Microbial community analysis in the gills of abalones suggested possible dominance of epsilonproteobacterium in *Haliotis gigantea*

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Abstract

Gills are important organs for aquatic invertebrates because they harbor chemosynthetic bacteria, which fix inorganic carbon and/or nitrogen and provide their hosts with organic compounds. Nevertheless, in contrast to the intensive researches related to the gut microbiota, much is still needed to further understand the microbiota within the gills of invertebrates. Using abalones as a model, we investigated the community structure of microbes associated with the gills of these invertebrates using next-generation sequencing. Molecular identification of representative bacterial sequences was performed using cloning, nested PCR, and fluorescence in situ hybridization (FISH) analysis with specific primers or probes. We examined three abalone species, namely *Haliotis gigantea*, *H. discus* and *H. diversicolor* using seawater and stones as controls. Microbiome analysis suggested that the gills of all three abalones had the unclassified Spirochaetaceae (1 OTU, 15.7±0.04%) and *Mycoplasma* sp. (1 OTU, 9.1±0.03%) as the core microbes. In most libraries from the gills of *H. gigantea*, however, a previously unknown epsilonproteobacterium species (1 OTU) was considered as the dominant bacterium, which

30 accounted for 62.2% of the relative abundance. The epsilonproteobacterium was only detected in

31 the gills of *H. diversicolor* at 0.2% and not in *H. discus* suggesting that it may be unique to *H.*

32 *gigantea*. Phylogenetic analysis performed using a near full-length 16S rRNA gene placed the

33 uncultured epsilonproteobacterium species at the root of the family Helicobacteraceae.

34 Interestingly, the uncultured epsilonproteobacterium was commonly detected from gill tissue

35 rather than from the gut and foot tissues using a nested PCR assay with uncultured

36 epsilonproteobacterium-specific primers. FISH analysis with the uncultured

37 epsilonproteobacterium-specific probe revealed that probe-reactive cells in *H. gigantea* had a

38 coccus-like morphology and formed microcolonies on gill tissue. This is the first report to show

39 that epsilonproteobacterium has the potential to be a dominant species in the gills of the coastal

40 gastropod, *H. gigantea*.

41

42 **Introduction**

43   Abalone is one of the worldwide valuable fisheries resources that is mainly consumed in

44 East Asia such as China, Japan, and South Korea. Farming abalone is particularly active in China

45 and South Korea, and has recently increased in South Africa (Cook 2016). Research on the

46 relationship between abalone and bacteria is important for stable abalone culture. The digestive

47 tract of abalone is more developed than bivalves, which are filter feeders, because abalones feed

48 brown algae that contains polysaccharides such as alginates. Therefore, the studies of bacteria

49 related to abalones are commonly focused on microbes associated to the gut (Tanaka et al. 2004,

50 Zhao et al. 2012, and Nam et al. 2018). However, it is still unclear on the role of microbes in

51 other important organs within abalone.

52   It has been reported that some marine invertebrates have chemoautotrophic bacteria in/on

53 their gills (Dubilier et al. 2008). Symbiotic relationships between marine invertebrates and

54 chemoautotrophic bacteria are characterized by the exchange of chemical molecules, such as

55 carbon dioxide, oxygen, hydrogen, hydrogen sulfide, nitrogen or methane, from the hosts,

56 through the seawater to symbionts. The symbionts then take up these molecules and provide their

57 hosts with organic compounds. Therefore, it can be said that gills are also play an important for

58 symbiotic bacteria as taking nutrients through seawater in addition to uptake oxygen for the hosts.

59   Since marine invertebrates with vestigial digestive tracts nutritionally depend on their

60 symbionts (Pennec et al. 1995), most of the studies on the microbial communities in invertebrate

61   gills have focused on deep-sea floor vent and cold seep environments where there are many

62   kinds of gutless organisms (Dubilier et al. 2008). Alternatively, it has also been reported that

63   bivalves in the coastal area have symbiotic bacteria in/on their gills, although the number of

64   reports is more scarce than one related to deep-sea floor. The members of bivalves in three

65   families, Solemyidae, Thyasiridae, and Lucinidae, are known as shallow-water invertebrates

66   having autotrophic sulfur-oxidizing bacteria in/on their gills (Duperron et al. 2013). These hosts

67   have autotrophic sulfur-oxidizing Gammaproteobacteria in common, but the symbiotic location

68   vary between the host species; the symbionts are intercellular in Solemyidae and Lucinidae

69   (Cavanaugh 1983, Frenkiel and Mouëza 1995), while most members of Thyasiridae have

70   extracellular symbionts on their gill tissue (Dufour 2005). Symbiotic bacteria play a nutritionally

71   important role in bivalves on coastal area as on deep-sea floor. *Solemya velum*, family

72   Solemyidae, derive more than 97% of their carbon from symbionts, although they are capable of

73   suspension feeding (Conway et al. 1989 and Krueger et al. 1992). Additionally, symbionts

74   associated with two species in Lucinidae, *Loripes lucinalis* and *Codakia orbicularis*, have been

75   reported to fix atmospheric nitrogen and synthesize amino acids for their hosts in addition to

76   carbon fixation and sulfur-oxidization (Petersen et al. 2016 and König et al. 2016). Meanwhile,

77   genomic information of symbiotic bacteria of *S. velum* suggests that they have both abilities to

78   function as endo-symbiotic and free-living lifestyle (Dmytrenko et al. 2014). According to Cúcio

79   et al. (2018), draft genomes closely related to the gill symbiont of *S. velum* were retrieved from

80   the rhizobiome of the seagrass where the clam inhabit, although Krueger et al. suggested that *S.*

81   *velum* vertically transmit their gill symbionts to offspring (1996). Furthermore, *C. orbiculata* can

82   acquire symbionts from the environment even if the clams are adult (Gros et al. 2012). These

83   symbiotic bacteria may have flexible lifestyles.

84   To date, only Gammaproteobacteria has been reported for symbionts on/in gills of

85   shallow-water invertebrates. On the other hand, Epsilonproteobacteria are known as symbionts

86   of invertebrates on the deep-sea floor. The deep-sea organisms such as *Petrasma* sp. (Rodrigues

87   et al. 2010), *Rimicaris exoculata* (Suzuki et al. 2005) or *Alviniconcha hessleri* (Urakawa et al.

88   2005 and Suzuki et al. 2006) have Epsilonproteobacteria in/on their gills that are known as

89   ecologically significant group of bacteria in deep-sea hydrothermal vents and cold seeps

90   (Nakagawa et al. 2005 and Takai et al. 2005). Gamma- and Epsilon-proteobacteria in/on the gills

91   have similar roles as carbon fixation and/or sulfide oxidation. Gamma- and Epsilon-

92 proteobacterial symbionts mediate the Calvin-Benson cycle (Stewart et al. 2006 and Kleiner et al.

93 2012) and the reductive tricarboxylic acid cycle (Takai et al. 2005), respectively, to fix CO_2.

94   Bivalves and marine gastropods have open circulatory system containing hemolymph,

95 which spread over their body including gills part. The hemolymph as the blood in invertebrates is

96 responsible for the transport of nutrients and the elimination of invaders, particularly bacteria. It

97 has been reported that bacteria were detected in the hemolymph of even healthy oysters (Schmitt

98 et al. 2012 and Lokmer and Wegner 2015), while the hemolymph of abalone have strict immune

99 system (Hooper et al. 2007).

100   In this study, we analyzed the microbial communities associated with the gills of

101 economically important abalones, such as *Haliotis gigantea*, *H. discus* and *H. diversicolor*, in

102 shallow-water environments. Additionally, this study investigated the phylogenetic

103 characteristics and placement of the unique bacteria, the uncultured epsilonproteobacterium, in

104 the gills of *H. gigantea*.

105

Materials & Methods

Sample preparation and DNA extraction

108   Five cultured giant abalones, *Haliotis gigantea*, were collected from the Minami-ise Farming

109 Fishery Center (Minami-ise, Mie, Japan) in October 2016. Wild abalone specimens (*H. gigantea,*

110 *H. discus* and *H. diversicolor*) were obtained from a fish-market in Mie, Japan, from April 2017

111 to May 2019. The purchased abalones were collected offshore along the coast of Mie prefecture

112 by scuba diver, and were transported in a water tank to the laboratory within 6 h of sampling.

113   Gill tissues from the cultured *H. gigantea* (n=5) specimens collected from the farm and wild

114 *H. discus* (n=3) and *H. diversicolor* (n=5) specimens collected from the fish-market were pooled

115 into three individual tubes, one for each species (sample code: Hgig1, Hdis1 and Hdiv1). Gill

116 tissues from other *H. gigantea* and *H. discus* specimens were prepared individually (sample

117 code: Hgig2-6, Hdis2-4). Gut and foot tissues were also collected from all *H. gigantea*

118 specimens, excluding Hgig1 from which no foot tissues were obtained. For all *H. discus* and *H.*

119 *diversicolor* specimens, only gill tissues were used. The collected gill, gut or foot tissues were

120 then homogenized in sterile artificial seawater using a bead beater homogenizer (4200 rpm, 30

121 seconds; Tietech Co., Nagoya, Japan) followed by a previously described method (Tanaka et al.

122 2004). Host tissues were removed from the homogenate by a quick centrifugation step (1 s, 8000

123   g), and the supernatant was transferred to new tubes and centrifuged for 20 min at 15000 g to

124   recover bacterial cells.

125   Seawater and stone samples were collected from the shore in Minami-ise (34.333958 N

126   136.692204 E) within 30 m from the Minami-ise Farming Fishery Center. Fifty milliliters of

127   seawater was filtered by passing through 0.22 μm filter paper and resuspended in sterile

128   phosphate-buffered saline (PBS: 130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4; pH 7.4) (sample

129   code: SW). Stones were shaken in sterile PBS (sample code: ST) and bacterial cells were

130   recovered from the SW and ST PBS samples by centrifuging for 20 min at 15000 g. Bacterial

131   genomic DNA from each bacterial pellet was extracted using a Promega DNA purification

132   system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

133

134   **PCR amplification, Illumina MiSeq sequencing, and Sequence processing**

135   All gill, SW, and ST samples were used for the analysis of microbial communities. The

136   V1-V2 region of 16S rRNA genes was amplified using Ex Taq (TaKaRa Biotechnology Corp.,

137   Kyoto, Japan). The first PCR step was performed using primers 27F-mod (5'-

138   ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGRGTTTGATYMTGGCTCAG-3') and

139   338R (5'-

140   GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT-3').

141   The amplification conditions were as follows: initial denaturation of 2 min at 94°C followed by

142   24 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, and primer

143   extension at 72°C for 30 s. The amplified PCR products were purified using a Wizard SV Gel

144   and PCR Clean-Up System (Promega Corp.) and used for the second PCR step, which was

145   performed using primers with a tag sequence. After the second PCR, the products were once

146   again purified using a Wizard SV Gel and PCR Clean-Up System before sequencing on a MiSeq

147   platform (Illumina Inc., San Diago, CA, USA).

148   The raw paired-end FASTQ reads were demultiplexed using the Fastq barcode splitter

149   (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and imported into the Quantitative Insights

150   Into Microbial Ecology 2 program (QIIME2, ver. 2019.7, https://qiime2.org/). Demultiplexed

151   reads were quality filtered, denoised, chimera checked and dereplicated using a DADA2 denoise-

152   paired plugin (Callahan et al. 2016). To be equal to sampling-depth, sequences were rarefied at

153   23,000 reads using qiime feature-table rarefy (Weiss et al. 2017). For preparing Upset diagram,

154   sequences from *H. gigantea* and *H. discus* were also combined into one for each host species

155   using feature-table merge plugin on Qiime2 in addition to prepare individual samples, and then

156   the sequences were resampled at 23,000 reads using qiime feature-table rarefy plugin on Qiime2

157   (Weiss et al. 2017). Next, the align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin

158   was used to perform multiple sequence alignment, remove phylogenetically uninformative or

159   ambiguously aligned sequences, and to generate unrooted and rooted phylogenetic trees (Lane

160   1991, Price et al. 2010, Katoh and Standley 2013). Diversity metrics were calculated using the

161   core-metrics-phylogenetic pipeline from the diversity plugin on QIIME2. For alpha diversity,

162   Shannon index (Shannon 1948), observed OTU number (DeSantis et al. 2006), Chao 1 index

163   (Chao 1984), and Good's coverage (Good, 1953) were calculated using the diversity alpha

164   command on QIIME2. For PCoA plots, unweighted UniFrac data and Shannon diversity values

165   were calculated by the core-metrics-phylogenetic pipeline on Qiime2, and they were combined

166   and visualized using the R packages "qiime2R", "tidyverse", "phyloseq" (McMurdie and

167   Holmes 2013) and "ggplot2" (Wickham 2009) in R (v3.5.0). Taxonomic assignments were

168   performed using the qiime feature-classifier classify-sklearn on Greengenes v_13.8 (McDonald

169   et al. 2012). Taxa bar plots were constructed using the plugin qiime taxa bar plot. For analysis of

170   core or unique microbiome (Reich et al. 2018 and Neu et al. 2019), Upset diagram was generated

171   using R package UpSetR (version 1.3.3) with grouped NGS sequence data by each host species

172   or environment (Lex et al. 2014). All of the data were deposited at the Sequence Read Archive

173   (SRA) under the accession number PRJDB8953.

174

175   **Obtaining the 16S rRNA gene sequences of the uncultured epsilonproteobacterium by**

176   **cloning**

177   PCR amplification of the bacterial 16S rRNA gene was performed using primer 27F

178   (5'-AGAGTTTGATCCTGGCTCAG-3', Lane 1991) and primer 1492R (5'-

179   GGTTACCTTGTTACGACTT-3', Lane 1991). The bacterial 16S rRNA gene clone library was

180   constructed using PCR amplicons obtained from Hgig1. PCR reaction mixtures contained 1×

181   PCR reaction buffer, 200 µM dNTP, 5 pmol of each primer, 2.5 units Ex Taq polymerase

182   (TaKaRa Biotechnology Corp.), and 10 – 100 ng of community DNA in a total volume of 50 µl.

183   PCR reactions were performed on a thermal cycler (iCycler; Bio-Rad Laboratories, Hercules,

184   CA, USA) using the following amplification conditions: initial denaturation of 4 min at 95°C

185 followed by 25 cycles of denaturation for 30 sec at 95°C, primer annealing for 30 sec at 55°C,

186 and primer extension at 72°C for 1.5 min. This was followed by a final extension reaction at

187 72°C for 7 min. PCR product was ligated into the TOPO TA cloning vector (Invitrogen Corp.,

188 Carlsbad, CA, USA) according to the manufacturers' instructions. Ligation products were

189 transformed into *Escherichia coli* One Shot TOP10 cells (Invitrogen Corp.) and clones were

190 amplified by PCR using vector-specific primers. Plasmid DNA with insertions was sequenced

191 using primer 27F. Partial sequencing was performed using the BigDye terminator cycle

192 sequencing method, and an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA).

193 The resulting chromatograms of DNA sequences were examined using Chromas 2.33. Homology

194 searches were performed using sequences and close relatives were identified using a BLAST

195 search of the GenBank database on the National Center for Biotechnology Information website

196 (http://www.ncbi.nlm.nih.gov/). The sequences identified as Epsilonproteobacteria by BLAST

197 search were selected from random clones generated during cloning. The phylogenetic

198 characterization of the selected sequence was estimated using the phylogenetic tree with

199 representative sequence of Epsilonproteobacteria by the Maximum Likelihood method in MEGA

200 7.0 software (Kumar et al. 2016).

201

202 **Nested PCR analysis and sequencing of the uncultured epsilonproteobacterium**

203   The nested PCR assay targeted 356 bp of the uncultured epsilonproteobacterial 16S rRNA

204 gene. At the first PCR, bacterial universal primers 27F and 1492R were used, and then the

205 uncultured epsilonproteobacterium-specific primers, Eps222F (5'-

206 CGCTAAGAGATTGGACTATAT-3') and Eps578R (5'-

207 GACTTAATAGGACACCTACATACC-3') (designed in this study) were used at the second

208 PCR. DNA samples extracted from gill, gut or foot tissue of each *H. gigantea* specimen were

209 used as DNA templates. The first-round master mixture contained the following: primers (27F

210 and 1492R, Lane 1991) at 0.2 μM (each), 25 μl volume of EmeraldAmp® PCR Master Mix

211 (TaKaRa Biotechnology Corp.), approximately 50 ng of DNA, and up to 50 μl with distilled

212 H_2O. Distilled H_2O was used as the template for negative controls. Cycle parameters were 98°C

213 for 10 sec; 10 cycles of 98°C for 10 sec, 55°C for 30 sec, and 72°C for 90 sec; and 72°C for 5

214 min. PCR reactions were performed using an iCycler (Bio-Rad Laboratories). A 2.5-μl aliquot of

215 the amplified PCR product was transferred to a new master mixture containing primers (Eps222F

216   and Eps578R) at 0.2 µM (each) in a 25 µl volume of EmeraldAmp® PCR Master Mix (TaKaRa

217   Biotechnology Corp.) made up to a volume of 50 µl with distilled H_2O. Cycle parameters were

218   98°C for 10 sec; 25 cycles of 98°C for 10 sec, 57°C for 30 sec, and 72°C for 60 sec; and 72°C

219   for 5 min. A 5-µl aliquot of the amplified PCR product was analyzed by agarose (1.5% [wt/vol]

220   prepared in TAE buffer) gel electrophoresis and Midori Green Direct (Nippon Genetic Europe

221   GmbH, Düren, Germany) staining, and the gel was photographed under UV light. The presence

222   of a band at around 350 bp was interpreted as a positive result. Sequencing and phylogenetic

223   analysis were performed as previously described in the subsection "Obtaining the 16S rRNA

224   gene sequences of the uncultured epsilonproteobacterium using cloning method" above.

225

226   **Fluorescence in situ hybridization localization of the uncultured epsilonproteobacterium**

227   Fluorescence in situ hybridization (FISH) was performed on the Hgig4 sample using a

228   previously described method (Tanaka et al. 2016). Gill tissue was obtained from Hgig4 and

229   placed in 75% ethanol at -30°C before being fixed in 4% (v/v) paraformaldehyde/PBS at 4°C

230   overnight. Following fixation, specimens were rinsed and dehydrated in a 50, 70, 80, 85, 90, 95,

231   and 100% ethanol series, followed by 100% xylene. The fixed gill specimens were then

232   embedded in paraffin and sliced into 10-µm transverse sections using a microtome (RV240,

233   Yamato, Japan), before being placed on APS-coated microscope slides (Matsunami, Japan), and

234   stored in slide boxes at room temperature until deparaffinization. Wax was removed, and tissue

235   was rehydrated in a decreasing ethanol series. The sections were dewaxed and hybridized at

236   47°C for 3 h using a solution containing 20% formamide, 0.9 M NaCl, 20 mM Tris–HCl (pH

237   7.4), 0.1% SDS, and 0.5 µM FITC-labeled Eub338 probe (5'-GCTGCCTCCCGTAGGAGT-3',

238   Amann et al. 1990) or 0.5 µM TAMRA-labeled Eps222 probe (5'-

239   CGCTAAGAGATTGGACTATAT-3'), which was designed to specifically target the 16S rRNA

240   gene of the uncultured epsilonproteobacterium (designed in this study). After hybridization, the

241   sections were washed twice with washing buffer containing 20 mM Tris-HCl (pH 7.4), 180 mM

242   NaCl and 0.01% SDS for 30 min at 48°C, and the sections were then rinsed with ddH_2O and air-

243   dried. An epifluorescence light microscope (Eclipse 400; Nikon Instech., Tokyo, Japan), was

244   used to observe the stained cells.

245

246   **Results**

247 **Microbial Community Analysis by next-generation sequencing (NGS)**

248   The microbial communities on the gills of various marine invertebrates were

249 characterized by sequencing the V1-V2 region of the 16S rRNA gene. A total of 473,349

250 quality-filtered sequence reads were obtained from 13 samples (PRJDB8953). Alpha diversity

251 index values for each sample are shown in supplementary Table 1. For all of the samples in this

252 study, observed OTUs and Chao1 were approximately the same value, and Good's coverage

253 value was higher than 99.99%, indicating that sequencing depth sufficient for capturing all

254 bacterial species and for downstream analysis. We identified 322 OTUs from 15 phyla, which we

255 taxonomically assigned with the Greengenes database v. 13_8. At the phylum level, the

256 microbial taxonomic composition of most samples, excluding Hgig5, had a high relative

257 abundance of Proteobacteria (67.8% on average). Hgig5 showed almost complete dominance of

258 Spirochaetes (68.4%).

259   In Proteobacteria, sequence reads were related to Alphaproteobacteria,

260 Gammaproteobacteria or Epsilonproteobacteria (Figure 1). Alphaproteobacteria was dominant in

261 Hgig1, Hgig5, Hdis2, Hdis4, SW and ST, (12.0-39.5%, Figure 1). The most abundant

262 Alphaproteobacteria in abalone were aligned with unclassified Rickettsiales (11.2% on average,

263 2 OTUs), while an unclassified Rhodobacteraceae (9.1%, 1 OTU) and *Nautella* sp. (12.5%, 1

264 OTU) were the most abundant Alphaproteobacteria-related sequences in the ST and SW

265 samples, respectively. The relative abundance of Gammaproteobacteria-related sequences was

266 more than 10% in all samples, except Hgig2. The genus *Vibrio* (Gammaproteobacteria) were

267 observed in all samples. In addition, the relative abundance of *Vibrio* spp. (6 OTUs) was more

268 than 10% in SW and all *H. discus* specimens, especially in Hdis4 (58.4%, 3 OTUs). In Hdiv1, an

269 unclassified Endozoicimonaceae (1 OTU) accounted for 25.1% of the relative abundance and

270 were more frequently recovered than *Vibrio* spp. In *H. gigantea* specimens, *Alcanivorax*

271 *dieselolei* (1 OTU) showed 11.8% relative abundance in Hgig1, while an unclassified

272 Francisellaceae (1 OTU) and an unclassified Gammaproteobacteria (1 OTU) were distributed at

273 9.4% and 5.9% in Hgig6, respectively. Gammaproteobacteria-related sequences in other Hgig

274 samples however were classified into OTUs with relative abundance less than 5%. Sulfur-

275 oxidized Gammaproteobacteria, which was reported as symbiotic bacteria, was not detected in

276 any samples. Epsilonproteobacteria-related sequences were dominantly recovered from SW and

277 all *H. gigantea* specimens, except Hgig5 (18.3-62.2%, Figure 1). All Epsilonproteobacteria-

278 related sequences in SW were affiliated with *Arcobacter* sp. (28.7%, 1 OTU). *Arcobacter* sp.

279 was also detected in the others samples, although the relative abundance was less than 5%. Other

280 Epsilonproteobacteria-related sequences were most closely related to unclassified

281 Epsilonproteobacteria (2 OTUs). One of these was detected in all *H. gigantea* specimens and

282 dominated in Hgig1 to Hgig4, accounting for $50.5 \pm 0.05\%$ of the relative abundance. The

283 unclassified Epsilonproteobacteria was not found in all Hdis, SW, and ST, although the related

284 sequences represented 0.2 % of the relative abundance in Hdiv1. Another unclassified

285 Epsilonproteobacteria was detected in Hgig4, Hgig5, Hgig6, Hdis1, and Hdiv1, accounting for

286 up to 7.1% of the relative abundance in Hgig4. Spirochaetes-related sequences were aligned with

287 an unclassified Spirochaetaceae (1 OTU). These sequences were observed in all abalone

288 specimens except in Hdis4. The unclassified Spirochaetaceae was abundance in Hgig5, Hdis1,

289 and Hdiv1, accounting for 68.4%, 28.1%, 19.9%, respectively. No Spirochaetes-related

290 sequences were detected in SW and ST. All members of the phylum Tenericutes were affiliated

291 with 1 OTU in the genus *Mycoplasma*. *Mycoplasma* sp. was present in every abalone, and most

292 prevalent in Hdis1 (21.9%), followed by Hdis3 (21.3%). In contrast, the sequences related to

293 *Mycoplasma* were never observed in SW and ST. Chloroplast-related sequences were only

294 detected in ST and SW, accounting for 22.6% (6 OTUs) and 4.5% (6 OTUs), respectively. Most

295 Chloroplast-related sequenceare associated with an unclassified Stramenopiles (1 OTU) and

296 accounted for up to 12.6% of the relative abundance in ST.

297   The similarity in the microbiota was supported by principal coordinates analysis (PCoA,

298 Figure 2). The microbiota in each invertebrate differed from those in the environment, such as

299 from the seawater and stones. The microbial community structure of *H. gigantea* was similar to

300 one of *H. discus*, while the microbe of *H. diversicolor* formed a cluster distinct from other

301 abalone species.

302   The idea of core and unique microbe was used to clarify the characteristics among

303 samples (Reich et al. 2018 and Neu et al. 2019). The largest numbers of unique OTUs was

304 obtained from Hgig with 64 OTUs (Figure 3). In descending order, the unique OTUs were 52, 49

305 and 28 OTUs in ST, Hdis and SW, respectively. The number of unique OTUs in Hdiv was the

306 smallest where 8 OTUs were obtained. The relative abundance of the unique OTUs showed low

307 value of 3.5%, 2.4%, 3.9%, and 7.6% in Hgig, Hdis, Hdiv, and SW, respectively (Table 1). On

308 the other hand, the unique OTUs in ST showed high proportion (26.2%), although the highest

309   relative abundance showed only 4.5%. 10 OTUs were obtained from all samples as core microbe,

310   and the relative abundance of the core microbe showed 12.6%, 36.3%, 12.2%, 44.5%, and 20.7%

311   in Hgig, Hdis, Hdiv, SW, and ST, respectively (Fig. 3 and Table1). *Arcobacter* sp. and a member

312   of genus *Vibrio* (1 OTU) were shared between all specimen as the core microbe. 14 OTUs were

313   detected as the core microbe in abalones, which was commonly obtained from Hgig, Hdis and

314   Hdiv, and their proportions were 31.3%, 32.6%, and 44.0%, in Hgig, Hdis, and Hdiv,

315   respectively (Table 1). The core microbe in abalones included the unclassified Spirochaetaceae

316   and *Mycoplasma* sp. The core microbe in environments, SW and ST, was 8 OTUs, including the

317   unclassified Stramenopiles, and their proportions were 2.7% and 25.3% in SW and ST,

318   respectively (Table 1).

319

320   **Phylogenetic analysis of 16S rRNA gene of the uncultured epsilonproteobacterium**

321   Cloning and sequencing of the long 16S rRNA gene sequence of the uncultured

322   epsilonproteobacterium found in *H. gigantea* were performed to clarify the phylogenetic position

323   of this bacterium. Four out of sixteen cloned sequences were completely matched to the

324   sequence of the uncultured epsilonproteobacterium obtained from the 16S rRNA gene amplicon

325   sequencing undertaken in this study. The longest sequence length was 1436 bp (LC511979).

326   BLAST analysis of the uncultured epsilonproteobacterial sequence revealed that the

327   sequence could be assigned to various described Epsilonproteobacteria species, such as

328   *Arcobacter canalis* strain F138-33 (87.43% identity), *Arcobacter marinus* strain CL-S1 (87.19%

329   identity), *Sulfurovum lithotrophicum* strain 42BKT (87.01% identity) and *Helicobacter pullorum*

330   strain ATCC51801 (86.78% identity), but these identity scores were low. Among the

331   environmental clones in public databases, the uncultured epsilonproteobacterium specimen was

332   most closely related to an uncultured clone sequence found in the gill tissue of a wood-feeding

333   gastropod in genus *Pectinodonta* (96.12-96.78% identity, Zbinden et al. 2010), followed by an

334   uncultured clone sequence from gill tissue of thyasirid bivalves from a cold seep in the Eastern

335   Mediterranean (94.86% identity, Brissac et al. 2011). In the phylogenetic analysis with

336   Maximum Likelihood method, the uncultured epsilonproteobacterium grouped within the order

337   Campylobacterales (Figure 4). However, the uncultured epsilonproteobacterium was not able to

338   be classified below the order, since the phylogenetic position of the sequence was at the root of

339   the family Helicobacteraceae.

340

Detection of the uncultured epsilonproteobacterium from each part of *H. gigantea*

342   Results of the 16S rRNA gene amplicon sequencing analysis revealed that the

343 uncultured epsilonproteobacterium was dominant only in the gill tissues of *H. gigantea*.

344 Therefore, in order to determine whether the uncultured epsilonproteobacterium was restricted to

345 the gill tissues, a PCR assay of gill, gut and foot tissues from *H. gigantea* was performed using

346 uncultured epsilonproteobacterium-specific primers. PCR products were obtained from gill tissue

347 from Hgig2, Hgig3 and Hgig6 (Table 2). Amplified products were also obtained from the gill

348 tissue from Hgig1, Hgig4, gut tissues from Hgig3 and foot tissues from Hgig4, but band

349 visibility was low. All amplification product sequences matched that of the uncultured

350 epsilonproteobacterial sequence obtained by cloning.

351

Localization of the uncultured epsilonproteobacterium on gill tissue of *H. gigantea*

353   In semi-thin section FISH analysis, Eps222-positive bacteria were observed on the gill

354 surface of Hgig4 (Figure 5) where they formed microcolonies on gill tissue (Figure 5B and C).

355 Eps222-positive bacteria showed a coccus-like morphology and measured ~1.0 μm in diameter

356 (Figure 5C).

357

Discussion

359   In this study, we analyzed the microbiota in the gills of three species of abalones. It was

360 previously considered that the bacterial community in the gills would be similar to that in

361 seawater because the gill filaments of aquatic organisms are exposed to seawater, particularly

362 during respiration. However, PCoA analysis suggested that the microbial composition and

363 characteristics of shellfishes differed from those of seawater and/or stone. In addition, when we

364 look at the bacterial community structure in detail, the dominant bacterial species were different

365 for each host species, even among congeneric hosts.

366   The member of the phylum Tenericutes, *Mycoplasma* sp., was detected in all abalone

367 samples thus, classified as the core microbe in abalone. It was suggested that abalones, the genus

368 *Haliotis,* harbor *Mycoplasma* sp. before their speciation. Additionally, the sequence of

369 *Mycoplasma* sp. obtained in this study showed 99.7% homology identity with uncultured clones

370 that were dominant in the gut of *H. d. hannai* (Tanaka et al. 2004). *Mycoplasma* sp. were also

371   expected to inhabit not only the gill tissues, but also gut tissues. However, the associations

372   between these potential symbiotic bacteria and their hosts is still unknown.

373   The sequence of the unclassified members of Spirochaetaceae in the class Spirocaetia also

374   classified as a core microbe in abalone, were closely related to the sequence detected in the cold-

375   water coral *Lophelia pertusa* in the northeastern Gulf of Mexico (91.2% identity, Kellogg et al.

376   2009). The relationship between uncultured *Spirochaeta* and *L. pertusa* was unknown, but

377   spirochetes have been reported to catalyze the synthesis of acetate from H_2 and CO_2 in the

378   termite gut (Leadbetter et al. 1999), as well as being a symbiont of the gutless worm,

379   *Oligochaeta loisae*, in the Australian Great Barrier Reef (Dubilier et al. 1999). Additionally,

380   *Spirochaeta* symbionts are detected in the gill tissues of cold-seep clams (Lucinidae) where it is

381   presumed that they are involved in autotrophy (Duperron et al. 2007). On the other hand,

382   *Spirochaeta perfilievii* isolated from a sulfur 'Thiodendron' mat in a saline spring are able to

383   oxidize sulfide, however, this was a heterotrophic species (Dubinina et al. 2011). The

384   unclassified bacteria belonging to Spirochaetaceae in abalones may therefore be a symbiont that

385   detoxified sulfide and/or carbon fixation.

386   Gammaproteobacteria was the dominant class of bacteria in *H. discus,* and most of these

387   bacteria were *Vibrio* spp. Some members of *Vibrio* are the core microbes but were also abundant

388   in seawater. Hence, it was considered that *Vibrio* in/on gills of abalones was infected from their

389   surrounding environment, particularly seawater. Non-motile *Vibrio* spp., called non-motile

390   fermenters (NMF) have been reported to be symbionts of abalones (Sawabe et al. 2003). These

391   authors reported that they fermented organic acids, especially acetate, from alginate of brown

392   algae in the abalone gut and provide the resulting organic acids to their hosts. However, since it

393   cannot be assumed that there is always alginate around the gills of abalone specimens, it is

394   proposed that *Vibrio* spp. on the gills originated in the gut and then adhered to the gills.

395   Alternatively, the *Vibrio* spp. found in the gills have different ecological roles from those found

396   in the gut.

397   Most symbionts in the gills of bivalve have not been isolated, and their 16S rRNA genes

398   show very low homology with characterized species (Dubilier et al. 2008). Therefore, these

399   symbionts are classified as uncultured Gammaproteobacteria. We searched for these symbiont-

400   related sequences in the uncultured Gammaproteobacteria sequences from our NGS analysis, but

401   we unable to identify them. Since some bivalves acquire their symbionts from the environments

402 in which they live like sediments (Gros et al. 2012 and Lim et al. 2019), while abalones inhabit

403 on reefs, we assumed that sulfur-oxidized Gammaproteobacteria was not detected in all samples.

404   The uncultured epsilonproteobacterium was dominant in all of the *H. gigantea* samples,

405 except Hgig5, by NGS analysis. Interestingly, this bacterium was not dominant in the congeneric

406 species, *H. discus* and *H. diversicolor*. The relative abundance of the uncultured

407 epsilonproteobacterium in Hgig6 was slightly lower than that in the other *H. gigantea* samples.

408 The high Shannon index value obtained for Hgig6 and the detection of less common bacterial

409 species in other *H. gigantea*, such as *Polaribacter* spp. and unclassified Flavobacteriaceae

410 species, indicates that the abundance of the uncultured epsilonproteobacterium in Hgig6 was

411 relatively low compared to that found in other *H. gigantea* specimens.

412   The class Epsilonproteobacteria in terrestrial environments is widely known for its

413 pathogenic genera; *Campylobacter*, *Helicobacter,* and to a lesser extent *Arcobacter,* (Gilbreath et

414 al. 2011). Some of the members of this class also inhabit at hydrothermal vents, where they often

415 associate with invertebrate hosts as ecto-, endo- or epi-symbionts; for example, polychaete worm

416 (*Alvinella pompejana*) (Haddad et al. 1995), shrimp (*Alvinocaris longirostris*) (Tokuda et al.

417 2008), crab (*Shinkaia crosnieri*) (Fujiyoshi et al. 2015) and gastropods (*Alviniconcha* spp.)

418 (Suzuki et al. 2005 and 2006, Urakawa et al. 2005). The members of this group are considered to

419 be symbionts that provide their hosts with organic carbon compounds by carbon fixation and

420 detoxify hydrogen sulfide. Although the 16S rRNA gene sequence of the uncultured

421 epsilonproteobacterium obtained from *H. gigantea* in this study formed a cluster in phylogenetic

422 analysis with members of Campylobacterale, the similarity score to described species was as

423 high as 87.43%. Thus, it is considered that the uncultured epsilonproteobacterium bacterium

424 obtained in this study could be, at least, a novel genus. BLAST analysis comparing the

425 uncultured epsilonproteobacterium with other uncultured bacterial clones revealed that they were

426 closely related to clones detected from the gills of a wood-feeding gastropod, *Pectinodonta* sp.

427 (Patellogastropoda, Mollusca) (96.12-96.78%, identity). Although the relationship between these

428 clones and their host was unknown, the epsilonproteobacterial clone sequence obtained from the

429 gills was not detected from the digestive system or foot of *Pectinodonta* sp. (Zbinden et al.

430 2010). The uncultured epsilonproteobacterium is therefore considered to specifically inhabit gill

431 tissues.

432   Results of the microbial community analysis revealed that the uncultured

433 epsilonproteobacterium was dominant in the gills of the gastropod, *H. gigantea*. However, it was

434 surmised that the bacterium was also presents in other body parts. Like the gills, the foot of *H.*

435 *gigantea* in in direct contact with seawater. Compared to gills or the foot, the gut is also a closed

436 space in which bacteria can be easily retained. The gut environment is also microaerobic, which

437 means that it is well suited for the growth of some members of Epsilonproteobacteria, such as

438 *Campylobacter* and *Arcobacter* (On et al. 2017). Therefore, PCR using a specific primer for the

439 uncultured epsilonproteobacterium was performed to confirm whether the bacterium also

440 inhabited tissues other than the gills. PCR amplification products of the uncultured

441 epsilonproteobacterium were obtained from all gill tissues from *H. gigantea*, but never or very

442 little, from the gut and the foot tissue, even when different tissues from the same individual were

443 used. These findings therefore suggested that the uncultured epsilonproteobacterium is generally

444 restricted to gill tissues. In addition, semi-thin section FISH analysis revealed micro-colonies of

445 the uncultured epsilonproteobacterium on *H. gigantea* gills. It is necessary to culture these

446 bacteria in order to better clarify their metabolism and the biological interactions that exist

447 between the uncultured epsilonproteobacterium and *H. gigantea*.

448

449 Conclusions

450   In this study, we analyzed the microbiota that colonize the gills of abalones such as *H.*

451 *gigantea*, *H. discus,* and *H. diversicolor,* using 16S rRNA amplicon sequencing. The findings

452 suggested that the gills of the abalones support specific bacterial communities compared to the

453 environment such as seawater and stones. The unclassified Spirochaetaceae (1 OTU) and

454 *Mycoplasma* sp. (1 OTU) were found in all abalone specimens as the core microbe in abalone. *H.*

455 *discus* showed dominance of *Vibrio* as the core microbe in all samples including seawater and

456 stones. An uncultured epsilonproteobacterium (1 OTU) was a unique microbe in the gills of *H.*

457 *gigantea*, except for detection of *H. diversicolor* (0.2%). Additionally, an uncultured

458 epsilonproteobacterium, which dominant only in *H. gigantea*, formed micro-colonies on the gills

459 of this species, but not in its gut or foot. This uncultured epsilonproteobacterium specifically

460 inhabits the gills of the shallow-water gastropod, *H. gigantea*.

461

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465

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The relative abundance of the core or unique microbes in each host species and environmental control.

The number of sequences is shown in parentheses.

	Hgig	Hdis	Hdiv	SW	ST
Core microbiome	12.6%	36.3%	12.2%	44.5%	20.7%
(No. of sequences)	(2890)	(8360)	(2805)	(10237)	(4770)
Core microbiome in abalones	31.3%	32.6%	44.0%		
(No. of sequences)	(7191)	(7508)	(10113)		
Core microbiome in environment				2.7%	25.3%
(No. of sequences)				(631)	(5809)
Unique microbiome	3.5%	2.4%	3.9%	7.6%	26.2%
(No. of sequences)	(807)	(551)	(898)	(1759)	(6033)
Others	52.7%	28.6%	39.9%	45.1%	27.8%

Detection of the uncultured epsilonproteobactrium in *H. gigantea* samples.

+, positive; w, weak (i.e. PCR products obtained but the bands were very feint); -, negative;
NT, not tested.

	Tissue		
Sample code	**Gill**	**Gut**	**Foot**
Hgig1	w	-	NT
Hgig2	+	-	-
Hgig3	+	w	-
Hgig4	w	-	w
Hgig5	-	-	-
Hgig6	+	-	-

Figure 1

Composition of the relative abundance of 16S rRNA gene amplicon reads (class-level taxa).

16S rRNA gene amplicon reads were obtained from the gills of three abalone species, *H. gigantea* (Hgig), *H. discus* (Hdis), and *H. diversicolor* (Hdiv), and Stone (ST) and Seawater (SW). Classes with relative abundance >10% in any sample are presented. Taxa with lower abundance and unassigned taxa are indicated as 'other'.
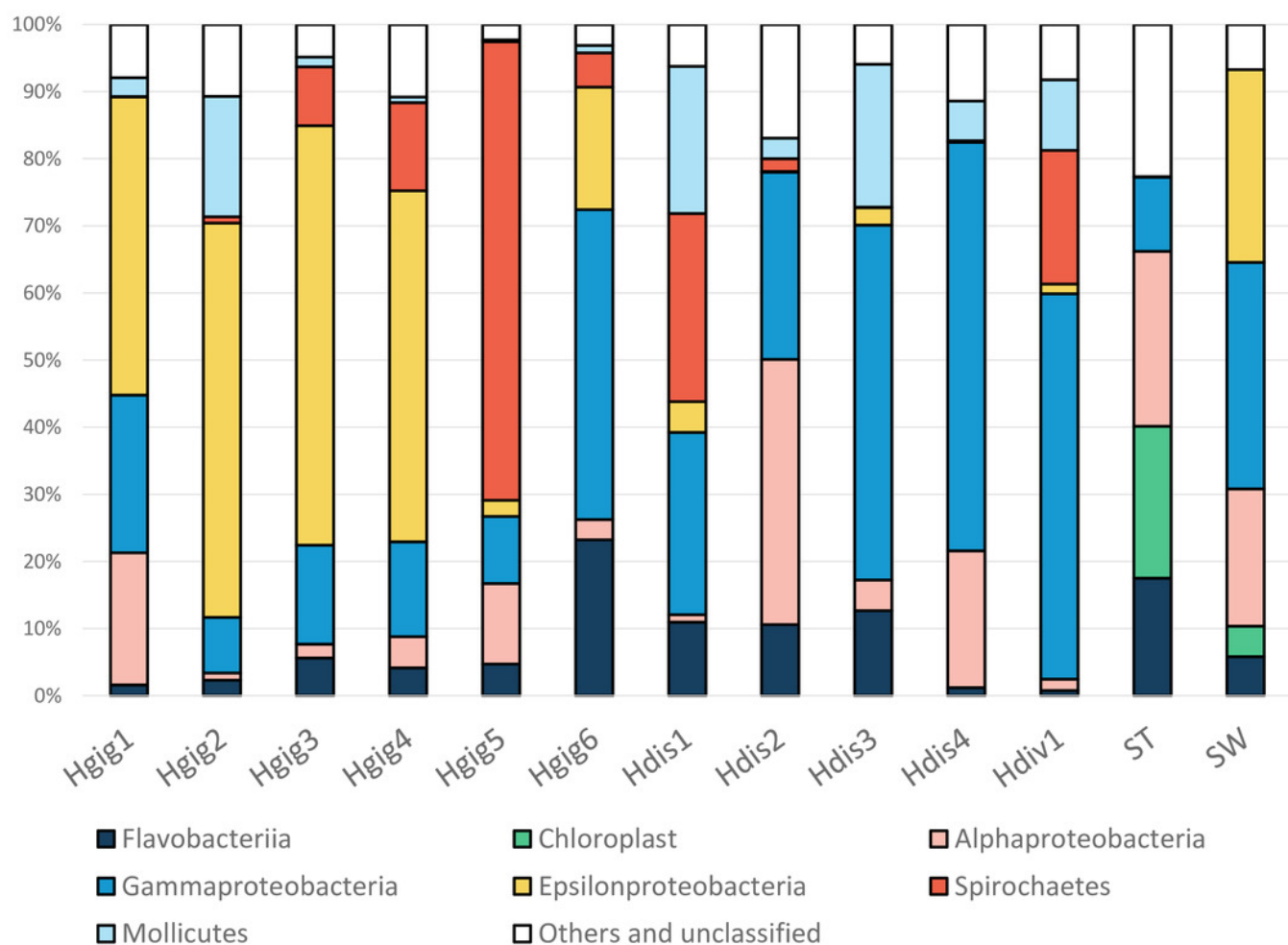
Figure 2

Principal coordinate analysis (PCoA) of unweighted UniFrac distances for *H. discus* (Hdis), *H. diversicolor* (Hdiv), *H. gigantea* (Hgig), Seawater, and Stone.
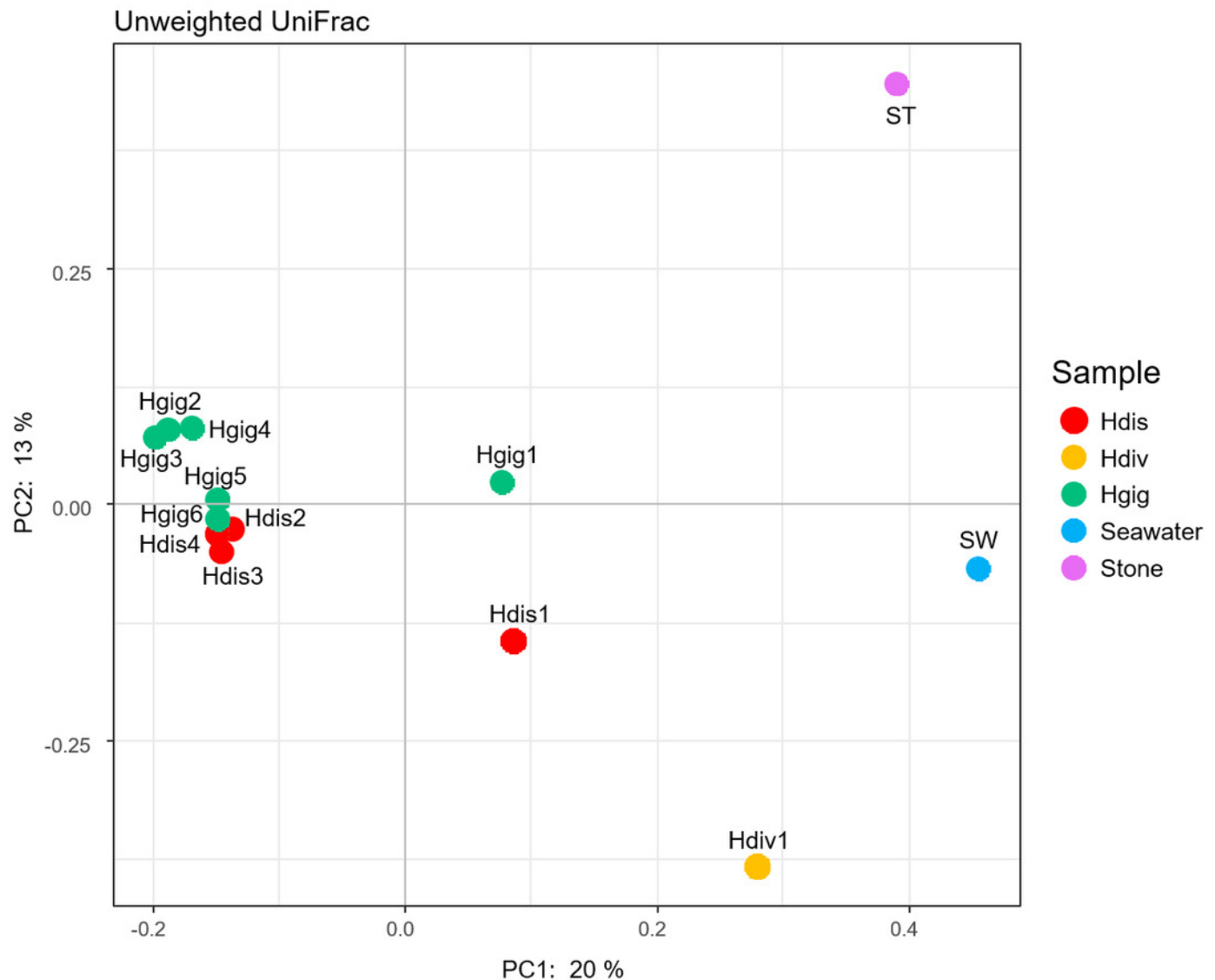
Figure 3

UpSet plot showing shared and unique OTUs in each host species and environmental samples.

Calculations are based on OTU table rarefied to 23,000 sequences/sample. The bars show the overlap between the indicated sample below.
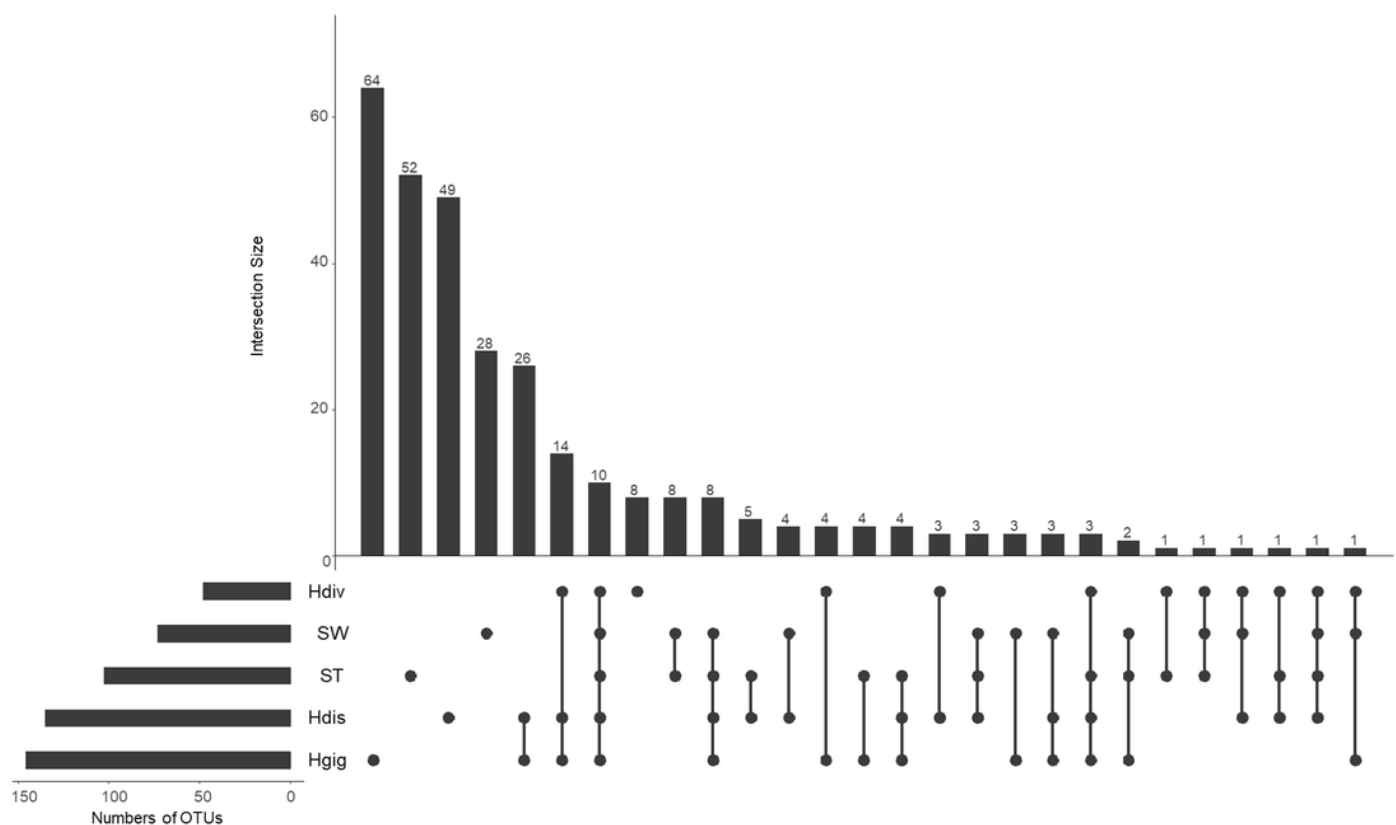
Figure 4

Phylogenetic tree showing the relationship between the uncultured epsilonproteobacterium and representative taxa of the Epsilonproteobacteria, based on a 1257 nucleotide sequence of the 16S rRNA gene.

The tree was inferred based on maximum likelihood. *Rhodobacter capsulatus* ATCC11166 in the Alphaproteobacteria was used as an outgroup. GenBank accession numbers are given in parentheses. Numbers at nodes are bootstrap values based on 1000 replicates. Bar: 0.02 nucleotide substitutions per site. Taxonomic classes of the order Campylobacterales are given on the right side of round brackets.
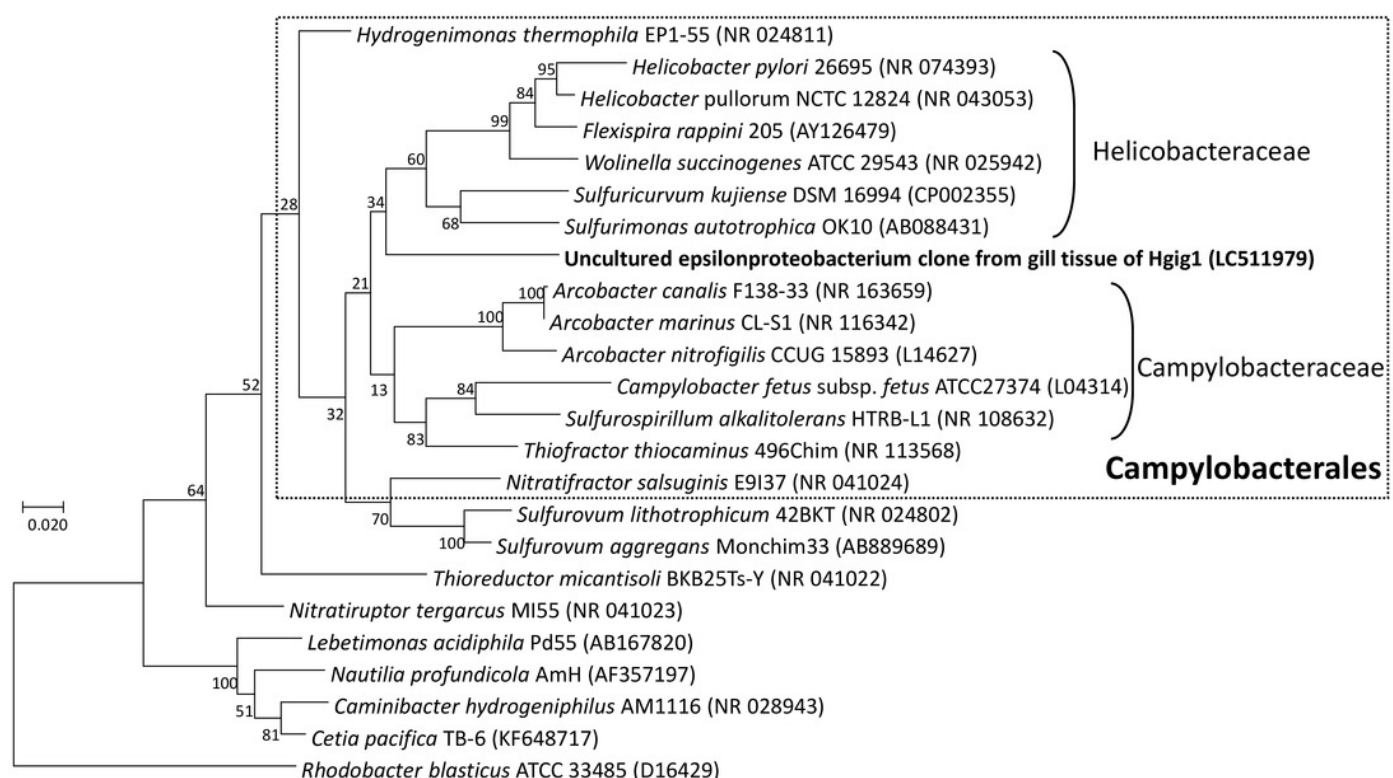
Figure 5

Micrographs of semi-thin gill sections of *H. gigantea*-4.

Micrographs showing the results of FISH analysis of gill sample by simultaneous application of probe TAMRA-Eps222 and FITC-Eub338. (A) ×40 micrographs; (B and C) ×1000 micrographs. White to magenta cells showed Eps222-positive cells. The yellow arrow indicates micro-colony (100–200 cells).