cells (3.2-8.0 × 10^3 cells mL^{-1}) have since been cultured from fish faeces (Castro-Sanguino & Sánchez, 2011). While there are few studies of epiphytic Symbiodiniaceae, the beneficial role that this habitat may play in provisioning corals with mutualistic symbionts contrasts with the generally negative view of macroalgae-coral interactions (McCook, Jompa, & Diaz-Pulido, 2001) and macroalgal removal strategies to aid coral reef restoration efforts (Ceccarelli et al., 2018). Curiously however, the C3 DIV was always the most abundant DIV sequence in macroalgae, which contrasts with its position as a minor DIV following the codominant C50b and C50p DIVs in *A. aspera*. This indicates that macroalgae may not be a source of *A. aspera* symbionts, and that other *Cladocopium* species of the C3 radiation reside on the thalli of *Padina*.

CONCLUSIONS

Recruitment and symbiont acquisition are inextricably bound processes for most coral species and thus the ecology of free-living Symbiodiniaceae is an indirect but important component of coral reef health. Here we uncover a genetically diverse pool of free-living Symbiodiniaceae with communities structured across common reef habitats. By integrating NGS with abundance assays we also highlight how Symbiodiniaceae potentially thrive within macroalgal habitats. Whether

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such patterns are consistent for other reef systems must be explored across a greater range of benthic habitats and environmental conditions to further unlock the intersections between Symbiodiniaceae and coral life histories.

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DATA ACCESSIBILITY STATEMENT

The detailed pipeline including tools used in the bioinformatic analysis for MiSeq sequences can be found on Github (github.com/nitschkematthew/Free_living_Symbiodiniaceae_HI). The raw

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FASTQ files produced in this study have been deposited in the NCBI Sequence Read Archive under the BioProject number PRJNA673006.

AUTHOR CONTRIBUTIONS

Designed research: LF, DJS, MS and MRN. Performed research: LF, DJS, MS, MRN, SGG and SG. Contributed new reagents or analytical tools: LF, MS, MB, TK, NS, and JS. Analyzed data: MRN, LF, TK and SW. Prepared figures: MRN. Wrote the paper: MRN, LF, DJS, MS. All authors contributed to subsequent editorials.

TABLES

Table 1: Symbiodiniaceae culture strains that were subjected to ITS2 amplification and next generation sequencing.

Strain (synonym)	**Genus**	**Species**	**Origin**
CCMP2468 (RT135)	*Fugacium*	*F. kawagutii*	Hawaii (USA)
CCMP2434	*Fugacium*	*Fugacium sp*	GBR (Australia)
CCMP2455 (RT133)	*Fugacium*	*Fugacium sp*	Jamaica
CCMP2430	*Symbiodinium*	*S. tridacnidorum*	GBR (Australia)
CCMP2461 (RT185)	*Symbiodinium*	*S. pilosum*	Jamaica
CCMP2469 (RT80)	*Symbiodinium*	*S. necroappetens*	Jamaica
CCMP2548 (HA3-5)	*Symbiodinium*	*S. natans*	Hawaii (USA)
CCMP2548-2 (HA3-5)	*Symbiodinium*	*S. natans*	Hawaii (USA)
CCMP2592	*Symbiodinium*	*Symbiodinium sp*	GBR (Australia)
Isolate_28 *Symbiodinium sp*	*Symbiodinium*	*Symbiodinium sp*	Unknown

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Isolate_29 *Symbiodinium sp*	*Symbiodinium*	*Symbiodinium sp*	Unknown
CCMP2556	*Durusdinium*	*D. trenchii*	Florida (USA)
RD03	*Durusdinium*	*D. trenchii*	Florida
RB7	*Durusdinium*	*Durusdinium sp*	Barbados

Table 2: Summary of PERMANOVA output, with partitioning of variance in a UniFrac distance-matrix of Hellinger-transformed ASV abundances. The model was specified with habitat (three levels), site (three levels), and season (two levels) as factors, and main and interactive effects with $P < 0.05$ are interpreted as significant (indicated with an *).

Factor	**F.Model**	**R2**	**Pr(>F)**
Habitat	18.36	0.32	0.001*
Site	1.65	0.03	0.113
Season	4.08	0.04	0.006*
Habitat:Site	1.22	0.04	0.229
Habitat:Season	7.09	0.12	0.001*
Site:Season	1.93	0.03	0.062
Habitat:Site:Season	1.03	0.03	0.401
Residuals		0.39	

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FIGURES

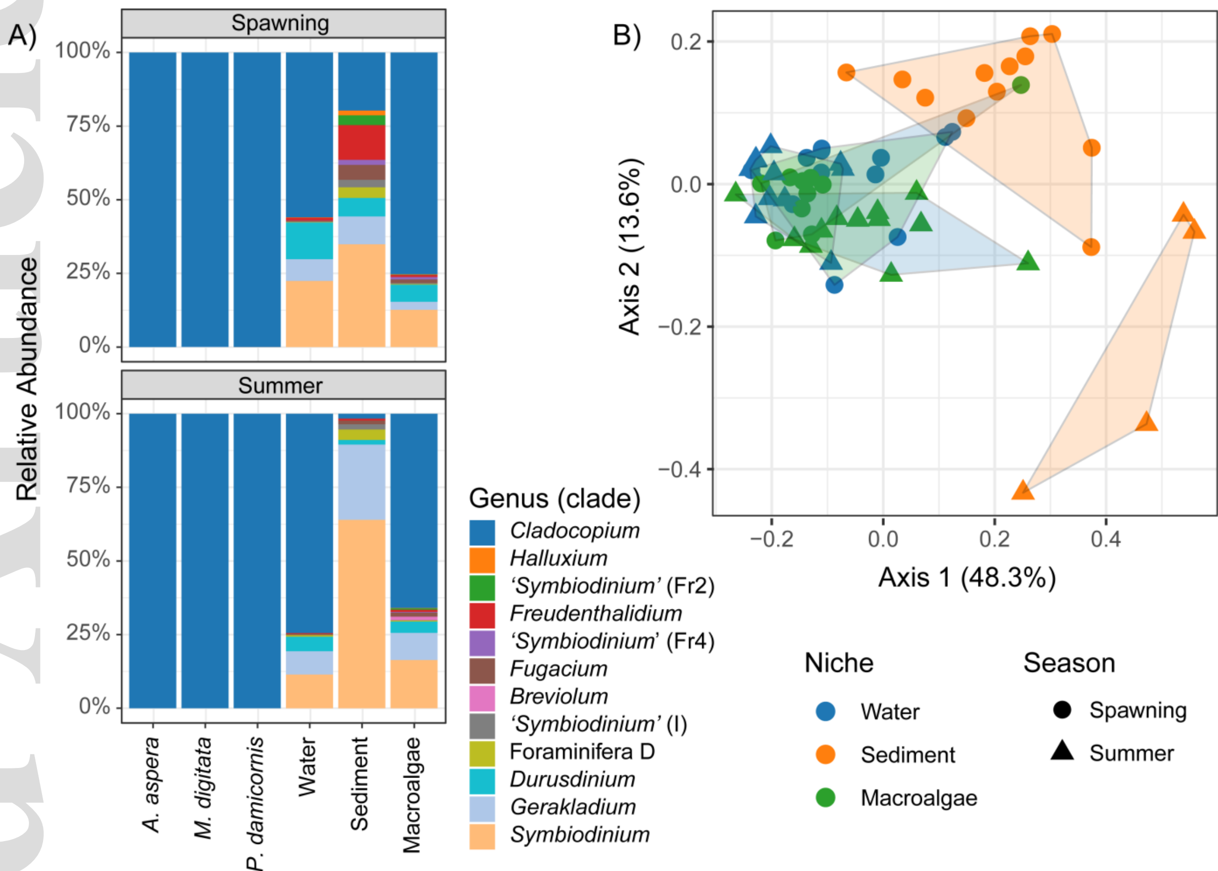

Figure 1: **Symbiodiniaceae community composition at Heron Island.** (A) Relative abundances of Symbiodiniaceae ASVs in each coral species and habitat across two seasons (spawning [October] versus summer [March]), with bars coloured according to genus-level taxonomic assignments. Values represent mean relative abundances across group samples. (B) Principle component analysis of weighted UniFrac distances of Hellinger transformed ASV counts in free-living habitats, in spawning (circles) and summer (triangles).

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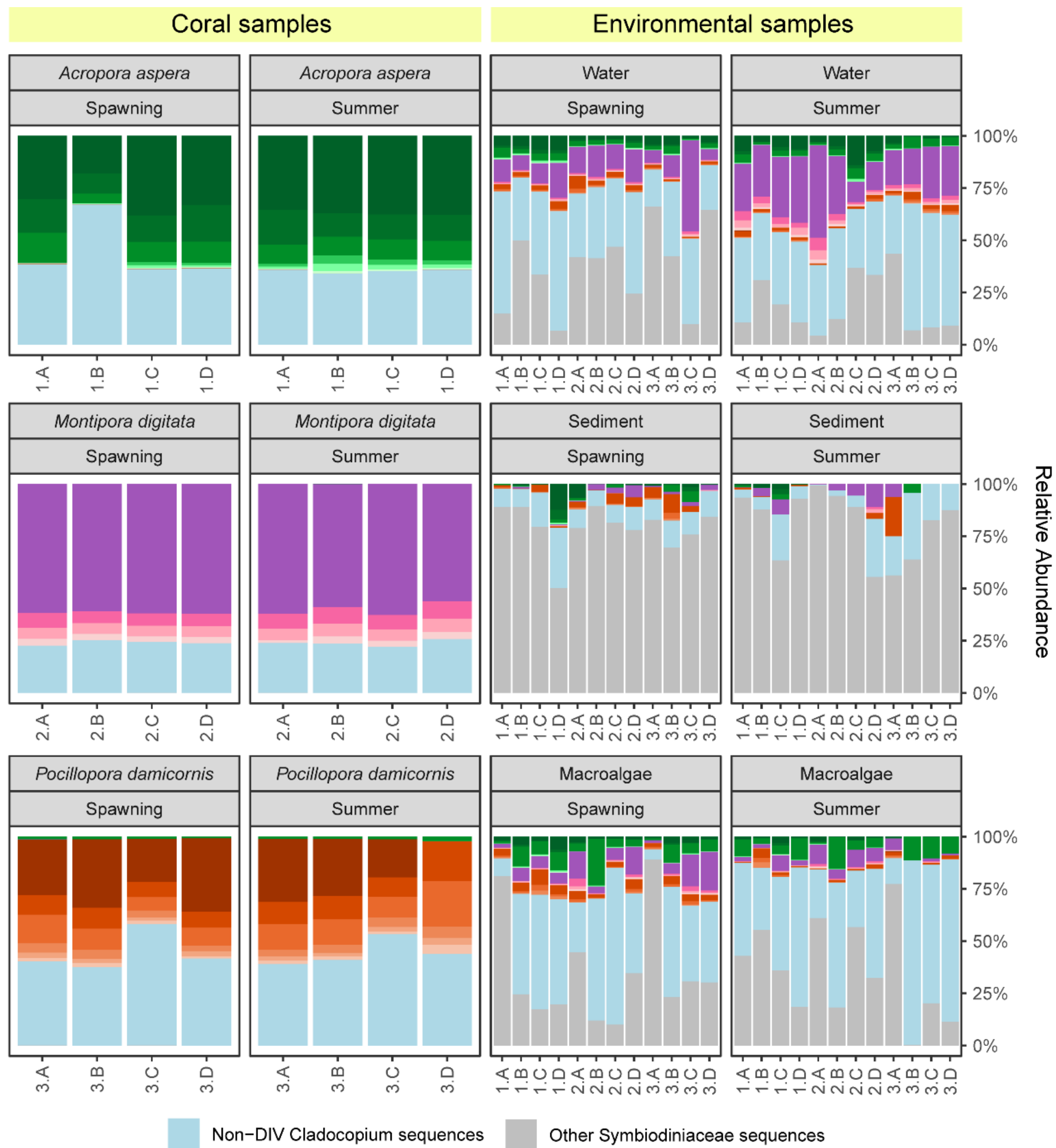

Figure 2: **Symportal DIV sequences in corals and free-living habitats.** *Cladocopium* DIV sequences generated for *Acropora aspera* (green palette), *Montipora digitata* (purple palette), and *Pocillopora damicornis* (orange palette). Values represent relative abundances in each sample (labelled according to site number and replicate, e.g. Site 1, replicate A = 1.A) across each coral species and habitat in spawning (October) versus summer (March). Non-DIV *Cladocopium* sequences are coloured light-blue and all other Symbiodiniaceae sequences from additional genera as grey.

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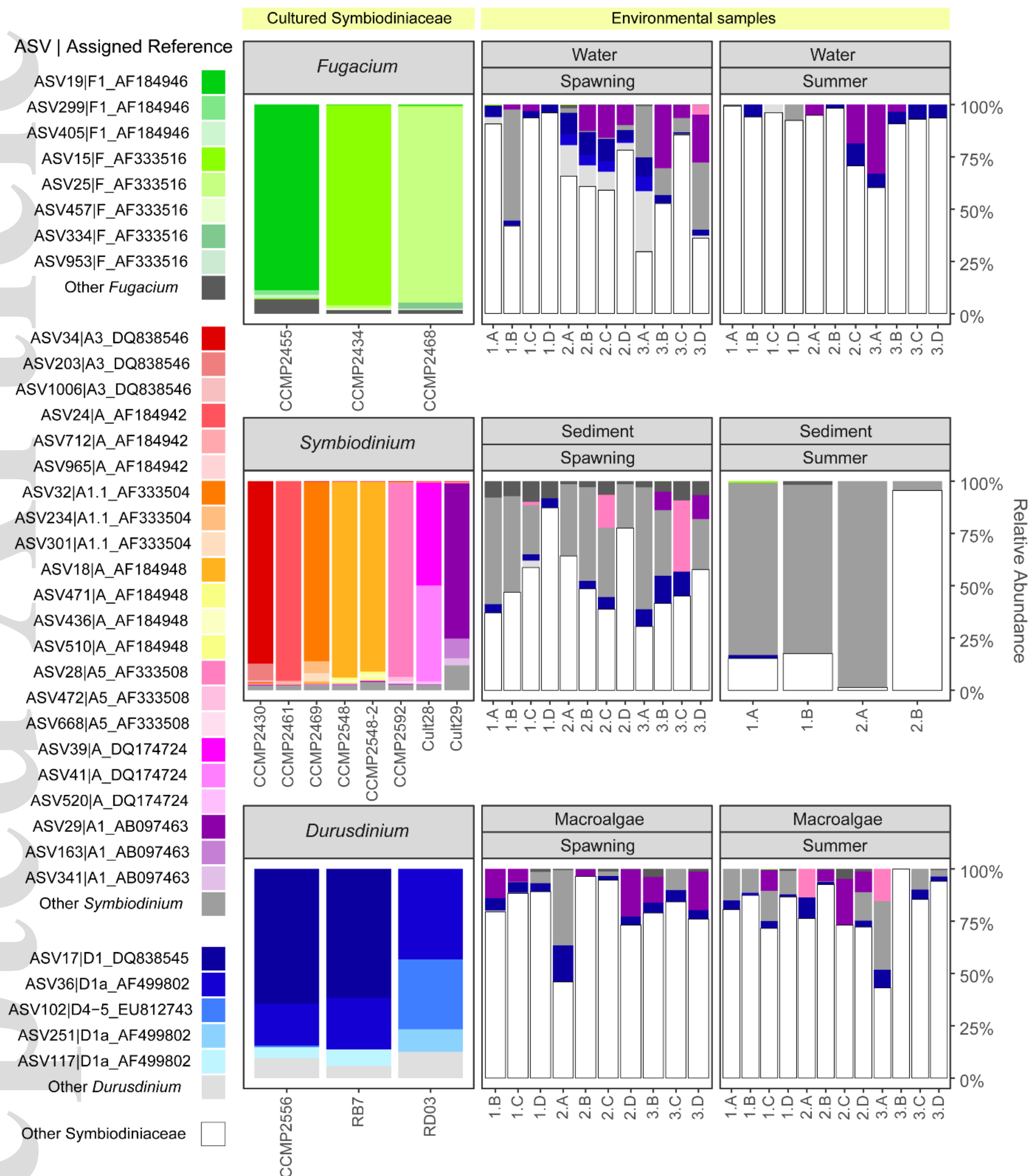

Figure 3: **Culture-based DIV profiles in corals and free-living habitats.** *Fugacium* (green palettes), *Symbiodinium* (red/orange/purple palettes) and *Durusdinium* (blue palettes) DIV profiles generated from cultures. Values represent relative abundances in each sample (labelled according to site number and replicate, e.g. Site 1, replicate A = 1.A) across each culture and habitat in spawning (October) versus summer (March). Non-DIV ASVs from *Fugacium*, *Symbiodinium*, and

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Durusdinium are shaded as dark, medium, and light grey, respectively, and all other Symbiodiniaceae genera as white.

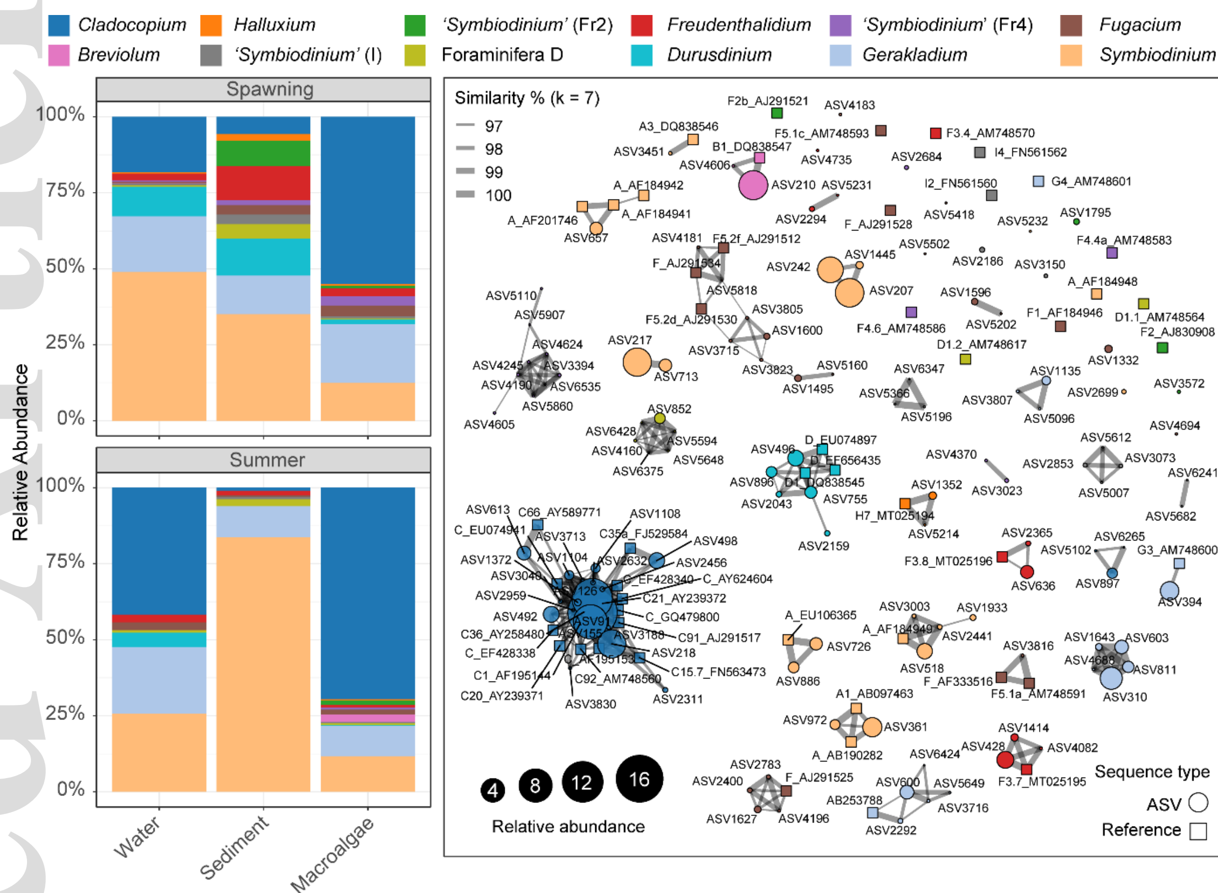

Figure 4: **Relative abundance and sequence similarity of non-DIV Symbiodiniaceae**: Relative abundances (left) of Symbiodiniaceae ASVs in each habitat across two seasons (spawning [October] versus summer [March]), with bars coloured according to genus-level taxonomic assignments. Values represent means across all group samples, after removal of non-DIV ASVs (i.e. all sequences in corals and cultures indicated by figures 2 and 3). Sequence similarity network (right) based on shared k-mers (k = 7) with edge weights (at a cut-off value of 97%) indicating sequence similarity between nodes (circles representing ASV sequences and squares reference database sequences that were assigned to one or more ASVs) which are also coloured by genus. ASV nodes (circles) are area-scaled according to habitat-wide relative abundances.

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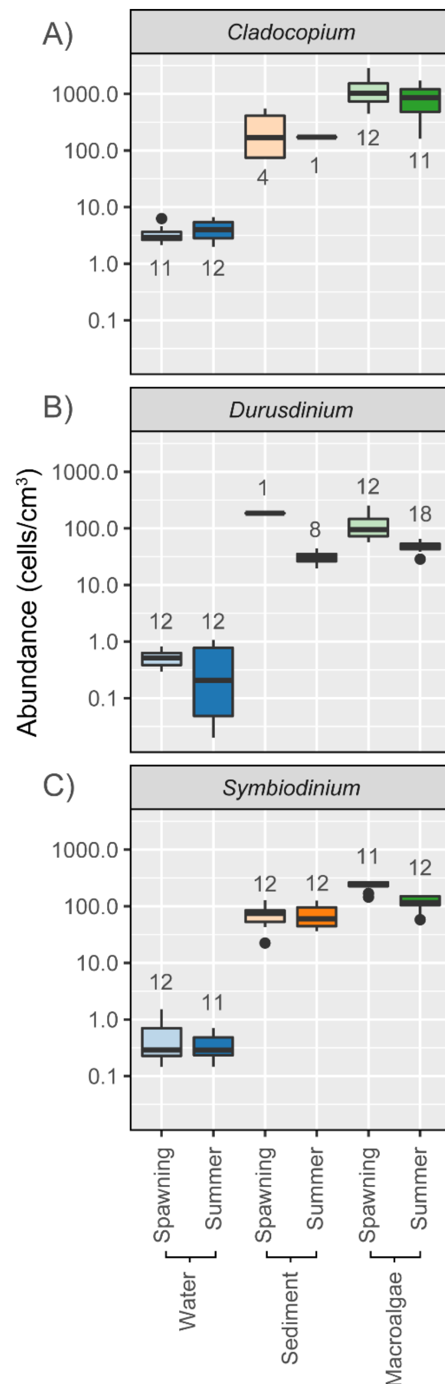

Figure 5: **Cell abundance of free-living Symbiodiniaceae.** Boxplots of Symbiodiniaceae cell abundance (cells/cm^3) for (A) *Cladocopium*, (B) *Durusdinium*, and (C) *Symbiodinium* in water, sediment, and macroalgae in spawning (October) versus summer (March). Lower and upper hinges correspond to the first and third quartiles. The upper and lower whiskers extend from the hinge to the largest and smallest value no further than 1.5 × the inter-quartile range, respectively.

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Outliers are indicated by points, and the number of samples that amplified to within the minimum-quantification range denoted by values adjacent to each box plot.

SUPPLEMENTARY INFORMATION CAPTIONS

SI.1: **Map of sampling locations, environmental data, and qPCR assay conditions.** The specific locations of Sites 1-3 (Figure S1) are provided, along with images of each habitat. Environmental data (Figure S2, sea-surface temperature, light) is used to contrast the two sampling periods (i.e. seasons). A full list of the final qPCR assay conditions are provided (accompanying SI.2).

SI.2: **Quantitative analysis of free-living Symbiodiniaceae cell abundances.** Specificity_tests: Symbiodiniaceae culture strains used for the efficiency and specificity check of the primer sets for qPCR assays. Assay_standards: Cell numbers used to produce culture-specific standard curves. _Raw qPCR data: Sample metadata, Cq values of standards and samples, plus technical replicate means and standard deviations (one sheet per genus, with rows colour coded according to habitat type). Values below the Minimum Quantification Range are highlighted in red. _Normalised data: Sample metadata, and factors (i.e. sample volumes) used to convert cell abundances to cells/cm^3 (one sheet per genus, with rows colour coded according to habitat type).

SI.3: **Differential abundance.** DeSeq2 output for genus-level differential abundance tests between levels of habitat, and then between seasons. Only significant results are shown (adjusted $p < 0.05$).

SI.4: **Complete Symportal output.** All output provided by Symportal.org, including sample metadata, pre and post-MED ITS2 sequences, ITS2 profiles, and count tables.

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SI.5: **qPCR statistical output.** Statistical output of Welch's one-way tests and Games-Howell pairwise comparisons, for the effects of Habitat and Season on Symbiodiniaceae abundances. All tests are conducted individually, genus by genus.

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