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Genetic diversity of the unicellular nitrogen-fixing cyanobacteria UCYN-A and its prymnesiophyte host

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Running title: UCYN-A genetic diversity

21 ***Summary***

22 Symbiotic interactions between nitrogen-fixing prokaryotes and photosynthetic
23 eukaryotes are an integral part of biological nitrogen fixation at a global scale. One of these
24 partnerships involves the cyanobacterium UCYN-A, which has been found in partnership with
25 an uncultivated unicellular prymnesiophyte alga in open-ocean and coastal environments.
26 Phylogenetic analysis of the UCYN-A nitrogenase gene (*nifH*) showed that the UCYN-A lineage
27 is represented by three distinct clades, referred to herein as UCYN-A1, UCYN-A2 and UCYN-
28 A3, which appear to have overlapping and distinct geographic distributions. The relevance of
29 UCYN-A's genetic diversity to its symbiosis and ecology was explored through combining flow
30 cytometric cell sorting and molecular techniques to determine the host identity, *nifH* expression
31 patterns, and cell size of one newly-discovered clade, UCYN-A2, at a coastal site. UCYN-A2
32 *nifH* expression peaked during daylight hours, which is consistent with expression patterns of the
33 UCYN-A1 clade in the open ocean. However, the cell size of the UCYN-A2 host was
34 significantly larger than UCYN-A1 and host, suggesting adaptation to different environmental
35 conditions. Like the UCYN-A1 host, the UCYN-A2 host was closely related to the genus
36 *Braarudosphaera*, however the UCYN-A1 and UCYN-A2 host rRNA sequences clustered into
37 two distinct clades suggesting co-evolution of symbiont and host.

38

39 Introduction

40 Symbioses between nitrogen (N_2)-fixing prokaryotes (diazotrophs) and photosynthetic
41 eukaryotes play an important role in global biological nitrogen fixation (BNF) (Carpenter and
42 Foster 2003; Houlton et al. 2008; Foster et al. 2011; Karl et al. 2012). In the oceans, these
43 symbioses usually involve diazotrophic cyanobacteria and photosynthetic eukaryotic plankton
44 and the partnerships range from obligate to facultative (Carpenter and Foster 2003; Lesser et al.
45 2004; Foster et al. 2006; Foster et al. 2011; Hilton et al. 2013).

46 Of the symbiotic marine diazotrophic cyanobacteria, the unicellular UCYN-A is
47 recognized for its global distribution (Goebel et al. 2010; Moisander et al. 2010; Zehr and
48 Kudela 2011) and significant contributions to local N_2-fixation (Montoya et al. 2004). The
49 genome of UCYN-A is extremely streamlined and lacks genes encoding the photosystem II
50 complex (PSII), carbon fixation pathways, and various other pathways including the TCA cycle
51 (Zehr et al. 2008; Tripp et al. 2010). A second UCYN-A genome was recently sequenced and is
52 missing the same pathways, suggesting that a streamlined genome and the absence of essential
53 metabolic pathways is a defining feature of the UCYN-A lineage (Bombar et al. *Submitted*). It
54 appears that the streamlined genome of UCYN-A is a result of genome degradation through
55 obligate symbiosis with a unicellular eukaryotic host. The eukaryotic host is from a lineage of
56 uncultivated prymnesiophytes related to *Braarudosphaera bigelowii*, a calcareous
57 phytoplankton, which was observed in symbiosis with UCYN-A in the open ocean (Thompson et
58 al. 2012) and in the coastal waters of Japan (Hagino et al. 2013). Incubation of seawater with
59 labeled nutrients demonstrated N transfer from the cyanobacterium to the host, and C transfer
60 from host to cyanobacterium (Thompson et al. 2012; Krupke et al. 2013), suggesting that the

61 basis of the symbiosis is nutrient exchange, specifically fixed N and fixed C, between the partner
62 species.
63 While it is recognized that UCYN-A is likely to be one of the major marine diazotrophs,
64 the genetic diversity of UCYN-A is not well known. For the very abundant marine non-
65 diazotrophic cyanobacteria, *Prochlorococcus* and *Synechococcus,* and the less abundant
66 diazotrophic cyanobacteria *Crocosphaera,* genetic diversity has clear relevance to ecological
67 function (Kettler et al. 2007; Webb et al. 2009; Coleman and Chisholm 2010; Malmstrom et al.
68 2010; Bench et al. 2013). In these cases, genetically-distinct clades (or, ecotypes) exhibit
69 phenotypic traits that reflect local environmental conditions including light availability, nutrient
70 composition, and temperature. Understanding the ecological function of the different ecotypes
71 has been critical to interpreting measurements of ecotype abundance and the content of
72 environmental sequence databases for these microbial groups. However, it has been difficult to
73 identify analogous ecotypes in UCYN-A due to its uncultivated status, relatively low
74 abundances, and poor to nonexistent representation in large metagenomic databases.
75 In this study, the genetic diversity of UCYN-A was assessed from phylogenetic analysis
76 of a large database of nitrogenase (*nifH*) sequences from marine systems, which revealed three
77 distinct clades of UCYN-A. One of the clades that emerged from the phylogenetic analysis is
78 well-represented in populations collected from the Scripps Institute of Oceanography (SIO) Pier
79 (San Diego, California, USA) and is the focus of this study.
80
81 **Results and Discussion**
82 *Divergence of UCYN-A into three distinct clades*

Phylogenetic analysis of UCYN-A nitrogenase (*nifH*) gene sequences revealed divergence of the UCYN-A lineage into multiple clades (Figure 1A). The clades were designated UCYN-A1, UCYN-A2, and UCYN-A3, with clade UCYN-A1 containing the original UCYN-A genome (Tripp et al. 2010). Variation between the sequences occurred predominantly in the third base pair position of each codon and clades could not be differentiated by *nifH* amino acid sequences (Figure 1B). Functional restraint on sequence variance in the nitrogenase protein likely explains the lack of divergence in the amino acid sequences. However, divergence of *nifH* at the nucleotide level may indicate that each UCYN-A clade was subject to different evolutionary pressures after divergence from a common ancestor. Indeed, this is consistent with the results of a comparative genomic study between UCYN-A1 and UCYN-A2, which shows that though over 96% of UCYN-A1 genes are present in UCYN-A2, the shared genes are on average only 86% similar at the amino acid level (Bombar et al. *Submitted*).

To determine if the UCYN-A clades occupied overlapping or distinct oceanic habitats, the geographic distribution of UCYN-A *nifH* sequences submitted to GenBank from clone-based studies was examined (Figure 2). UCYN-A1 *nifH* sequences have been recovered from many of the major ocean basins, however all clades were widely distributed (Figure 2). UCYN-A2 *nifH* sequences were present where large numbers of UCYN-A1 had been reported from clone libraries, with the exception of the Gulf of Catalina (this study), Heron Reef (Hewson et al. 2007), and the Eastern Mediterranean Sea (Man-Aharonovich et al. 2007). UCYN-A3 *nifH* sequences have also been reported in regions where other UCYN-A clades were present, such as the Cape Verde Islands in the Eastern Atlantic, but also in the South Pacific Gyre where cyanobacterial diazotrophs are scarce (Halm et al. 2012; Turk-Kubo et al. 2013). Several sequences from the Eastern Atlantic, where diazotroph diversity is known to be rich (Turk et al.

106 2011), did not cluster clearly with any clade (Figure 1 and 2D), indicating that additional clades
107 may exist.
108 The discovery of additional UCYN-A clades has implications for estimates of the
109 contribution of UCYN-A to oceanic N_2 fixation. The most frequently used quantitative assay for
110 UCYN-A was designed from Station ALOHA UCYN-A1 sequences (Church et al. 2005) and
111 has been used to assay UCYN-A abundances in other ocean basins (Goebel et al. 2010;
112 Moisander et al. 2010). However, this assay (now referred to as the UCYN-A1 *nifH* assay) does
113 not detect UCYN-A2 or UCYN-A3 due to mismatches at several positions (Supporting
114 Information - Table S1). As a result, the true population size of UCYN-A and their N_2 fixation
115 rates has been underestimated.
116 We investigated the characteristics of one of the newly recognized UCYN-A clades,
117 UCYN-A2, in the Gulf of Santa Catalina, a region where UCYN-A1 has not previously been
118 reported (Figure 2). Subsequent sampling indicated that UCYN-A2 was abundant at the Scripps
119 Institute of Oceanography (SIO) Pier in the Gulf of Santa Catalina (Supporting Information -
120 Table S2) and clone libraries indicated that UCYN-A *nifH* sequences recovered from these
121 samples were exclusively UCYN-A2.
122
123 *Diel nitrogenase expression by UCYN-A2*
124 The expression of nitrogenase during the day is a feature of UCYN-A1 that sets it apart
125 from most other unicellular N_2-fixing cyanobacteria such as *Crocosphaera* and *Cyanothece*, that
126 fix N_2 at night to protect nitrogenase from oxygen (Schneegurt et al. 1997; Church et al. 2005).
127 Daytime N_2 fixation by UCYN-A1 is likely possible because genes responsible for oxygen
128 generation via photosynthesis are missing (Zehr et al. 2008) and UCYN-A may be physically

129 isolated from its photo-oxygenic host by membrane(s) boundaries, as has been shown for a
130 UCYN-A isolated in coastal Japan (Hagino et al. 2013). To determine whether UCYN-A2 is
131 similar to UCYN-A1 in diel patterns of N_2 fixation, UCYN-A2 *nifH* gene expression was
132 measured over three consecutive diel cycles at the SIO Pier. Maximum *nifH* expression occurred
133 during the morning between 0600 and 1200 hours, which is consistent with the timing of *nifH*
134 expression by UCYN-A1 at Station ALOHA (Figure 3) and the lack of oxygen-evolving
135 pathways in the genome of UCYN-A2 (Bombar et al. *Submitted*). When comparing the peak-to-
136 peak amplitude of gene expression, UCYN-A2 *nifH* expression at SIO spanned 4 orders of
137 magnitude while UCYN-A1 *nifH* expression at the open ocean station ALOHA spanned only 2
138 orders of magnitude (Figure 3). This difference may be due to different sampling depths between
139 the two stations. SIO Pier samples were collected at the surface while Station ALOHA samples
140 were collected at 25 m depth (Church et al. 2005). At depth, diel fluctuations in light availability
141 are dampened due to the attenuation of light in water with depth. This observation may suggest
142 that UCYN-A is sensitive to light availability despite the absence of major photosynthetic
143 machinery such as the Photosystem II complex. Whether these transcriptional responses are
144 mediated through the host, or autonomously, remains unclear.
145
146 *UCYN-A2 host identification*
147 Genetic specificity between host and symbiont pairings can indicate co-evolution
148 between symbiotic partners. To determine if the UCYN-A clades associate with different hosts,
149 cell sorting and qPCR were applied to untreated samples from the SIO Pier in order to identify
150 the UCYN-A2 host. Distinct phytoplankton populations were analyzed by flow cytometry and
151 isolated by sorting from five sort gates in October 2012 and June 2013. Cells from sort gates A

152 and B were positive for UCYN-A2 *nifH*, while gates C, D, and E were negative (Figure 4). The
153 highest proportion of *nifH* gene copies amplified per sorted cell was from sort gate A. Thus, sort
154 gate A was targeted for subsequent sorts, microscopy, and screenings of single phytoplankton
155 cells for UCYN-A2 host identification.

156 UCYN-A2 *nifH* was amplified from only 9.5% of single phytoplankton cells (10 cells)
157 from gate A, suggesting that gate A contained a mix of cell types, only some of which contained
158 UCYN-A. Microscopic analysis of sorted cells from gate A revealed diatoms, dinoflagellates,
159 unidentified flagellated and non-flagellated cells, however, the host could not be positively
160 identified among these cells (Supporting Information - Figure S1). Together microscopy and
161 flow cytometry indicated that the UCYN-A host contained chlorophyll but not phycoerythrin and
162 ranged from 7 – 10 μm in diameter (Figure 4, Supporting Information - Figure S1). This size is
163 significantly larger than the estimates of UCYN-A1 host diameter at 1 – 3 μm (Thompson et al.
164 2012; Krupke et al. 2013). Differences in size may indicate different nutrient acquisition
165 strategies and requirements of the host associated with each lineage of UCYN-A. The
166 importance of cell size to nutrient acquisition in phytoplankton has been tested explicitly for
167 diatoms and iron where cell size was inversely correlated with iron uptake rates (Sunda and
168 Huntsman 1995; Sunda and Huntsman 1997). Similar effects are observed for a range of
169 phytoplankton species, including the prymnesiophyte *E. huxleyi* (Sunda and Hardison 2010).

170 Ten UCYN-A2-positive single phytoplankton cells from gate A were subjected to nested
171 PCR using universal 18S rRNA gene primers in order to identify the host. Seven of the cells
172 yielded prymnesiophyte sequences (GenBank accession numbers KF771248-KF771254) that
173 were 100% identical (6 cells) and 99.1% (1 cell) to *Braarudosphaera bigelowii* strains
174 Yatsushiro 1 (GenBank accession number AB478414), Furue 15 (GenBank accession number

175 AB478413), and TP05-6-a (GenBank accession number AB058358) (Figure 5). The three
176 remaining nested PCRs yielded only non-marine Chinese white pine (*Pinus armandii*) 18S rRNA
177 gene sequences that were also present in negative controls in this and previous studies
178 (Thompson et al. 2012). The UCYN-A2 host 18S rRNA gene is closely related to, but only has
179 only 98% nucleotide similarity across the amplified region to that of the UCYN-A1
180 prymnesiophyte host (Gen Bank accession number JX291893). This result confirms a recent
181 study that amplified UCYN-A 16S rRNA genes from an isolated specimen of Intermediate
182 Form-B *B. bigelowii* (Hagino et al. 2013).

183 The prymnesiophytes are abundant, ecologically relevant, and diverse eukaryotic
184 phytoplankton (Jardillier et al. 2010) and *Braarudosphaera* is no exception. Extant
185 *Braarudosphaera* have been recovered from diverse environments including coastal Japan
186 (Takano et al. 2006; Hagino et al. 2009), the Bering Sea (Konno et al. 2007), the Sargasso Sea
187 (Gaarder 1954; Hulburt 1962) and the Mediterranean Sea (Borsetti and Cati 1972;
188 Knappertsbusch 1993) (see Konno et al. (2007) for additional references and distribution map).
189 *B. bigelowii* is known to exist as at least five different genotypes and 18S rRNA gene sequences
190 have been recovered from calcareous pentalith-forming *B. bigelowii* in coastal Japanese waters
191 (Takano et al. 2006; Hagino et al. 2009). Thus, it is particularly intriguing that the coastally-
192 derived UCYN-A2 host sequence from this study (SIO Pier) clusters with *B. bigelowii* 18S
193 rRNA gene sequences from coastal Japan (Takano et al. 2006; Hagino et al. 2009; Hagino et al.
194 2013) while the UCYN-A1 host sequence clusters with sequences derived from the open-ocean
195 (Figure 5). There is now evidence that both *B. bigelowii* Intermediate form B (Hagino et al.
196 2013) and an open-ocean close relative of *B. bigelowii* (Thompson et al. 2012) harbor UCYN-A.
197 This study demonstrates that at least for UCYN-A1 and UCYN-A2, the host-symbiont

198 relationships are formed between genetically-distinct UCYN-A and *B. bigelowii* lineages.
199 However, it is still unknown whether other types of *B. bigelowii* (Takano et al. 2006; Hagino et
200 al. 2009) form symbioses with UCYN-A. If symbionts are always present, it is possible that the
201 genetic and morphological diversity of *B. bigelowii* corresponds to the diversity of UCYN-A,
202 and that there has been co-evolution of *B. bigelowii* and UCYN-A strains.

203 In previous studies, the UCYN-A1 host was not genetically identical to cells where the
204 presence of plates was confirmed, therefore the calcareous nature of the UCYN-A1 was
205 speculative (Thompson et al. 2012). However, the 100% genetic match of the UCYN-A2 host
206 sequence to *B. bigelowii* cells known to carry calcareous plates and a recent microscopic
207 observation of UCYN-A within a pentalith-carrying cell demonstrate unequivocally that the
208 UCYN-A host is capable of forming calcareous plates (Hagino et al. 2013). However,
209 picoeukaryotes with calcareous plates were not apparent following microscopic observation of
210 UCYN-A2-positive phytoplankton from sorted samples in this study (Supporting Information -
211 Figure S1). One possible explanation is that *B. bigelowii* passes through a non-calcifying life
212 stage during which this study was conducted (June and October). Indeed, other calcifying
213 prymnesiophytes carry out heteromorphic life cycles. *Emiliana huxleyi*, for example, exists in a
214 diploid calcifying form, a diploid non-calcifying form, and a haploid non-calcifying form. Work
215 on cultured isolates and naturally-occurring populations of *E. huxleyi* demonstrate that each life
216 stage exhibits distinct physiological and genetic characteristics such as light physiology and viral
217 susceptibility (Houdan et al. 2005; von Dassow et al. 2009; Frada et al. 2012). Similar
218 physiological changes may be part of the UCYN-A host life cycle.

219 The patterns of *B. bigelowii* calcification and the impacts of this host process on the UCYN-
220 A symbiont are relevant in the context of changing carbonate chemistry and rising atmospheric

221 carbon dioxide (CO_2) concentrations. For an organism such as *E.huxleyi*, the relationship
222 between calcification and CO_2 concentration is not straightforward (Beaufort et al. 2011). The
223 case may be similar for *B. bigelowii,* as this group is present in diverse ecosystems with varied
224 chemical gradients (Takano et al. 2006; Hagino et al. 2009). If host calcification sensitivities and
225 patterns influence UCYN-A N_2 fixation rates, there may be as of yet unexplored consequences of
226 global climate change to the global nitrogen cycle (Doney et al. 2009).
227

228 *UCYN-A2 and host abundance at the SIO Pier*

229 The abundances of UCYN-A2 and its host were measured in two sets of samples taken at
230 different times from the SIO Pier using qPCR targeting *nifH* and the 18S rRNA gene,
231 respectively. A monthly sampling series revealed the highest abundances of UCYN-A2 between
232 May and October 2011 (up to 1.9 x10^5 *nifH* copies per L) while the lowest abundances occurred
233 between November and April 2011 (1.3 x 10^3 *nifH* copies per L) (Supporting Information - Table
234 S2). An hourly sampling series from a 3-day period in October 2012 revealed stable abundances
235 of UCYN-A2 and *B. bigelowii* despite tidal flux and shifts in temperature, chlorophyll *a*
236 concentration, and salinity (Figure 6, Supporting Information - Figure S2). UCYN-A2
237 abundances averaged 1.36 x 10^5 *nifH* copies per L during the three-day period and host
238 abundances remained constant with an average of 4.3 x 10^4 18S rRNA gene copies per L of
239 seawater. Comparable ranges of UCYN-A1 abundances have been measured at Station ALOHA
240 (Church et al. 2005). Their persistent abundance through physical changes in the environment
241 may suggest that *B.bigelowii* abundance is more heavily controlled by nutrient availability (not
242 measured) than light availability and temperature, however, the influence of the environment on
243 *B. bigelowii* ecology is poorly understood.

244 The relative abundance of two symbiotic partners is an important aspect of the ecology
245 and evolution of host-symbiont interactions. In the case of UCYN-A and its host, the relative
246 abundances of the partners will aid in estimating contributions of UCYN-A to N_2 fixation taking
247 into account host N requirements, nutrient and metabolite exchange between the two cells, and
248 the mechanism of UCYN-A transfer between host generations. In this study, the ratio of UCYN-
249 A2 to *B. bigelowii* abundance in bulk DNA samples ranged from 0.2 to 11.0 over three days,
250 averaging 3.3 UCYN-A2 cells per host (derived from data in Figure 6, and assuming one copy of
251 *nifH* in UCYN-A2 and one copy if the 18S rRNA gene in *B. bigelowii*). It must be noted that
252 these ratios are estimates as qPCR is not reliable for absolute quantification of single gene
253 copies, and *B. bigelowii* may have multiple copies of the 18S rRNA gene like many other
254 picoeukaryotes (Prokopowich et al. 2003; Zhu et al. 2005). Previously, application of
255 Halogenated *In Situ* Hybridization (HISH) and Fluorescence *In Situ* Hybridization (FISH) to
256 seawater samples detected 1-2 UCYN-A1 per host (Thompson et al. 2012; Krupke et al. 2013).
257 A microscopy study of *B. bigelowii* in coastal Japan waters showed that there were either one or
258 two UCYN-A cells per host (Hagino et al. 2013). These independent lines of evidence suggest
259 that at least 1-2 UCYN-A symbionts are associated with each host, however it remains unclear if
260 the UCYN-A cells are sister cells and how their division might be synchronized with host cell
261 division and life cycles.
262
263 *Conclusions*
264 The results of this study showed that one of the major lineages of marine diazotrophic
265 cyanobacteria, UCYN-A, is represented by three genetically-distinct clades. Two of these clades,
266 UCYN-A1 and UCYN-A2, were compared and were similar in their day-time expression of *nifH*

267 and symbiosis with a *B.bigelowii*-like prymnesiophyte. However, the study demonstrated that
268 UCYN-A1 and UCYN-A2 associate with specific host genotypes suggesting coevolution of
269 symbiont and host and the adaptation of each host-symbiont pair to a specific environmental
270 niche. In some cases, these niches are physically distant and distinct in nutrient availability and
271 oceanographic conditions, such as in the case of UCYN-A1 from Station ALOHA in the open
272 ocean and UCYN-A2 from the coastal SIO Pier. However, in most of the environments that have
273 been sampled, multiple clades of UCYN-A are present, thus specific host-symbiont pairs may
274 occupy overlapping ecological niches as well. Future work will reveal if the genetic distinction
275 between lineages of the UCYN-A symbiont and prymnesiophyte host have implications to
276 patterns of biological nitrogen fixation in the marine environment.
277
278 Experimental Procedures
279 *Sample and oceanographic data collection*
280 Seawater samples were collected with a bucket from the end of the Scripps Institution of
281 Oceanography (SIO) Ellen Browning Scripps Memorial Pier in La Jolla, CA. After collection,
282 sample water was gently poured into a 10 L polypropylene bottle then placed in a dark cooler
283 and transported to the lab (5 minutes). The first set of samples was collected monthly between
284 September 2010 and May 2011, then they were shipped overnight to Santa Cruz, CA for
285 filtration and DNA analysis. The second set of samples was collected October 1st - 4th 2012,
286 May 8th 2013, and June 10 -13th 2013 and was processed immediately for flow cytometry, gene
287 expression, and DNA analysis. Oceanographic data for chlorophyll concentration, temperature,
288 and salinity were collected from the Southern California Coastal Ocean Observation System
289 website (www.sccoos.org/) for the dates sampled.

290

291 *Flow cytometry*

292 Flow cytometry was performed using the Influx (Becton Dickinson, San Jose, CA, USA)
293 high-speed cell sorter equipped with a small particle detector and a 488 nm laser (Sapphire
294 Coherent, Coherent Inc., Santa Clara, CA, USA). In the course of the work presented here, two
295 different cytometers were used, thus cytometry settings vary based on the instrument used for
296 data collection and the experimental purpose of the measurements. For example, the 3 µm beads
297 in Figure 4A and B are used for qualitative reference rather than calibration of the forward
298 scatter (FSC) signal in each panel. BioSure (Grass Valley, CA, USA) Sheath Solution was used
299 at 1X concentration with either 70 or 100 µm diameter nozzles. Data collection and sorting was
300 triggered with light scattered in the forward direction (FSC). Sortware (Becton Dickinson) data
301 collection software was used to identify populations of cells for gating and cell sorting. Beads of
302 0.75, 3, 10, and 25 µm diameter Polysciences Fluoresbrite ® YG Microspheres and/or 3.8 µm
303 diameter Ultra Rainbow Fluorescent Particles (Spherotech, Warrington, PA, USA) were used for
304 internal reference. Data analysis and figures were created in Flow Jo (TreeStar, Ashland, OR,
305 USA).

306 Prior to cell analysis and sorting, seawater samples were either untreated (not filtered or
307 frozen, no preservatives) or were agitated. Untreated seawater was used to study the intact
308 UCYN-A2/host complex for host identification and characterization experiments. Agitated
309 seawater samples were used for experiments to study UCYN-A2 when separated from the host.
310 Agitation involved vacuum filtration (5 – 10 psi) onto 0.22 µm pore-size type GV 47 mm
311 diameter filters (Millipore, Billerica, CA, USA), 100X concentration by re-suspension of the

312 filter in sterile-filtered seawater, brief vortex mixing, flash-freezing in liquid nitrogen, thawing at
313 room-temperature, and 1 minute of vortex mixing before analysis.
314
315 *Nucleic acid extraction and complementary DNA synthesis*
316 RNA and DNA samples were collected by vacuum filtration of replicate 500 mL volumes
317 of seawater at 10 psi pressure onto 47 mm, 0.22 µm pore-size, Supor filters (Pall Corporation,
318 Port Washington, NY, USA). Filters were placed in sterile 2 mL bead-beating tubes with sterile
319 glass beads (both DNA and RNA samples). RNA samples were amended with the lysis buffer
320 RLT (Qiagen, Valencia, CA, USA) and β-mercaptoethanol before storage. Samples were stored
321 at -80 °C until extraction. Care was taken to process RNA samples within 15 minutes of seawater
322 sampling.
323 DNA extractions were carried out using a modification of the Qiagen DNeasy Plant Kit
324 (Moisander et al. 2008). Samples were subjected to 2 minutes of bead-beating, three sequential
325 freeze-thaw cycles using liquid nitrogen and a 60 °C water bath, a one hour proteinase K
326 treatment, and column purification using the QIAcube automated extraction platform (Qiagen).
327 RNA extraction was performed using a modified version of the Qiagen RNeasy Plant
328 Mini Kit, which included an automated on-column DNase treatment step. The QuantiTect
329 Reverse Transcription Kit (Qiagen) was used for additional removal of genomic DNA and
330 synthesis of complementary DNA (cDNA) (Turk-Kubo et al. 2012).
331
332 *Quantitative PCR (qPCR) assay design and application*
333 Two Taqman® quantitative PCR (qPCR) assays, one targeting the UCYN-A2
334 nitrogenase (*nifH*) gene and the other targeting the *Braarudosphaera sp.* 18S rRNA gene

335 sequence present at the SIO Pier, were developed to quantify UCYN-A2 and host from DNA
336 samples, whole sorted cells, and cDNA (UCYN-A2 only).

337 Nested PCR using degenerate *nifH* primers (Zehr and McReynolds 1989; Zani et al.
338 2000) was applied to the first set of DNA samples (DNA collected monthly). Sequences
339 (GenBank accession numbers KF806604-KF806612) belonged exclusively to the UCYN-A2
340 clade and were used to inform design of a UCYN-A2-specific qPCR assay. Primers and probe
341 sequences for the UCYN-A2 *nifH* gene assay (96 bp amplicon) are as follows: Forward, 5'-
342 GGTTACAACAACGTTTTATGTGTTGA-3'; Reverse, 5'-ACCACGACCAGCACATCCA-3';
343 Probe, 5'-FAM-TCTGGTGGTCCTGAGCCCGGA-TAMRA-3'. Nested PCR with universal
344 18S rRNA gene primers (see below), were used to amplify the 18S rRNA gene of UCYN-A2
345 host cells (*Braarudosphaera bigelowii*), which were used to design the UCYN-A2 host qPCR
346 assay. Primers and probe sequences sequences for the SIO *B. bigelowii* 18S rRNA gene assay
347 (96 bp amplicon) are as follows: Forward, 5'-GGTTTTGCCGGTCTGCCGTT-3'; Reverse, 5'-
348 ATCCGTCTCCGACACCCACTC-3'; Probe, 5'-FAM-CTGGTGCGAGCGTCCTTCCT-
349 TAMRA-3'.

350 Each assay used TaqMan® Gene Expression MasterMix (Invitrogen, Carlsbad, CA,
351 USA) at 1X concentration along with 0.4 μM forward and reverse primers and 0.2 μM probe.
352 Both assays were initially incubated for 10 minutes at 95 °C to relax target DNA and data was
353 collected at the end of each of 45 staged repeats of 15 seconds at 95 °C and 60 seconds at either
354 64 °C (UCYN-A2 *nifH* gene) or 60 °C (SIO *Braarudosphaera* 18S rRNA gene). Standards for
355 each assay were generated using linear plasmids containing clones of PCR amplified gene from
356 environmental samples containing either UCYNA-A2 *nifH* (359 bp) or *Braarudosphaera* sp. 18S
357 rRNA (733bp).

358

359 *Identification of target cell populations by flow cytometry and qPCR*

360 The UCYN-A2-specific qPCR assay was used to screen flow-sorted events (individual
361 phytoplankton cells) to identify populations enriched in UCYN-A2 (sorts of 50-500 events) or
362 individual phytoplankton (1 event) that could contain both UCYN-A2 and its host organism.
363 Cells were sorted into aliquots of 10 µl, 5 kDa-filtered, nuclease-free water then amended for
364 "whole cell" qPCR as above. Assays were run with an Applied Biosystems 7500 Real Time PCR
365 thermal cycler.

366

367 *Confocal Microscopy*

368 Once the seawater was sorted and the presence of *nifH* gene was confirmed by qPCR, the
369 positive *nifH* gene fraction was imaged live in a chamber slide at 600X final magnification at the
370 Laser Scanning Confocal Microscope A1R (Nikon Instrument Inc. Japan). The nucleic stain
371 DAPI, was added to the seawater fraction at a final concentration of 10 µg mL^{-1}. The sample was
372 imaged using the 405 nm (DAPI) laser, the 488 nm (phycoerythrin) laser and the 648 nm
373 (chlorophyll) laser.

374

375 *Nested PCR on single cells with universal 18S rRNA gene primers*

376 Single events (individual phytoplankton cells) from a sort gate enriched in UCYN-A2
377 were sorted into individual wells of 96-well qPCR plates on October 4, 2012 and June 12, 2013.
378 Plates were screened for UCYN-A2 using qPCR alongside standards and no template controls
379 (NTC). Wells identified as positive for UCYN-A2 *nifH* were selected and the entire reaction

380 volume (25 µl) was used as template in a nested PCR series using universal 18S rRNA gene
381 primers.
382 The nested PCR series consisted of two sequential reactions: the first was primed with
383 external primers EukA/EukB (Medlin et al. 1988) and the second was primed with internal
384 primers Euk555F/Univ1269R (López-García et al. 2003). EukA/EukB reactions were run in 100
385 µl volumes containing 75 µl of master mix added to the 25 µl volume of a UCYN-A2 positive
386 qPCR. PCR using Euk555F/Univ1269R primers was then performed in 50 µl volumes with 2 µl
387 of the EukA/EukB reaction as template. All PCR was performed with ExTaq DNA polymerase
388 (Takara Bio, Inc., Otsu, Shiga, Japan) with 25 mM $MgCl_2$. Gel electrophoresis was used to
389 visualize PCR product from the inner primers. Amplified DNA bands of approximately 700 bp
390 were cut and purified from the gel. The pGEM-T Vector System (Promega, Madison, WI, USA)
391 was used for cloning of 18S rRNA gene products and Sanger sequencing was performed at the
392 UC Berkeley DNA Sequencing Center (Berkeley, CA). Trimming of vectors, removal of poor
393 quality sequences, and sequence comparisons were performed using Sequencer version 5.1
394 (Gene Codes Corporation, Ann Arbor, MI, USA). GenBank accession numbers for the 7 UCYN-
395 A2 host 18S rRNA gene sequences that were recovered are KF771248-KF771254.
396
397 *Phylogenetic analysis*
398 UCYN-A-like sequences were identified using an auto-curated publically available
399 database (http://pmc.ucsc.edu/~wwwzehr/research/database/). This database was populated with
400 all *nifH* sequences submitted to GenBank's nr/nt database as of September 6, 2013. Amino acid
401 sequences were aligned using the Hidden Markov Model profile Fer4_NifF_fs (Pfam PF00142)
402 in HMMer v3.1b1 (Finn et al., 2010), and nucleotide sequences were back-aligned to the aligned

403 amino acids. UCYN-A-like sequences were identified based on their amino acid similarity to the
404 UCYN-A1 genome (Tripp et al. 2010) and were clustered at 97% nucleic acid identity, using the
405 CD-HIT package (Li and Godzik 2006). Representative sequences of the resulting clusters were
406 chosen for the construction of nucleic acid and amino acid trees (Figure 1A, B). Maximum
407 likelihood trees of partial *nifH* nucleic acid and amino acid sequences were constructed in
408 MEGA6.06 (Ludwig et al 2004). Evolutionary distances were inferred using the Tamura-Nei
409 (307 positions) and JTT-matrix-based (101 positions) models for nucleotide and amino acid
410 trees, respectively, and bootstrap values were determined using 1000 replicates. Bayesian
411 analysis was conducted to provide further support for the nucleic acid phylogeny using Mr.
412 Bayes (Ronquist et al., 2011), using a GTR substitution model with gamma distributed rate
413 variation, and branches supported by this analysis are indicated with black squares (Fig. 1A).
414 Maximum-likelihood trees of 18S rRNA genes were constructed using PhyML (Guindon et al.
415 2010) to analyze phylogeny of the UCYN-A hosts and other publically-available related
416 sequences. Phylogenetic trees were visualized in Tree View (Page 1996).
417

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427 the maker of the Influx cell sorter used in this study.
428
429 ***Figure legends***
430 Figure 1. Maximum likelihood trees of representative partial nitrogenase (*nifH)* UCYN-A
431 sequences. Representative sequences were determined by clustering all UCYN-A sequences
432 deposited in NCBI's Genbank database at 97% nucleotide similarity using CD-HIT-EST. A)
433 Nucleic acid tree showing at least three phylogenetically-distinct clusters of UCYN-A. Clades
434 are designated UCYN-A1 (initially characterized at Station ALOHA and includes the UCYN-A
435 genome - (Zehr et al. 2008)), UCYN-A2, and UCYN-A3, and are color-coded. Black text
436 indicates sequences that do not clearly cluster with any of the clades defined in this study.
437 Genbank accession numbers are in parenthesis and the number of clones represented by each
438 sequence is in bold brackets. Bootstrap values are indicated on branches with bootstrap support
439 of >500 out of 1000 permutations. Branches supported by Bayesian analysis are noted with a
440 black triangle. B) Amino acid tree that shows no obvious clustering of UCYN-A sequences.
441 Sequences are color-coded to reflect their clustering in (A).
442
443 Figure 2. Distribution and counts of UCYN-A *nifH* sequences from GenBank. A) UCYN-A1, B)
444 UCYN-A2, C) UCYN-A3, and D) other UCYN-A sequences not included in clades defined here.
445 The size of the circle is proportional to the number of sequences that fall into each clade
446 according to the legend in panel D.
447

448 Figure 3. Comparison of *nifH* gene expression between UCYN-A1 at ALOHA (dotted line)
449 (Church et al. 2005) and UCYN-A2 at SIO (solid line) (This study). Gene expression is
450 calculated relative to gene copy number from DNA (cDNA/DNA). Gene expression at each
451 time-point is normalized to the median gene expression over the entire time-course for each
452 study. Black bars represent dark periods over the diel cycles.
453
454 Figure 4. Flow cytometry analysis of phytoplankton populations at the SIO Pier. (A) Populations
455 present in untreated seawater. UCYN-A2 was detected by qPCR in sort gates A and B and absent
456 from sort gates C, D, E, and F. (B) Populations present in agitated and concentrated seawater
457 (See Methods). UCYN-A2 was detected in the population indicated using qPCR. Standard beads
458 are present for FSC reference only as different data collection settings were utilized for each
459 panel. 3 µm diameter beads in panel A are full-spectrum ultra-bright beads and thus are more
460 fluorescent than the 3 µm diameter YG beads used in panel B. 10 µm diameter beads were the
461 same in both samples (See Experimental Procedures).
462
463 Figure 5. Maximum-likelihood tree showing the phylogeny of the UCYN-A2 host 18S rRNA
464 gene sequences (green box) from single cells relative to other publically-available sequences
465 from isolates (black), environmental sequences (green and blue type), and the UCYN-A1 host
466 sequence (blue box). Sequences amplified from the oligotrophic environments of the North
467 Pacific (Stn. ALOHA) and Eastern South Pacific are typed in blue. Sequences amplified from
468 coastal environments of Japan (*Braarudosphaera* sp.), the Cariaco Basin, and coastal Southern
469 California (SIO Pier) are typed in green.
470

471 Figure 6. (A) Abundance of UCYN-A2 *nifH* and *Braarudosphaera* 18S rRNA genes at the SIO

472 Pier over three diel cycles. Black bars represent dark periods over the diel cycles.

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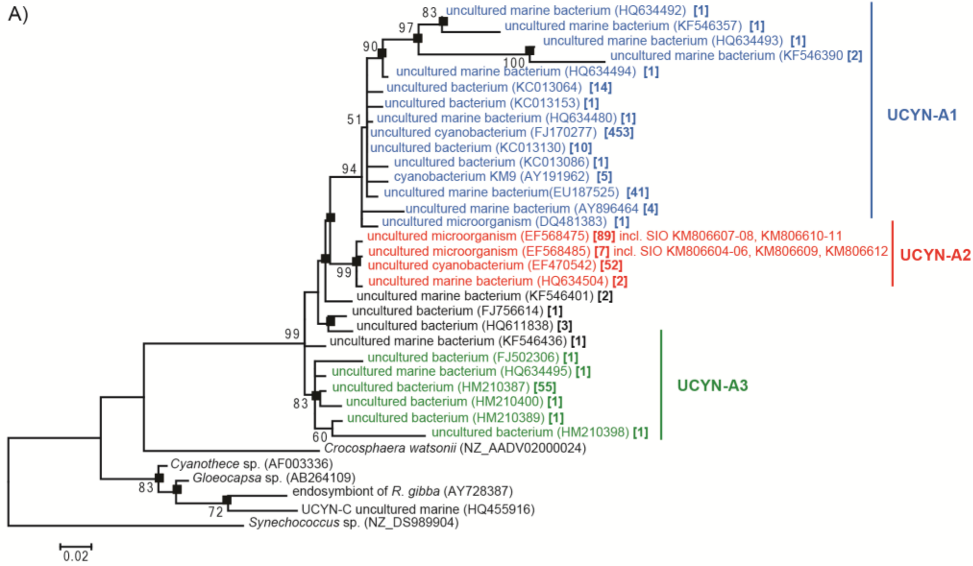
Figure 1

A)



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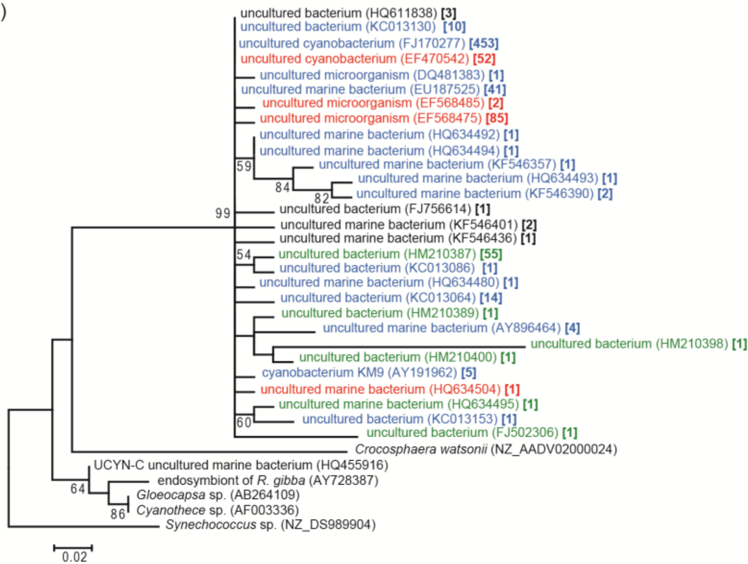


Figure 2

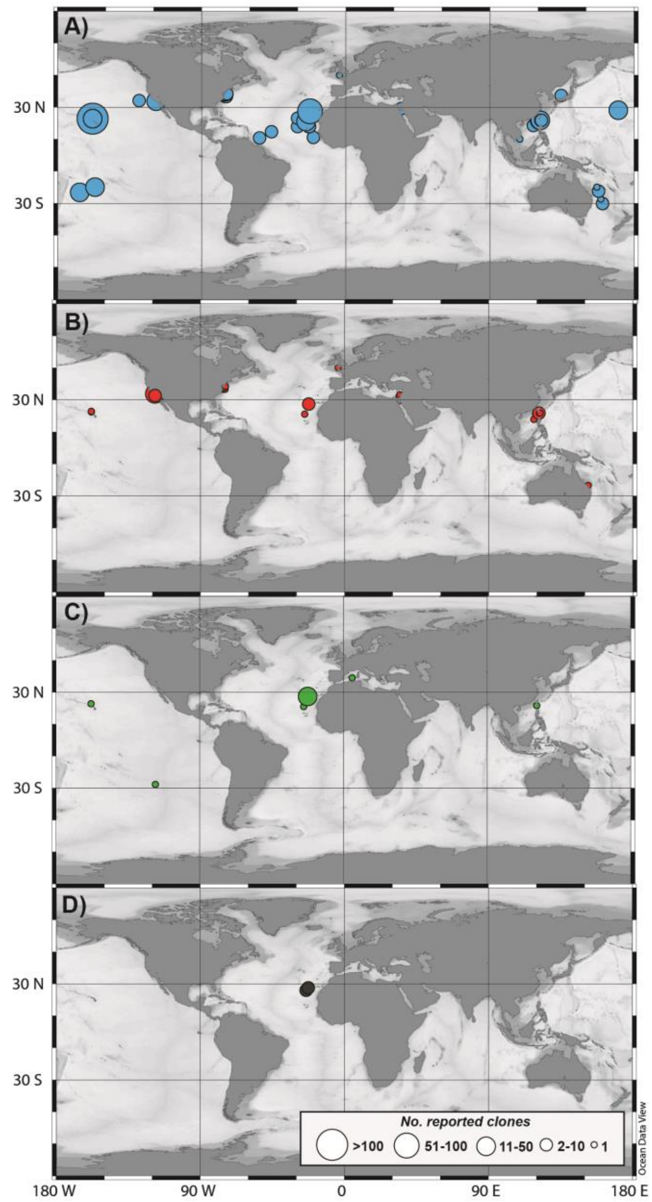
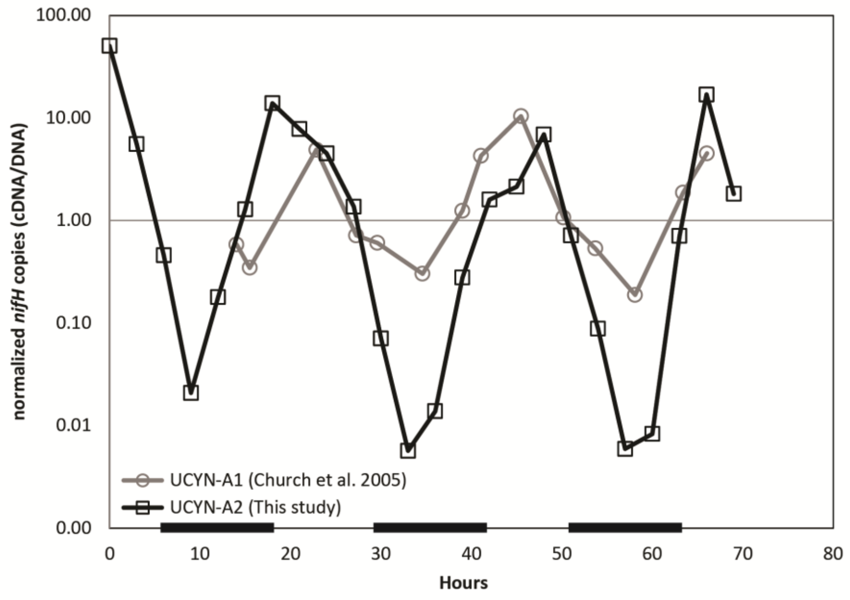


Figure 3



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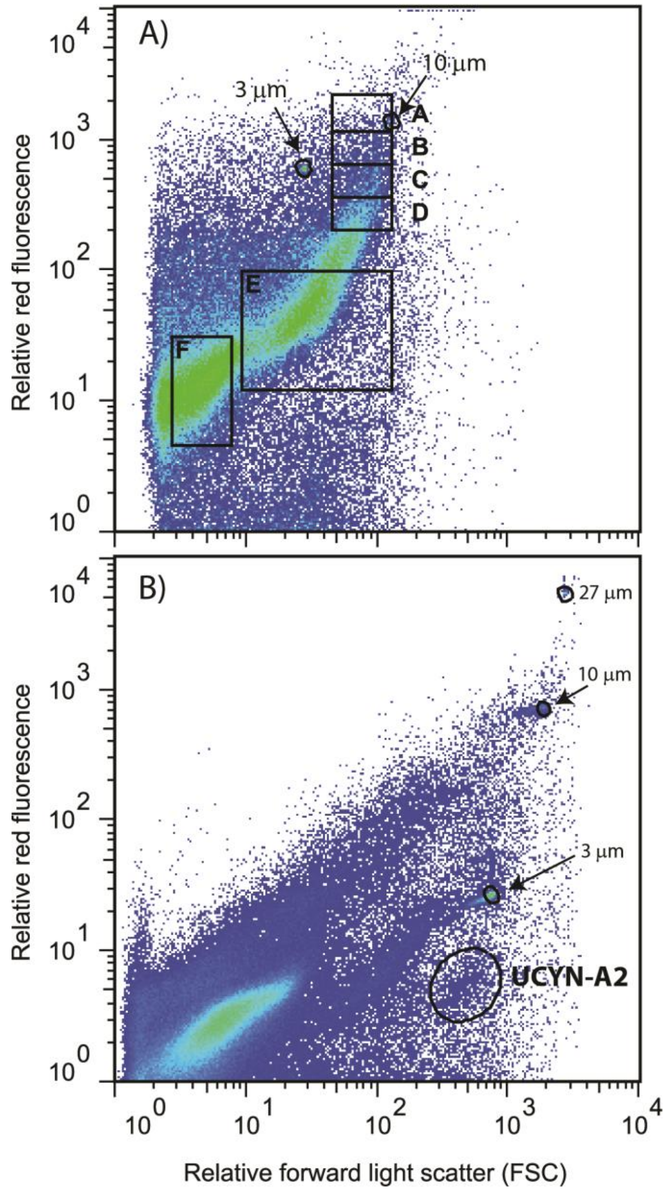

Figure 5

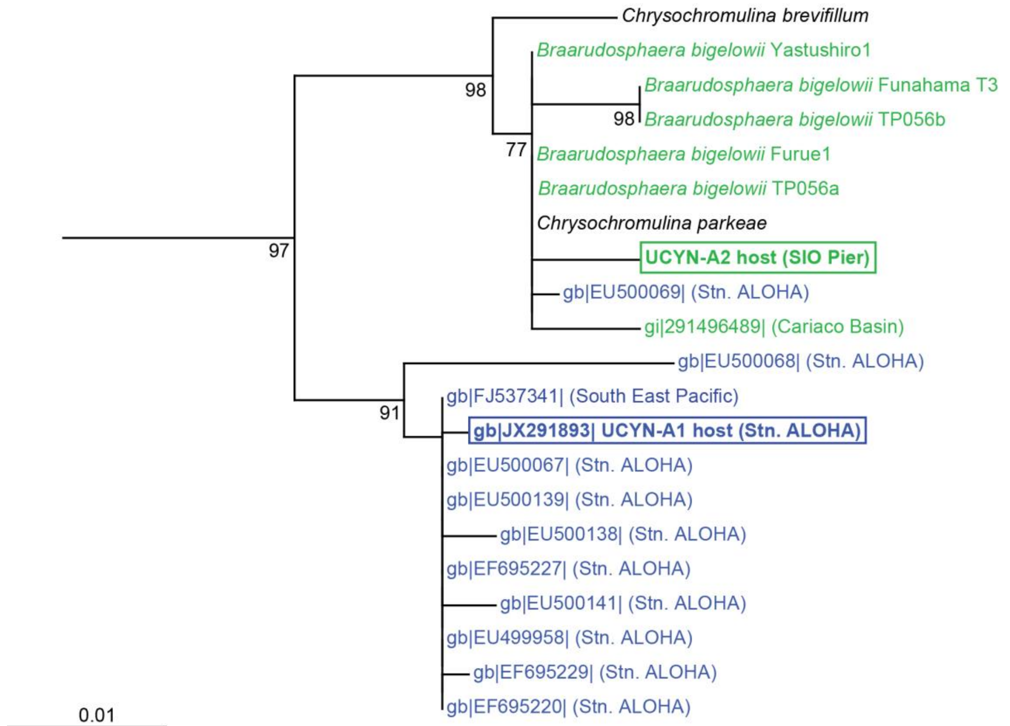

Figure 6