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ORIGINAL ARTICLE

Differential specificity between closely related corals and abundant *Endozoicomonas* endosymbionts across global scales

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Reef-building corals are well regarded not only for their obligate association with endosymbiotic algae, but also with prokaryotic symbionts, the specificity of which remains elusive. To identify the central microbial symbionts of corals, their specificity across species and conservation over geographic regions, we sequenced partial SSU ribosomal RNA genes of Bacteria and Archaea from the common corals *Stylophora pistillata* and *Pocillopora verrucosa* across 28 reefs within seven major geographical regions. We demonstrate that both corals harbor *Endozoicomonas* bacteria as their prevalent symbiont. Importantly, catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD–FISH) with *Endozoicomonas*-specific probes confirmed their residence as large aggregations deep within coral tissues. Using fine-scale genotyping techniques and single-cell genomics, we demonstrate that *P. verrucosa* harbors the same *Endozoicomonas*, whereas *S. pistillata* associates with geographically distinct genotypes. This specificity may be shaped by the different reproductive strategies of the hosts, potentially uncovering a pattern of symbiont selection that is linked to life history. Spawning corals such as *P. verrucosa* acquire prokaryotes from the environment. In contrast, brooding corals such as *S. pistillata* release symbiont-packed planula larvae, which may explain a strong regional signature in their microbiome. Our work contributes to the factors underlying microbiome specificity and adds detail to coral holobiont functioning.
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Introduction

Coral reefs are declining globally at unsustainable rates (Descombes *et al.*, 2015), driven by stressors including increasing sea surface temperatures, overfishing and anthropogenic inputs (De'ath *et al.*, 2012). Devising strategies for mitigating future reef loss is challenging because corals are 'metaorganisms', comprised not only of the host itself, but also

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symbiotic algae of the genus *Symbiodinium*, viruses, bacteria, archaea and fungi. This complex consortium is referred to as the coral holobiont (Rohwer *et al.*, 2002; Knowlton and Rohwer, 2003). The best-studied members of the coral holobiont are the coral animal host and the *Symbiodinium* algae, which provide most of the host's energy requirements through photosynthates. In return, the symbiotic algae receive a safe refuge, consistent sunlight and nutrients (Goodson *et al.*, 2001).

Although coral-prokaryotic associations have been recognized since the late 1970s (Ducklow and Mitchell, 1979; Williams *et al.*, 1987), recent advancements in sequencing technology have revealed that microbes associated with corals are distinct from those in the seawater and are diverse, totaling several hundred species (Rohwer *et al.*, 2001; Kellogg, 2004; Wegley *et al.*, 2004; Sunagawa *et al.*, 2009; Roder *et al.*, 2013, 2014; Bayer *et al.*, 2013b; Pantos *et al.*, 2015). The high diversity of

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these microorganisms, with many belonging to uncultured genera, makes untangling the complicated interactions between microbes, the coral host and the algal dinoflagellate partners challenging. However, certain microbial species in the coral holobiont can fix nitrogen (Lesser et al., 2004; Lema et al., 2014), metabolize sulfur (Raina et al., 2009), provide antibiotic compounds (Reshef et al., 2006; Ritchie, 2006) and probably have roles in various other biogeochemical cycles (Kimes et al., 2010). Moreover, particular microbes appear to be 'core' symbionts of the holobiont, always associating with a specific coral host, and likely providing important functional benefits to the coral holobiont (Rohwer et al., 2001; Speck and Donachie, 2012; Jessen et al., 2013; Rodriguez-Lanetty et al., 2013; Bayer et al., 2013b; Ainsworth et al., 2015).

One of the potential 'core' symbionts of many corals are the Endozoicomonas, members of the Gammaproteobacteria's Oceanospirillaceae family (Morrow et al., 2012; Speck and Donachie, 2012; Apprill et al., 2013; Jessen et al., 2013; Rodriguez-Lanetty et al., 2013; Bayer et al., 2013b; Lesser and Jarett, 2014; Morrow et al., 2014; Pantos et al., 2015). These bacteria also frequently associate with other marine organisms, including gorgonians (Correa et al., 2013; La Rivière et al., 2013; Vezzulli et al., 2013; Bayer et al., 2013a; Ransome et al., 2014), ascidians (Dishaw et al., 2014), tube worms (Forget and Juniper, 2013), mollusks (Jensen et al., 2010; Hyun et al., 2014) and fish (Mendoza et al., 2013). Although the sequence-based evidence for Endozoicomonas as a core microbiome member of certain corals is compelling, most studies have used corals from only one geographic location and often methods across studies are not standardized, making comparative analyses difficult. A comprehensive study of global reefs, with fine-scale attention to Endozoicomonas genotypes, is required to understand worldwide patterns of association between Endozoicomonas symbionts and corals.

Here, we examine the microbiomes of Stylophora pistillata and Pocillopora verrucosa across their global distribution to better understand the bacterial and archaeal community composition and governing principles, as well as the fine-scale specificity of their core symbionts. We predict that if microorganisms are important to healthy coral functioning and have co-evolved with corals, then coral microbiomes should be similar worldwide. Indeed, both coral species harbor Endozoicomonas as their abundant symbiont, which was found to reside in aggregates within coral tissues. Furthermore, S. pistillata displayed a highly geographically structured microbiome and was associated with Endozoicomonas genotypes unique to each geographic region. In contrast, P. verrucosa exhibited a weakly geographically structured microbiome and contained similar Endozoicomonas symbionts across large spatial scales. This suggests that microbial structure in corals may be linked to life history traits, and

advances our understanding of coral holobiont acquisition and functioning.

Materials and methods

Site description and sampling
A total of 28 reefs were sampled in seven major geographical regions across the global range of the corals S. pistillata and P. verrucosa (Figure 1; Supplementary Table 1). A map of the sample sites was created in python using the package 'basemap', and the code to recreate the map is available at https://github.com/neavemj/microbiomeMap. At each reef, replicate colonies of each coral species were sampled (if present) using snorkel or SCUBA at depths between 2 and 10 m. Colonies were sampled by removing ~ 5 cm² fragments using a hammer and chisel, which were then placed into Whirl-Pak bags (Nasco, Salida, CA, USA) underwater. At the surface, one fragment designated for DNA extraction was wrapped in aluminum foil and immediately frozen in liquid nitrogen, except for the Indonesian samples, which were preserved in 95% ethanol. A second coral fragment was placed into a vial of 4% paraformaldehyde for microscopy analysis. Back at the laboratory, the DNA samples were frozen to − 80 °C and the paraformaldehyde samples were stored at 4 °C.

At all of the reefs except those in Indonesia, the physiochemical properties of the seawater were investigated. Temperature, salinity, dissolved oxygen and pH were measured using a YSI EXO1 Multi-Parameter Water Quality Sonde (Yellow Springs, OH, USA), except at the Red Sea sites, which were measured using a WTW Multi 3500i multimeter (Weilheim, Germany). In addition, seawater surrounding the reefs was sampled for inorganic nutrients (250 ml, frozen to − 20 °C) and planktonic cell counts (1 ml, fixed with 4% paraformaldehyde and frozen in liquid nitrogen) and analyzed as previously reported (Apprill and Rappé, 2011). For seawater microbial community analysis, several liters of seawater was collected, placed on ice and passed through 25 mm, 0.2 μm filters (Millipore, Billerica, MA, USA) until saturated using a peristaltic pump. The seawater samples and filters were then stored at − 80 °C until analysis.

DNA extraction and host phylogeny
Coral fragments were removed from the − 80 °C freezer and immediately airbrushed using cold 1 × phosphate-buffered saline (PBS). DNA was extracted from the airbrushed tissue and seawater filters with the PowerPlant Pro DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. S. pistillata host genotypes were assessed by amplifying the cytochrome oxidase I (COI) gene using the primer set LCO1: GGTCAACAAATCATAAAGATATTGG and HCO2:
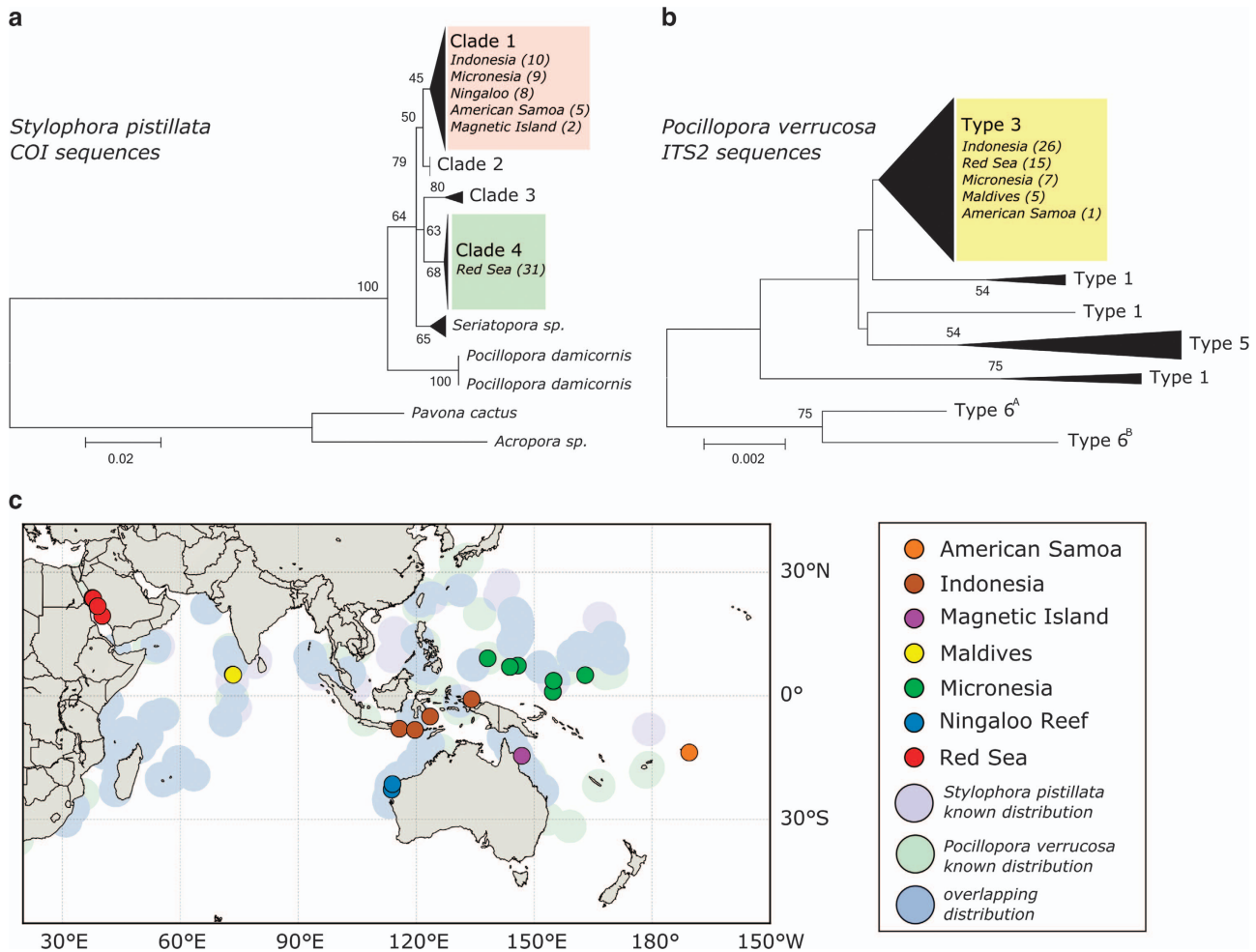
Figure 1 Coral host distribution and phylogenetic relationships of *S. pistillata* and *P. verrucosa* across global scales. Maximum Likelihood phylogenetic comparison of *S. pistillata* COI sequences with 'clades' from Keshavmurthy *et al.* (2013) (**a**), and Minimum Evolution comparison of *P. verrucosa* ITS2 sequences with 'types' from Pinzón *et al.* (2013) (**b**), and map of the reefs where corals were sampled in relation to their known distributions (**c**) obtained from the Ocean Biogeographic Information System (www.iobis.org).

TAAACTTCAGGGTGACCAAAAAATCA following Folmer *et al.* (1994). *P. verrucosa* host genotypes were determined using the internal transcribed spacer region 2 (ITS2) with the coral-specific primers ITSc2-5: AGCCAGCTGCGATAAGTAGTG and R28S1: GCTG CAATCCCAAACAACCC following Flot *et al.* (2008).

Amplified COI genes from the *S. pistillata* specimens were sequenced in the forward direction using an ABI3730xl genetic analyzer (GENEWIZ, South Plainfield, NJ, USA). Sequences were then trimmed for quality using CodonCode Aligner v3.7.1.2 (Centerville, MA, USA) and aligned with COI sequences previously reported (Keshavmurthy *et al.*, 2013) using MUSCLE (Edgar, 2004). A Maximum Likelihood phylogenetic tree with 1000 bootstraps was created from the alignment using MEGA 6 (Tamura *et al.*, 2013). The ITS2 genes from *P. verrucosa* specimens were sequenced in both the forward and reverse direction using an ABI3730xl genetic analyzer (GENEWIZ). The forward and reverse sequences were both required because more than one haplotype was often seen per

individual and both sequences were needed to deconstruct the chromatograph into individual alleles (Champuru v1.0 (Flot, 2007)). Deconstructed alleles were then aligned with sequences from Pinzón *et al.* (2013) using MUSCLE (Edgar, 2004) and a Minimum Evolution phylogenetic tree with 1000 bootstraps was created using MEGA 6 (Tamura *et al.*, 2013). All sequences were deposited in GenBank (COI accession numbers KR105650— KR105714, ITS accession numbers KR105715— KR105768).

Single-cell SSU rRNA gene sequencing was conducted on cells isolated from Red Sea corals collected from Al Fahal in February 2013 (Supplementary Table 1). Briefly, fragments of *S. pistillata* colonies were collected as previously described, placed on ice and immediately taken to the laboratory where they were airbrushed with cold PBSE ($1 \times$ PBS, 10 mM EDTA). The slurry was then gently spun down, resuspended in glyTE ($10 \times$ TE, 50% glycerol) and frozen to $-80\,^{\circ}C$. The cells were shipped on dry ice to the Bigelow Single Cell

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Genomics Center (East Boothbay, ME, USA), where they were sorted using fluorescence-activated cell sorting (FACS). The DNA was then extracted and amplified using multiple displacement amplification (MDA), and the full-length SSU rRNA gene from single cells was sequenced (GenBank accession numbers KR233695-KR233752) as described by Stepanauskas and Sieracki (2007).

Microbiome sequencing and analysis
Bacterial and Archaeal V4 SSU rRNA gene fragments were amplified from the coral and seawater DNA using the primer set 515F: GTGCCAGCMGCCG CGGTAA and 806RB: GGACTACNVGGGTWTC TAAT (Caporaso *et al.*, 2012; Apprill *et al.*, 2015) combined with an Illumina (San Diego, CA, USA) adapter, 8 bp index sequence, 10 bp pad sequence and 2 bp linker sequence in order to use the dual-index sequencing strategy outlined by Kozich *et al.* (2013). The V4 SSU rRNA binding site for the reverse primer was modified as discussed by Apprill *et al.* (2015) to more efficiently capture bacteria from the SAR11 clade. We did not utilize the 515F primer optimized for Thaumarchaeota (Parada *et al.*, 2015); however, using a metagenomics approach in *S. pistillata* and *P. verrucosa*, we found that Archaea were far less abundant than Bacteria (unpublished). The PCR amplicons were sequenced over two 250-bp paired-end Illumina MiSeq runs. To check for bias across the two runs, 11 of the samples were identically prepared and sequenced on both.

De-noising and other quality filtering procedures were completed in mothur v1.34.4 (Schloss *et al.*, 2009). The *make.contigs* command was used to combine the reads, *screen.seqs* was used to remove sequences if they contained ambiguous positions or were longer than 255 bp, *classify.seqs* was used with the 'knn' method to classify the contigs against the SILVA database v119 (Quast *et al.*, 2013), and the *remove.lineage* command removed contigs classified as chloroplast, mitochondria, eukaryota or were unknown. Finally, chimeras were removed using UCHIME (Edgar *et al.*, 2011) with abundant sequences as the reference. The error rate of the sequencing was determined using *seq.error* and mock community samples, and was found to be 0.19%. The sequenced negative controls returned few reads that were removed in the cleaning and filtering steps. The sequences were then subsampled to 7974, which captured most of the diversity and kept most samples. Raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA280923.

Two different approaches were applied for sorting the cleaned sequences into taxonomic units: pairwise similarity (1% and 3%) and Minimum Entropy Decomposition (Eren *et al.*, 2014) (MED). The MED approach uses Shannon entropy to identify nucleotide positions in a sequence alignment that are useful for discriminating operational taxonomic units (OTUs) and was able to discern finer scale differences in sequences compared with pairwise similarity approaches. For example, using 3% OTU clustering, the most abundant overall OTU was present in both Micronesian and Red Sea *S. pistillata*, the MED technique, however, decomposed this sequence into OTUs that were specific to each geographic region (Figure 2; bottom boxplots). A custom python script was used to convert the cleaned fasta and group files from mothur into a MED-compatible format (available at: https://github.com/neavemj/globalCoral Microbiomes/ 'headersMED.py'). MED was then run on the sequences using default settings and a minimum substantive abundance criterion (*M*) of 158 (1 578 852 sequences/10 000) as recommended (Eren *et al.*, 2014). For comparative purposes, the same sequences were used to make 1% and 3% OTUs in mothur (Schloss *et al.*, 2009). The sequences were aligned to the SILVA database v119 (Quast *et al.*, 2013) using *align.seqs*, then *pre. cluster* was used with a difference of 2 bps to reduce the dataset size. The sequences were re-classified using the SILVA database v119 to use *cluster.split* with the taxonomy option and a taxlevel of 5 to cluster the sequences. Following the clustering, 1% and 3% OTU tables were created using *make.shared*. To ensure accurate comparisons, the *remove.rare* command was used to remove OTUs with an abundance lower than 158, matching the minimum abundance of MED OTUs above. The representative MED OTUs and 1% and 3% OTU sequences were classified in mothur (Schloss *et al.*, 2009) using the 'knn' method and the SILVA database v119. Analysis of molecular variance was used to look for significant differences in the microbiomes across sites using a Bray-Curtis similarity matrix and *amova* in mothur (Schloss *et al.*, 2009).

All remaining statistical and graphical analyses were completed in R (R Development Core Team, 2010). In the interest of reproducibility, an R markdown document containing complete commands for the analysis is available here: https:// github.com/neavemj/globalCoralMicrobiomes/ 'coralMicrobiomes.Rmd'. Briefly, after the OTU matrices and metadata were imported into R, alpha diversity measures were calculated using 3% pairwise OTUs with the command *plot_richness* in the package 'phyloseq' (McMurdie and Holmes, 2013), and the measures were plotted as a boxplot in 'ggplot' (Wickham, 2009). To check for significant differences between the alpha diversity measures, the R base package 'stats' was used to perform a Kruskal–Wallis test and *post hoc* pairwise comparisons were conducted using a Dunn's test (Dinno, 2015) with Bonferroni corrections for multiple testing. Phyloseq was used to create barcharts with *plot_bar* and ordinations with *plot_ordination* using square-root transformed Bray–Curtis similarity matrices on MED OTUs (McMurdie and Holmes, 2013). Physiochemical data were correlated with the
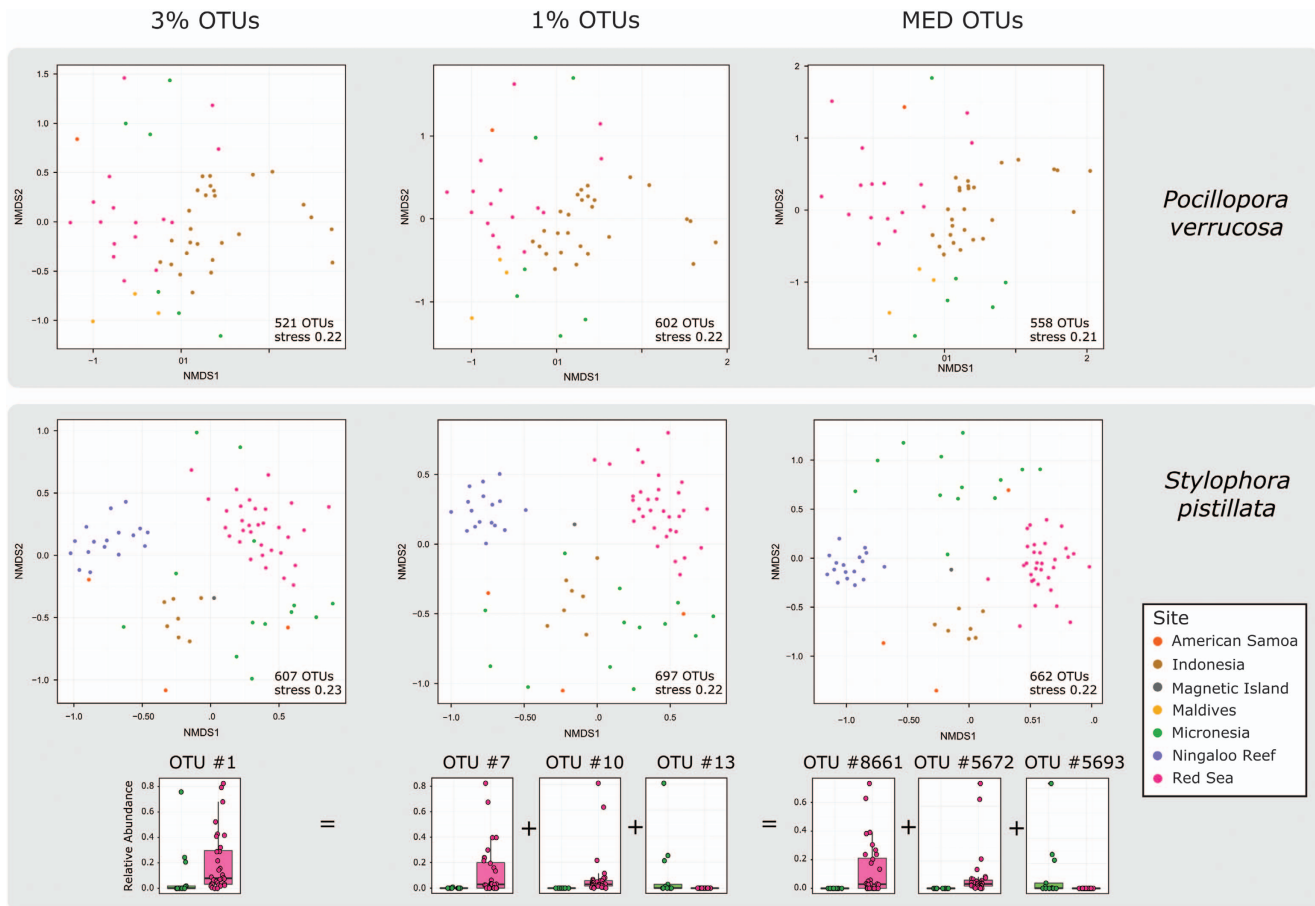
Figure 2 Clustering of coral specimens based on microbiome composition. Non-metric multidimensional scaling comparisons of *S. pistillata* and *P. verrucosa* microbiomes, comparing pairwise OTU clustering with the MED OTU approach to clustering SSU rRNA gene sequences (Eren *et al.*, 2014). The bottom boxplots show how sequences that formed the most abundant OTU with 3% clustering (left) are divided into 1% OTUs (middle) and MED OTUs (right), for two sample sites (Micronesia and Red Sea). This indicates that 3% clustering detects no difference between the most abundant OTU within Micronesian and Red Sea corals, whereas 1% OTUs and MED OTUs clearly show site-specificity.

microbiome ordinations using *envfit* in the R package 'vegan' and 999 permutations to test for significance (Oksanen *et al.*, 2013), and SIMPROF analysis was run with 999 permutations using the R package 'clustsig' (Whitaker and Christman, 2014).

Endozoicomonas phylogeny
Endozoicomonas bacteria were found to be the most prevalent symbionts associated with both corals and were analyzed further. The MED OTUs that classified to *Endozoicomonas* were aligned using the SINA alignment service (www.arb-silva.de/aligner/) (Pruesse *et al.*, 2012), and imported into ARB (Ludwig *et al.*, 2004). After manual refinement of the alignment, including exclusion of partially represented regions, a Maximum Likelihood tree containing the OTUs, plus *Endozoicomonas* sequences from several previous studies, was constructed. The tree was imported into R and metadata was added to the tree tips using *plot_tree* in phyloseq (McMurdie and Holmes, 2013). A heatmap of *Endozoicomonas* OTUs was also created using

phyloseq and the *plot_heatmap* command. To check for significant differences in *Endozoicomonas* abundance across sites, negative binomial modeling was conducted using DESeq2 (Love *et al.*, 2014; McMurdie and Holmes, 2014).

Catalyzed reporter deposition—fluorescence in situ hybridization (CARD–FISH)
Coral fragments that were preserved for microscopy analysis were removed from the 4% paraformaldehyde and rinsed three times in phosphate-buffered saline (PBS) for 20 min each. Samples were decalcified at 4 °C in 20% (w/v) tri-sodium EDTA (pH 7–8) in distilled water changed daily for up to 10 days. The tissues were then dehydrated for 20 min each in 70%, 90% and 95% and two times in 100% ethanol, followed by three 20-min washes in HistoClear (National Diagnostics, Atlanta, GA, USA). The tissues were then embedded in paraffin and sectioned to 5 or 10 μM using an 820 Rotary Microtome (American Optical, Buffalo, NY, USA). The sections were deparaffinated by heating to 60 °C and washing

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for 10 min in HistoClear three times, then 10-min washes of 95%, 80% and 70% ethanol. The sections were permeabilized in 0.2 M HCl for 12 min, 20 mM Tris/HCL for 10 min, 0.5 µg ml^{-1} proteinase K for 5 min at 37 °C and a final rinse of 20 mM Tris/HCL for 10 min. Next, sections were covered in hybridization buffer (35% formamide, 0.9 M NaCl, 20 mM Tris/HCL (pH 7.4), 0.01% SDS, 1% blocking reagent (Roche, Basel, Switzerland), 10% (w/v) dextran sulfate) and placed in a humidity chamber for 20 min at 35 °C. The tissues were then hybridized for 3 h with horseradish peroxidase-labeled 0.5 ng µl^{-1} Endo-zoi663: 5′-GGAAATTCCACACTCCTC-3′ (Bayer et al., 2013b) or horseradish peroxidase-labeled EUB338: 5′-GCWGCCWCCCGTAGGWGT-3′ (Daims et al., 1999) in hybridization buffer at 35 °C. In addition, control samples were hybridized with horseradish peroxidase-labeled NON338 probe: 5′-ACTCCTACGGGAGGCAGC-3′ (Amann et al., 1990), as well as no probe, under the same conditions. Following hybridization, the slides were washed two times for 15 min at 35 °C in fresh hybridization wash (80 mM NaCl, 20 mM Tris/HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.01% SDS), then for 15 min in 1× PBS at room temperature. For CARD amplification, a fresh solution of 0.15% H$_2$O$_2$ in 1× PBS was mixed in a ratio of 1:100 with amplification buffer (1× PBS, 0.1% blocking reagent (Roche), 2 M NaCl, 10% (w/v) dextran sulfate). Cy3-labeled tyramide (1 mg ml^{-1}) was then added to the mix at a ratio of 1:500, applied to the slides and incubated for 20 min at 37 °C in a dark humidity chamber. Following the amplification, the slides were washed in 1× PBS at room temperature for 15 min, then for 1 min in distilled water. To reduce background interference from the naturally autofluorescent coral tissues, the slides were covered with 6% H$_2$O$_2$ in ethanol overnight (14–16 h) according to Fukatsu et al. (2007) and mounted using the Vectashield DAPI mounting strain (Vector Laboratories, Burlingame, CA, USA).

Samples were imaged using a ×63 objective under ×1.6 optovar on a Zeiss Axio Observer.Z1 fluorescent microscope with an AxioCam MRm camera using the Zen Pro 2011 software (Carl Zeiss Microscopy, Thornwoods, NY, USA) and Cy3, DAPI and PA-GFP emission detected at 570, 395 and 495 nm, respectively. For each colony (eight S. pistillata from Ningaloo and Micronesia, three P. verrucosa from Maldives and Micronesia), 10–41 sections were examined with the Endozoicomonas probe, and compared with control specimens (no probe, nonsense probe) of selected adjacent sections of the same samples. Hematoxylin and eosin staining (ClearView, BBC Biochemical, Mount Vernon WA, USA) was conducted on sections adjacent to those that were positive for Endozoicomonas, and sections were imaged under ×4 and ×60 objectives using an EVOS FL Cell Imaging System (Life Technologies, Carlsbad, CA, USA).

Results

Coral host phylogeny

To ensure valid comparisons of coral species across distant geographic regions, host genotype was resolved using the cytochrome oxidase I (COI) gene for S. pistillata and the internal transcribed spacer region (ITS2) for P. verrucosa (Figure 1) (Flot et al., 2008; Keshavmurthy et al., 2013). The rapidly evolving ITS2 region was used to identify P. verrucosa because many Pocilloporid corals cannot be differentiated using the slower evolving COI gene, including P. damicornis and P. verrucosa, two closely related species that were sympatric within our study range (discussed by Pinzón et al. (2013) and Flot et al. (2008)). Species of Stylophora, on the other hand, can be differentiated using the COI gene (see Keshavmurthy et al. (2013)), eliminating the need to sequence the ITS2 region for our Stylophora specimens. All S. pistillata colonies formed a monophyletic clade, except for those from the Red Sea, which formed a sister branch (Figure 1a). These results correspond to clade 1 and clade 4 from Keshavmurthy et al. (2013) and suggest that Red Sea S. pistillata are genetically isolated from the Indo-Pacific populations. The taxonomy of P. verrucosa was more complicated owing to higher diversity across the ITS2 gene and the presence of heterozygous individuals. Nevertheless, most of the samples we identified in the field as P. verrucosa could be assigned to Pocillopora 'type 3' (which includes P. verrucosa) sensu Pinzón et al. (2013) (Figure 1b). Pocillopora colonies that did not cluster with the 'type 3' samples were not used in subsequent microbiome analyses. Hereafter, the 'type 3' Pocillopora will be referred to as Pocillopora verrucosa. This investigation of host genotypes provided some confidence that the global coral samples could be examined in a comparative framework. It should be noted, however, that coral phylogeny is a frequently changing field and that host subspecies cannot be ruled out.

Fine-scale SSU rRNA diversity analysis

High-throughput sequencing of hypervariable V4 region SSU rRNA gene amplicons (Sogin et al., 2006; Caporaso et al., 2012) was used to examine the bacterial and archaeal communities in corals (73 colonies S. pistillata, 53 colonies P. verrucosa) and the seawater surrounding those colonies (Supplementary Table 1). We examined two different approaches for categorizing the microbial sequences into OTUs for fine-scale specificity characterizations across global scales: pairwise similarity (both at 1% and 3% cutoffs) and MED (Eren et al., 2014) (Figure 2). We found that MED resolved finer clusters than the pairwise similarity approaches, particularly when compared with 3% pairwise similarity (Figure 2). In addition, we conducted single-cell sorting of microbial cells and subsequent full-length

SSU rRNA gene Sanger sequencing for *S. pistillata* acquired from the Red Sea. To date, few studies have used single-cell genomics for multicellular animals and several challenges were encountered, largely arising owing to the complex consortium of cells within the coral holobiont, such as coral mitochondria, viruses, fungi, *Symbiodinium* and naturally fluorescent coral cells that interfere with the sorting process. Nevertheless, through careful selection of bacteria-like size ranges during the fluorescence-

activated cell sorting, we recovered 67 bacterial cells from a 384-well plate, 66 of which were identified as *Endozoicomonas*. Convincingly, the abundant Red Sea MED OTUs were identical to the SSU rRNA gene sequences obtained from single-cell sequencing (Figure 3, single-cell types A and B), suggesting that MED OTUs obtained from high-throughput sequencing indeed represent biologically relevant units. For these reasons, we used MED OTUs for further global comparisons, except for the calculation of alpha
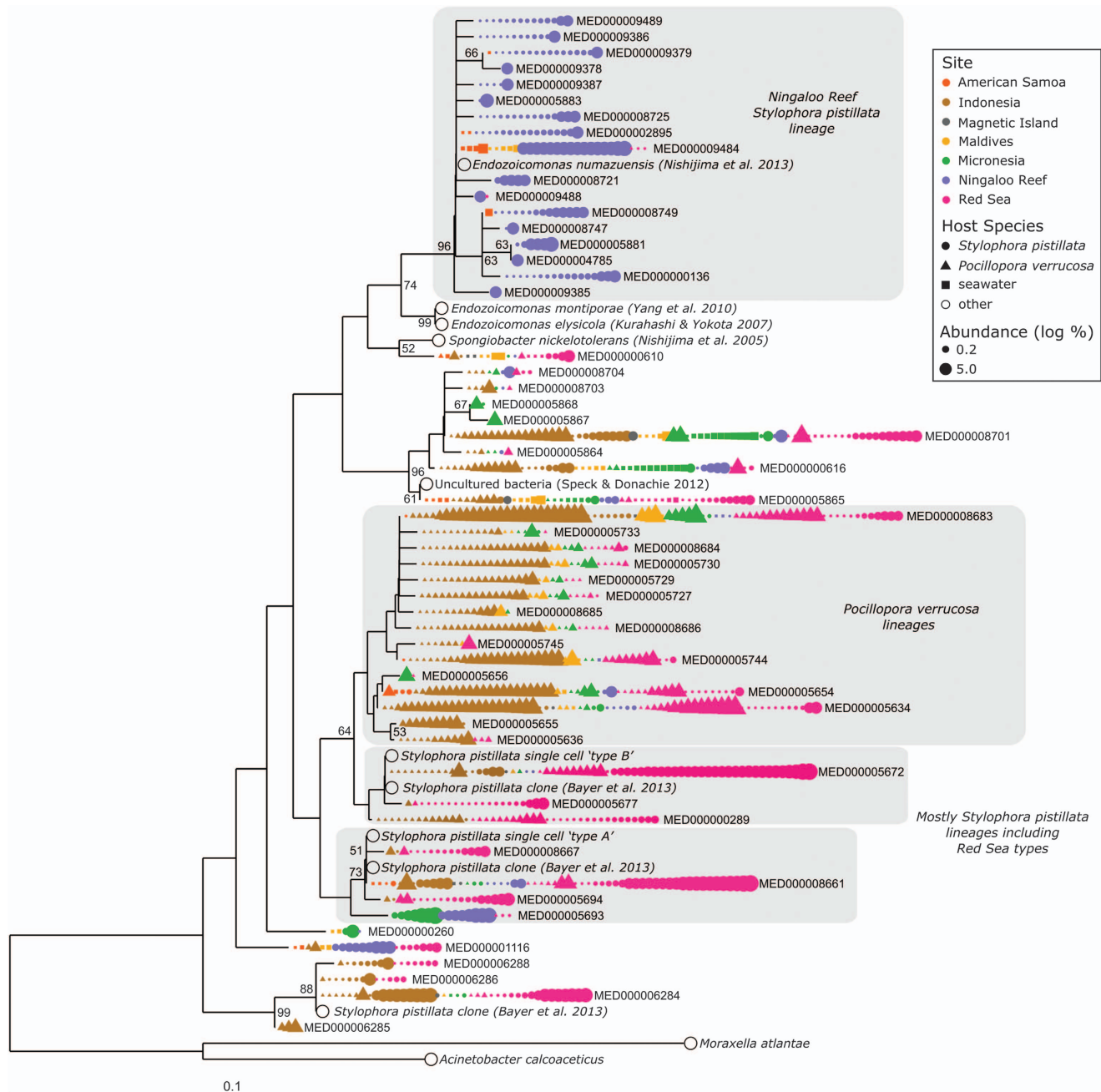
Figure 3 Phylogeny of coral-associated *Endozoicomonas* symbionts. Neighbor-joining tree containing all *Endozoicomonas* MED OTUs detected in the study, overlaid with the host species and geographic location of sequences clustered within the OTUs. Sequences detected in seawater are also represented, as well as the V4 region from full-length *Endozoicomonas* SSU rRNA gene sequences from the single-cell sorts and other coral studies. The scale bar corresponds to the estimated number of nucleotide changes per sequence position.

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diversity measures, which are not yet implemented for MED methodology.

Corals are highly selective for their microbiome compared with seawater

Comparisons of Chao1 and Simpson diversity indices between the coral and seawater microbial communities revealed that, on a global scale, corals harbored a more selective community of microorganisms compared with those in the surrounding seawater, as evidenced by significantly lower richness and evenness compared with the seawater communities (Supplementary Figure 1). This trend was consistent among the reefs, despite some variation in environmental parameters, such as the concentrations of inorganic nutrients (0.04–2.0 μM nitrate+nitrite) and temperature (24.7–30.5 °C), as well as among concentrations of picoeukaryotes (341–23 809 cells per milliliter) in the waters surrounding the corals (Supplementary Tables 2 and 3). For *S. pistillata* and *P. verrucosa*, alpha diversity of the associated microbes was not significantly different (Supplementary Figure 1), and the Chao1 richness metric predicted an average of 217 (min 26, max 851) coral-associated microbial taxa using 3% OTU clustering.

Different geographic structuring of the microbiome in S. pistillata and P. verrucosa, yet selection for Endozoicomonas bacteria

Multivariate analysis of the SSU rRNA gene MED OTUs revealed that the microbiomes of *S. pistillata* and *P. verrucosa* partitioned differently according to geographic location (Figure 2). Specifically, *S. pistillata* microbiomes were strongly geographically segregated, but this structuring was weaker for *P. verrucosa*. Analyses of molecular variance (AMOVA) demonstrated that the *S. pistillata* microbiomes were significantly distinct from each other at four of the six major geographic locations, including Indonesia, Micronesia, Ningaloo Reef and the Red Sea (Supplementary Table 4). In contrast, the *P. verrucosa* microbiomes did not show a clear segregation according to geographic location (Figure 2), and the only significantly different corals were from the Red Sea (Supplementary Table 4). These patterns were also seen in a Similarity Profile Analysis (SIMPROF), which clustered samples without any *a priori* assumptions regarding sample origin (Supplementary Figure 2).

To test for significant environmental correlations with microbiome composition, the environmental variables were fitted to the microbiome ordinations (Supplementary Figure 3). The Red Sea sites had higher salinity, Ningaloo Reef sites had higher silicate concentrations, and the Micronesian sites displayed higher temperatures compared with the other sites, which correlated with the microbiome composition. Although these correlations are difficult to interpret in a cause-and-effect framework, it may suggest that environmental factors had some influence on the coral microbiomes. However, this influence is unlikely to account for the different microbiome specificity seen between *S. pistillata* and *P. verrucosa*, because the corals were collected at the same reefs at the same time and presumably experienced the same physiochemical conditions.

Taxonomically, the microbiomes of both *S. pistillata* and *P. verrucosa* were dominated by bacteria belonging to the *Gammaproteobacteria* and *Alphaproteobacteria* (Supplementary Figures 4 and 5). Higher variability between the corals was seen at the genus level, although *Endozoicomonas* was the most prevalent taxa for both corals. *Endozoicomonas* OTUs (relative abundance > 1%) were detected in 79% of *S. pistillata* samples and 85% of *P. verrucosa* samples across their global distribution, suggesting that these bacteria are prevalent microbiome members. Along with the widespread *Endozoicomonas* associations, the corals also contained a diverse community of prokaryotic groups (Supplementary Figures 4 and 5). For example, the *S. pistillata* samples from Ningaloo Reef contained unusually large abundances of *Pseudomonas* (average 32%, min 10%, max 68%), and *P. verrucosa* from several Indonesian sites contained substantial amounts of *Prochlorococcus* (average 15%, min 9%, max 21%). Although some of these prokaryotic associates may have important roles in the coral holobiont, here, we focused on the *Endozoicomonas* owing to their consistent prevalence and the opportunity to assess host specificity for *Endozoicomonas* across global scales. The coral-associated microbes were different to those in the seawater, which was dominated by members of the Cyanobacteria and SAR11 clade of *Alphaproteobacteria* (Supplementary Figure 6). Archaea were generally rare in both corals and seawater, although members of the Euryarchaeota occasionally accounted for up to 25% of the seawater microbial community (Supplementary Figure 6).

Intimate association of Endozoicomonas bacteria with their coral hosts

The spatial location of the *Endozoicomonas* endosymbionts within coral tissues was examined using CARD–FISH (Figure 4). Microscopic CARD–FISH images revealed that *Endozoicomonas* symbionts were located deep within coral tissues, confirming an important and intimate association with their hosts (Figure 4) (Bayer *et al.*, 2013b). In both coral species, the *Endozoicomonas* cells were arranged in cyst-like, dense cellular aggregations, spanning ~ 50 μM in diameter, which were confirmed with hematoxylin and eosin staining (Supplementary Figures 7 and 8). In *S. pistillata*, the *Endozoicomonas* cyst-like aggregations were intratentacular, bordering the epidermis and gastrodermis (Figure 4, Supplementary Figure 7), whereas in *P. verrucosa*, they were predominantly located within gastrodermal
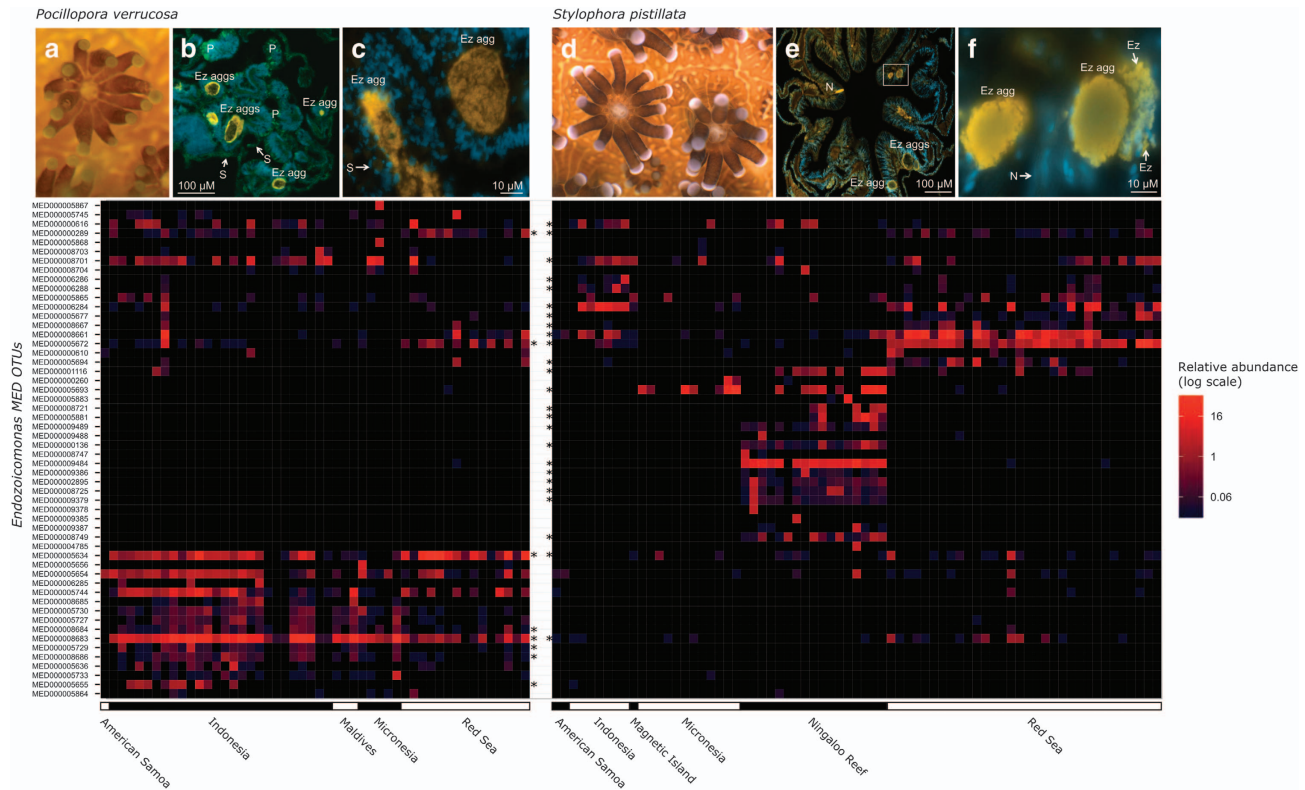
Figure 4 Light microscopy and FISH imaging of *Endozoicomonas* within the gastrodermal tissues of *P. verrucosa* (**a–c**) and within the tentacles surrounding the oral disc of *S. pistillata* (**d–f**), and a heatmap of *Endozoicomonas* OTU abundance and diversity in *P. verrucosa* and *S. pistillata* (bottom panel). *Endozoicomonas* cells are hybridized with the horseradish peroxidase-labeled probe targeting the *Endozoicomonas* bacteria and appear yellow. The blue staining is a general nucleic acid stain (DAPI) and (**b**) is additionally imaged under fluorescein isothiocyanate excitation and the green coloration is coral and *Symbiodinium* autofluorescence. Select *Endozoicomonas* cells (Ez) and cell aggregations (Ez agg), nematocysts (N), *Symbiodinium* cells (S) and coral pigment cells (P) are denoted. (**f**) is an inset of (**e**), and (**c**) is a representative magnified image from *P. verrucosa*. For the FISH images, non-cellular yellow coloration is present owing to coral tissue autoflurescence as well as probe sticking to nematocysts (N). The bottom panel is a heatmap of the abundance (log-scale base 4) of *Endozoicomonas* MED OTUs detected in *P. verrucosa* and *S. pistillata*. Asterisks indicate *Endozoicomonas* OTUs that had significantly different abundances ($P < 0.05$) across the sites based on negative binomial modeling in DESeq2.

tissues and absent from the tentacles (Figure 4, Supplementary Figure 8).

Fine-scale coral–Endozoicomonas specificity across species and globally distant reefs

Almost all of the corals examined contained a dominant *Endozoicomonas* MED OTU ($> 75\%$), and most also contained several less abundant types (Figure 4). *S. pistillata* and *P. verrucosa* were associated with host-specific *Endozoicomonas* MED OTUs, and few OTUs were present in both coral hosts. Moreover, the *Endozoicomonas* in *P. verrucosa* showed only slight geographic partitioning, whereas *Endozoicomonas* in *S. pistillata* were strongly grouped by geographic location, with 8 and 25 OTUs, respectively, demonstrating significantly different geographic abundances ($P < 0.05$, Figure 4), reflecting the total community results (Figure 2).

Phylogenetic analysis of the *Endozoicomonas* OTUs revealed site- and host-specificity patterns

within this bacterial genus (Figure 3). As observed in Figure 4, *Endozoicomonas* associated with *S. pistillata* were generally distinct OTUs at the geographically distant reefs. Particularly distinct were *Endozoicomonas* OTUs from Ningaloo Reef of Australia, which formed a monophyletic branch and the only other geographic location represented here were from sequences recovered from seawater (Figure 3). Interestingly, *Endozoicomonas* from the other geographic locations did not always belong to the same genetic lineage. For example, an abundant Red Sea OTU (MED000005672) was more closely related to *P. verrucosa's Endozoicomonas* from the Red Sea and Indonesia, than to the other abundant *S. pistillata* Red Sea OTUs (MED000008661 and MED000006284). Several other *Endozoicomonas* types within Indonesia, Micronesia and Ningaloo Reef corals belonged to disparate lineages, suggesting that a number of genetically distinct *Endozoicomonas* OTUs coexist in these corals. In contrast, most *Endozoicomonas* taxa associated with

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P. verrucosa belonged to the same lineage and were shared across geographic areas (Figure 3).

Discussion

Different microbiome structuring for S. pistillata and P. verrucosa

S. pistillata and *P. verrucosa* are closely related coral species both belonging to the family *Pocilloporidae*. They probably diverged relatively recently, near the beginning of the Neogene period around 23 million years ago (Park *et al.*, 2012). Despite this phylogenetic similarity, we found significant differences in the microbiomes of the two corals and in the apparent forces structuring the microbiome. Specifically, *S. pistillata* microbiomes were strongly clustered according to geographic location, whereas in *P. verrucosa*, the groupings were much weaker. These differences may have arisen because of the distinctive reproductive strategies of the corals; *S. pistillata* is a brooder (Hall and Hughes, 1996; Shlesinger *et al.*, 1998) and *P. verrucosa* is a broadcast spawner (Pinzón *et al.*, 2013). In fact, some authors have suggested that the divergence between *Stylophora* and *Pocillopora* was established by the evolution of these different reproductive modes (Schmidt-Roach *et al.*, 2014). Previous studies of the broadcast spawning coral, *P. meandrina*, found that larvae less than 3 days old were sterile of prokaryotes (Apprill *et al.*, 2009) and some of their microbiome originates from the surrounding seawater (Apprill *et al.*, 2012). The gametes of other broadcast spawning corals are also sterile of prokaryotic cells, which are acquired later from the water column (Sharp *et al.*, 2010; Ceh *et al.*, 2013). In contrast, the brooding coral, *Porites astreoides*, passed microbes vertically from parent to offspring (Sharp *et al.*, 2012). Assuming these patterns hold true for the corals studied here, it could explain the observed geographic structuring patterns. Namely, the brooding coral *S. pistillata* may vertically transfer microbes to their offspring, thereby tightly controlling the microbiome and its evolution, and resulting in strong geographic structuring. Conversely, if *P. verrucosa* larvae were sterile and their microbial associates were acquired from seawater, symbiont mixing between distinct reefs is likely, resulting in weaker geographic structuring. Moreover, *Pocillopora* species have unusually large areas of genetic connectivity and low species diversity compared with other cosmopolitan coral species, such as *S. pistillata* (Pinzón *et al.*, 2013; Robitzch *et al.*, 2015). This potentially high gene flow between *Pocillopora* species is probably facilitated by exceptionally long planktonic stages. For example, *P. damicornis*, which is closely related to *P. verrucosa*, has a planktonic stage of more than 100 days in aquaria (Richmond, 1987), which would allow mixing of populations from relatively distant oceanic basins. High connectivity between hosts would almost certainly provide opportunities for symbiont sharing, potentially explaining the weaker geographic signature in *P. verrucosa* microbiomes.

The geographic structuring pattern for the overall microbial communities was even more pronounced for *Endozoicomonas*, the most abundant symbiont in both corals. For *S. pistillata*, each large geographic region contained unique *Endozoicomonas* OTUs. This was not seen in *P. verrucosa*, which often harbored the same *Endozoicomonas* OTUs across large geographic ranges. Reproductive differences in the corals may again account for this pattern. If *Endozoicomonas* are passed vertically in the brooder *S. pistillata,* this could produce strong regional clustering, whereas if *Endozoicomonas* are acquired from the seawater in *P. verrucosa*, the endosymbiont types may be shared. The higher genetic connectivity of *Pocillopora* (Pinzón *et al.*, 2013; Robitzch *et al.*, 2015) would also allow for greater sharing of *Endozoicomonas* genotypes. Interestingly, Hester *et al.* (2015) studied three spawning coral species (*Acropora rosaria, A. hyacinthus* and *Porites lutea*) and did not find stable symbionts that were universally associated with one host, similar to *P. verrucosa* here. Our study was able to extend this analysis by including the brooder, *S. pistillata*, allowing us to consider the mode of symbiont transmission as an influence on microbiome structure, and also used a deeper sequencing depth to more thoroughly characterize the microbiome.

Endozoicomonas fine-scale specificity resembles coral–algae and other symbiotic systems

Endozoicomonas appear to be 'core' microbiome members of both *S. pistillata* and *P. verrucosa,* and were detected in 79% and 85% of the coral samples, respectively. Although these percentages provide support that *Endozoicomonas* are core microbiome members, these calculations are dependent on the sequence identity used in the formation of OTUs, which can alter the results (for discussion, see Shade and Handelsman (2012)). Although colonies of *S. pistillata* and *P. verrucosa* both generally associated with a single dominant *Endozoicomonas* genotype, often several *Endozoicomonas* OTUs were present, frequently from distinct lineages (Figure 3). This reflects the population structure of the most conspicuous coral endosymbiont, the *Symbiodinium* algae. Although most coral colonies are dominated by a single *Symbiodinium* type, several genotypes are generally present (Arif *et al.*, 2014; Quigley *et al.*, 2014). Moreover, in times of heat stress, some corals can undergo a process of 'symbiont shuffling', whereby the current symbionts are replaced with *Symbiodinium* clades or types that have a higher thermotolerance (Silverstein *et al.*, 2014). It is possible that different *Endozoicomonas* types are also adapted to different conditions and become abundant at certain times. To test the stability of *Endozoicomonas*–host associations, we compared

our Red Sea MED OTUs with *Endozoicomonas* sequences from a previous study of *S. pistillata* from nearby Red Sea reefs collected in June 2009 (Bayer *et al.*, 2013b) (Figure 3). All of the most abundant sequences from the earlier study had correspondingly abundant MED OTUs (Figure 3), suggesting that the most prevalent *Endozoicomonas* types maintained their dominance for at least the last 4.5 years. Nevertheless, future studies should explore whether rare *Endozoicomonas* genotypes become abundant depending on time of day and season, or whether their abundance correlates with the fluctuations of different *Symbiodinium* clades.

This differential host specificity in *Endozoicomonas* parallels the specificity seen between *Apidae* bees and their symbionts. The honeybee, *Apis mellifera*, and the bumblebee, *Bombus* spp., both contain two abundant symbionts, *Snodgrassella alvi* and *Gilliamella apicola* (Kwong *et al.*, 2014). Despite this similarity, strong host specificity exists for different symbiont strains; for example, *S. alvi* strains isolated from honeybees could not colonize bumblebees (Kwong *et al.*, 2014). The specificity was driven by host–microbe and microbe–microbe interactions and transmission differences, which may also structure the *Endozoicomonas* specificity seen in *S. pistillata* and *P. verrucosa*.

Potential functional roles of Endozoicomonas endosymbionts
Despite the abundance and worldwide distribution of *Endozoicomonas* in multiple marine organisms, their function is unknown. A suggested role is DMSP breakdown (Raina *et al.*, 2009; Lema *et al.*, 2013; Pike *et al.*, 2013; Vezzulli *et al.*, 2013; Ransome *et al.*, 2014), however, sequenced *Endozoicomonas* genomes do not contain known DMSP breakdown pathways (Neave *et al.*, 2014). Others have suggested a nutritional symbiosis (La Rivière *et al.*, 2013), interactions with the algal symbiont *Symbiodinium* (Morrow *et al.*, 2012; Pantos *et al.*, 2015) or roles in producing antimicrobial compounds (Bourne *et al.*, 2008). Although most studies propose that *Endozoicomonas* are beneficial symbionts, Mendoza *et al.* (2013) found that cyst-like aggregations of *Endozoicomonas* were responsible for epitheliocystis in fish and caused mass mortalities. This suggests that different *Endozoicomonas* genotypes may have disparate roles in their diverse hosts. Genomic sequencing revealed that *Endozoicomonas* genomes are large (>5 Mbs) and are not streamlined for an obligate endosymbiotic lifestyle, implying they have free-living stages (Neave *et al.*, 2014). The genomes are suggestive of an aerobic heterotrophic lifestyle with the ability to metabolize diverse carbon sources, including amino acids and nucleic acids. The genomes also code for a large number of transport molecules, which they may use to interact with compounds produced by the host or by the *Symbiodinium* algae.

CARD–FISH revealed the spatial location of *Endozoicomonas* in the tentacles of *S. pistillata* and in the gastrodermis of *P. verrucosa*, which underlines that different host species show distinct association patterns further supporting that it is (i) a highly intimate relationship and (ii) a highly specialized relationship. Further, the aggregation patterns observed here suggest that *Endozoicomonas* may utilize metabolic products from other cells within the aggregation, or a component of their metabolism may rely on quorum sensing (Waters and Bassler, 2005). Further, the presence of intratentacular *Endozoicomonas* aggregates in *S. pistillata* suggests that they may have a role in prey acquisition. In line with this, Schuett *et al.* (2007) found comparable *Endozoicomonas* aggregations in the tentacles of a sea anemone. This potentially suggests similar functional roles for some *Endozoicomonas* genotypes across host species, although further microscopy studies examining the location and arrangement of *Endozoicomonas* cells in a variety of hosts are required. It should be noted that the rod-shaped *Endozoicomonas* cells imaged here are distinct from those previously reported for *S. pistillata* (Bayer *et al.*, 2013b), and the use of CARD–FISH has greatly aided our ability to localize specific cells within highly autofluorescent coral tissues. Altogether, we provide further evidence that *Endozoicomonas* have an important role in the coral holobiont, however, their precise function remains to be determined.

Conclusions

Global profiling of microbial community structure in a multi-species framework revealed that *Endozoicomonas* bacteria are prevalent and often the most abundant prokaryotic symbionts residing intimately within the tissues of two common corals across their global distribution. These differences in *Endozoicomonas* specificity map onto the different reproductive strategies of the coral hosts. *S. pistillata* is a brooder and may control the evolution and specificity of its microbiome through the vertical transfer of symbionts, while *P. verrucosa* is a spawner and acquires its prokaryotic symbionts from the water column, thus allowing for greater variability within and less spatial structure across *Endozoicomonas* genotypes. Our study introduces a coral–bacterial relationship that features a consistent, cosmopolitan, highly aggregated endosymbiont that exhibits some taxonomic variability, and is an important component of the endosymbiotic relationships of the coral holobiont that should be examined with attention to evolutionary and ecological interactions and constraints.

Conflict of Interest

The authors declare no conflict of interest.

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Acknowledgements

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