Supplement

The microbiome of the Red Sea coral *Stylophora pistillata* is dominated by tissue-associated *Endozoicomonas* bacteria

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Supplementary Methods

Evaluation of 16S rRNA gene primers for bacterial amplification in coral DNA extractions

The use of universal bacterial 16S primers for assessments of coral bacterial diversity are challenging because the existing primer sets frequently amplify the coral host 18S gene, the *Symbiodinium* 18S gene, or the 16S gene of the *Symbiodinium* plastid genome. In order to assess amplification fidelity as well as the amount of side products amplified, four published primer sets (Suppl. Table 1) were tested on DNA extracts from 22 different coral species (Suppl. Table 2). An in silico analysis based on the Ribosomal Database Project database excluded many other primer sets due to their potential amplification of chloroplast sequences. In order to be suitable for 454 sequencing using the Titanium FLX chemistry, all tested primers were intended to amplify a fragment of between 270-450 bp.

Coral genomic DNA was extracted as described below. PCR conditions were as follows: 30 ng template DNA were used in a 25 µl PCR reaction using the Qiagen Multiplex PCR kit. Primers were diluted to a final concentration of 0.2 µM. Cycling conditions were 95°C for 15 min and then 30 cycles of 95°C for 30 s, annealing temperature for 1.5 min and 72°C for 1.5 min, and a final step of 72°C for 10 min. The annealing temperatures are given in Suppl. Table 1.

To assess the specificity of the PCR, the PCR product was run on an Agilent Bioanalyzer DNA chip. The different products (18S or bacterial 16S amplicons) possess different sizes, and thus this method makes it possible to quantify the product and unwanted side products without cloning and sequencing, but with higher resolution than an agarose gel.

The primer set developed by Andersson et al. (1) was found to produce the lowest amount of non-target PCR products in the greatest number of samples tested (Suppl. Table 2). This primer sets was therefore chosen for the main experiment.

Stylophora pistillata sampling

Stylophora pistillata samples for the 16S rRNA gene sequencing and fluorescence in situ hybridization (FISH) experiments were collected in the southern Red Sea during June, 2009 by SCUBA at depths between 2 and 5 m. Single colonies of healthy appearing corals without signs of disease or tissue necrosis were selected from the following sites: site 5, 18°40'30.36"N, 40°44'21.18"E; site 12, 19°10'35.14"N, 40°16'27.78"E; site 14, 19°53'52.74"N, 40° 0'53.46"E; site 15, 19°53'15.42"N, 40°9'23.94"E; and site 17, 20°8'58.38"N, 40°14'7.50"E (Suppl. Fig. 1). Samples were rinsed with sterile seawater, frozen in liquid nitrogen, shipped frozen in liquid nitrogen dry shippers to Woods Hole, MA, where they were stored at -80°C until analysis.

S. pistillata DNA extractions and sequencing (454 and Sanger)

S. pistillata tissue was removed from the skeleton using an airbrush and 0.2 µm filtered phosphate buffered saline solution. The biomass (0.02 - 0.06 g) was pelleted, and the DNA extracted using the PowerPlant DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's protocol with modifications listed in Sunagawa et al. (2). DNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Rockfod, IL). Prior to conducting 454 pyrosequencing of the 16S rRNA gene on the same samples, a range of primers was examined. This was done to avoid sequencing a large fraction of genes from the coral or dinoflagellate (i.e., (3)). The 784F and 1061R primer pair (1) was found to produce the least cross amplification over a range of coral species, and it

amplifies a 277 bp fragment that includes variable regions 5-6 of the 16S rRNA gene. To enable 454 library generation from the PCR products the primers 5' CTATGCGCCTTGCCAGCCCGCTCAGtaAGGATTAGATACCCTGGTA 3' (784F) and 5' CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNNctCRRCACGAGCTGACGAC 3' (1061R) were used. The primers included the 454 adapter sequences (underlined), an eight base pair barcode (4) (shown as N) and a linker sequence (lowercase). The forward primer did not contain a barcode sequence. For amplification of the 16S rRNA region for pyrosequencing, the DNA concentration was adjusted so each reaction contained 30 ng and the primers were adjusted to 0.2 µM in the final concentration. PCR was performed in triplicate per *S. pistillata* sample, in 30 µl reactions using the Multiplex PCR kit (Qiagen, Hilden, Germany) and the following cycling protocol: Initial denaturation of 15 min at 95°C followed by 27 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 40 sec and extension at 72°C for 40 sec and a final extension step of 10 min at 72°C. PCR efficiency was assessed by gel electrophoresis of 3 µl aliquots. Triplicates were pooled for purification with the Qiagen MinElute PCR Purification kit to yield one PCR product per specimen. The cleaned PCR products were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, USA) and equal amounts PCR products from all specimens were pooled to yield a single mixture for sequencing library generation. This PCR product pool was run on an electrophoresis gel and purified by cutting out the respective band and cleaned using a Qiagen MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). This step allowed separating the bona fide PCR products from primer dimers that might have otherwise interfered with sequencing. Libraries were generated using the GS FLX Titanium emPCR Kit (Lib-A) (Roche, Branford, USA) according to the manufacturer's instructions, and sequenced on ¼ of a picotiter plate using the Titanium FLX chemistry.

In order to compare 454 data with full-length sequences, PCR was performed to amplify the 16S rRNA gene with the 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') primer pair. PCR conditions included an initial denaturation step of 15 min at 94°C followed by 27 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 90 sec, and extension at 72°C for 90 sec. To facilitate cloning, a final extension step of 30 min at 72°C was conducted to produce A overhangs. PCR performance was assessed using gel electrophoresis, and the amplified products were cleaned using the Qiagen MinElute PCR Purification Kit and subsequently cloned using the Qiagen PCR Cloning Kit. Plasmids from 96 clones obtained from each sample were sequenced from both directions on an ABI3730XL Capillary Sequencer (Applied Biosystems, Foster City, USA). A total of 457 sequenced for all 5 samples remained after removal of low quality sequences and chimeras.

SSU rRNA gene data analysis

Analysis of pyrosequencing data was conducted using the mothur software version v.1.24.1 (5). Sequencing resulted in a total of 287,488 reads with a median length of 320 basepairs (bp). The reads were split according to barcodes, and error corrected and quality trimmed using PyroNoise (6) as implemented in mothur. These steps reduced the number of sequences to 134,692, which were then aligned to the SILVA database v102 (7) within mothur. Any sequences that did not cover positions 25,437 to 34,113 (*E. coli* variable regions 5 and 6) were discarded. To reduce sequencing noise a pre-clustering step as implemented in mothur (two base pairs difference) was performed (8) in addition to a check for chimeric sequences using UCHIME (9) as implemented in mothur. The resulting dataset of 131,421 sequence reads was used for all analyses and is available in the NCBI Sequence Read Archive under accession number PRJNA189184 (see Table 1 for read numbers per specimen). The sequences were clustered into OTUs at the 97% similarity level, and for alpha and beta

diversity analyses were subsampled to the sample size of the specimen with the least number of reads (18,676; Sp1). For taxonomic classification, sequences were searched against the 2011 version of the Greengenes database (10) using the method from Wang et al. (11) as implemented in mothur, with a bootstrap cutoff of 60%.

Full-length clone sequences were vector- and quality-trimmed with Codon Code Aligner 3.7.1 (CodonCode, Dedham, USA). The resulting contigs were imported into the mothur software, checked for chimeras against the SILVA v102 database as provided by mothur. The "classify.seqs" function was used to classify all sequences against the RDP database release 10 (12) as provided on the mothur webpage with a bootstrap cutoff of 80% to identify sequences originating from the coral host or *Symbiodinium*. These non-bacterial sequences were removed. The same function was used to classify all remaining sequences against the 2011 Greengenes database (10) as provided on the mothur webpage with a bootstrap cutoff of 60%. Contigs were aligned with the SILVA database v102 and any contigs that did not align at positions 1,044 to 43,116 were removed. The remaining contigs were clustered into operational taxonomic units (OTUs) with a 3% distance cutoff criterion. The OTUs were subsequently imported into ARB (13) and aligned against SILVA database v106 (7) that also contained custom sequences. The OTUs were aligned using the integrated 'fast aligner' and the alignment was manually examined and improved using the ARB edit 4 alignment tool. To produce phylogenetic trees, sequences that clustered together with the *Endozoicomonas* and *Burkholderia* OTUs, as well as representative sequences from each major taxonomic group, were selected. Trees were constructed for each bacterial group using both neighbor-joining and the maximum parsimony methods with 1,000 bootstrap replicates in ARB. The tree building process used custom filters that were designed for each dataset that excluded nucleotide positions not well represented in the alignments. Full-length *Endozoicomonas* and *Burkholderia* sequences are available on GenBank under accession numbers KC668823 to KC669277.

Sampling and 16S rRNA gene cloning and sequencing of other Red Sea coral species

In order to examine the ubiquity of *Endozoicomonas* in other coral species from the Red Sea, healthy specimens of *Acropora humilis* and *Pocillopora damicornis* (three of each species) were sampled for 16S rRNA gene cloning and sequencing. Colonies were sampled in July 2010 in the same area as the *S. pistillata* samples (19°52'24.48"N, 40°04'46.14"E, brown reef) (Supp. Fig. 1). Samples were rinsed in filtered seawater and frozen and stored at -80°C. For DNA extraction, the tissue was scraped off the skeleton and crushed in liquid nitrogen using a mortar and pestle. The DNA was then extracted from the tissue slurry using the Qiagen DNeasy Plant Mini Kit following the manufacturer's protocol. Amplification, cloning and sequencing, chimera checking and classification of the full-length 16S rRNA gene were performed as described above. A total of 412 sequences for these samples are available on GenBank under the accession numbers KC668414 to KC668822.

Design and testing of *Endozoicomonas* FISH probes

Probes targeting the SSU rRNA gene of the *Endozoicomonas* were designed using the Probe Design feature of the ARB software (13) and a custom database. This database included a total of 17,302 published 16S rRNA sequences of cultivated bacteria from the 'All Species Living Tree' project, version LTPs104 SSU (14). It also included over 2000 16S rRNA sequences of coral bacteria and *Endozoicomonas* spp. obtained from the SILVA 106 SSU database (7). A single probe was desired, but was not achievable, and the two following probes were identified as nearly specific for the *Endozoicomonas*:

Endozoi663 (5' AGGAGUGUGGAAUUUCC 3') and Endozoi736 (5' CUCUGGUCUGACACUGAC 3'). The specificity of the probes to *Endozoicomonas* 16S rRNA genes was examined by 1) comparison with the Test Probe tool against the SILVA SSU r108 database (>1200bp) (http://www.arb-silva.de/search/testprobe/), 2) BLASTn against the microbial 16S rRNA gene database of NCBI (15, 16), and 3) the 16S database using the Probe Match tool of the Ribosomal Database Project (17). Several sequences outside of the *Endozoicomonas* were found to theoretically hybridize with the probes, but no groups were previously found associated with corals. Probes were constructed by Eurofins MWG Operon and were labeled with the fluorochrome Cy3.

In order to develop stringent hybridization conditions for the probes, cultures of *Endozoicomonas elysicola* [DSMZ-22380] (18) and *E. montiporae* [LMG-24815] (19) were utilized, as well as an isolate of *Vibrio splendidus* [2B10] as a hybridization control. Cells (10^3 cells ml^{-1}) were fixed in 4% (v/v) paraformaldehyde (PFA) for 4–6 hours, filtered onto 25mm diameter, 0.2μm pore-sized polycarbonate membrane filters, and attached to microscope slides using Tough-Spots (USA Scientific). Hybridization solution (5, 15, 25, 35, 40, or 45% formamide - initially used for *E. elysicola* to evaluate probe stringency at different concentrations and based on results, 35 and 40% formamide only were then used for all cultures; 0.9M NaCl; 20mM Tris/HCl [pH 7.4]; 0.01% sodium dodecyl sulfate [SDS]) and probes (5 – 20 ng/μl) were added to each slide (with no-probe controls containing only hybridization solution) and incubated at 46°C for 16 – 18h in a moist chamber. Next, the filters were washed twice, each for 10 min at 48°C in 0.2 μm-filtered wash buffer with NaCl concentrations varying dependent on the formamide concentration (NaCl 0.636, 0.318, 0.159, 0.080, 0.056, or 0.040M for 5, 15, 25, 35, 40 or 45% formamide, respectively; 20mM Tris/HCl [pH 7.4]; 6mM EDTA [pH 8.0]). Filters were subsequently counter-stained with 4',6-diamidino-2-phenylindole (DAPI) solution (Sigma-Aldrich, Germany) (5 μg/ml in wash solution) for 10 min at 4°C, followed by a 10 min rinse in wash solution at 4°C. The hybridizations were examined under UV (for DAPI) and Cy3-specific filters using an Axioplan 2 Imaging epifluorescent microscope (Carl Zeiss, Germany), and cells were counted using the Zeiss AxioVision software (version 4.8.2). For each hybridization treatment and control, 500 cells were randomly counted under the UV and Cy3 filters (~20 locations on the slide). From these calculations, it was determined that hybridization solution containing 35% formamide and wash solution with 0.080M NaCl provided the highest efficiency and stringency hybridizations of Endozoi663 and Endozoi736 to *E. elysicola* (DSMZ-22380) and *E. montiporae* (LMG-24815) (100% of cells; Suppl. Fig. 5). Additionally, these experiments demonstrated that the probes did not hybridize to *Vibrio splendidus* 2B10 (0% of cells; Suppl. Fig. 6). The amount of probe was not found to alter these results.

Stylophora pistillata FISH analysis

The deep-frozen *Stylophora pistillata* samples were thawed and tissues fixed in 4% paraformaldehyde in 0.2 μm filter sterilized phosphate buffered saline (PBS) overnight. The tissues were then rinsed three times, each for 20 min with PBS, then decalcified at 4°C using 20% (w/v) tri-sodium EDTA (pH 7-8) in distilled water. The EDTA mix was changed daily until the samples were completely decalcified (no longer than 14 days). Following decalcification, the tissues were dehydrated using 30%, 50%, and 70% ethanol in PBS, before being stored in 70% ethanol at 4°C.

S. pistillata decalcified tissues were sectioned into ~1 mm pieces with a Zeiss SteREO Discovery dissecting microscope and a sterile blade and then rehydrated in series of 60% and 30% ethanol in 0.2 μm-filtered PBS solution. The tissues were then rinsed 3x in PBS for 10 min each. Next, samples were rinsed in hybridization solution (35% formamide, 0.9 M NaCl,

20 mM Tris/HCl [pH 7.4], 0.01% SDS) at room temperature for 10 min, followed by a second rinse for 1.5 - 2 h in the solution at the temperature of hybridization (46°C). The samples were then hybridized overnight (14-16 h) with a mixture of Endozoi663-Cy3, Endozoi736-Cy3 and EUB338-Cy5 (5' GCWGCCWCCCGTAGGWGT 3'; (20)) or Endozoi736-Cy3 and EUB338-Cy5 in hybridization solution at 46°C (final probe concentration 1ng/μl). Control samples were also exposed to the NON338 probe (5'ACTCCTACGGGAGGCAGC 3'; (21)), as well as no probe (only hybridization solution) at the same conditions. Following hybridization, samples were washed twice in pre-warmed hybridization wash (0.080M NaCl, 20 mM Tris/HCl [pH 7.4], 6 mM EDTA [pH 8.0], 0.01% SDS) for 20-30 min each, followed by a rinse (5 min) in the same solution, and a final rinse (2 min) in 100% isopropanol. Samples were then placed in 100 μl of antifading mounting medium (Citifluor, Ted Pella, Inc.) and stored on ice until examination. Samples were imaged under a 63x objective on a Zeiss LSM 710 confocal laser-scanning microscope using the Zen 2009 software. Cy3 emission was detected at 577 nm and Cy5 at 667 nm. For each *S. pistillata* colony (n = 5), 5-6 specimens were examined by imaging in 3-4 areas of the specimen, and compared with control specimens (no probe, nonsense probe) of the same samples. Zen 2009 Light Edition and ImageJ software (National Institute of Health) were used for scaling and compiling images from larger z-stacks.

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Suppl. Table 1: Primers tested for 16S rRNA gene amplification from coral DNA

Primer set	Variable regions amplified	Fwd primer name	Rev primer name	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	PCR product length*	T_A [°C]	Reference
Andersson	V5-V6	784F	1061R	AGGATTAGATACCCTGGTA	CRRCACGAGCTGACGAC	277	55	(1)
Nossa	V3-V4	347F	803R	GGAGGCAGCAGTRRGGAAT	CTACCRGGGTATCTAATCC	456	53	(22)
Chelius	V5-V6	799F	1115R	AACMGGATTAGATACCCKG	AGGGTTGCGCTCGTTG	335	53	(23)
27F-338R	V1-V2	27F	338R	AGAGTTTGATCCTGGCTCAG	TGCTGCCTCCCGTAGGAGT	311	55	(24)

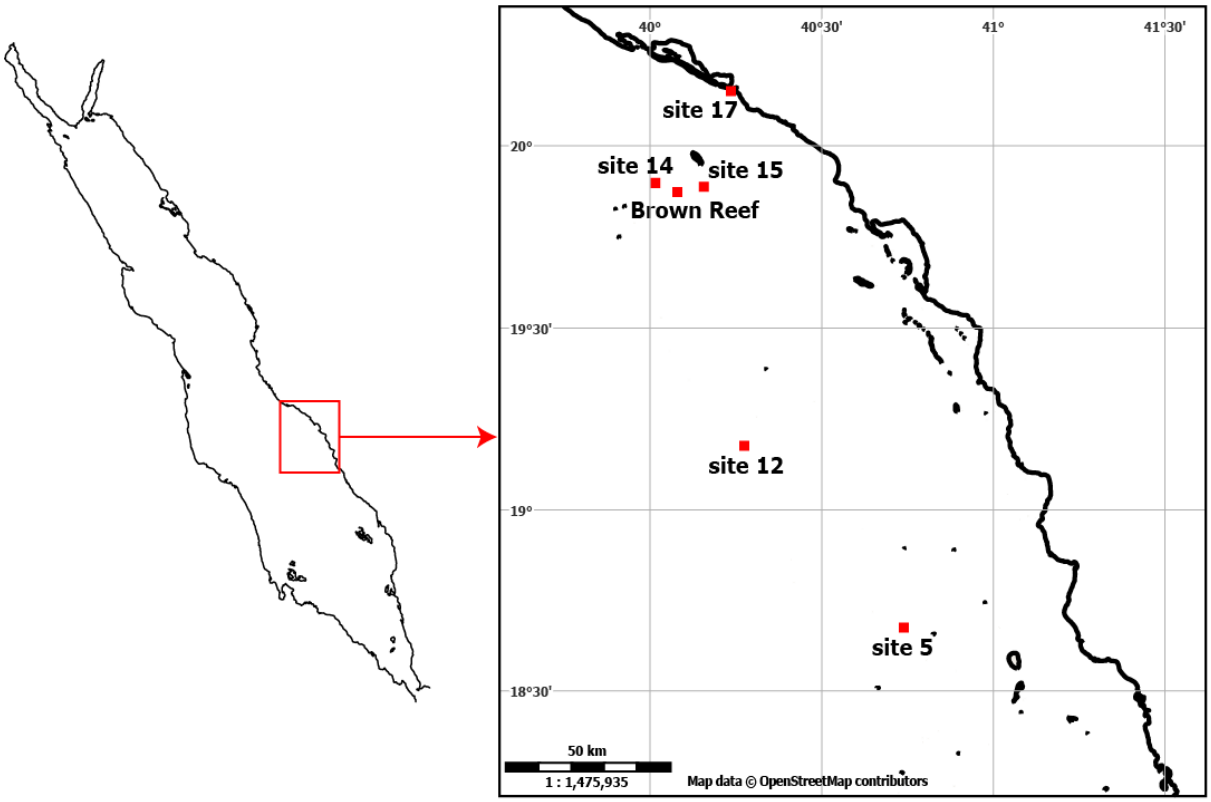
* PCR product length in *E. coli*; T_A = annealing temperature

Suppl. Table 2: Summary of primer fidelity. For each combination of primer set and coral species, the result was categorized as either suitable for analysis ("++") or not suitable ("--"). All suitable tests are shown in green; those that were not suitable are in red. In addition, primer sets that amplify the 16S gene of bacteria but have a disadvantage such as low amounts of side products, or a generally low amplification, are shown in yellow. Additional information is given in the notes in the table. All specimens were collected in the Red Sea unless otherwise noted. See Suppl. Table 1 for primer details.

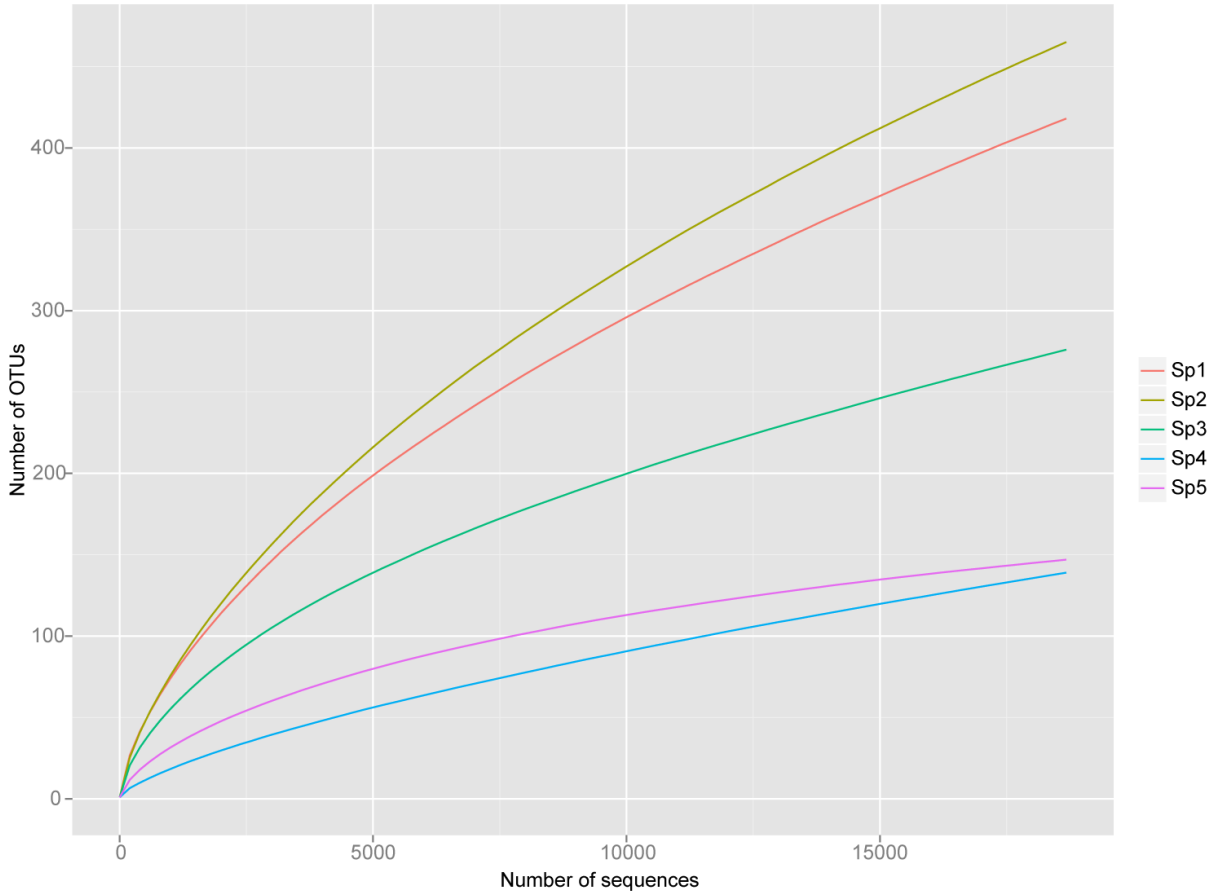
	Andersson	Nossa	Redford	27F-338R
Acropora hemprichii	++	--	++	+ [2]
Acropora humilis	++	+ [1]	++	++
Acropora sp.	+ [9]	--	+ [9]	++
Acropora sp.	-- [10]	--	-- [7]	-- [7]
Diploastrea heliopora	++	--	+	+ [2,8]
Echinophyllia sp.	++	--	+	+ [2,8]
Echinopora fruticulosa	--	--	-- [7]	-- [7]
Fungia sp.	+ [4]	+ [5]	+ [6]	++
Galaxea sp.	++	--	++	+ [2,8]
Goniastrea peresi	++	--	+	+ [2]
Leptoseris sp.	--	--	+	+ [2]
Montastrea sp.	++	--	+	+ [2,8]
Oulophyllia sp.	++	--	+	+ [8]
Pavona sp. (Gulf of Thailand)	++	--	-- [7]	-- [7]
Pocillopora sp.	++	--	+ [3]	++
Porites monticulosa	++	--	+	+ [2]
Porites nodifera	+ [8]	--	-- [7]	+ [2]
Porites verrucosa	+ [4]	--	+ [6]	+ [2]
Stylophora pistillata	++	--	++	+ [2]
Turbinaria sp.	++ [8]	--	-- [7]	-- [7]

[1] several close peaks ~410-500 bp
[2] several close peaks ~320-420 bp
[3] low amount side product 410 bp
[4] low amount side product 350 bp
[5] low amount side product 300 bp
[6] low amount side product 400 bp
[7] no product
[8] low product amount
[9] smear around 600
[10] low amount side product 210 bp

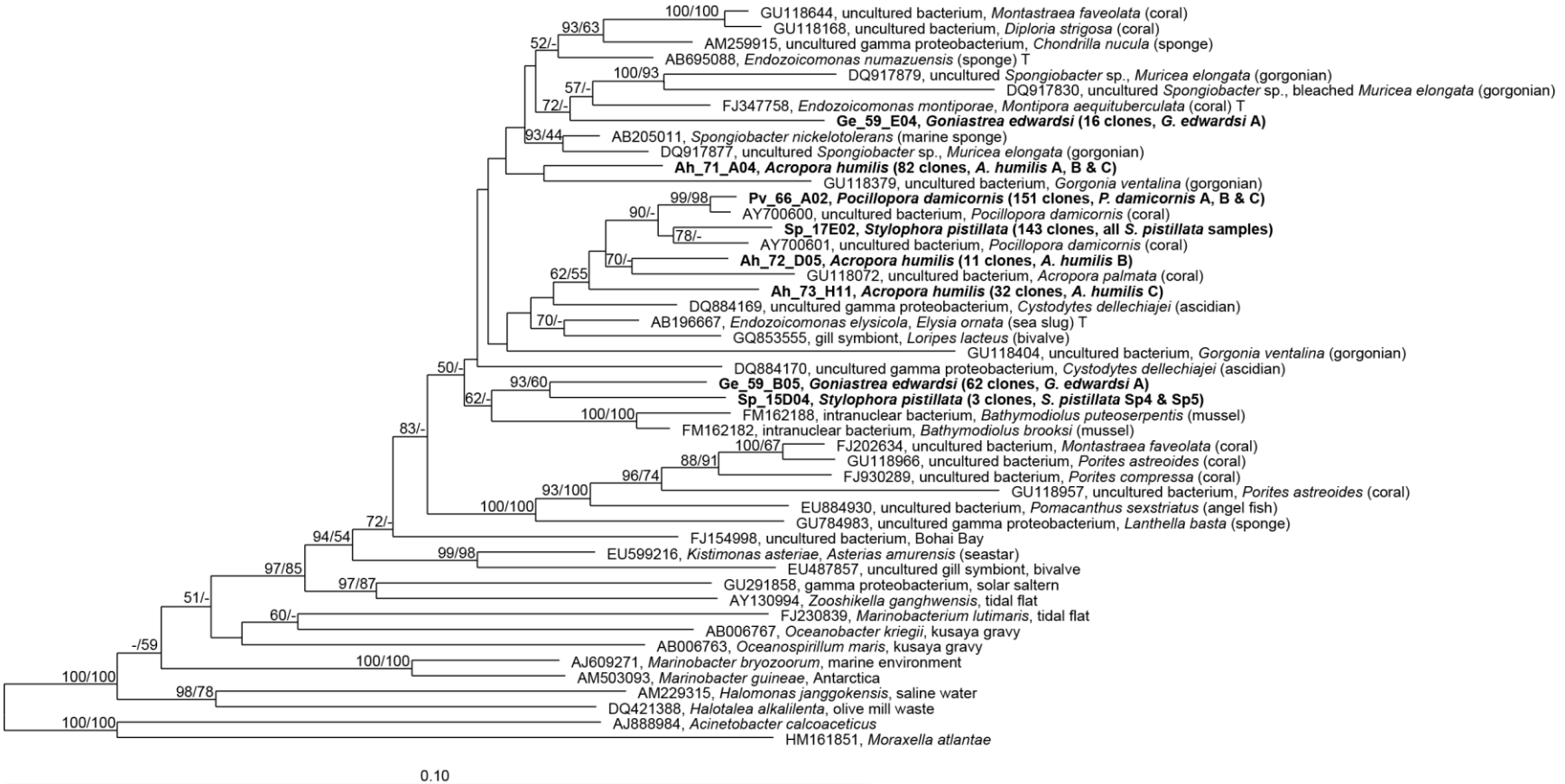
Supplementary figures



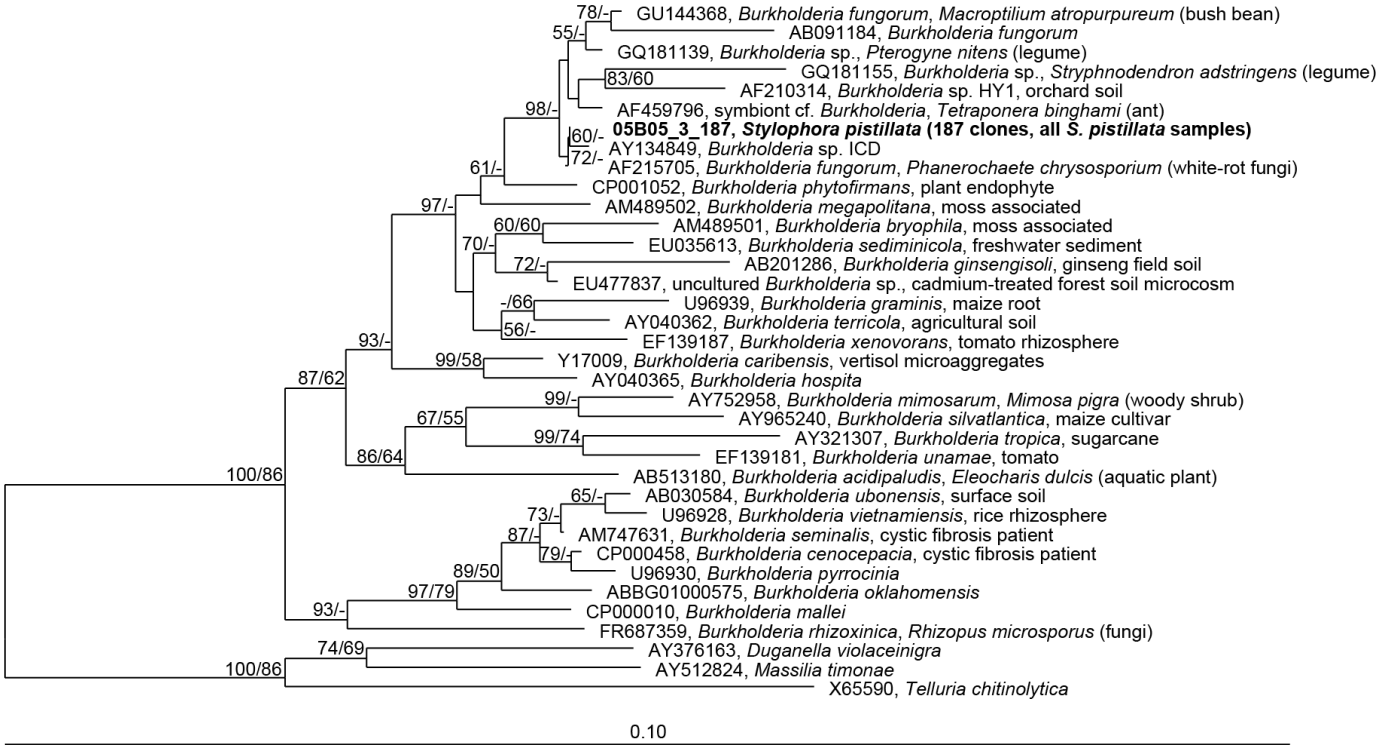
Suppl. Figure 1: Map of Red Sea sampling locations for corals examined in this study. Numbered sites correspond to the sampling locations of *Stylophora pistillata* for 454 sequencing and FISH analysis, "Brown Reef" was the sampling site for *Acropora humilis* and *Pocillopora damicornis*. The base map on the left is reprinted from Natural Earth. The base map on the right is reprinted from OpenStreetMap (copyright OpenStreetMap contributors), under the Open Database License.



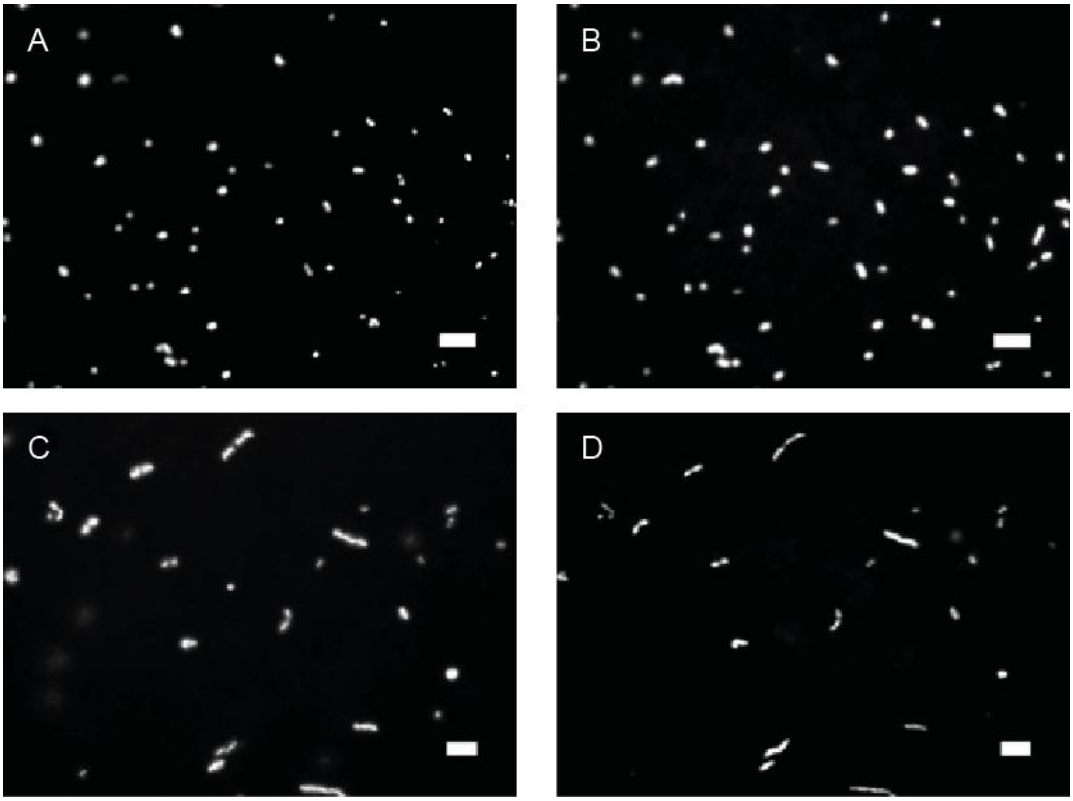
Suppl. Figure 2: Rarefaction plots for OTUs identified in *Stylophora pistillata* 454 16S rRNA gene sequencing.



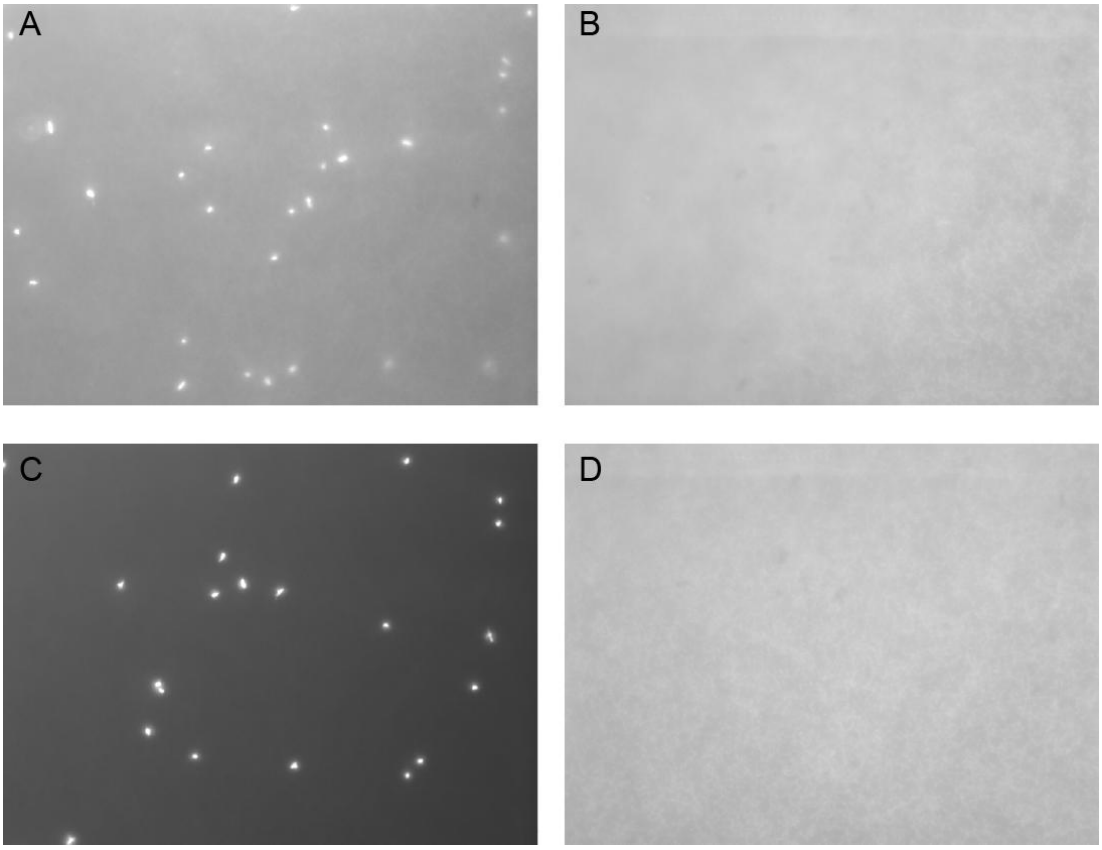
Suppl. Figure 3: Phylogenetic relationships between SSU rRNA gene clones from this study (in bold, with colony names provided), other members of the *Endozoicomonas* and representative sequences from the *Oceanospirillaceae* and *Gammaproteobacteria*. Numbers shown on branches are bootstrap values (1000 bootstraps) for neighbor joining and maximum parsimony, respectively. Bootstrap values less than 50% are not shown. The scale bar corresponds to 0.10 substitutions per nucleotide position.



Suppl. Figure 4: Phylogenetic relationships between SSU rRNA gene clones from this study (in bold) and other members of the *Burkholderia*. Numbers shown on branches are bootstrap values (1000 bootstraps) for neighbor joining and maximum parsimony, respectively. Bootstrap values less than 50% are not shown. The scale bar corresponds to 0.10 substitutions per nucleotide position.



Suppl. Figure 5: Evaluation of the hybridization stringency of *Endozoicomonas* specific probes using cultivated isolates. Micrographs of *E. elysicola* (DSMZ-22380) (A, B) and *E. montiporae* (LMG-24815) (C, D) stained with the nucleic acid stain DAPI (A, C) compared to Cy3 fluorescence from the specific probes Endozoi663 (B) and Endozoi736 (D).



Suppl. Figure 6: Evaluation of the hybridization stringency of *Endozoicomonas* specific probes using cultivated isolates. Micrographs of *Vibrio splendidus* 2B10 stained with the nucleic acid stain DAPI (A, C) compared to Cy3 fluorescence from the specific probes Endozoi736 (B) and Endozoi663 (D).