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A Specific Mix of Generalists: Microbial Symbionts in Mediterranean *Ircinia* spp.

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ABSTRACT

Microbial symbionts form abundant and diverse components of marine sponge holobionts, yet the ecological and evolutionary factors that dictate their community structure are unresolved. Here, we characterized the microbial symbiont communities of 3 sympatric host species in the genus *Ircinia* from the NW Mediterranean Sea, using electron microscopy and replicated 16S rRNA gene sequence clone libraries. All *Ircinia* host species harbored abundant and phylogenetically diverse symbiont consortia, comprised primarily of sequences related to other sponge-derived microbes. Community-level analyses of microbial symbionts revealed host species-specific genetic differentiation and structuring of *Ircinia*-associated microbiota. Phylogenetic analyses of host sponges showed a close evolutionary relationship between *I. fasciculata* and *I. variabilis*, the 2 host species exhibiting the most similar symbiont communities. In addition, several symbiont OTUs were exclusive to *I. variabilis* and *I. oros*, the 2 host species inhabiting semi-sciophilous communities in more cryptic benthic habitats. The generalist nature of individual symbionts and host-specific structure of entire communities suggest that: 1) a "specific mix of generalists" framework applies to microbial symbionts in *Ircinia* hosts, and 2) factors specific to each host species contribute to the distinct symbiont mix observed in *Ircinia* hosts, including habitat-specific conditions (e.g., irradiance and competition) and host evolutionary relatedness.

INTRODUCTION

Sponges are sessile, filter-feeding invertebrates that inhabit diverse marine ecosystems and host remarkable microbial symbiont populations (Taylor et al., 2007;

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45 Webster & Taylor, 2011), in some hosts accounting for up to 35% of sponge biomass
46 (Vacelet et al., 1975) and consisting of hundreds to thousands of symbiont taxa (Webster
47 et al., 2010; Lee et al., 2011). These diverse symbiont communities may enhance sponge
48 holobiont metabolism through non-metazoan processes, including photosynthesis (Erwin
49 & Thacker, 2008), nitrification (López-Legentil et al., 2010b) and sulfate reduction
50 (Hoffmann et al., 2005), and can produce defensive secondary metabolites (Flatt et al.,
51 2005) that decrease the susceptibility of host sponges to predation and fouling (Paul &
52 Ritson-Williams, 2008). In turn, sponge-associated microbes may benefit from the unique
53 microenvironment within host tissues, potentially nourished by the ammonia-rich end
54 products of animal metabolism and protected from open-ocean grazing pressures.
55 Although empirical evidence for symbiont benefit is scarce (Taylor et al., 2007), the high
56 biodiversity of sponge-associated microbes and their exclusivity to host sponges suggest
57 these niche habitats are fertile grounds for the evolutionary diversification of marine
58 microorganisms. To date, few host sponges have been investigated for microbial
59 symbionts and future research in sponge microbiology will further reveal the impact of
60 these symbioses on the ecological success of host sponges and the global biodiversity of
61 marine microorganisms (Taylor et al., 2004).

62 The study of sponge microbiology has benefited greatly from the application of
63 modern DNA sequencing technology, allowing for greater access to elusive symbiont
64 communities via culture-independent characterization and revealing striking trends in the
65 distribution and specificity of microbial symbionts. Perhaps most prominent is molecular
66 evidence for sponge-specific microbial lineages, distinct clusters of sponge-derived
67 sequences that represent major components of sponge-associated microbial communities

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68 but are absent, or found in extremely low abundances (i.e., rare biosphere; Webster et al.,
69 2010; Lee et al., 2011), in ambient bacterioplankton (Taylor et al., 2007). While specific
70 to host sponge microenvironments, these symbiont lineages often exhibit a generalist
71 distribution among sponge hosts, occurring in taxonomically distinct hosts from distant
72 geographic regions (Olson & McCarthy, 2005; Hill et al., 2006; Sipkema et al., 2009) and
73 prompting early hypotheses on uniform microbial communities in marine sponges
74 (Hentschel et al., 2002). Typically, sponge-specific clades are common in high-microbial-
75 abundance (HMA) sponge hosts and absent in low-microbial-abundance (LMA) sponge
76 hosts (Hentschel et al., 2006; Weisz et al., 2007; Schmitt et al., 2011), where symbiont
77 communities are less diverse and contain more specialized (host species- or genus-
78 specific) microbes (Gernert et al., 2005; Holmes & Blanch, 2007; Schmitt et al., 2008).
79 An additional benefit of sequence-based approaches to characterize the sponge
80 microbiota is the accessibility of individual datasets and cumulative databases for
81 comparative studies and meta-analyses. A comprehensive review of sponge-derived 16S
82 rRNA gene sequences revealed that nearly one-third (32%) of all symbiont sequences
83 were associated with sponge-specific phylogenetic clades (Taylor et al., 2007).
84 From a host perspective, the composition and structure of microbial symbiont
85 communities have been reported as species-specific, despite the presence of sponge-
86 specific clusters within these communities (Webster et al., 2010; Lee et al., 2011). The
87 apparent conflict of distinct, host-specific symbiont communities that consist of shared,
88 sponge-specific symbiont lineages is explained by: 1) the selective presence (and relative
89 abundance) of putatively cosmopolitan symbionts among host sponges, and 2) the high
90 genetic diversity within sponge-specific sequence clusters. Indeed, the widespread

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91 distribution of many sponge symbionts does not imply these symbionts are a ubiquitous
92 component of all HMA sponge hosts. For example, symbionts from the sponge-specific
93 cluster SC8 (*Chloroflexi*) exhibit an inter-ocean distribution, recovered from a Great
94 Barrier Reef sponge (*Rhopaloeides odorabile*; Webster et al., 2001b) and two Bahamian
95 sponges (*Agelas digitata* and *Plakortis* sp.; Taylor et al., 2007), and yet are absent in the
96 HMA sponge *Ircinia ramosa* collected at the same depth and habitat (within 100 m^2) as
97 *R. odorabile* (Webster et al., 2010). Further, the definition of a sponge-specific symbiont
98 lineage does not require a sequence identity minimum to delineate sequence clusters
99 (Hentschel et al., 2002). Thus, the high genetic diversity exhibited within these microbial
100 sequence clusters, in some cases exceeding 20% sequence divergence, can encompass
101 multiple microbial OTUs from the species to the family level.
102 The factors that drive the observed differences in symbiont communities among
103 host sponge species are not fully resolved, with previous reports suggesting a role for
104 both environmental and host-specific factors. Multiple environmental factors have been
105 hypothesized to influence microbial symbiont composition in marine sponges, including
106 abiotic (temperature, nutrient levels, heavy metals) and biotic (competition, predation,
107 disease) factors (Webster et al., 2001a; Mohamed et al., 2008b; Webster et al., 2008a;
108 Webster et al., 2008b; Anderson et al., 2010; Turque et al., 2010; Angermeier et al.,
109 2011; Webster et al., 2011); however, several studies have shown that different host
110 species from the same habitat harbor distinct symbiont communities (Lee et al., 2009b;
111 Radwan et al, 2010), including the first 2 studies applying pyrosequencing technology to
112 the characterization of sponge-associated microorganisms (Webster et al., 2010; Lee et
113 al., 2011). Together, these results indicate that environmental conditions may influence

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114 symbiont community structure in some host sponges, yet environmental factors alone are
115 not sufficient to fully explain the observed patterns of symbiont community structure.
116 Host-specific factors may also affect the composition of microbial symbiont in marine
117 sponges, although few studies have targeted congeneric hosts from the same habitat (Lee
118 et al., 2009b) or utilized molecular techniques to resolve host sponge phylogenies
119 (Erpenbeck et al., 2002; Thacker & Starnes, 2003; Thacker et al. 2007; Sipkema et al.,
120 2009).

121 To investigate the structuring and specificity of microbial symbionts in closely
122 related HMA host sponges, we studied 3 sympatric species in the genus *Ircinia* from the
123 Mediterranean Sea. The genus *Ircinia* is a chemically diverse and symbiont rich sponge
124 taxon that exhibits high species richness, occurring in shallow to deep-water habitats of
125 tropical and temperate marine environments. The chemical diversity of *Ircinia* spp.
126 includes diverse terpenoid compounds, most commonly sesterterpenoids (Cafieri et al.,
127 1972; Liu et al., 2006). These secondary metabolites have been shown to exhibit both
128 ecological functioning (e.g., anti-predatory activity; Pawlik et al., 2002) and biological
129 activity (e.g., anti-tumor cytotoxicity; Choi et al., 2004). The microbial diversity of
130 *Ircinia* spp. is consistent with other HMA host sponges, composed largely of sponge-
131 specific sequences from *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Nitrospira*,
132 *Poribacteria* and *Proteobacteria* (Schmitt et al., 2007; Schmitt et al., 2008; Mohamed et
133 al., 2008a, Mohamed et al., 2008b, Mohamed et al., 2010; Webster et al., 2010; Yang et
134 al., 2011). These studies have focused on the microbiota in Caribbean and Indo-Pacific
135 host species, whereas the molecular diversity of microbial symbionts in Mediterranean

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136 *Ircinia* hosts has been addressed by a single study that focused specifically on
137 cyanobacteria (Usher et al., 2004).
138 In this study, we characterized the microbial communities in the common
139 Mediterranean species, *Ircinia fasciculata*, *I. variabilis* and *I. oros*, using 16S rRNA gene
140 sequence clone libraries and compared the richness, diversity, structure and specificity of
141 symbiont communities among these congeneric and sympatric hosts. Phylogenetic
142 analyses were conducted to compare symbionts in Mediterranean *Ircinia* spp. with
143 previously described sponge-associated microbes, including sequences derived from
144 Caribbean *Ircinia* spp. In addition, we resolved the phylogenetic relationships among the
145 3 host sponges using ribosomal and mitochondrial DNA markers, thus allowing for the
146 determination of symbiont specificity within a well-defined host phylogenetic context.
147

148 **MATERIAL AND METHODS**

149 <u>Sample Collection</u>

150 The marine sponges *Ircinia fasciculata* (PALLAS 1766; Fig. 1a), *I. variabilis*
151 (SCHMIDT, 1862; Fig. 1b) and *I. oros* (SCHMIDT, 1864; Fig. 1c) were collected from
152 shallow (< 20 m) littoral zones at 3 neighboring sites (< 12 km apart) along the Catalan
153 Coast (Spain) in the northwestern Mediterranean Sea. *I. fasciculata* colonies were
154 sampled at Punta de S'Agulla (Blanes; 41º 40' 54.87" N, 2º 49' 00.01" E), *I. variabilis* at
155 Mar Menuda (Tossa de Mar; 41º 43' 13.62" N, 2º 56' 26.90" E) and *I. oros* at Punta
156 Santa Anna (Blanes; 41º 40' 21.48" N, 2º 48' 13.55" E) by SCUBA during 3 consecutive
157 days in March 2010. At each site, ambient seawater samples (500 ml) were collected
158 simultaneously and in close proximity (< 1 m) to sampled sponges. Sponge and seawater

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159 samples were transported in an insulated cooler to the laboratory (ca. 2 hrs. transit time),
160 where sponge samples were preserved in 100% ethanol and stored at -20 ºC and seawater
161 samples were concentrated on 0.2 μm filters and stored at -80 ºC.
162

163 Transmission Electron Microscopy (TEM)

164 To visualize the microbial diversity present in *I. variabilis*, *I. fasciculata* and *I.*
165 *oros,* small ectosomal and choanosomal tissue pieces (ca. 4 mm^3) were dissected and
166 fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde, buffered with
167 filtered seawater. Samples were incubated in the fixative mixture overnight at 4ºC and
168 subsequently rinsed with filtered seawater to remove fixative, then dehydrated in a
169 graded ethanol series and embedded in Spurr resin at room temperature. A Reichert
170 Ultracut microtome was used to produce ultra-thin sections (ca. 60 nm) that were
171 contrasted with uranyl acetate and lead citrate for ultrastructural observation (Reynolds,
172 1963). TEM observations were performed at the Microscopy Unit of the Scientific and
173 Technical Services of the University of Barcelona on a JEOL JEM-1010 (Tokyo, Japan)
174 coupled with a Bioscan 972 camera (Gatan, Germany).
175

176 DNA Extraction

177 Metagenomic DNA extracts were prepared separately for 3 individuals of each
178 host sponge species (including ectosomal and choanosomal tissue) and 3 samples of
179 concentrated seawater (1 from each collection site) using the DNeasy® Blood & Tissue
180 Kit (Qiagen®). Full-strength and 1:10 diluted DNA extracts were used as templates in
181 PCR amplifications.

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16S rRNA Gene Sequence Clone Libraries

The universal bacterial forward primer 8F (5'-AGA GTT TGA TCA TGG CTC AG-3')(Reysenbach et al., 1994) and reverse primer 1509R (5'-GGT TAC CTT GTT ACG ACT T-3')(Martínez-Murcia et al., 1995) were used to amplify approximately 1,500 bp fragments of bacterial 16S rRNA gene sequences from all sponge and seawater extracts. Total PCR reaction volume was 50 μl, including 10 pmol of each primer, 10 nmol of each dNTP, 1X Reaction Buffer (Ecogen) and 5 units of BIOTAQ™ polymerase (Ecogen). Thermocycler reaction conditions were an initial denaturing time of 2 min at 94ºC, followed by 30 cycles of 1 min at 94ºC, 0.5 min at 50ºC, and 1.5 min at 72ºC, and a final extension time of 2 min at 72ºC. To minimize PCR amplification biases, a low annealing temperature and low cycle number were used and 3 separate reactions were conducted for each sample. PCR amplification products were gel-purified and cleaned using the QIAquick Gel Extraction Kit (Qiagen®). Triplicate PCR products were combined and quantified using a Qubit™ fluorometer and Quant-iT™ dsDNA Assay Kit (Invitrogen™). Purified PCR products (25 ng) were ligated into plasmids using the pGEM®-T Vector System (Promega).

Individual clones were PCR-screened using vector primers and clones with approximately 1,500 bp inserts were purified and sequenced at Macrogen Inc. A single sequencing reaction was performed for all clones to recover the 5'-end of 16S rRNA gene sequences using the primer 800R (5'-TAC CAG GGT ATC TAA TCC-3'). Ambiguities on sequencing reaction ends were excluded by trimming sequences at the 5'-end to the highly conserved *E. coli* site 54 and at the 3'-end to *E. coli* site 754, yielding sequences

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205 ranging from 613 to 725 bp (average length = 683 bp) that were used for diversity
206 calculations and phylogenetic metrics. In addition, bi-directional sequencing reactions
207 with vector primers were performed to recover near full-length 16S rRNA gene
208 sequences (range = 1,423 to 1,523 bp; average = 1,491 bp) of representative clones (*n* =
209 39) for phylogenetic analyses. Representative clones consisted of bacterial OTUs that
210 were either: 1) 'common' – occurring at least twice in clone libraries, or 2) 'unique'–
211 exhibiting greater than 5% sequence divergence from any known sequence. Raw
212 sequence data were processed in Geneious (Drummond et al., 2010) and low quality
213 sequencing reads were discarded. Sequences were screened for sequencing anomalies
214 (e.g., chimeras) using Mallard (Ashelford et al., 2006) and a reference 16S rRNA gene
215 sequence from *E. coli* (GenBank accession no. U00096). Putative sequence anomalies
216 were subsequently confirmed or refuted using Pintail (Ashelford et al., 2005) and two
217 related reference 16S rRNA gene sequences. All confirmed chimeras were removed from
218 the dataset. High quality sequences are archived in GenBank under accession nos.
219 JN655200-JN655511.
220

221 Operational Taxonomic Unit (OTU) Assignment and Composition

222 Clone library sequences were ascribed to OTUs calculated at different sequence
223 identity percentages (99%, 97%, 95%, 90%, 85% and 80%) using the nearest neighbor
224 algorithm, as implemented in the mothur software package (Schloss et al., 2009). The
225 observed OTU richness (S_{obs}) for each microbial community was compared across
226 different OTU thresholds, calculated as total (combined sequence data by source) and
227 average values (separated sequence data by samples). All subsequent OTU-based metrics

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228 were conducted using an OTU classification at 99% sequence identity. Representative
229 sequences from each 99% OTU were analyzed by using a nucleotide-nucleotide BLAST
230 search (Altschul et al., 1990) to find the most closely related sequence, and by using the
231 Ribosomal Database Project II (Cole et al., 2007) sequence classifier to assess taxonomic
232 affiliations.
233
234 Microbial Community Diversity
235 The diversity of recovered bacterial communities in *Ircinia* spp. and ambient
236 seawater were compared using multiple metrics for OTU richness, dominance and
237 evenness, calculated in the mothur software package. Richness calculations included
238 observed species richness (99% OTUs), rarefaction analysis and expected species
239 richness using the Chao1 estimator (S_{Chao1}) to determine both the sampled diversity and
240 total expected diversity of bacterial communities. In addition, the effect of increase
241 sequencing effort on OTU richness was estimated using the Boneh calculation (Boneh et
242 al., 1998). Dominance metrics included Simpson's inverse index ($1/D$) and the Berger-
243 Parker index (d), the former a reciprocal version of Simpson's heterogeneity index ($D =$
244 $\sum p_i^2$, where p_i is the proportion of individuals in species i) interpretable as the number of
245 equally common species that would produce the observed heterogeneity, and the latter a
246 simple metric that calculates the relative abundance of the most dominant OTU.
247 Evenness calculations included the Simpson's evenness measure ($E_{1/D}$), based on
248 Simpson's inverse index ($1/D$), and Smith & Wilson's evenness index (E_{VAR}). Both
249 indices provide an evenness measure independent of species richness that ranges 0 to 1;
250 however, $E_{1/D}$ places equal weight on both common and rare taxa, while with E_{VAR}

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251 common species received higher weighting (i.e., greater influence on the evenness
252 measure) than rare species.
253 Genetic diversity of symbiont communities was compared among host species
254 and seawater sources using nonparametric tests for homogeneity of molecular variance
255 (HOMOVA) and an analysis of molecular variance (AMOVA; Stewart & Excoffier,
256 1996). These tests provide OTU-independent assessments of genetic variation and
257 differentiation within and among bacterial communities inhabiting *Ircinia* spp. and
258 seawater. HOMOVA tests whether the genetic variation observed within each population
259 (i.e., bacterial community) differs among sources (i.e., host sponge species or seawater),
260 thus providing a comparative measure of genetic diversity within each population.
261 AMOVA tests whether the genetic diversity observed within each population differs from
262 the total genetic diversity resulting from pooling the populations, thus providing a
263 comparative measure of genetic differentiation among populations. Distances were
264 calculated for AMOVA using the Tajima and Nei algorithm with $\alpha = 0.05$. Using the
265 Arlequin software package, version 3.5 (Excoffier & Lischer, 2010), a hierarchical
266 partitioning of genetic variation was assessed across different levels (among sources,
267 among replicates within sources and among sequences within each replicate) and
268 pairwise variation among sources was computed as F_{ST}, with statistical significance based
269 on 1000 permutations. Distributions of unique lineages among bacterial communities
270 were examined using a phylogenetic lineage-sorting test (P-test; Martin, 2002), also
271 referred to as the parsimony test (Schloss, 2008).
272
273 Microbial Community Structure & Similarity

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274 To determine the distribution of bacterial OTUs within each community, OTU
275 rank-abundance plots were constructed and compared to a fitted log series and geometric
276 series distributions by calculating the Kolmogorov-Smirnov test statistic (D_{max}). The
277 significance of D_{max} statistics were determined by comparison to critical values calculated
278 at α=0.05 and 0.01. Community similarity among sources was calculated as Bray-Curtis
279 similarity values and visualized in complete linkage similarity dendrograms using
280 PRIMER v6 (Plymouth Marine Laboraroty, UK) computer software. Finally, the integral
281 form of LIBSHUFF (∫-LIBSCHUFF; Schloss et al., 2004) was used to test pairwise
282 differences in microbial communities from each source. ∫-LIBSHUFF analysis was
283 chosen because it represents a relatively 'generic' test of differences between microbial
284 communities, based on both membership and structure (Schloss, 2008). ∫-LIBSHUFF
285 analyses were implemented in the mothur software package, with significance values
286 determined by 100,000 randomizations and adjusted using Bonferroni corrections to
287 maintain an experiment-wise critical value of 0.05 across multiple pairwise comparisons
288 (Sokal & Rohlf, 1995).
289
290 Phylogenetic analysis
291 Phylogenetic analyses of 16S rRNA gene sequences were conducted to determine
292 the affiliations between sequences recovered from *Ircinia* spp. herein and previously
293 characterized sponge symbionts. In particular, sequences from recent studies
294 characterizing sponge-associated bacterial communities in *Ircinia* species from the
295 Caribbean were targeted. Publicly available datasets for *I. felix* (Schmitt et al., 2007;
296 Schmitt et al., 2008) and *I. strobilina* (Mohamed et al., 2008b; Yang et al., 2011) were

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297 retrieved from GenBank and grouped into 99% OTUs, following the methods employed
298 for the clone libraries herein. Representative sequences from each OTU for *I. felix* (*n* =
299 102) and *I. strobilina* (*n* = 156), top matching sequences from BLAST searches (*n* =
300 189), and near full-length 16S clones (*n* = 39) and partial 16S clones (*n* = 87) from this
301 study were aligned to the greengenes reference database (DeSantis et al., 2006) using the
302 mothur software package, with an outgroup sequence from Archaea (*Haloarcula*
303 *vallismortis*, GenBank accession no. D50581). Maximum likelihood phylogenetic trees
304 were constructed in RAxML (Stamatakis et al., 2005) using the General Time Reversible
305 model with a gamma distribution of variable substitution rates among sites (GTR+G).
306 Data were resampled using 100 bootstrap replicates and a thorough ML search was
307 conducted to optimize the topology and recover the best-scoring tree. Due to the variable
308 length of 16S rRNA gene sequences being compared (422 to 1,526 bp), a binary
309 backbone constraint tree was constructed from long (>1,000 bp) sequences and used to
310 restrict topology changes when introducing short (<1,000 bp) sequences into the
311 phylogeny. This method allowed for: 1) accurate reconstruction of deeper nodes, based
312 on the most informative sequences, and 2) precise placement of short 16S gene sequence
313 fragments near terminal nodes, for comparative analysis with previous *Ircinia* sp. datasets
314 (e.g., excised and sequenced DGGE bands; Schmitt et al., 2007).
315
316 Molecular Identification of Host Sponges
317 Sponge samples were identified to species using morphological observations,
318 including gross morphology and fiber characteristics. In addition, ribosomal and
319 mitochondrial molecular markers were used to objectively test morphological

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320 characterizations, as some controversy surrounds the taxonomic status of *Ircinia*
321 *fasciculata* and *I. variabilis* (Pronzato et al., 2004) and these species exhibit high
322 phenotypic plasticity that can confound their identification in the field (Maldonado et al.,
323 2010). A segment of nuclear ribosomal DNA corresponding to the 3'-end of the 5.8S
324 subunit, the entire second internal transcribed spacer (ITS-2) region and the 5'-end of the
325 28S subunit were PCR-amplified following the methods of Erwin & Thacker (2007). A
326 segment of mitochondrial DNA corresponding to the cytochrome oxidase subunit I (COI)
327 was PCR-amplified using a degenerated version of the universal barcoding forward
328 primer dgLCO1490 (5'-GGT CAA CAA ATC ATA AAG AYA TYG G-3')(Meyer et al.,
329 2005) and the reverse primer COX1-R1 (5'-AAT ACT GCT TTT TTT GAT CCT GCC
330 GG-3'). This primer combination yielded a segment of mtDNA encompassing both the
331 standard barcoding fragment (i.e., "Folmer" partition)(Folmer et al., 1994, Herbert et al.,
332 2003) and the I3-M11 partition (Erpenbeck et al., 2006), which spans the internal loop 3
333 to the transmembrane domain 11. The resulting PCR amplification products were gel-
334 purified and cleaned using the QIAquick Gel Extraction Kit (Qiagen®) and ligated into
335 plasmids using the pGEM®-T Vector System (Promega). Individual clones were PCR-
336 screened using vector primers, purified and sequenced at Macrogen Inc. Bi-directional
337 sequencing reactions were performed for all clones using vector primers to recover the
338 entire cloned amplicons. Raw sequence data were processed in Geneious and low quality
339 sequencing reads were discarded. High quality sequences are archived in GenBank under
340 accession nos. JN655171-JN655199.

341 For COI fragments, 2 to 4 clones were recovered per sponge individual (5 to 9
342 clones per species) and used to construct consensus sequences. For rDNA fragments, 2 to

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3 clones were recovered per sponge individual (6 to 8 clones per species) and consensus sequences were processed separately for each clone, due to the potential for intragenomic variation among the multiple ITS-2 copies in tandemly repeated rDNA clusters (Wörheide et al., 2004). Consensus sequences for rDNA fragments were aligned using MAFFT (Katoh et al., 2002) with outgroup sequences from *Smenospongia aurea* (Dictyoceratida; Thorectidae)(Erwin & Thacker, 2007). Alignment of consensus COI sequences was unequivocal, due to the protein coding nature of these sequences, and included the congeneric Caribbean species *I. strobilina* (GenBank accession no. GQ337013; Erpenbeck et al. 2009) and the outgroup species *Hippospongia lachne* (Dictyoceratida; Spongiidae)(EU237484; Lavrov et al., 2008). Pairwise genetic distance matrices (uncorrected p-distance) were constructed using the software package mothur. For rDNA fragments, maximum likelihood (ML) phylogenies were constructed using PHYML (Guidon & Gascuel, 2003) and the Hasegawa-Kishino-Yano model with a gamma distribution of variable substitution rates among sites (HKY+G), as suggested by FINDMODEL (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) based on the Akaike information criterion; data were resampled using 100 bootstrap replicates. Neighbor-Joining trees were constructed using Geneious and the HKY model of nucleotide substitution; data were resampled using 1000 bootstrap replicates.

RESULTS

Transmission Electron Microscopy

Electron microscopy observations of host sponge tissue revealed a high density of bacterial cells (Fig. 1). Characteristic of high-microbial-abundance (HMA) sponges,

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366 examined sections were comprised primarily of bacterial cells, with only occasional
367 sponge cells (archeocytes) and structural elements (spongin and collagen fibers). In *I.*
368 *fasciculata* and *I. variabilis*, ectosomal (peripheral) tissue sections revealed dense
369 populations of "*Candidatus* Synechococcus spongiarum" identifiable by their
370 characteristic spiral thylakoid membranes encompassing the perimeter of the cells. *S.*
371 *spongiarum* cells dominated the ectosomal regions of host tissue and were observed to be
372 actively dividing, exhibiting several stages of binary fission (Fig. 1d,e). In *I. oros*, no *S.*
373 *spongiarum* symbionts or other cyanobacterial cells were observed in ectosomal tissue,
374 rather a high density of heterotrophic bacteria occurred consisting of multiple bacterial
375 morphotypes, some of which were also showing active cell division (Fig. 1g). Sections
376 from deeper tissue regions (choanosome) of *I. variabilis* revealed the absence of *S.*
377 *spongiarum* cells and the proliferation of heterotrophic bacteria cells, many with similar
378 morphotypes to those observed in *I. oros* ectosomal tissue (Fig. 1f).
379
380 Microbial Community OTUs and Coverage
381 Bacterial 16S rRNA gene sequences recovered from *I. fasciculata* (*n* = 77), *I.*
382 *variabilis* (*n* = 80), *I. oros* (*n* = 82) and ambient seawater (*n* = 73) grouped into 124
383 OTUs, defined by 99% or greater sequence similarity. Grouping sequences according to
384 lower identity thresholds reduced the number of OTUs within each source community;
385 however, the same trend in comparative OTU richness among communities was observed
386 (Fig. S1). Coverage estimates revealed that sampled sponge-associated bacterial
387 communities represented the majority of expected diversity (76.3% in *I. variabilis*, 80.5%
388 in *I. fasciculata*, 81.7% in *I. oros*) and doubling the sampling effort conducted herein

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389 were predicted to produce few (5 to 6) new OTUs. In contrast, recovered seawater
390 bacterial communities represented less than half (43.8%) of the total expected diversity
391 and doubling the sampling effort was predicted to produce 12 new OTUs, indicating
392 more extensive sampling is required to fully characterize bacterioplankton diversity.
393 Similarly, rarefaction analyses showed the sponge-associated bacterial communities
394 beginning to reach OTU saturation, whereas seawater bacterial communities continued to
395 rapidly accumulate new OTUs (Fig. S2).
396

397 Microbial Community Composition

398 Sponge-associated microbial communities exhibited high diversity and were
399 comprised of 9 phyla, including representatives from 4 classes of *Proteobacteria* (*Alpha*-,
400 *Beta*-, *Gamma*- and *Deltaproteobacteria*; Table 1). Sequences affiliated with
401 *Proteobacteria* and *Bacteroidetes* were recovered from all 3 *Ircinia* host species and
402 seawater, whereas *Acidobacteria*, *Nitrospira*, *Chloroflexi* and *Gemmatimonadetes* were
403 exclusively found in *Ircinia*-associated communities. Within the sponge-associated
404 microbiota, *Deltaproteobacteria*-affiliated sequences comprised an abundant component
405 of microbial communities in all 3 host species (>15% total clones), although phylum-
406 level differentiation of symbiont communities among hosts was apparent, with a high
407 relative abundance of *Cyanobacteria* in *I. fasciculata*, *Nitrospira* in *I. variabilis* and
408 *Acidobacteria* in *I. oros* (Table 1). Ambient seawater microbes represented 10 phyla and
409 included 4 phyla not detected in sponge-associated communities (*Chlorophyta*,
410 *Firmicutes*, *Planctomycetes* and *Verrucomicrobia*; Table 1).

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411 The vast majority of 16S rRNA gene sequences recovered from *I. fasciculata*
412 (87.0%), *I. variabilis* (85.0%) and *I. oros* (81.0%) matched most closely to other sponge-
413 derived bacterial sequences, generally at high sequence identity levels (≥ 97%; Fig. 2).
414 Sequences from *Ircinia* spp. not associated with sponge-derived clones were most
415 commonly matched to sequences from marine sediment (*n* = 19), corals (*n* = 4) and
416 seawater (*n* = 3). Most sequences obtained from ambient seawater bacteria (98.6%) were
417 closely related to sequence from other bacterioplankton sources, with nearly all
418 sequences (90.3%) matching at very high sequence identity levels (≥ 99%; Fig. 2). In
419 fact, only 3 sequences from seawater clone libraries did not exhibit a top match to another
420 bacterioplankton clone, with 2 matching to coral-associated bacteria and 1 to a bacterium
421 from a sulfate-reducing bioreactor.
422
423 Microbial Community Diversity
424 Seawater bacterioplankton communities were clearly differentiated from the
425 sponge-associated bacterial communities, exhibiting higher OTU richness (observed and
426 expected), lower dominance indices and higher evenness indices (Table 2). The most
427 dominant OTU is the seawater community accounted for 8.0% of the total community
428 and singleton OTUs (*n* = 41) accounted for over 80% of all OTUs. In fact, only 5 OTUs,
429 (9.4%) were recovered more than twice in seawater clone libraries.
430 Among the 3 host sponges, bacterial communities in *I. variabilis* and *I. oros*
431 exhibited very similar richness and evenness values (Table 2), suggesting a similar
432 diversity of symbionts among these host species. Dominant OTUs in *I. variabilis* and *I.*
433 *oros* accounted for 15% of each community, with the top 3 most abundant OTUs

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434 accounting for one-third of the total community. Approximately half of the OTUs
435 recovered from *I. variabilis* (n = 19, 55.9%) and *I. oros* (n = 15, 45.5%) were singletons.
436 By comparison, the bacterial community in *I. fasciculata* exhibited lower OTU richness
437 and a less even (more dominant) community structure (Table 2), driven by a single OTU
438 (corresponding to the cyanobacterium *Synechococcus spongiarum*) that accounted for
439 over one-fourth of all recovered clones. The top 3 bacterial OTUs accounted for nearly
440 half (48.1%) of all recovered clones and singleton OTUs were common (n = 15, 51.7%).
441 Notably, although *I. fasciculata* hosted a comparatively less diverse community,
442 overlapping confidence intervals for index values were observed among all *Ircinia* hosts
443 (Table 2), indicating similar symbiont diversity across the 3 host species.
444 Genetic diversity analyses revealed similar trends to OTU-based metrics (Table
445 3). Microbial communities in seawater exhibited significantly higher genetic variation
446 compared to those in *Ircinia* sponges (HOMOVA, P < 0.005), indicating higher genetic
447 diversity in the bacterioplankton communities. No significant differences in genetic
448 variation among communities were observed in pairwise comparisons of host sponge
449 species (HOMOVA, P > 0.237), indicating similar levels of genetic diversity in *Ircinia*-
450 associated bacterial communities. AMOVA revealed significant genetic differentiation
451 among all 4 sources (P < 0.001), which accounted for the majority (62.1%) of genetic
452 variation, and among replicates within each source (P < 0.001), which accounted for the
453 remaining genetic variation (37.9%). Further, distinct phylogenetic lineages of symbionts
454 (P-test, P < 0.001) were observed among all pairwise comparisons between source
455 communities (Table 3).
456

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Microbial Community Structure and Similarity

In addition to differences in diversity between bacterioplankton and sponge-associated communities, minimal overlap in OTU composition was observed between seawater and sponge-derived sequences. Of the 124 bacterial OTUs recovered, only 2 OTUs were shared between sponges and seawater (Fig. 3), both in *I. variabilis*, and accounted for a small portion of the total *I. variabilis* (2.5%) and seawater (5.3%) clone libraries. Comparisons among the sponge-associated bacterial communities revealed varying levels of OTU overlap and host-specificity (Fig. 3). Four bacterial OTUs were recovered from all 3 host sponge species and represented dominant symbionts, accounting for nearly one-fourth of all clones from each host library (23.4% in *I. fasciculata*, 27.5% in *I. variabilis*, and 23.2% in *I. oros*). An additional 7 OTUs were shared between *I. fasciculata* and *I. variabilis*, accounting for an additional 40.3% and 31.3% of each library, respectively, and 6 OTUs were shared between *I. variabilis* and *I. oros*, accounting for 15.0% and 32.9% of each library, respectively. No additional microbial OTUs were shared between *I. fasciculata* and *I. oros*. Finally, the majority of OTUs recovered for each host species' community consisted of specific symbionts (i.e., recovered from a single host species)(n = 18 in *I. fasciculata*, n = 15 in *I. variabilis*, n =23 in *I. oros*) and represented rare OTUs, commonly appearing only once (65.5% of specific OTUs) or twice (25.5%) in clone libraries. Rank-abundance analyses showed that the presence of few, dominant OTUs and numerous, rare OTUs were consistent with a log-series distribution of OTUs within each clone library (D_{max}, $P > 0.05$), and differed significantly from a geometric distribution (D_{max}, $P < 0.01$).

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479 Consistent with patterns of symbiont OTU overlap among host sponges, overall
480 bacterial community similarity values were lowest between *I. fasciculata* and *I. oros*
481 (16.7%) and the symbionts in these two hosts differed significantly in community
482 structure (LIBSHUFF, $P < 0.001$; Table 3). The microbiota in *I. variabilis* exhibited
483 higher similarity to *I. fasciculata* symbiont communities (36.6%), where no significant
484 difference in symbiont structure were detected ($P > 0.639$; Table 3), than to microbial
485 symbionts in *I. oros* (31.1%), where significant differences in community structure were
486 detected ($P < 0.05$; Table 3).
487
488 <u>Phylogenetic analysis</u>
489 Phylogenetic analysis revealed that sequences recovered from the *I. fasciculata*, *I.*
490 *variabilis* and *I. oros* formed 56 monophyletic sequence clusters (Fig. 4). Nearly half
491 (48.2%) of these clusters were comprised exclusively of sponge-associated bacterial
492 sequences, with an additional 11 clusters (19.6%) consisting of sponge and coral-
493 associated clones. The remaining 18 clades (32.1%) contained non-symbiotic
494 representatives, most commonly derived from sediment ($n = 8$) and seawater
495 bacterioplankton ($n = 5$). Previously described bacterial sequences from Caribbean
496 *Ircinia* spp. were present in 21 of the 56 clades (37.5%), with *I. felix* from Florida ($n =$
497 16) and *I. strobilina* from the Bahamas ($n = 12$) more commonly presenting related
498 symbionts than *I. strobilina* from Florida ($n = 3$). Only 1 of the 56 clades (1.8%) was
499 comprised of sequences exclusively from *Ircinia* spp. (Fig. 4c). Notably, symbiont
500 sequences from the unrelated host sponge species *Aplysina aerophoba* (Order Verongida)
501 from the Mediterranean, *Ancorina alata* (Order Astrophorida) from New Zealand,

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Rhopaloeides odorabile (Order Dictyocertida, Family Spongiidae) from Australia and *Xestospongia muta* (Order Haplosclerida) from the Caribbean were also prevalent in these 56 clusters (32.1%, 19.6%, 14.3% and 10.7% of clusters, respectively; Fig. 4).

Phylogenetic analysis was also used to compare the host-specificity of bacteria observed among the 3 *Ircinia* spp. with host-specificity on a broader scale. The 4 common bacterial OTUs (i.e., generalist symbionts) present in all 3 Mediterranean *Ircinia* spp. were related to sequences derived from unrelated host sponge species, non-sponge (coral) hosts and environmental (sediment) clones (Fig. 4). These generalist symbionts corresponded to 1 *Deltaproteobacterium* (IRC001) and 3 *Gammaproteobacteria* (IRC006, IRC012 and IRC019), with only IRC006 forming a sequence cluster comprised exclusively of sponge-derived clones. The remaining generalist symbiont OTUs formed sequence clusters with not only sponge-derived clones, but also coral-derived (IRC001) and sediment-derived (IRC012 and IRC016) bacteria. Further, even the bacterial OTUs identified as specific to a single species of *Ircinia* in the clone libraries constructed herein (Fig. 3) were closely related to sequences derived from unrelated sponge hosts and environmental samples (Fig. 4), suggesting a more generalist distribution of these symbionts.

Molecular Identification of Host Sponges

Consensus COI sequences from *I. fasciculata* (n = 3) and *I. variabilis* (n = 3) and *I. oros* (n = 2) individuals yielded a 1,213 bp fragment encompassing the standard barcoding region ("Folmer" partition; 676 bp) and the I3-M11 region (537 bp). No intra-specific variation was observed among host species and *I. fasciculata* and *I. variabilis*

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525 exhibited identical sequences across the entire fragment length (Table 4). *I. oros* was
526 clearly differentiated from *I. fasciculata* and *I. variabilis*, exhibiting 1.8% sequence
527 divergence across the entire fragment with higher variability observed in the Folmer
528 partition (Table 4). Interestingly, *I. fasciculata* and *I. variabilis* sequences were more
529 closely related to the Caribbean species *I. strobilina* (0.6% divergence) than the
530 sympatric Mediterranean species *I. oros* (1.8% divergence). The outgroup species
531 *Hippospongia lachne*, representing a different family in the order Dictyoceratida,
532 exhibited 4.2 to 4.6% sequence from *Ircinia* spp (Table 4).

533 Consensus rDNA sequences from *I. fasciculata* (*n* = 6), *I. variabilis* (*n* = 7) and *I.*
534 *oros* (*n* = 8) clones yielded a 650 to 654 bp fragment encompassing the 3'-end of the 5.8S
535 subunit (50 bp), the entire ITS-2 region (243 to 248 bp) and the 5'-end of the 28S subunit
536 (356 to 357 bp). Low levels of intra-genomic polymorphisms (IGPs) were observed and
537 variable sites occurred in all rDNA subunits and regions as inconsistent point mutations
538 (i.e., occurring at different positions). Similar to COI sequences, partial 28S rDNA
539 sequences differentiated *I. oros* from *I. fasciculata* (1.16% divergence) and *I. variabilis*
540 (1.10% divergence), but did not resolve the latter two species, as intra-specific variation
541 within *I. fasciculata* (average = 0.28%, range = 0.00 – 0.56%) and *I. variabilis* (average =
542 0.16%, range = 0.00 – 0.56) was equal or greater than inter-specific variability among
543 these species (average = 0.22%, range = 0.0 – 0.56%). ITS-2 sequences exhibited the
544 highest variability and clearly differentiated *I. fasciculata* from *I. variabilis* (2.97% ±0.38
545 divergence), with variability among species (range = 2.43 – 3.63%) consistent greater
546 than variability within *I. fasciculata* (range = 0.40 – 2.02%) and *I. variabilis* (range =
547 0.00 – 0.00%). Phylogenetic analysis of combined ITS-2 and 28S rDNA sequences

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548 resolved each species into monophyletic clades with high bootstrap support and showed
549 *I. fasciculata* and *I. variabilis* were more closely related to each other than to *I. oros* (Fig.
550 S3), similar to the microbial symbiont communities inhabiting these hosts species (Fig.
551 5).
552

553 **DISCUSSION**

554 The Mediterranean sponges *I. fasciculata*, *I. variabilis* and *I. oros* were shown to
555 host dense communities of phylogenetically diverse microbial symbionts, as occurs in
556 congeneric hosts in the Caribbean and more distantly related HMA host sponges.
557 Microbial communities associated with *Ircinia* spp. were clearly differentiated from
558 ambient bacterioplankton communities, in terms of richness, diversity and composition,
559 and were comprised primarily of sponge-specific symbionts related to bacterial sequences
560 derived from related and unrelated host sponge species. Despite the generalist nature of
561 these symbionts among sponge hosts, each species of *Ircinia* harbored a unique microbial
562 consortium. These differences in symbiont communities among congeneric and sympatric
563 sponges suggest that factors specific to each host play a role in structuring microbial
564 symbiont communities in marine sponges.

565 Determining significant and ecologically relevant differences in complex
566 microbial communities is aided by the application of modern sequence-based and
567 phylogenetic statistical methods (Hughes et al., 2001; Martin, 2002; Schloss, 2008). In
568 the present study, the microbial communities in *I. fasciculata*, *I. variabilis* and *I. oros*
569 exhibited variable levels of symbiont overlap and specificity. Nearly one-fourth of each
570 symbiont community was shared among all 3 sponge hosts, representing 4 dominant

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571 bacterial OTUs; however, the majority of bacterial OTUs were exclusive to a single host
572 species. Coupling symbiont clone libraries with phylogenetic metrics revealed species-
573 specific differences in symbiont communities among *I. fasciculata*, *I. variabilis* and *I.*
574 *oros*. While each host species harbored a similar diversity of microbial symbionts, each
575 symbiont community was comprised of specific phylogenetic lineages and exhibited clear
576 genetic differentiation based on host species. Further, symbiont community structure
577 differed significantly between *I. oros* and the other two hosts, but not between *I.*
578 *fasciculata* and *I. variabilis*. Consistent with these results, *I. fasciculata* and *I. variabilis*
579 shared the most symbiont sequences (63.7% of clones) and OTUs (n = 11) of all pairwise
580 comparisons among hosts.

581 Host-specific symbiont structuring in *Ircinia* sponges is particularly notable since
582 the vast majority these microbial symbiont communities were closely related (>97%
583 identity) to sequences derived from other host sponges. These hosts included congeneric
584 hosts from the Caribbean, as well as more distantly related sponges from the
585 Mediterranean (e.g., *Aplysina aerophoba*) and the Great Barrier Reef (e.g., *Rhopaloeides*
586 *odorabile*). Indeed, symbionts characterized as 'specific' to one *Ircinia* host species
587 herein were commonly observed in sponge hosts from different oceans when compared to
588 other sponge-derived microbial sequences. For example, IRC046 represents an
589 *Actinobacteria*-affiliated symbiont OTU exclusively associated with *I. oros* in the current
590 study, yet matched closely (99%) to a symbiont sequence from the Indo-Pacific sponge
591 *Theonella swinhoei*. These observed generalist patterns of specificity among host sponges
592 for individual symbionts, combined with the host species-specific structure of symbiont
593 assemblages revealed by community-level analyses, suggest that each host *Ircinia* species

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594 harbors a specific mix of generalist sponge symbionts. A similar trend was observed in a
595 meta-analysis of bacterial symbionts in 10 host sponges that revealed significant pairwise
596 differences in symbiont communities among all host species, despite the predominance of
597 generalist (i.e., sponge-specific) symbionts comprising each microbiota (Taylor et al.,
598 2007).

599 The persisting question is which factors account for the observed similarities and
600 dissimilarities in microbial community structure among different host sponges. To
601 classify the numerous physical, chemical and biological conditions that may structure
602 symbiont communities, a framework is presented that divides putative factors into 4
603 categories based on their source (external environment vs. host sponge related) and
604 prevalence among host sponge species (shared vs. exclusive; Fig. 6). The influence and
605 number of factors in each category depends on the geographic and taxonomic scope of
606 each study. Congeneric and sympatric host species were examined herein, thus numerous
607 factors were shared among these species, including regional environmental conditions
608 and a common evolutionary trajectory, and these factors cannot account for the reported
609 differences in symbiont communities among hosts. However, factors exclusive to
610 different host species, including habitat-specific conditions and host-specific factors, and
611 their variable influence on each host may represent a source of differentiation for *Ircinia*-
612 associated microbial communities.

613 The host species investigated exhibit different zonation patterns within littoral
614 benthic landscapes and environmental factors specific to distinct habitats may play a role
615 in structuring symbiont communities. *I. variabilis* and *I. oros* occur preferentially in
616 semi-sciophilous communities, commonly inhabiting vertical relief structures where

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617 much of the sponge diversity in these habitats exists. In contrast, *I. fasciculata* is more
618 prevalent in photophilic communities, characterized by high irradiance conditions and
619 dense algal growth. Irradiance levels are primary factor in structuring sponge
620 assemblages in the Mediterranean, likely as an indirect consequence of stimulating algal
621 growth and competitive pressures (Uriz et al. 1992). The distribution of host species
622 within the local landscape may thus have ecological consequences for sponge-symbiont
623 interactions and dictate symbiont composition by imposing functional performance
624 pressures. For example, the dense populations of cyanobacteria in *I. fasciculata* may
625 contribute to host nutrition and growth via the transfer of photosynthetic by-products
626 (Arillo et al., 1994; Erwin & Thacker 2008, Freeman & Thacker 2011), thereby
627 enhancing competitive ability and allowing this species to thrive in algal-dominated
628 habitats.

629 In addition to habitat-specific factors, a recent and shared evolutionary past may
630 influence the composition of symbiotic microbial communities in sponge hosts, as
631 suggested by the observed correlation between *Ircinia* host phylogenies and symbiont
632 community similarity. Vertical transmission of microbial symbionts, or direct parent-to-
633 offspring passage, has been observed in several marine sponges (Usher et al., 2001;
634 Ereskovsky et al., 2005; Oren et al., 2005; Enticknap et al., 2006; Caralt et al., 2007;
635 Sharp et al., 2007; Steger et al, 2008; Lee et al., 2009a; Webster et al., 2010), including
636 *Ircinia* hosts (Schmitt et al., 2007; Schmitt et al., 2008), and provides a mechanism to
637 maintain a similar microbiota mix in related sponges where successive host generations
638 are seeded as larvae with diverse microbial symbionts. Thus, while periodic horizontal
639 transmission of microbial symbionts (i.e., environmental acquisition) may contribute to

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640 the homogenization of symbiont communities in unrelated sponge hosts (Schmitt et al.
641 2008), recurrent vertical transmission may dictate symbiont composition over shorter
642 evolutionary time scales due to legacy effects of the microbial inheritance.

643 External environmental factors and host sponge related factors are not mutually
644 exclusive and may act in concert to structure microbial symbiont communities in marine
645 sponges. In the case of *Ircinia* hosts from the Mediterranean, a shared evolutionary past
646 may contribute more strongly to symbiont structure compared to habitat-specific
647 conditions, considering that the microbiota of *I. variabilis* exhibited higher similarity to
648 the more closely related species, *I. fasciculata*, than to the neighboring species, *I. oros*.
649 However, a portion of the microbial community in *I. variabilis* was exclusively shared
650 with *I. fasciculata* (7 OTUs) or *I. oros* (6 OTUs), suggesting an interactive effect of these
651 factors to produce an intermediate or transitional community between the clearly
652 differentiated symbiont communities in *I. fasciculata* and *I. oros*.

653 Discerning the contribution of common ancestry among hosts to the microbial
654 structure of symbiont communities requires accurate resolution of host phylogenies based
655 on molecular data. To date, no universal molecular marker has been discovered that
656 offers species-level resolution for all sponge taxa, as barcoding genes commonly
657 employed in the molecular identification of other animal taxa (e.g., COI) are conserved
658 and unable to distinguish different species in some sponge groups (Erpenbeck et al.,
659 2007; López-Legentil et al., 2010a; Pöppe et al., 2010). Consistent with these findings,
660 the current study showed that COI mtDNA sequences were unable to differentiate *I.*
661 *fasciculata* and *I. variabilis*, despite extended coverage of the I3-M11 partition, a region
662 of the COI gene suggested to offer greater resolution for lower metazoan taxa (Erpenbeck

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663 et al., 2006). The utility of the I3-M11 region in differentiating related sponge species
664 (Reveillaud et al. 2011) and as a population genetics marker for intra-specific
665 differentiation in sponges (López-Legentil & Pawlik, 2009; Xavier et al., 2010), suggest
666 that this gene region is either highly conserved within the genus *Ircinia* or that *I.*
667 *fasciculata* and *I. variabilis* are very closely related species. Indeed, some degree of
668 controversy surrounds the taxonomic status of *I. fasciculata* and *I. variabilis* (Pronzanto
669 et al., 2004) and these species can be difficult to differentiate in the field, resulting in
670 some ecological studies grouping these two putative species into a single taxon
671 (Maldonado et al., 2010).

672 Analysis of the second internal transcribed spacer region (ITS-2) of the nuclear
673 ribosomal operon revealed consistent genetic differentiation between *I. fasciculata* and *I.*
674 *variabilis*, indicating some molecular markers can delineate these species, and resolved
675 each *Ircinia* host into well-supported monophyletic clades. ITS-2 sequences have also
676 been utilized in previous phylogenetic analyses (Erwin & Thacker 2007) and population
677 genetics of marine sponges (Wörheide et al., 2002; Duran et al., 2004). The results herein
678 suggest this region offers higher fine-scale phylogenetic resolution than COI sequence
679 data for some sponge taxa. While few species of *Ircinia* have been investigated using
680 molecular phylogenetics (Pöppe et al., 2010), it is interesting to note that COI sequences
681 in *I. fasciculata* and *I. variabilis* were more closely related to *I. strobilina* from the
682 Caribbean (0.6% sequence divergence) than to the sympatric species *I. oros* (1.8%).
683 Clearly, additional phylogenetic studies and greater sampling of the high species diversity
684 within this genus are required to resolve the relationships among these species and
685 determine how these relationships affect symbiont community structure.

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The sponge-specific nature of individual symbionts, host-specific structure of symbiont communities and diverse environmental and host-related factors that dictate the structure of sponge-associated microbes result in a complex picture of host-symbiont specificity in HMA sponges. Based on the generalist nature of individual symbionts and host-specific structure of entire communities, our results suggest a 'specific mix of generalists' framework applies to host-symbiont specificity in *Ircinia* spp. from the Mediterranean, and possibly other HMA sponge hosts. Further, by comparing symbiont communities in congeneric species from the same environment, the results herein suggest that factors specific to the each host species play a role in structuring microbial symbiont communities in marine sponges, including host evolutionary history and habitat-specific abiotic and biotic environmental factors. Additional research on the spatio-temporal dynamics of microbial symbionts in sponges and controlled experimental manipulations of sponge holobionts are required to further unravel the multiple and potentially interactive factors that structure the complex sponge microbiota.

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971 **TABLES**

972

973 Table 1. Composition of microbial communities in *Ircinia* spp. and ambient seawater.

974 Percentage of total clones is shown by bacterial phyla (classes of Proteobacteria shown in

975 parentheses). Values in parenthesis depict the number of 99% OTUs within each lineage.

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Phylum (Class)	***I. fasciculata***	***I. variabilis***	***I. oros***	**Seawater**	**Total**
Proteobacteria	45.4 (16)	61.3 (22)	56.1 (17)	56.2 (28)	54.5 (65)
(α-proteobacteria)	6.5 (4)	11.3 (6)	12.2 (4)	31.5 (16)	15.0 (26)
(β-proteobacteria)	-	1.3 (1)	-	2.7 (2)	1.0 (3)
(γ-proteobacteria)	20.8 (10)	33.8 (11)	28.0 (10)	22.0 (10)	26.1 (31)
(δ-proteobacteria)	18.2 (2)	15.0 (4)	15.9 (3)	-	12.4 (5)
Cyanobacteria	32.5 (2)	7.5 (1)	-	1.4 (1)	10.2 (3)
Acidobacteria	5.2 (3)	5.0 (3)	18.3 (3)	-	7.3 (8)
Bacteroidetes	9.1 (3)	3.8 (2)	3.7 (2)	9.6 (7)	6.7 (14)
Bacillariophyta	-	2.5 (2)	9.8 (5)	8.2 (4)	5.4 (11)
Nitrospira	2.6 (1)	15.0 (1)	2.4 (1)	-	5.1 (2)
Actinobacteria	-	3.8 (2)	3.7 (2)	12.3 (3)	4.8 (7)
Chloroflexi	3.9 (3)	-	4.9 (2)	-	2.2 (5)
Chlorophyta	-	-	-	4.1 (3)	1.0 (3)
Gemmatimonadetes	1.3 (1)	1.3 (1)	1.2 (1)	-	1.0 (3)
Firmicutes	-	-	-	1.4 (1)	0.3 (1)
Planctomycetes	-	-	-	1.4 (1)	0.3 (1)
Verrucomicrobia	-	-	-	1.4 (1)	0.3 (1)
Uncertain Classification	-	-	-	4.0 (2)	1.0 (2)

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979 Table 2. Diversity metrics comparing the richness, dominance and evenness of microbial
980 communities in *Ircinia* spp. and seawater. Lower and upper 95% confidence intervals are
981 shown in parentheses where available.
982

Diversity Metric	Measure	*I. fasciculata*	*I. oros*	*I. variabilis*	Seawater
Richness	Observed OTUs S_{obs}	29	33	34	51
	Expected OTUs S_{Chao1}	40 (32 – 64)	43 (36 – 65)	53 (40 – 91)	188 (105 – 394)
Dominance	Berger-Parker d	26.0%	14.6%	15.0%	8.2%
	Simpson Index $1/D$	10.3 (7.1 – 19.1)	19.4 (13.6 – 34.0)	19.9 (14.0 – 33.9)	58.4 (36.0 – 155.2)
Evenness	Smith & Wilson E_{var}	0.14	0.24	0.20	0.70
	Simpson Index $E_{1/D}$	0.32	0.48	0.47	0.64

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Table 3. Pairwise statistical comparisons of genetic diversity and community structure of bacterial communities in *Ircinia fasciculata* (*IF*), *I. variabilis* (*IV*), *I. oros* (IO) and seawater (H2O).

Test	Statistic	*IF – IV*	*IV – IO*	*IO – IF*	*IF* – H2O	*IV* – H2O	*IO* – H2O
AMOVA	F_{ST}	0.0312	0.0203	0.0444	0.0827	0.0794	0.0692
	P-value	***	***	***	***	***	***
HOMOVA	*B*	0.0018	0.0500	0.0697	1.3499	1.2813	0.8456
	P-value	0.862	0.321	0.237	***	***	**
P-Test	Score	17.0	40.0	27.0	15.0	16.0	33.0
	P-value	***	***	***	***	***	***
LIBSHUFF	ΔC_{XY}	0.0018	0.0043	0.0181	0.0686	0.0787	0.0911
	ΔC_{YX}	0.0014	0.0031	0.0367	0.0850	0.0661	0.0691
	P-value $_{XY}$	0.639	*	***	***	***	***
	P-value $_{YX}$	0.802	0.172	***	***	***	***

* = $P < 0.05$, ** = $P < 0.005$, *** = $P < 0.001$

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994 Table 4. Pairwise genetic distance (p-distance) among *Ircinia* spp. and *Hippospongia*
995 *lachne* (Order Dictyoceratida, Family Spongiidae) for the 5'-end 'Folmer' partition
996 (upper right; 676 bp fragment) and the I3-M11 partition (lower left; 537 bp fragment) of
997 the mitochondrial gene cytochrome oxidase subunit I. Values shown as percentages.
998

	I. fasciculata	*I. variabilis*	*I. strobilina*	*I. oros*	*H. lachne*
I. fasciculata	-	0	0.74	1.18	5.47
I. variabilis	0	-	0.74	1.18	5.47
I. strobilina	0.37	0.37	-	1.33	5.33
I. oros	2.61	2.61	2.24	-	5.47
H. lachne	3.17	3.17	2.79	3.54	-

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1001 **FIGURE LEGENDS**

1002

1003 **Figure 1**. *In situ* photographs and electron micrographs of the external morphology of
1004 host sponge species (A–C) and density and diversity of bacterial symbionts (D–G).
1005 Underwater photographs highlight the distinct coloration, growth forms and surface
1006 texture of *I. fasciculata* (A), *I. variabilis* (B) and *I. oros* (C) individuals sampled in this
1007 study. Representative micrographs show the density the symbiotic cyanobacterium,
1008 *Synechococcus spongiarum*, in ectosomal (peripheral) tissues of *I. fasciculata* (D) and *I.*
1009 *variabilis* (E) that is absent in *I. oros* tissue (G). Heterotrophic bacterial symbionts
1010 dominate the choanosomal (interior) tissue of *I. variabilis* (F) and exhibit similar
1011 morphotypes to symbiotic bacteria present in *I. oros* (G). Scale bars represent 2 cm (A–
1012 C), 2 μm (D, E) and 1 μm (F, G).

1013

1014 **Figure 2**. Similarity of bacterial 16S rRNA gene sequences recovered from *Ircinia*
1015 *fasciculata* (A), *I. variabilis* (B), *I. oros* (C) and seawater (D) to sequences in the
1016 GenBank database. Top matches from BLAST searches are grouped by sequence identity
1017 bins and sequence source: sponge-associated, coral-associated, seawater, sediment and
1018 other.

1019

1020 **Figure 3**. Specificity of microbial communities in *I. fasciculata*, *I. variabilis* and *I. oros*.
1021 Pie charts depict the percentage of total clones corresponding to each specificity
1022 category; numbers denote the total OTUs accounting for each partition.

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1024 **Figure 4**. Phylogeny of bacterial 16S rRNA gene sequences from *Ircinia fasciculata*, *I.*
1025 *oros*, *I. variabilis* and ambient seawater. Tree topology was constructed using maximum
1026 likelihood criteria and numbers on nodes depict bootstrap support (< 50% not shown).
1027 Terminal nodes denote the sequence source, bold values correspond to sequences from
1028 this study and indicate the OTU and host species followed by the total number of clones
1029 (in parentheses). For condensed clades (white triangles), the total number of sequences
1030 (in parentheses) and bootstrap support (%) are shown. Gray boxes delineate clusters
1031 containing sequences from *Ircinia* spp. herein, with black triangles indicating *Ircinia*-
1032 derived sequences from other studies. Asterisks (*) indicate clones for which near full-
1033 length sequences were recovered. BAH = Bahamas, FLO = Florida.
1034

1035 **Figure 5.** Phylogenetic tree of host sponges (left) and similarity dendrogram of bacterial
1036 symbiont communities (right) in *I. fasciculata*, *I. variabilis* and *I. oros*. Tree topology
1037 was constructed using maximum likelihood (ML) analysis of ITS-2/28S rDNA gene
1038 sequences. ML (upper) and neighbor-joining (lower) bootstrap support values are shown
1039 on internal node labels; bold values indicate support for species-level clades (gray boxes).
1040 Scale bar represents 0.03 substitutions per site. Dendrogram (right) was constructed from
1041 Bray-Curtis community similarity values among microbial symbionts in each host
1042 species. Scale represents percentage similarity values. Arrows point to outgroup taxa
1043 (left) and seawater bacterial communities (right). Full sponge phylogeny available as
1044 supplemental material (Fig. S3).
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Figure 6. Theoretical framework of the putative factors that structure microbial symbiont communities in marine sponges. Factors are first divided based on their source, occurring in the ambient external environment or within the host sponge microenvironment. Factors are further categorized by their impact on the host sponges investigated (shared among all hosts vs. exclusive to one or a subset of hosts) to distinguish factors that may homogenize (shared) or differentiate (exclusive) microbial symbiont communities among hosts.

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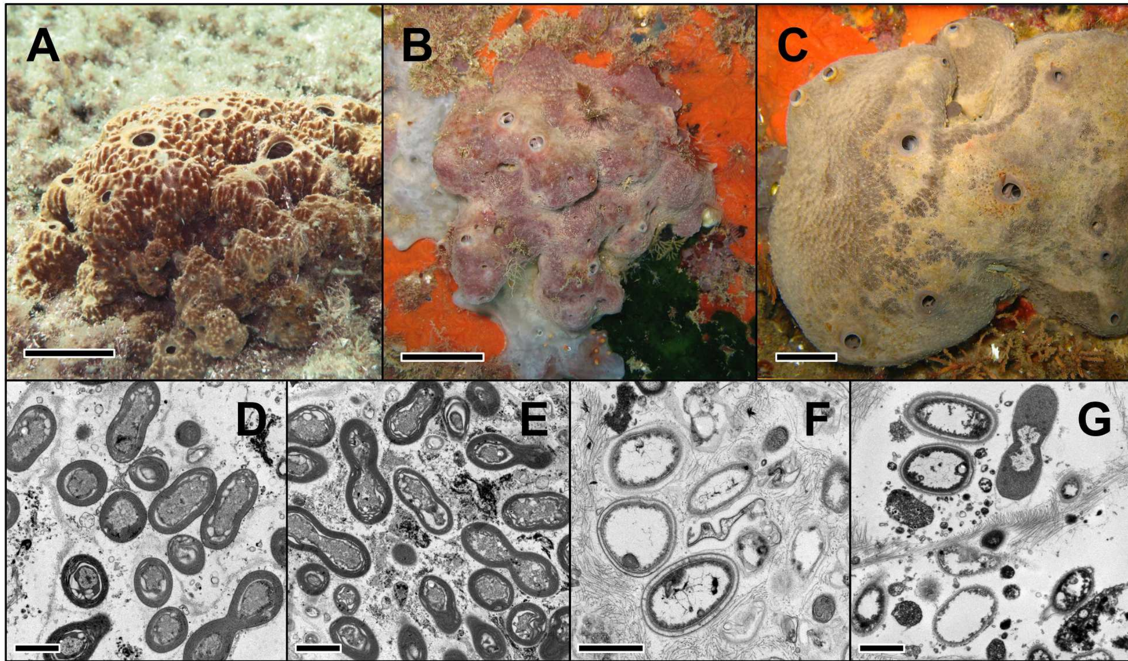
1054 **FIGURES**



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1056 **Fig. 1**

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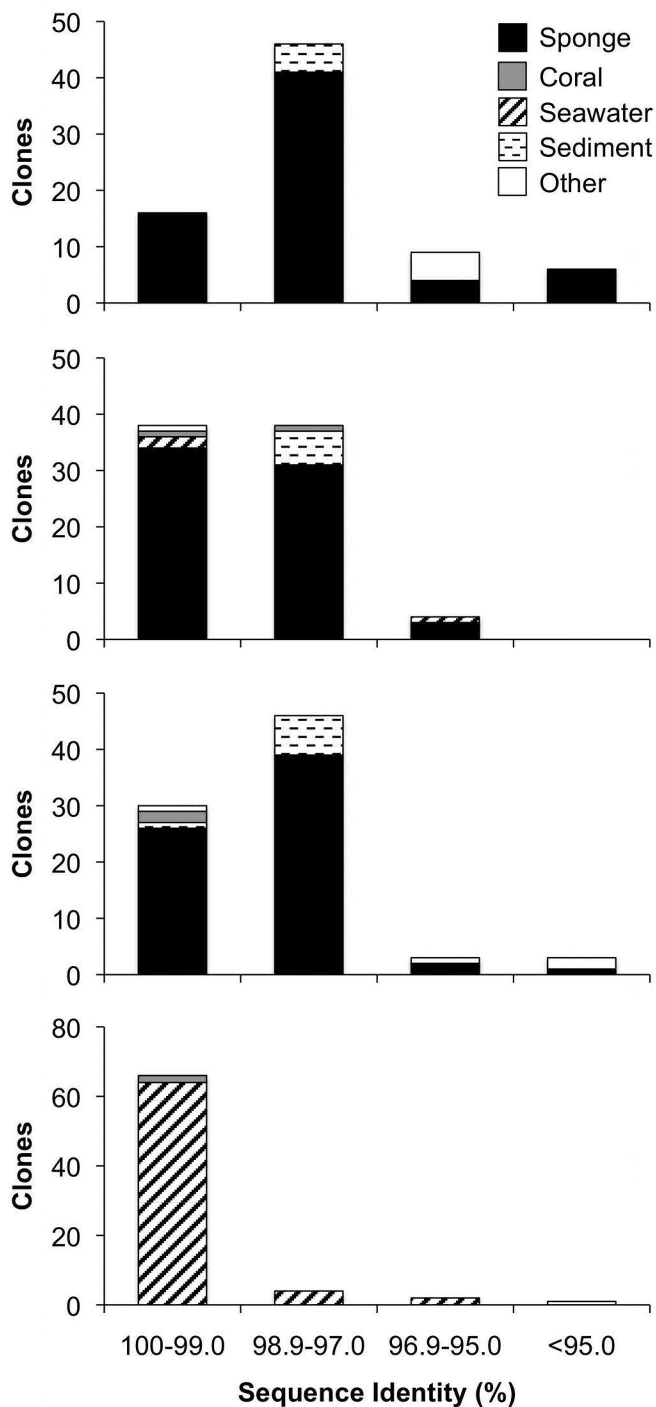

Fig. 2

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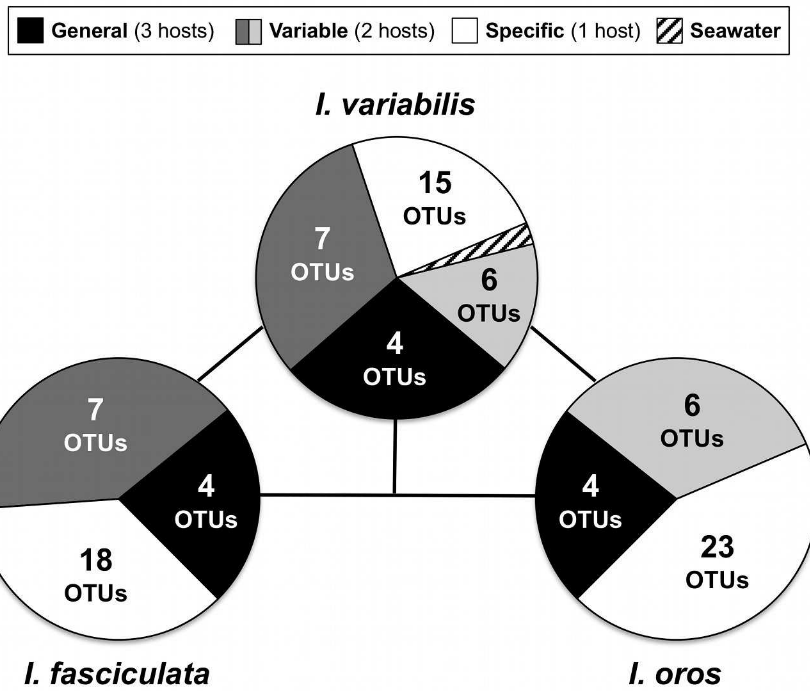

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1061 **Fig. 3**

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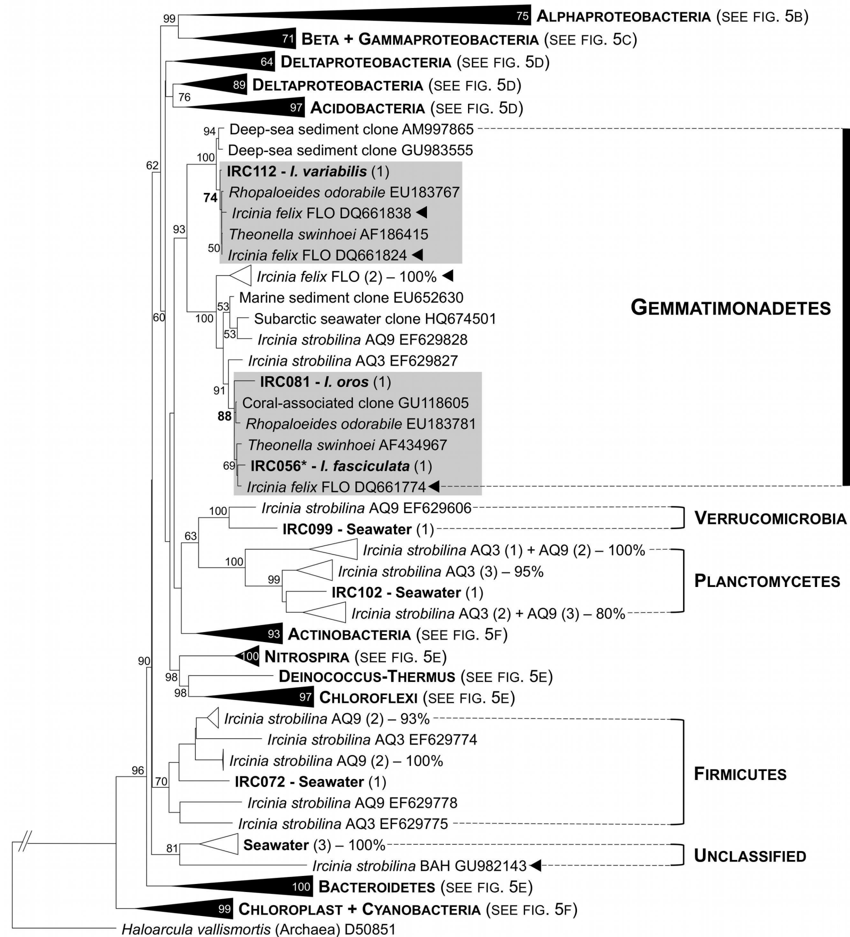

Fig. 4a

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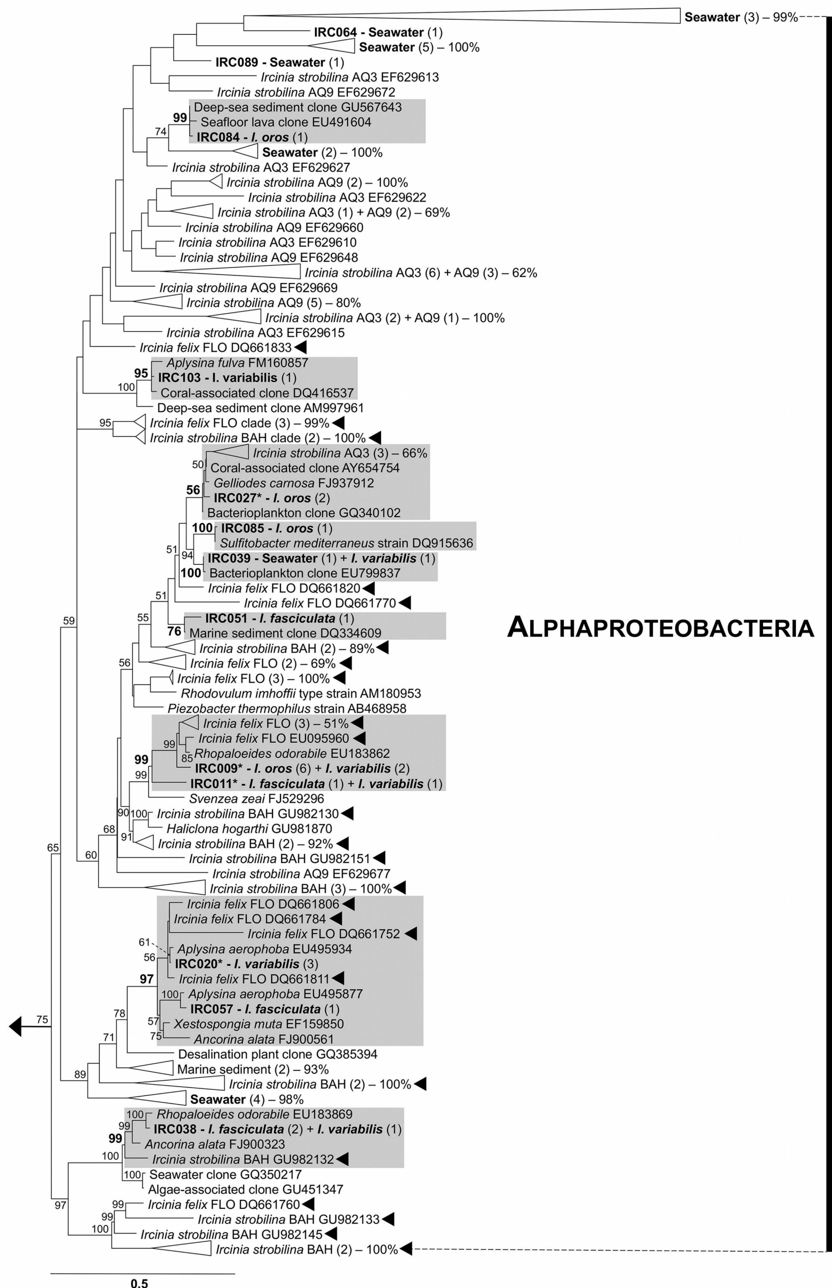

Fig. 4b

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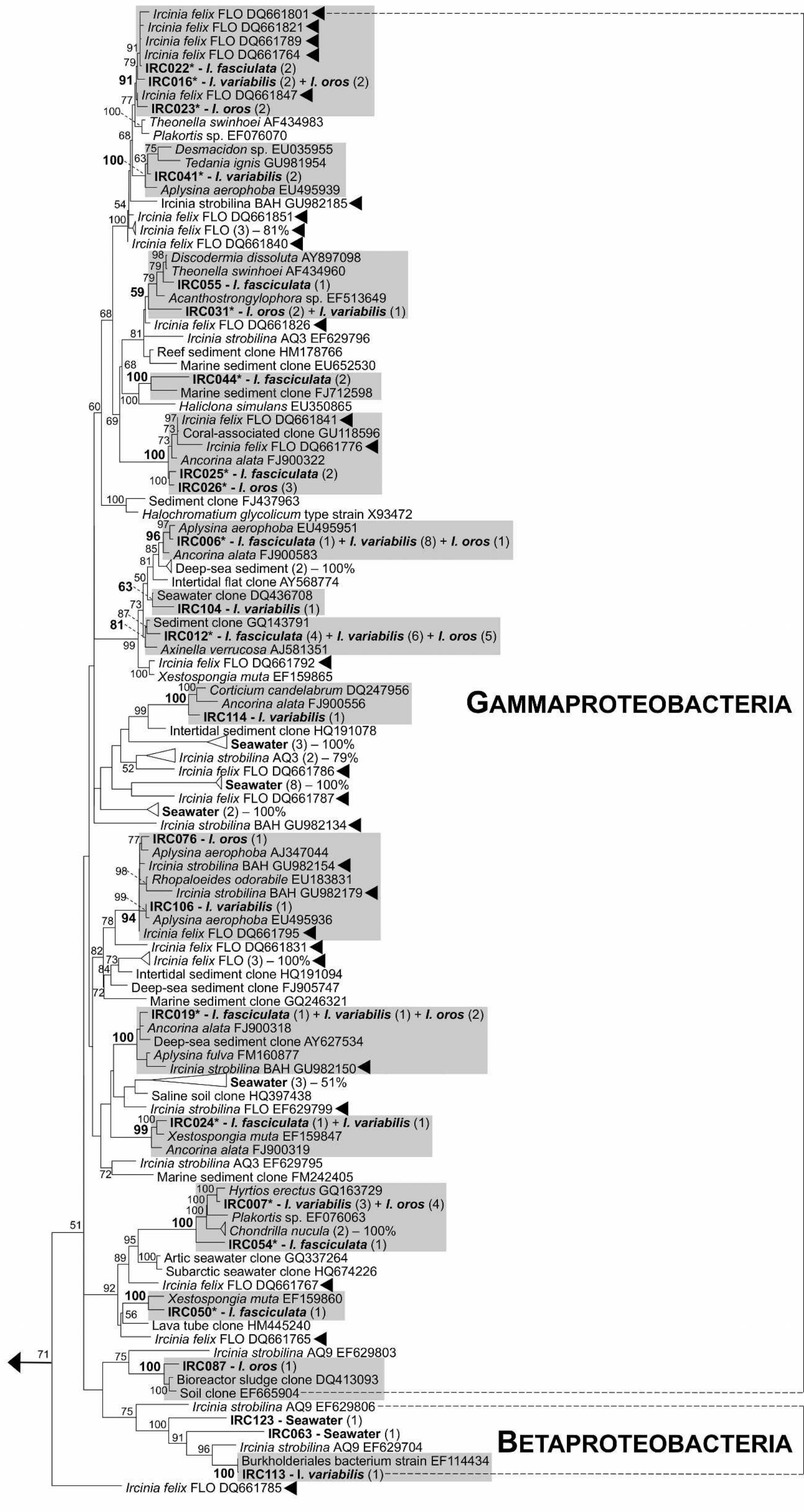

Fig. 4c

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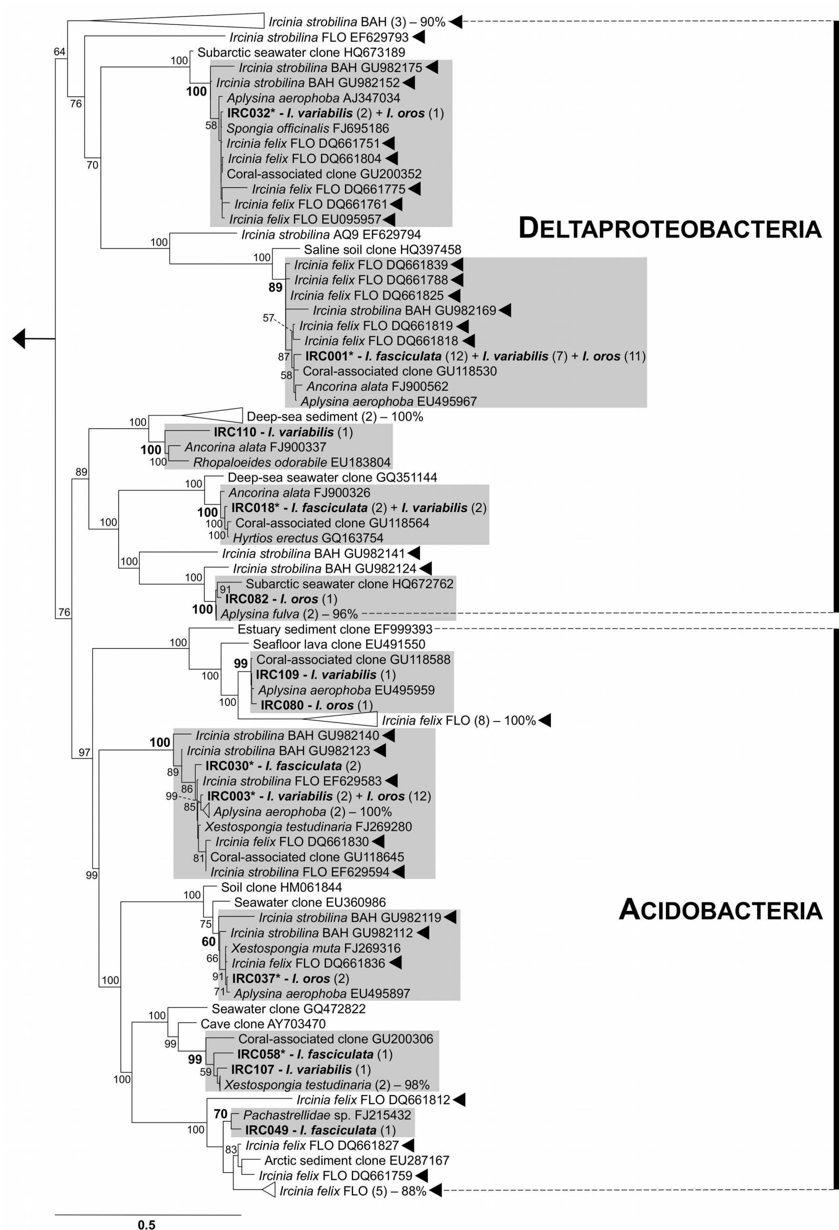

Fig. 4d

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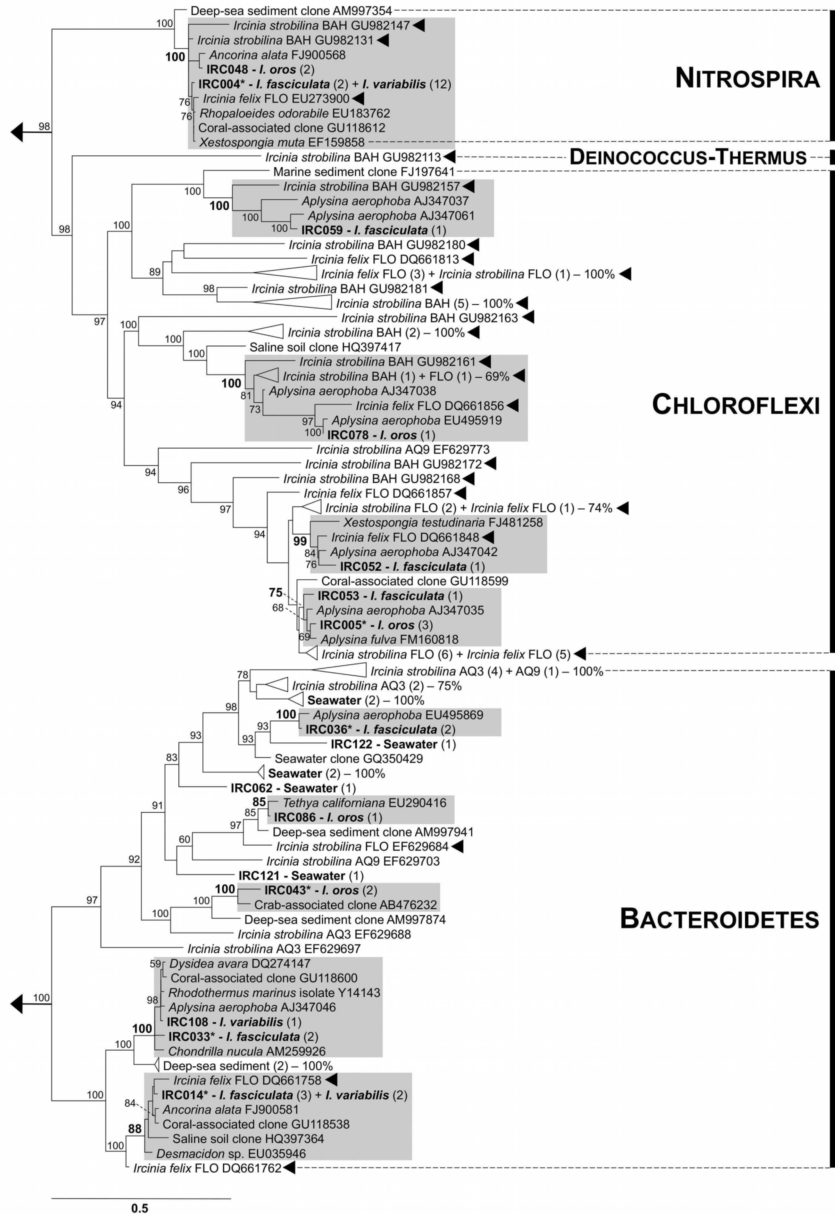

Fig. 4e

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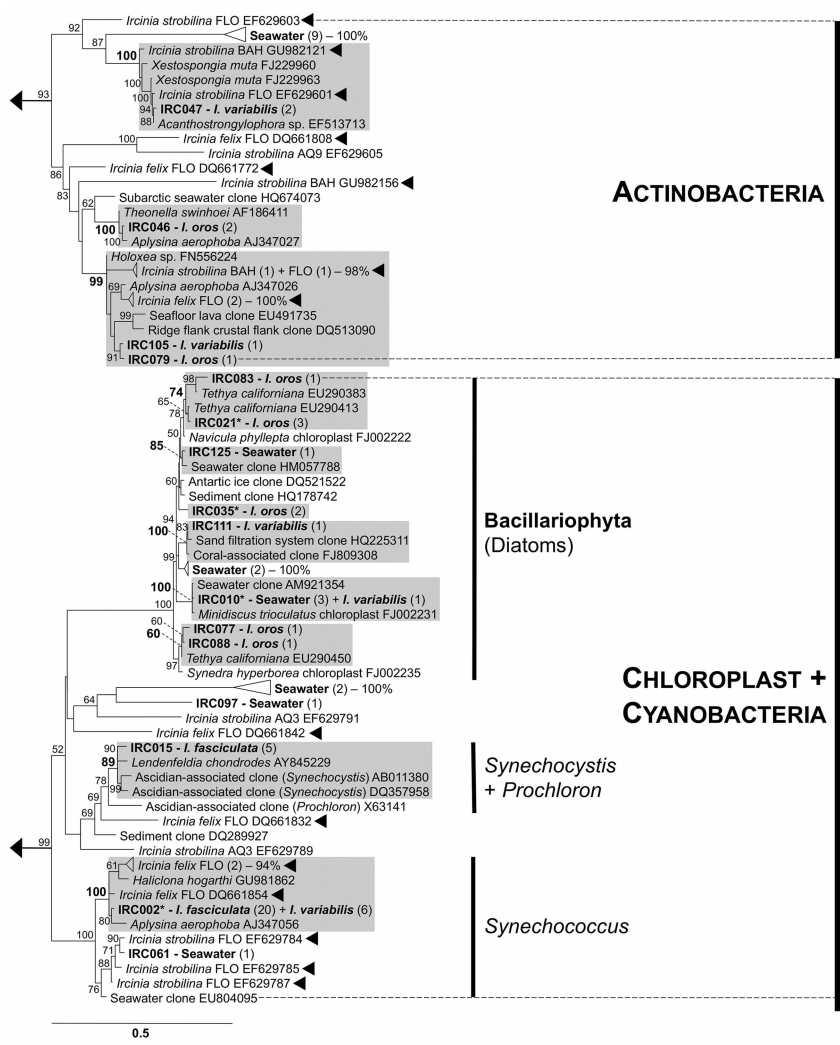

Fig. 4f

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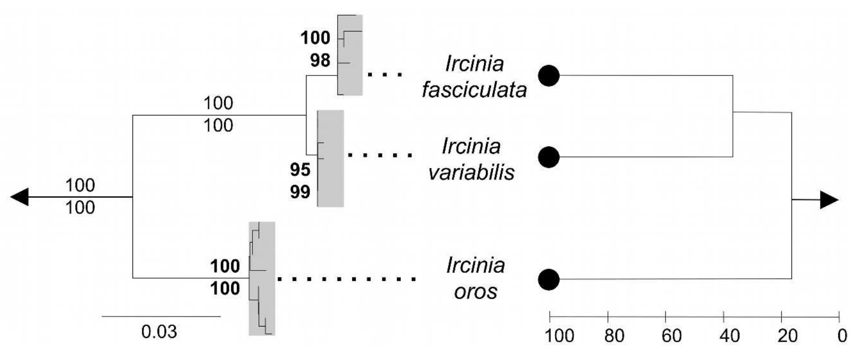

Fig. 5

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	Shared	Exclusive
Environment	Regional conditions • Temperature • Salinity • POM [Coastal NW Mediterranean Sea]	Habitat specific • Competition • Irradiance • Predation [Photophilic vs. sciophilous communities]
Host Sponge	Internal microenvironment • Nutrients • Oxygen • Irradiance [choanosome vs. ectosome, internal gradients]	Host species-specific • Evolutionary History • Microbial Inheritance [Species relatedness, vertical transmission]

Fig. 6

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Erwin PM, López-Legentil S, González-Pech R, Turon X (2012) A specific mix of generalists: Bacterial symbionts in Mediterranean Ircinia spp.
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