

1 **Assessment of Molecular Detecting of Anaerobic Ammonium Oxidizing**
2 **(Anammox) Bacteria in Different Environmental Samples Using PCR**
3 **Primers based on 16S rRNA and Functional Genes**

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26 **Abstract**

27 Eleven published PCR primer sets for detecting genes encoding 16S rRNA, hydrazine
28 oxidoreductase (HZO), cytochrome *cd₁*-containing nitrite reductase (NirS) and hydrazine
29 synthase subunit A (HzsA) of anammox bacteria were assessed for the diversity and
30 abundance of anammox bacteria in samples of three environments, waste water treatment
31 plant (WW), wetland of Mai Po Nature Reserve (MP), and the South China Sea (SCS).
32 Consistent phylogenetic results of three biomarkers (16S rRNA, *hzo*, and *hzsA*) of
33 anammox bacteria were obtained from all samples. WW had the lowest diversity with
34 *Candidatus* Kuenenia dominating while the SCS was dominated by *Ca. Scalindua*. MP
35 showed the highest diversity of anammox bacteria including *Ca. Scalindua*, *Ca. Kuenenia*
36 and *Ca. Brocadia*. Comparing different primer sets, no significant differences in specificity
37 for 16S rRNA gene could be distinguished. Primer set CL1 showed relatively high
38 efficiency in detecting anammox bacteria *hzo* gene from all samples, while CL2 showed
39 greater selectivity for WW samples. The recently reported primer sets of *hzsA* gene resulted
40 in high efficiencies in detecting anammox bacteria while *nirS* primer sets were more
41 selective for specific samples. Results collectively indicate that the distribution of
42 anammox bacteria is niche-specific within different ecosystems and primer specificity may
43 cause biases on the diversity detected.

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45 **Key words:** anammox bacteria; 16S rRNA; *hzo*; *nirS*; *hzsA*; diversity; nitrogen cycle

46 **Introduction**

47 The anaerobic ammonium-oxidizing (anammox) bacteria grow lithotrophically coupling the
48 energy derived from oxidation of ammonium to the reduction of nitrite into dinitrogen (N₂)
49 gas in the absence of oxygen. The existence of such microorganisms capable of ammonium
50 oxidation with nitrite as an electron acceptor was predicted based on thermodynamics
51 calculations in 1977 (Broda 1977). Almost 20 years later, the anammox process was first
52 described in bioreactors of wastewater treatment plants in 1995 (Mulder et al. 1995; van de
53 Graaf et al. 1995). As one of the latest additions to the biogeochemical nitrogen cycle,
54 anammox bacteria have been extensively studied. Five anammox genera have been described,
55 including the genera of *Brocadia* (Kartal et al. 2008; Oshiki et al. 2011; Park et al. 2017),
56 *Anammoxoglobus* (Kartal et al. 2007), *Kuenenia* (Schmid et al. 2000; Strous et al. 2006),
57 *Scalindua* (Kuypers et al. 2003; Schmid et al. 2003; Speth et al. 2017; van de Vossenberg et
58 al. 2008) and *Jettenia* (Ali et al. 2015; Quan et al. 2008). All anammox bacteria belong to the
59 same monophyletic branch named the *Brocadiaceae* and are related to the phylum
60 *Planctomycetes* and have been detected in various ecosystems including extreme
61 environments (Byrne et al. 2009; Jaeschke et al. 2009; Zhu et al. 2015), terrestrial ecosystems
62 (Zopfi et al. 2009), freshwater and marine ecosystems (Hirsch et al. 2011; Schmid et al.
63 2007), mangrove (Li et al. 2011c; Meyer et al. 2005; Wang et al. 2012c), oil field (Li et al.
64 2010a), and also in wastewater treatment systems (Araujo et al. 2011). PCR amplification of
65 anammox bacterial 16S rRNA and functional genes are commonly used for the detection and
66 identification (Chouari et al. 2003; Junier et al. 2010; Li and Gu 2011; Penton et al. 2006;
67 Quan et al. 2008; Schmid et al. 2005; Sonthiphand and Neufeld 2013; Wang et al. 2015).
68 Fluorescence *in situ* hybridization (FISH) and stable isotope labeling also serve as useful
69 methods to confirm the presence and activity of anammox bacteria in natural environments
70 (Schmid et al. 2007).

71 The approaches using the 16S rRNA gene as a phylogenetic biomarker with universal
72 bacterial PCR primer sets often show disadvantages due to the high sequence divergence of
73 anammox bacteria (Schmid et al. 2005). Thus, 16S rRNA gene targeting PCR primers with

74 high specificity for *Planctomycetes* or different genera of anammox bacteria have been
75 designed (Kuypers et al. 2003; Neef et al. 1998; Penton et al. 2006). As no general primer set
76 for anammox bacterial detection has been agreed upon (Jetten et al. 2009), a combination of
77 results obtained from different genera specific primer sets are often used to provide
78 information about anammox bacterial abundance, diversity and distribution in natural and
79 engineered ecosystems. Nevertheless, since the 16S rRNA gene as a molecular marker is not
80 necessarily related to the physiology of anammox bacteria, the use of functional gene markers
81 may provide good alternatives (Junier et al. 2010). Therefore, analysis of the functional genes
82 encoding for enzymes involved in specific anammox biochemical reactions could
83 significantly increase the detection efficiency and specificity (Kartal et al. 2011a). With the
84 hypothetical metabolic pathway proposed based on this genome sequence of “*Candidatus*
85 *Kuenenia stuttgartiensis*” (Strous et al. 2006) and later fully supportive experimental evidence
86 (Kartal et al. 2011b), it is reported that first, nitrite is reduced to nitric oxide by a cytochrome
87 *cd₁*-type nitric oxide/nitrite oxidoreductase (NirS), and then a hydrazine synthase (HZS)
88 produces hydrazine from nitric oxide and ammonium, and finally hydrazine is oxidized to
89 produce dinitrogen gas by a hydrazine oxidoreductase (HZO).

90 The first functional gene used for detecting anammox bacteria is HZO encoding *hzo* gene.
91 Several different primer sets (Schmid et al. 2008) have been reported to detect anammox
92 bacteria from different environmental samples including coastal and deep-ocean sediments
93 (Dang et al. 2010; Hirsch et al. 2011; Li et al. 2010b), mangrove sediments (Li et al. 2011c;
94 Wang et al. 2012c), rice paddy (Wang and Gu 2013), oilfields (Li et al. 2010a) or wastewater
95 treatment plants (Quan et al. 2008). Results from these studies imply that the *hzo* gene is a
96 reliable qualitative functional biomarker for the investigation of anammox bacteria in natural
97 environments (Li et al. 2010b).

98 The primer set ScnirS was used to detect the cytochrome *cd₁*-containing nitrite reductase
99 encoding gene (*nirS*) in anammox bacteria belonging to the *Scalindua* in Peruvian oxygen
100 minimum zone, surface and subsurface sediments in the South China Sea (Lam et al. 2009; Li
101 et al. 2013a; Li et al. 2013b). Another recently designed primer set, AnnirS, has also been

102 shown to successfully amplify *nirS* genes in other genera of anammox bacteria from coastal
103 to deep ocean samples (Li et al. 2011b). By combining information from these two primer
104 sets, the *nirS* gene can be considered as another functional biomarker for investigation of
105 anammox bacteria in natural environments (Li et al. 2011b). In addition to *hzo* and *nirS*, the
106 hydrazine synthase protein encoded by the *hzsCBA* gene cluster (kuste2859-kuste2861) has
107 been successfully purified (Kartal et al. 2011b) and is considered to be a very good biomarker
108 for anammox bacteria. PCR primer sets targeting the new unique functional *hzsA* were
109 developed and were successfully tested on engineered sample and on a variety of
110 environments indicating broad diversity (Borin et al. 2013; Harhangi et al. 2012). In addition,
111 primer sets for *hzsB* genes have also been developed and applied to Pearl River Estuary,
112 paddy soil, ocean sediments, coastal wetland sediments and wastewater treatment plant
113 (WWTP) samples (Wang et al. 2012a; Wang et al. 2012b; Zhou et al. 2017), indicating a
114 wide coverage for all known anammox genera.

115 Red granules of anammox bacteria were reported in a full-scale wastewater treatment
116 plant with simultaneous partial nitrification, anaerobic ammonium oxidation and
117 denitrification (SNAD) process, and anammox bacteria in the reactor were investigated with
118 16S rRNA gene marker (Wang et al. 2010). The South China Sea (SCS), as an oligotrophic
119 ecosystem and the largest semi-enclosed marginal sea basin of the West Pacific with an area
120 of about 3.5×10^6 km² (Chen et al. 2001; Ning et al. 2009), has been studied extensively to
121 examine the diversity and distribution of anammox bacteria in both surface and subsurface
122 sediments. A high micro-diversity of anammox bacteria with *Scalindua* dominating in SCS
123 has been demonstrated (Han and Gu 2013; Hong et al. 2011; Li et al. 2013a). The Mai Po
124 Nature Reserve of Hong Kong, the largest area of wetland in Hong Kong, consists of
125 intertidal mudflats, mangroves, traditionally operated shrimp ponds, fishponds, reed beds and
126 drainage channels in between Shenzhen River and Yuen Long River (Nelson 1993). Diversity
127 and distribution of anammox bacteria have been extensively studied spatially and seasonally
128 (Chen and Gu 2017; Li et al. 2011a; Li et al. 2011c; Wang et al. 2012c; Wang and Gu 2012;
129 Zhou et al. 2017).

130 In this study, eleven in total primer sets targeting anammox bacterial 16S rRNA (3 sets),
131 *hzo* (4 sets), *nirS* (2 sets) and *hzsA* (2 sets) genes were selected to study the diversity of
132 anammox bacteria in samples of three environments from a wastewater treatment plant (WW),
133 the South China Sea (SCS) and the Mai Po Nature Reserve (MP). The objectives were to 1)
134 compare the diversity and abundance of anammox bacteria by 16S rRNA and functional
135 biomarkers from these three different environments, and 2) examine the efficiencies and
136 specificities of phylogenetic and functional markers in recovering the diversity and
137 abundance of anammox bacteria, thus providing useful information for primer selection in
138 analysing anammox bacterial community structure in future studies. This is the first time that
139 16S rRNA gene and core functional gene markers have been applied in one single study for
140 samples from three distinct environments.

141 **Materials and Methods**

142 **Sampling and chemical analysis**

143 Samples from three environments were selected for the current study: 1) Red granules
144 from a long-term, simultaneous partial nitrification, anammox and denitrification (SNAD)
145 wastewater treatment system (WW) in Taiwan (Kumar and Lin 2010; Lin et al. 2011; Ni et al.
146 2011; Wang et al. 2010). Red granules were collected from the influent of the wastewater
147 treatment system in Apr. 2011. Samples were taken in triplicate and stored at -20 °C in a
148 refrigerator after collection, 2). Mai Po Nature Reserve wetland sediment (MP) (N 22°29, E
149 114°01), an estuarine coastal wetland influenced by the discharge of the Pearl River, was
150 selected based on the reported information of ammonia-oxidizing microorganisms (Li et al.
151 2011a; Li et al. 2011c). The Mai Po sediment samples were collected in June 2011 and kept
152 in an ice-cooled container for transportation back to the laboratory within 2 hours for further
153 chemical analysis and molecular processing, and 3). Deep-sea surface sediments from the
154 South China Sea (SCS), a pristine marine ecosystem, were also chosen with the samples
155 collected from a depth of 2350 meters at 19°38' N, 115°31'E (Li et al. 2011a). Surface
156 sediments (0-1 cm) were taken in triplicate and stored at -80 °C in a refrigerator after
157 collection on board during the South China Sea Open Cruise by R/V Shiyun 3 in 2008.

158 Concentration of ammonia, nitrite, and nitrate in pore water of red granule and sediment
159 samples were analyzed using the standard flow injection analysis (FIA) technique with
160 QuickChem (Milwaukee, Wisconsin, USA) based on the protocol by Ariza (Ariza et al.
161 1992).

162 **DNA extraction and PCR amplification**

163 Thawed and homogenized granules or sediments (approximately 0.5 g, wet weight) were
164 transferred into sterile 1.5 mL centrifuge tubes. The total genomic DNAs were extracted
165 using the Power Soil Isolation Kit (Mo Bio, Carsbad, California) according to the
166 manufacturer's instructions. All extracted DNAs were separately stored at -20°C for further
167 analysis.

168 Primer sets used in this study and the PCR annealing temperatures are listed in Tab. 1. In
169 a final volume of 25 μ l, a PCR reaction mixture contained: 1 μ L of extracted template DNA
170 (1-10 ng), 5 μ L of 5 \times GoTaq Flexi buffer (Promega, Hong Kong) and 2.5 μ L of MgCl₂ (25
171 mM, Promega), 2 μ L of dNTPs (2.5 mM, Invitrogen, Hong Kong), 0.5 μ L of each forward
172 and reverse primer (20 μ M) and 0.2 μ L of GoTaq Flexi polymerase (5 U ml⁻¹, Promega,
173 Hong Kong). PCR products were analyzed by agarose gel electrophoresis in 1% agarose gels
174 in TAE (20 mM Tris-acetate pH 8.0; 0.5 mM EDTA) at 130 V, 400 mA for 20 min
175 (Amersham Biosciences, Electrophoresis Power Supply 301). The gels were stained by
176 addition of GelRed nucleic acid stain (Biotium) and photographed by using a Bio-Rad[®]
177 GelDoc[™] station.

178 **Cloning, sequencing and phylogenetic analysis**

179 Clone libraries were constructed from the PCR products according to the methods
180 described by Friedrich (Friedrich et al. 2001). Briefly, the amplified PCR products were
181 analyzed by gel electrophoresis as described above, and then purified using a Qiaex II Gel
182 Extraction Kit (QIAGEN, Hilden, Germany). Ligation with a pMD[®] 18-T vector (Takara,
183 Japan) was performed at 16°C for 10 hours in a 8 μ L reaction volume consisting of 4 μ L of
184 Solution 1 (containing T4 ligase and buffer), 3.5 μ L of purified PCR products and 0.5 μ L of

185 pMD[®] 18-T vector. Transformation of the vectors to competent cells of *E. coli* was
186 performed (Chung et al. 1989) afterwards. The cloned single colonies were then randomly
187 picked for PCR amplification using the primer sets M13 (M13F:
188 5'-TGTAACGACGGCCAGT-3'; M13R: 5'-CAGGAAACAGCTATGACC-3') in a 25
189 µL PCR reaction volume consisting of the same components as described above. The PCR
190 program consisted of 95°C of initial denaturation for 5 min, 35 cycles of 95°C for 30 s, 55°C
191 for 30 s, 72°C for 80 s, followed by 10 min of final elongation at 72°C. Positive PCR
192 products were sent for DNA sequencing performed with the Big Dye Terminator Kit
193 (Applied Sciences, Foster City, CA) on an ABI Prism 3730 DNA analyzer.

194 **Construction of phylogenetic trees**

195 Clone sequences were checked on their sequence similarity with known references using
196 the NCBI Blast nucleotide database. 16S rRNA gene sequences were inspected before
197 alignment using the Black Box Chimera Check tool (Gontcharova et al. 2010) for the
198 presence of chimerical sequences. Sequences were aligned and phylogenetic trees were
199 constructed using MEGA version 5.0 (Tamura et al. 2011) and subjected to phylogenetic
200 inference using the neighbor-joining algorithm followed by 1000 times of bootstrap.

201 **Quantitative Analysis**

202 The abundance of anammox bacteria in the three samples was estimated by the presence
203 of the anammox bacterial 16S rRNA, *hzo* and *hzsA* genes with the primer sets Brod, CL1 and
204 HZSS respectively. Quantitative polymerase chain reaction (q-PCR) were performed to
205 qualify the copy numbers of 16S rRNA, *hzo* and *hzsA* genes for anammox bacteria in
206 triplicate using the Applied Biosystems StepOnePlus[™] Real-Time PCR System. The
207 quantification was based on the fluorescent dye SYBR-Green I. Each reaction was performed
208 in a 20-µL volume containing 10 µL of Power SYBR Green PCR Master Mix (Applied
209 BioSystems), 1 µL of DNA template (1-10 ng), 0.2 µL of each primer (20 µM), and 8.8 µL of
210 autoclaved DD water. The PCR profile for the 16S rRNA and *hzsA* gene was started with 10
211 min at 95 °C, followed by a total of 40 cycles of 50 s at 95 °C, 50 s at 54 °C, and 50 s at

212 72 °C. The PCR cycle for the *hzo* gene was started 10 min at 95 °C, followed by a total of 48
213 cycles of 50 s at 95 °C, 1 min at 48 °C, and 1 min at 72 °C. Standard plasmids carrying the
214 targeted gene fragments were generated by amplifying from sample DNA templates of MP
215 and cloning into pMD 18-T Vector. The plasmid DNA concentration was determined, and the
216 copy numbers of the target genes were calculated. Tenfold serial dilutions of a known copy
217 number of the plasmid DNA were subjected to a Q-PCR assay in triplicate to generate an
218 external standard curve. The correlation coefficients (r^2) were all greater than 0.99 for the
219 three targeted genes.

220 **Statistical analysis**

221 All sequences associated with anammox or putative anammox bacterial gene sequences
222 were analyzed using the DOTUR (Distance-Based OTU and Richness) program to compare
223 their diversity and richness (Schloss and Handelsman 2005). Operational taxonomic units
224 (OTUs) were defined at 3% variation in nucleic acid sequences for 16S rRNA genes and 5%
225 for protein sequences. In order to examine the relationship between the distribution of these
226 various genes among samples, all clone libraries of each target gene, namely 16S rRNA, *hzo*,
227 *nirS* and *hzsA*, were analyzed with UniFrac (<http://bmf.colorado.edu/unifrac/>) using the
228 principal coordinates analysis tool (PCoA) (Lozupone and Knight 2005). Correlations of the
229 anammox clusters with environmental factors were also explored with canonical
230 correspondence analysis (CCA) using the software Canoco (version 4.5; Microcomputer
231 Power).

232 **Nucleotide sequence accession numbers**

233 Partial sequences of 16S rRNA, *hzo*, *nirS* and *hzsA* genes from WW, MP and SCS have
234 been deposited to GenBank under accession numbers JQ822788-JQ823004 (WW),
235 JQ822258-JQ822427 (MP) and JQ822536-JQ822787 (SCS), respectively.

236 **Results**

237 **Characteristics of the samples**

238 The physiochemical characteristics of the three types of samples from WW, MP, and
239 SCS are shown in Tab. S1. There was no significant pH difference among samples. WW
240 showed the highest concentration of NH_4^+ , but is relatively lower in concentrations of NO_3^-
241 and NO_2^- . MP showed a much lower concentration of NH_4^+ than WW, but higher
242 concentrations of NO_3^- and NO_2^- . SCS shows the lowest concentrations of NH_4^+ , NO_2^- and
243 NO_3^- among all samples.

244 **PCR amplification and sequencing**

245 All primer sets with few exceptions resulted in positive amplification products from all
246 three samples (Tab. 1 and S2). These exceptions included primer set CL2 worked only for
247 sample WW., and two primer sets for *nirS* gene, ScnirS and AnnirS, worked with SCS and
248 MP respectively. All three 16S rRNA gene primer sets demonstrated high efficiency (> 80%)
249 in detecting anammox bacteria in all samples, except for MP-Brod (64.3%). Three *hzo* gene
250 based primer sets (CL1, H4F and Ana) showed high efficiency (> 80%) in detecting
251 anammox bacteria in all samples except for MP-CL1 (78.9%) and MP-Ana (44.4%). Primer
252 CL2 only worked with sample WW with 100% efficiency. The two *nirS* gene based primer
253 sets showed selectivity for sample SCS (ScnirS, 93.3% efficiency) and MP (AnnirS, 43.5%
254 efficiency). For sample WW, both of *nirS* primer sets showed 100% efficiency. Both *hzsA*
255 primer sets worked with all samples while primer set HZSL showed relatively lower
256 efficiency at 33.0% for MP and 35.0% for the SCS.

257 **Diversity and community structure of anammox bacteria by 16S rRNA**

258 Retrieved 16S rRNA gene sequences confirmed to be affiliated with the anammox
259 bacteria were aligned with known reference sequences for the phylogenetic tree construction
260 (Fig. 1a). Three major groups are visualized: i) the *Scalindua* group, ii) a combined group
261 including *Kuenenia*, *Brocadia*, *Jettenia* and *Anammoxoglobus*, and iii) a putative anammox
262 bacteria group by MP sequences. The *Scalindua* group included all SCS and 87.6% MP
263 (71/87) sequences which share at least 96% sequence similarity with the known *Scalindua*
264 species. Sequences within the *Scalindua* group were also subdivided into six clusters (brodae,

265 arabica, wagneri, zhenghei- I , zhenghei- II and zhenghei-III) as described previously (Hong
266 et al. 2011). Most (93.5%, 86/92) SCS sequences (with primer sets AMX, BS and Brod) were
267 clustered to the zhenghei- I cluster. The brodae cluster contained the remainder (6.5%, 6/92)
268 of the SCS sequences (with primer sets AMX, BS and Brod) and 2.3% (2/87) of the MP
269 sequences (with primer sets AMX and BS). Sequences from MP contributed exclusively to
270 the other three clusters, there are 31.0% (27/87) MP sequences (with primer sets AMX, BS
271 and Brod) included in zhenghei- II , 2.3% (2/87) (with primer set BS) in zhenghei- II and
272 46.0% (40/87) (with primer sets AMX, BS and Brod) in the wagneri cluster. No sequences
273 obtained in this study affiliated with the arabica cluster. Only sequences from MP and WW
274 contributed to the second group. The *Kuenenia* cluster contained 9.2% (8/87) MP sequences
275 (with primer sets AMX and BS) and 85.7% (54/63) WW (with primer sets AMX, BS and
276 Brod) sequences. The rest, 14.3% (9/63) WW sequences (with primer sets AMX, BS and
277 Brod) contributed to the *Brocadia* cluster. Finally, eight sequences (with primer set AMX)
278 from the MP grouped to a separated cluster (a new cluster).

279 All retrieved 16S rRNA gene sequences were analyzed with UniFrac principal
280 coordinates analysis (PCoA) (Fig. S1a). The phylogenetic differences within the different
281 clone libraries led to a separation into different single groups, with each accounting for a
282 different sample type, WW, MP, and SCS. For SCS and MP clusters, the Brod libraries are
283 relatively far from the other two (AMX and BS), indicating apparent differences of primer
284 specificity for anammox bacteria detection in SCS and MP ecosystems.

285 **Diversity and community structure of anammox bacteria by *hzo* gene**

286 Deduced amino acid sequences obtained from all *hzo* gene clone libraries were aligned
287 with known reference protein sequences of anammox bacteria to build a phylogenetic tree, in
288 which three separated groups were indicated (Fig. 1b). Five (5.4%, 5/93) SCS sequences
289 (with primer set CL1) form their own cluster, which shared only 77% to 81% similarity with
290 sequences retrieved from an anammox reactor (Li et al. 2009), and thus suggesting a new
291 phylogenetic group. Cluster 1a consists of the rest (94.6%, 88/93) of the SCS sequences (with
292 primer sets CL1, H4F and Ana) and half (12/24) of the MP sequences (with primer sets CL1,

293 H4F and Ana). Cluster 1b was contributed to by 33.3% (8/24) of the MP sequences (with
294 primer set CL1). Cluster 1a and cluster 1b were assembled as the *Scalindua* group. In the
295 second group, cluster 2a contained 16.7% (4/24) MP sequences (with primer sets H4F and
296 Ana). Cluster 2b was contributed to by 5.7% (4/70) of the WW sequences (with primer sets
297 CL1), cluster 2c was formed by 80% (56/70) WW sequences (with primer sets CL1, H4F and
298 Ana) and cluster 3 by 14.3% (10/70) WW sequences (with primer set CL2). Cluster 3 is an
299 independent cluster, consisting of 10 WW sequences sharing 99% of the amino acid sequence
300 similarity with *Kuenenia stuttgartiensis*. In addition, cluster 3 is relatively distant from the
301 above clusters, indicating an apparent specificity in the *Kuenenia*.

302 The phylogenetic differences among clone libraries formed three clusters for the three
303 sample types (Fig. S1b). Within each of the clusters, clone libraries constructed with the ANA
304 and the H4F are relatively closer to one another than to CL1 libraries. The WW-CL2 is
305 located far away from all other three libraries, indicating a greater diversity of HZO proteins.

306 **Diversity and community structure of anammox bacteria by *nirS* gene**

307 Deduced amino acid sequences obtained were aligned with known reference sequences
308 of *nirS* genes of both anammox and denitrifying bacteria to build a phylogenetic tree (Fig. 1c).
309 Five clusters form anammox's *nirS* group which is separated from the denitrifier's *nirS* group.
310 Sequences obtained from each of the samples formed a different cluster: cluster 1b (SCS with
311 primer set ScnirS), cluster 1c (MP with primer set AnnirS) and cluster 2 (WW with primer
312 sets ScnirS and AnnirS). In addition, more than half (56.5%, 13/23) of the MP sequences
313 (with primer set AnnirS) were affiliated with denitrifiers. Retrieved *nirS* gene sequences were
314 analyzed with PCoA (Fig. S1c), resulting in separated libraries in terms of sample type,
315 consistent with the phylogenetic analysis.

316 **Diversity and community structure of anammox bacteria by *hzsA* gene**

317 Deduced amino acid sequences of all six *hzsA* gene clone libraries were aligned with the
318 known reference sequences of anammox bacteria to build two phylogenetic trees (Figs. 1d
319 and 1e). Since products amplified with primer HZSL and HZSS were not from the same

320 region of the *hzsA* gene, two trees were constructed separately. Sequences obtained with
321 primer HZSL and reported sequences could be divided into 5 clusters within two large groups.
322 For sequences obtained with primer HZSS, SCS sequences contributed to cluster 1a (11.8%,
323 2/17), cluster 1b (64.7%, 11/17) and cluster 1c (23.6%, 4/17). MP sequences contributed to
324 cluster 1c (33.3%, 5/15), cluster 1d (20%, 3/15) and cluster 2a (46.7%, 7/15). All WW
325 sequences were included in cluster 2a and were closely related to hydrazine synthase subunit
326 A from *Ca. Kuenenia stuttgartiensis*. Two independent PCoA analyses show similar
327 community structures for anammox bacteria except MP, which is more diverse, and distant,
328 from *Scalindua* in the HZSS clone library (Fig. S1e).

329 **Abundance of Anammox Bacteria**

330 The abundance of anammox bacteria was estimated by the copy numbers of the 16S
331 rRNA, *hzo* and *hzsA* genes (Tab. 2). Results showed that 16S rRNA gene abundance was
332 1.69×10^6 to 2.51×10^6 copies per gram of dry sediment and the *hzo* gene and *hzsA* gene were
333 1.21×10^5 to 1.11×10^6 and 1.67×10^5 to 1.26×10^6 copies per gram of dry sediment, respectively.
334 In addition, the ratio of copies of 16S rRNA gene to *hzo* gene ranged from 2.26 (WW) to 13.9
335 (MP), the ratio of copies of 16S rRNA gene to *hzsA* gene ranged from 1.60 (SCS) to 10.12
336 (MP).

337 **The relationship of environmental factors and anammox communities**

338 Correlations of the anammox diversity based on 16S rRNA phylogenetic analysis with
339 environmental parameters were analyzed via CCA (Fig. 2). The environmental variables in
340 the first two CCA dimensions (CCA1 and CCA2) explained 97.4% of the total variance in the
341 anammox species composition and 82.3% of the cumulative variance of the anammox
342 environment relationship. Correlations of the anammox bacterial assemblages with
343 environmental parameters analyzed by CCA indicated that ammonia has a strong relationship
344 with the amount of *Kuenenia* and *Brocadia* in certain environmental samples, that means
345 much of the distribution of *Kuenenia* and *Brocadia* in some samples can be explained by
346 ammonia concentration. In addition, the concentration of nitrite and nitrate could also
347 contribute to the distribution of clusters zhenghei- I , zhenghei-II and wagneri, the nitrite to

348 nitrite plus nitrate ratio significantly affected brodae and zhenghei-III clusters.

349 **Discussion**

350 A closer look at the samples reveals that WW was by far the easiest sample to amplify
351 anammox bacterial sequences with all primer sets. Based on the phylogenetic analysis with
352 16S rRNA, *hzo* and *hzsA* gene clone libraries, WW was dominated by *Kuenenia* while
353 *Brocadia* was also detected, representing agreement with previous studies (Wang et al. 2010).
354 Mai Po showed the highest diversity of anammox bacteria among all three samples. All
355 primer sets, except for CL2 and ScnrS, were able to amplify anammox bacterial gene
356 sequences, showing several different clusters within the *Scalindua*, *Kuenenia*, and *Brocadia*,
357 as well as some sequences of putative anammox bacteria, representing agreement with
358 previous studies (Li et al. 2011a). The SCS showed micro-diversity with two clusters
359 (zhenghei- I and Brodae), all belonging to *Scalindua* based on phylogenetic analyses of the
360 16S rRNA gene sequences obtained. The SCS-CL1 new cluster based on the phylogenetic
361 analyses of HZO proteins also indicated the possibility of hidden diversity of anammox
362 bacteria in the marine environment. Most sequences obtained with all primer pairs used
363 showed a similarity of 97-100% to *Scalindua* type anammox bacteria from previous studies
364 of marine environment (Hong et al. 2011; Woebken et al. 2008).

365 The simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification
366 (SNAD) process has been frequently applied in nitrogen removal and in bioreactors (Chen et
367 al. 2009; Daverey et al. 2012; Lin et al. 2011; Wang et al. 2010). Previous studies have
368 revealed the existence and dominance of anammox bacteria in SNAD bioreactors (Lin et al.
369 2011; Wang et al. 2010). Phylogenetic analysis based on 16S rRNA and functional genes in
370 this study support this finding. However, it should be noted that many questions remain on
371 how the conditions and processes shape the anammox bacterial community structure, and
372 how the interactions among anammox bacteria, nitrifiers and denitrifier's regulate removal of
373 organic carbon and nitrogen waste.

374 The diversity and community structure of anammox bacteria have been studied

375 seasonally (Li et al. 2011a; Wang et al. 2012c), spatially (Li et al. 2010b) and also on a basis
376 of soil types (Wang et al. 2012c) from the mangrove sediments and mudflats in the area of
377 the Mai Po marsh. Since the Mai Po marsh is a typical wetland with a mixture of freshwater,
378 soil, seawater, wetlands and mangrove trees, it is not unexpected that a higher diversity of
379 anammox bacteria was retrieved with almost all described genera. In contrast, the dominance
380 of *Scalindua* in the South China Sea samples may be a function of substrate limitation
381 contributing to the competitive fitness of this bacterium in the marine realm. Recent work
382 based on metagenomic studies suggests that *Scalindua* may be well adapted to the marine
383 environments (van de Vossenberg et al. 2013), but further studies are clearly warranted.
384 Recent findings concerning the micro-diversity and niche-specific distribution of anammox
385 bacteria in the South China Sea (Han and Gu 2013; Hong et al. 2011) may inspire the need of
386 more detailed physiological studies.

387 The consistency of phylogenetic analysis of 16S rRNA, *hzo* and *hzsA* genes from all
388 three samples suggests that these three biomarkers could be successfully applied in detecting
389 anammox bacteria (Fig. 1). Nevertheless, functional gene markers may reflect more
390 physiological information: 1) distinctive WW-CL2 sequences indicate *Kuenenia*-specific
391 HZO proteins; 2) the SCS-CL1 new cluster may indicate higher diversity of
392 *Scalindua*-specific HZO proteins; 3) the large differences in the ratios of 16S rRNA gene
393 copies to functional genes copies among the three samples suggest even more complicated
394 metabolic mechanisms involved in anaerobic ammonia oxidation. To date, genomic
395 information is available for *Kuenenia* (Speth et al. 2012; Strous et al. 2006), *Jettenia* (Hira et
396 al. 2012; Hu et al. 2012), *Scalindua* (Speth et al. 2017; van de Vossenberg et al. 2013) and
397 *Brocadia* (Park et al. 2017) and the core metabolic mechanisms have been described.
398 However, the presence of eight highly divergent *hzo* genes in the genome of *Ca. Kuenenia*
399 *stuttgartiensis* (Strous et al. 2006), and the identification of copper-containing nitrite
400 reductase NirK in *Ca. Jettenia asiatica* (Hu et al. 2012) and organism KSU1 (Hira et al. 2012)
401 instead of cytochrome *cd₁*-type nitrite reductase NirS identified in *Ca. Kuenenia*
402 *stuttgartiensis* and *Ca. Scalindua profunda*, indicate variable mechanisms involved in

403 metabolism of anammox bacteria in terms of nitrite reduction to NO, and further complicate
404 interpretation of the retrieved sequence data.

405 All three 16S rRNA gene based primer sets showed high efficiency in recovering
406 anammox bacterial amplicons with high specificity (Tab. S2). In contrast to former studies
407 using these primer sets (Kuypers et al. 2003; Penton et al. 2006; Schmid et al. 2000), no
408 significant differences in specificity on the genus level. PCoA suggested that AMX and BS
409 clone libraries of each sample, and Brod except in the MP sample, are clustered very closely
410 to each other. The formerly reported *Scalindua* specific Brod541F (Penton et al. 2006) was
411 combined with the *Brocadia* and *Kuenenia* specific Amx820R primer (Schmid et al. 2000)
412 and described as the Brod primer pair in this study. This combination could also amplify most
413 of the different genera of known anammox bacteria, but the *Kuenenia* type clade detected
414 with the other two 16S rRNA gene primer sets from MP could not be recovered with the
415 Brod primer pair. In general, primer set BS showed the best coverage in detecting all
416 recovered clusters (Fig.1a).

417 All three *hzo* gene based primer sets showed their ability in detecting taxa closely related
418 to anammox bacteria from the three samples used in this study (Fig. 1b). There are also
419 primer-specific clusters obtained, namely the new SCS cluster, MP cluster 1b and WW
420 cluster 2b with primer set CL1, and WW cluster 3 with primer set CL2. In addition, cluster 2a
421 is a CL1 and CL2 exclusive cluster with only sequences obtained by primer set Ana and H4F.
422 In particular, the new cluster of marine anammox bacterial *hzo* genes (SCS new cluster)
423 shares only up to 81% sequence identity to the known anammox organisms (Dalsgaard et al.
424 2003; Kuypers et al. 2003; Ward et al. 2009), indicating hidden diversity of anammox
425 bacteria in marine ecosystems and the relatively higher efficiency of primer set CL1. The
426 CL2 primer set amplified solely a specific sequence of the open reading frame *kustc1061*
427 encoding one of the *hzo* genes of *K. stuttgartiensis* from the WW. In general, primer CL1 has
428 a higher efficiency in recovering most of the anammox genera from different samples,
429 indicating similar patterns found by 16S rRNA gene diversity analyses. CL2 only worked
430 with sample WW, indicating the existence of the *hzo* gene in the *K. stuttgartiensis* genome is

431 more diverse and needs further study.

432 The specificity of the ScnirS primer set for *Scalindua* (Lam and Kuypers 2011) has also
433 been observed in this study, as demonstrated by SCS sequences which all fall into a
434 *Scalindua* cluster 1b (Fig..1c). However, MP sequences determined with the AnnirS primer
435 set did contribute to another *Scalindua* cluster 1c. Cluster 2 contained exclusively sequences
436 from sample WW, which was dominated by *Kuenenia*. Compared to ScnirS, the AnnirS
437 primer set performed better in detecting anammox bacteria from sample MP, while a large
438 portion (56.5%, 13/23, Tab. S2) of denitrifier *nirS* sequence was also obtained due to the
439 lower specificity. In general, both of the *nirS* primer sets showed their selectivity, however,
440 there is a need to improve the efficiency and specificity of both ScnirS and AnnirS in
441 detecting anammox bacteria.

442 Two sets of primers for *hzsA* gene amplification share the same reverse primer, while the
443 length of the HZSL product is much longer than HZSS (Tab. 1). Both of the primer sets have
444 been used recently (Harhangi et al. 2012) and were compared in this study. Based on the
445 efficiency results and phylogenetic analyses, HZSS was more effective in detecting anammox
446 bacteria than HZSL. Even though the length of PCR products with HZSS is much shorter
447 than that with HZSL, sequences obtained with HZSS contained sufficient meaningful
448 phylogenetic information. However, neither of the *hzsA* primer sets recovered genes affiliated
449 with *Brocadia* from the WW and MP samples, as shown in phylogenetic analyses based on
450 16S rRNA gene sequences and HZO protein sequences, indicating the limitation of this
451 functional gene marker.

452 In conclusion, diversity and community structure of anammox bacteria were studied with
453 16S rRNA and three functional gene markers from samples of three environments. Results
454 showed agreement with previous studies while new functional gene clusters indicated the
455 diverse metabolic mechanisms among different genera of anammox bacteria. Primers were
456 also assessed and most are of high efficiencies and specificities in detecting anammox
457 bacteria while some showed selectivity and lower specificities. In general, 16S rRNA gene

458 targeting primer set BS, *hzo* gene targeting primer set CL1 and *hzsA* gene targeting primer set
459 HZSS are highly recommended.

460

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466

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473

474 **References**

- 475 Ali M, Oshiki M, Awata T, Isobe K, Kimura Z, Yoshikawa H, Hira D, Kindaichi T, Satoh H, Fujii T, Okabe S (2015)
476 Physiological characterization of anaerobic ammonium oxidizing bacterium '*Candidatus Jettenia caeni*'. Environ
477 Microbiol 17(6):2172-2189 doi:10.1111/1462-2920.12674
- 478 Araujo JC, Campos AC, Correa MM, Silva EC, Matte MH, Matte GR, Von Sperling M, Chernicharo CA (2011)
479 Anammox bacteria enrichment and characterization from municipal activated sludge. Water Sci Technol
480 64(7):1428-1434 doi:10.2166/wst.2011.632
- 481 Ariza AC, Linares P, de Castro MD, Valcarcel M (1992) Flow-injection analysis for on-line monitoring of nutrients
482 (ammonia and nitrite) in aquaculture. J Automat Chem 14(5):181-183 doi:10.1155/S1463924692000348
- 483 Borin S, Mapelli F, Rolli E, Song B, Tobias C, Schmid MC, De Lange GJ, Reichart GJ, Schouten S, Jetten M, Daffonchio
484 D (2013) Anammox bacterial populations in deep marine hypersaline gradient systems. Extremophiles
485 17(2):289-299 doi:10.1007/s00792-013-0516-x
- 486 Broda E (1977) Two kinds of lithotrophs missing in nature. Z Allg Mikrobiol 17(6):491-493
- 487 Byrne N, Strous M, Crepeau V, Kartal B, Birrien JL, Schmid M, Lesongeur F, Schouten S, Jaeschke A, Jetten M, Prieur D,
488 Godfroy A (2009) Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal
489 vents. ISME J 3(1):117-123 doi:10.1038/ismej.2008.72
- 490 Chen CTA, Wang SL, Wang BJ, Pai SC (2001) Nutrient budgets for the South China Sea basin. Mar Chem 75(4):281-300
- 491 Chen H, Liu S, Yang F, Xue Y, Wang T (2009) The development of simultaneous partial nitrification, ANAMMOX and
492 denitrification (SNAD) process in a single reactor for nitrogen removal. Bioresour Technol 100(4):1548-1554
493 doi:S0960-8524(08)00766-9
- 494 Chen J, Gu J-D (2017) Faunal Burrows Alter the Diversity, Abundance, and Structure of AOA, AOB, Anammox and
495 n-Damo Communities in Coastal Mangrove Sediments. Microb Ecol 74(1):140-156 doi:
496 10.1007/s00248-017-0939-5.
- 497 Chouari R, Le Paslier D, Daegelen P, Ginestet P, Weissenbach J, Sghir A (2003) Molecular evidence for novel
498 planctomycete diversity in a municipal wastewater treatment plant. Appl Environ Microbiol 69(12):7354-7363
- 499 Chung CT, Niemela SL, Miller RH (1989) One-step preparation of competent *Escherichia coli*: transformation and
500 storage of bacterial cells in the same solution. Proc Natl Acad Sci U S A 86(7):2172-2175
- 501 Dalsgaard T, Canfield DE, Petersen J, Thamdrup B, Acuna-Gonzalez J (2003) N₂ production by the anammox reaction in
502 the anoxic water column of Golfo Dulce, Costa Rica. Nature 422(6932):606-608 doi:10.1038/nature01526
- 503 Dang H, Li J, Chen R, Wang L, Guo L, Zhang Z, Klotz MG (2010) Diversity, abundance, and spatial distribution of
504 sediment ammonia-oxidizing Betaproteobacteria in response to environmental gradients and coastal
505 eutrophication in Jiaozhou Bay, China. Appl Environ Microbiol 76(14):4691-4702 doi:AEM.02563-09
- 506 Daverey A, Su SH, Huang YT, Lin JG (2012) Nitrogen removal from opto-electronic wastewater using the simultaneous
507 partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) process in sequencing batch
508 reactor. Bioresour Technol 113:225-231 doi:10.1016/j.biortech.2011.12.004
- 509 Friedrich A, George RL, Bridges CC, Prasad PD, Ganapathy V (2001) Transport of choline and its relationship to the
510 expression of the organic cation transporters in a rat brain microvessel endothelial cell line (RBE4). Biochim
511 Biophys Acta 1512(2):299-307 doi:S0005273601003339 [pii]
- 512 Gontcharova V, Youn E, Wolcott RD, Hollister EB, Gentry TJ, Dowd SE (2010) Black Box Chimera Check (B2C2): a
513 Windows-Based Software for Batch Depletion of Chimeras from Bacterial 16S rRNA Gene Datasets. Open
514 Microbiol J 4:47-52 doi:10.2174/1874285801004010047
- 515 Han P, Gu J-D (2013) More refined diversity of anammox bacteria recovered and distribution in different ecosystems.

516 Appl Microbiol Biotechnol 97(8):3653-3663 doi:10.1007/s00253-013-4756-6

517 Harhangi HR, Le Roy M, van Alen T, Hu BL, Groen J, Kartal B, Tringe SG, Quan ZX, Jetten MS, Op den Camp HJ
518 (2012) Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of
519 anammox bacteria. Appl Environ Microbiol 78(3):752-8 doi:AEM.07113-11

520 Hira D, Toh H, Migita CT, Okubo H, Nishiyama T, Hattori M, Furukawa K, Fujii T (2012) Anammox organism KSU-1
521 expresses a NirK-type copper-containing nitrite reductase instead of a NirS-type with cytochrome *cd₁*. FEBS
522 Lett 586(11):1658-1663 doi:S0014-5793(12)00330-4

523 Hirsch MD, Long ZT, Song B (2011) Anammox bacterial diversity in various aquatic ecosystems based on the detection
524 of hydrazine oxidase genes (*hzxA/hzoB*). Microb Ecol 61(2):264-276 doi:10.1007/s00248-010-9743-1

525 Hong YG, Li M, Cao H, Gu J-D (2011) Residence of habitat-specific anammox bacteria in the deep-sea subsurface
526 sediments of the South China Sea: analyses of marker gene abundance with physical chemical parameters.
527 Microb Ecol 62(1):36-47 doi:10.1007/s00248-011-9849-0

528 Hu Z, Speth DR, Francoijs KJ, Quan ZX, Jetten MS (2012) Metagenome Analysis of a Complex Community Reveals the
529 Metabolic Blueprint of Anammox Bacterium "*Candidatus* Jettenia asiatica". Front Microbiol 3:366
530 doi:10.3389/fmicb.2012.00366

531 Jaeschke A, Op den Camp HJ, Harhangi H, Klimiuk A, Hopmans EC, Jetten MS, Schouten S, Sinninghe Damste JS (2009)
532 16S rRNA gene and lipid biomarker evidence for anaerobic ammonium-oxidizing bacteria (anammox) in
533 California and Nevada hot springs. FEMS Microbiol Ecol 67(3):343-350
534 doi:10.1111/j.1574-6941.2008.00640.x

535 Jetten MS, Niftrik LV, Strous M, Kartal B, Keltjens JT, Op den Camp HJ (2009) Biochemistry and molecular biology of
536 anammox bacteria. Crit Rev Biochem Mol Biol:1-20 doi:909087210

537 Junier P, Molina V, Dorador C, Hadas O, Kim OS, Junier T, Witzel JP, Imhoff JF (2010) Phylogenetic and functional
538 marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. Appl Microbiol
539 Biotechnol 85(3):425-440 doi:10.1007/s00253-009-2228-9

540 Kartal B, Geerts W, Jetten MS (2011a) Cultivation, detection, and ecophysiology of anaerobic ammonium-oxidizing
541 bacteria. Methods Enzymol 486:89-108 doi:B978-0-12-381294-0.00004-3

542 Kartal B, Maalcke WJ, de Almeida NM, Cirpus I, Gloerich J, Geerts W, Op den Camp HJ, Harhangi HR, Janssen-Megens
543 EM, Francoijs KJ, Stunnenberg HG, Keltjens JT, Jetten MS, Strous M (2011b) Molecular mechanism of
544 anaerobic ammonium oxidation. Nature 479(7371):127-130 doi:10.1038/nature10453

545 Kartal B, Rattray J, van Niftrik LA, van de Vossenberg J, Schmid MC, Webb RI, Schouten S, Fuerst JA, Damste JS, Jetten
546 MS, Strous M (2007) *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of
547 anaerobic ammonium oxidizing bacteria. Syst Appl Microbiol 30(1):39-49 doi:S0723-2020(06)00050-6

548 Kartal B, van Niftrik L, Rattray J, van de Vossenberg JL, Schmid MC, Sinninghe Damste J, Jetten MS, Strous M (2008)
549 *Candidatus* 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol
550 Ecol 63(1):46-55 doi:FEM408

551 Kumar M, Lin JG (2010) Co-existence of anammox and denitrification for simultaneous nitrogen and carbon
552 removal--Strategies and issues. J Hazard Mater 178(1-3):1-9 doi:S0304-3894(10)00110-X

553 Kuypers MM, Sliemers AO, Lavik G, Schmid M, Jorgensen BB, Kuenen JG, Sinninghe Damste JS, Strous M, Jetten MS
554 (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422(6932):608-611
555 doi:10.1038/nature01472

556 Lam P, Kuypers MM (2011) Microbial nitrogen cycling processes in oxygen minimum zones. Ann Rev Mar Sci
557 3:317-345

558 Lam P, Lavik G, Jensen MM, van de Vossenberg J, Schmid M, Woebken D, Gutierrez D, Amann R, Jetten MS, Kuypers

559 MM (2009) Revising the nitrogen cycle in the Peruvian oxygen minimum zone. Proc Natl Acad Sci U S A
560 106(12):4752-4757 doi:0812444106

561 Li H, Chen S, Mu BZ, Gu J-D (2010a) Molecular detection of anaerobic ammonium-oxidizing (anammox) bacteria in
562 high-temperature petroleum reservoirs. Microb Ecol 60(4):771-783 doi:10.1007/s00248-010-9733-3

563 Li M, Cao H, Hong YG, Gu J-D (2011a) Seasonal Dynamics of Anammox Bacteria in Estuarial Sediment of the Mai Po
564 Nature Reserve Revealed by Analyzing the 16S rRNA and Hydrazine Oxidoreductase (*hzo*) Genes. Microbes
565 Environ 26(1):15-22 doi:JST.JSTAGE/jsme2/ME10131

566 Li M, Ford T, Li X, Gu J-D (2011b) Cytochrome *cd₁*-containing nitrite reductase encoding gene *nirS* as a new functional
567 biomarker for detection of anaerobic ammonium oxidizing (Anammox) bacteria. Environ Sci Technol
568 45(8):3547-3553 doi:10.1021/es103826w

569 Li M, Gu J-D (2011) Advances in methods for detection of anaerobic ammonium oxidizing (anammox) bacteria. Appl
570 Microbiol Biotechnol 90(4):1241-1252 doi:10.1007/s00253-011-3230-6

571 Li M, Hong Y, Cao H, Gu J-D (2013a) Community Structures and Distribution of Anaerobic Ammonium Oxidizing and
572 *nirS*-Encoding Nitrite-Reducing Bacteria in Surface Sediments of the South China Sea. Microb Ecol
573 66(2):281-296 doi:10.1007/s00248-012-0175-y

574 Li M, Hong Y, Cao H, Klotz MG, Gu J-D (2013b) Diversity, abundance, and distribution of NO-forming nitrite
575 reductase-encoding genes in deep-sea subsurface sediments of the South China Sea. Geobiology 11(2):170-179
576 doi:10.1111/gbi.12020

577 Li M, Hong Y, Klotz MG, Gu J-D (2010b) A comparison of primer sets for detecting 16S rRNA and hydrazine
578 oxidoreductase genes of anaerobic ammonium-oxidizing bacteria in marine sediments. Appl Microbiol
579 Biotechnol 86(2):781-790 doi:10.1007/s00253-009-2361-5

580 Li M, Hong YG, Cao HL, Gu J-D (2011c) Mangrove trees affect the community structure and distribution of anammox
581 bacteria at an anthropogenic-polluted mangrove in the Pearl River Delta reflected by 16S rRNA and hydrazine
582 oxidoreductase (HZO) encoding gene analyses. Ecotoxicology 20(8):1780-1790
583 doi:10.1007/s10646-011-0711-4

584 Li XR, Du B, Fu HX, Wang RF, Shi JH, Wang Y, Jetten MS, Quan ZX (2009) The bacterial diversity in an anaerobic
585 ammonium-oxidizing (anammox) reactor community. Syst Appl Microbiol 32(4):278-289
586 doi:10.1016/j.syapm.2009.03.002

587 Lin JG, Lan CJ, Kumar M, Wang CC (2011) Development of simultaneous partial nitrification, anammox and
588 denitrification (SNAD) process in a sequential batch reactor. Bioresource Technol 102(9):5514-5519

589 Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ
590 Microbiol 71(12):8228-8235 doi:71/12/8228

591 Meyer RL, Risgaard-Petersen N, Allen DE (2005) Correlation between anammox activity and microscale distribution of
592 nitrite in a subtropical mangrove sediment. Appl Environ Microb 71(10):6142-6149

593 Mulder A, Vandegraaf AA, Robertson LA, Kuenen JG (1995) Anaerobic Ammonium Oxidation Discovered in a
594 Denitrifying Fluidized-Bed Reactor. FEMS Microbiol Ecol 16(3):177-183

595 Neef A, Amann R, Schlesner H, Schleifer KH (1998) Monitoring a widespread bacterial group: in situ detection of
596 planctomycetes with 16S rRNA-targeted probes. Microbiology 144 (Pt 12):3257-3266

597 Nelson JG (1993) Conservation and use of the Mai Po marshes, Hong Kong. Nat Areas J 13:5

598 Ni SQ, Gao BY, Wang CC, Lin JG, Sung S (2011) Fast start-up, performance and microbial community in a pilot-scale
599 anammox reactor seeded with exotic mature granules. Bioresour Technol 102(3):2448-2454
600 doi:S0960-8524(10)01808-0

601 Ning X, Lin C, Hao Q, Liu C, Le F, Shi J (2009) Long term changes in the ecosystem in the northern South China Sea

602 during 1976-2004. Biogeosciences 6(10):2227-2243

603 Oshiki M, Shimokawa M, Fujii N, Satoh H, Okabe S (2011) Physiological characteristics of the anaerobic
604 ammonium-oxidizing bacterium '*Candidatus* Brocadia sinica'. Microbiology 157(Pt 6):1706-1713
605 doi:mic.0.048595-0

606 Park H, Brotto AC, van Loosdrecht MC, Chandran K (2017) Discovery and metagenomic analysis of an anammox
607 bacterial enrichment related to *Candidatus* "Brocadia caroliniensis" in a full-scale glycerol-fed
608 nitritation-denitritation separate centrate treatment process. Water Res 111:265-273
609 doi:10.1016/j.watres.2017.01.011

610 Penton CR, Devol AH, Tiedje JM (2006) Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing
611 bacteria in freshwater and marine sediments. Appl Environ Microbiol 72(10):6829-6832 doi:72/10/6829

612 Quan ZX, Rhee SK, Zuo JE, Yang Y, Bae JW, Park JR, Lee ST, Park YH (2008) Diversity of ammonium-oxidizing
613 bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. Environ Microbiol
614 10(11):3130-9 doi:10.1111/j.1462-2920.2008.01642.x

615 Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and
616 estimating species richness. Appl Environ Microb 71(3):1501-1506

617 Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer KH, Wagner M (2000)
618 Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation.
619 Syst Appl Microbiol 23(1):93-106

620 Schmid M, Walsh K, Webb R, Rijpstra WI, van de Pas-Schoonen K, Verbruggen MJ, Hill T, Moffett B, Fuerst J, Schouten
621 S, Damste JS, Harris J, Shaw P, Jetten M, Strous M (2003) *Candidatus* "Scalindua brodae", sp. nov., *Candidatus*
622 "Scalindua wagneri", sp. nov., two new species of anaerobic ammonium oxidizing bacteria. Syst Appl Microbiol
623 26(4):529-538

624 Schmid MC, Hooper AB, Klotz MG, Woebken D, Lam P, Kuypers MM, Pommerening-Roeser A, Op den Camp HJ,
625 Jetten MS (2008) Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase
626 genes of aerobic and anaerobic ammonium-oxidizing bacteria. Environ Microbiol 10(11):3140-3149
627 doi:EMI1732

628 Schmid MC, Maas B, Dapena A, van de Pas-Schoonen K, van de Vossenberg J, Kartal B, van Niftrik L, Schmidt I, Cirpus
629 I, Kuenen JG, Wagner M, Sinninghe Damste JS, Kuypers M, Revsbech NP, Mendez R, Jetten MS, Strous M
630 (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. Appl Environ
631 Microbiol 71(4):1677-84 doi:71/4/1677

632 Schmid MC, Risgaard-Petersen N, van de Vossenberg J, Kuypers MM, Lavik G, Petersen J, Hulth S, Thamdrup B,
633 Canfield D, Dalsgaard T, Rysgaard S, Sejr MK, Strous M, den Camp HJ, Jetten MS (2007) Anaerobic
634 ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. Environ
635 Microbiol 9(6):1476-1484 doi:EMI1266

636 Sonthiphand P, Neufeld JD (2013) Evaluating primers for profiling anaerobic ammonia oxidizing bacteria within
637 freshwater environments. PLoS One 8(3):e57242 doi:10.1371/journal.pone.0057242

638 Speth DR, Hu B, Bosch N, Keltjens JT, Stunnenberg HG, Jetten MS (2012) Comparative genomics of two independently
639 enriched "*Candidatus* kuenenia stuttgartiensis" anammox bacteria. Front Microbiol 3:307
640 doi:10.3389/fmicb.2012.00307

641 Speth DR, Lagkouvardos I, Wang Y, Qian PY, Dutilh BE, Jetten MS (2017) Draft Genome of *Scalindua rubra*, Obtained
642 from the Interface Above the Discovery Deep Brine in the Red Sea, Sheds Light on Potential Salt Adaptation
643 Strategies in Anammox Bacteria. Microb Ecol doi:10.1007/s00248-017-0929-7

644 Strous M, Pelletier E, Manganot S, Rattei T, Lehner A, Taylor MW, Horn M, Daims H, Bartol-Mavel D, Wincker P, Barbe

645 V, Fonknechten N, Vallenet D, Segurens B, Schenowitz-Truong C, Medigue C, Collingro A, Snel B, Dutilh BE,
646 Op den Camp HJ, van der Drift C, Cirpus I, van de Pas-Schoonen KT, Harhangi HR, van Niftrik L, Schmid M,
647 Keltjens J, van de Vossenberg J, Kartal B, Meier H, Frishman D, Huynen MA, Mewes HW, Weissenbach J,
648 Jetten MS, Wagner M, Le Paslier D (2006) Deciphering the evolution and metabolism of an anammox
649 bacterium from a community genome. *Nature* 440(7085):790-794 doi:nature04647

650 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics
651 Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol*
652 *Evol* 28(10):2731-2739

653 van de Graaf AA, Mulder A, de Bruijn P, Jetten MS, Robertson LA, Kuenen JG (1995) Anaerobic oxidation of ammonium
654 is a biologically mediated process. *Appl Environ Microbiol* 61(4):1246-1251

655 van de Vossenberg J, Rattray JE, Geerts W, Kartal B, van Niftrik L, van Donselaar EG, Sinninghe Damste JS, Strous M,
656 Jetten MS (2008) Enrichment and characterization of marine anammox bacteria associated with global nitrogen
657 gas production. *Environ Microbiol* 10(11):3120-3129 doi:10.1111/j.1462-2920.2008.01643.x

658 van de Vossenberg J, Woebken D, Maalcke WJ, Wessels HJ, Dutilh BE, Kartal B, Janssen-Megens EM, Roeselers G, Yan
659 J, Speth D, Gloerich J, Geerts W, van der Biezen E, Pluk W, Francoijs KJ, Russ L, Lam P, Malfatti SA, Tringe
660 SG, Haaijer SC, Op den Camp HJ, Stunnenberg HG, Amann R, Kuypers MM, Jetten MS (2013) The
661 metagenome of the marine anammox bacterium '*Candidatus Scalindua profunda*' illustrates the versatility of
662 this globally important nitrogen cycle bacterium. *Environ Microbiol* 15(5):1275-1289
663 doi:10.1111/j.1462-2920.2012.02774.x

664 Wang CC, Lee PH, Kumar M, Huang YT, Sung S, Lin JG (2010) Simultaneous partial nitrification, anaerobic ammonium
665 oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant. *J Hazard Mater*
666 175(1-3):622-628 doi:S0304-3894(09)01703-8

667 Wang J, Gu J-D (2013) Dominance of *Candidatus Scalindua* species in anammox community revealed in soils with
668 different duration of rice paddy cultivation in Northeast China. *Appl Microbiol Biotechnol* 97(4):1785-1798
669 doi:10.1007/s00253-012-4036-x

670 Wang S, Hong Y, Wu J, Xu XR, Bin L, Pan Y, Guan F, Wen J (2015) Comparative analysis of two 16S rRNA gene-based
671 PCR primer sets provides insight into the diversity distribution patterns of anammox bacteria in different
672 environments. *Appl Microbiol Biotechnol* 99(19):8163-8176 doi:10.1007/s00253-015-6814-8

673 Wang S, Zhu G, Peng Y, Jetten MS, Yin C (2012a) Anammox bacterial abundance, activity, and contribution in riparian
674 sediments of the pearl river estuary. *Environ Sci Technol* 46(16):8834-8842 doi:10.1021/es3017446

675 Wang Y, Zhu G, Harhangi HR, Zhu B, Jetten MS, Yin C, Op den Camp HJ (2012b) Co-occurrence and distribution of
676 nitrite-dependent anaerobic ammonium and methane-oxidizing bacteria in a paddy soil. *FEMS Microbiol Lett*
677 336(2):79-88 doi:10.1111/j.1574-6968.2012.02654.x

678 Wang YF, Feng YY, Ma X, Gu J-D (2012c) Seasonal dynamics of ammonia/ammonium-oxidizing prokaryotes in oxic and
679 anoxic wetland sediments of subtropical coastal mangrove. *Appl Microbiol Biotechnol* 97(17):7919-7934
680 doi:10.1007/s00253-012-4510-5

681 Wang YF, Gu J-D (2012) Higher diversity of ammonia/ammonium-oxidizing prokaryotes in constructed freshwater
682 wetland than natural coastal marine wetland. *Appl Microbiol Biotechnol* 97(15):7015-7033
683 doi:10.1007/s00253-012-4430-4

684 Ward BB, Devol AH, Rich JJ, Chang BX, Bulow SE, Naik H, Pratihary A, Jayakumar A (2009) Denitrification as the
685 dominant nitrogen loss process in the Arabian Sea. *Nature* 461(7260):78-81 doi:nature08276

686 Woebken D, Lam P, Kuypers MM, Naqvi SW, Kartal B, Strous M, Jetten MS, Fuchs BM, Amann R (2008) A
687 microdiversity study of anammox bacteria reveals a novel *Candidatus Scalindua* phylotype in marine oxygen

688 minimum zones. *Environ Microbiol* 10(11):3106-3119 doi:10.1111/j.1462-2920.2008.01640.x
689 Zhou Z, Chen J, Meng H, Dvornyk V, Gu J-D (2017) New PCR primers targeting hydrazine synthase and cytochrome c
690 biogenesis proteins in anammox bacteria. *Appl Microbiol Biotechnol* 101(3):1267-1287
691 doi:10.1007/s00253-016-8013-7
692 Zhu GB, Xia C, Shanyun W, Zhou LL, Liu L, Zhao SY (2015) Occurrence, activity and contribution of anammox in some
693 freshwater extreme environments. *Env Microbiol Rep* 7(6):961-969 doi:10.1111/1758-2229.12341
694 Zopfi A, Humbert S, Bagnoud A, Tarnawski S, Conen F, Seth B (2009) Anammox in terrestrial ecosystems: Distribution,
695 diversity and activity. *Geochim Cosmochim Ac* 73(13):A1538-A1538
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697 **Table 1** PCR primer sets used for amplification of the four anammox bacterial marker genes
 698 chosen in this study

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Target gene	Primer pair	Primer name	Sequence 5'-3'	Product size (bp)	Annealing temperature	References
16S rRNA	AMX	AMX368F	CCTTTCGGGCATTGCGAA	452	56°C	(Kuypers et al. 2003)
		AMX820R	AAAACCCCTCTACTTAGTGCCC			(Schmid et al. 2000)
	BS	AMX368F	CCTTTCGGGCATTGCGAA	452	56°C	(Kuypers et al. 2003)
		BS820R	TAATTCCCTCTACTTAGTGCCC			(Kuypers et al. 2003)
	Brod	Brod541F	GAGCACGTAGGTGGGTTTGT	279	56°C	(Penton et al. 2006)
		AMX820R	AAAACCCCTCTACTTAGTGCCC			(Schmid et al. 2000)
<i>hzo</i>	CL1	hzocl1F1	TGYAAGACYTGAYCAYTGG	470	48°C	(Schmid et al. 2008)
		hzocl1R2	ACTCCAGATRTGCTGACC			
	H4F	hzoF1	TGTGCATGGTCAATTGAAAG	1000	53°C	(Li et al. 2010b)
		hzoR1	CAACCTCTTCWGCAGGTGCATG			
	Ana	Ana-hzo1F	TGTGCATGGTCAATTGAAAG	1000	53°C	(Quan et al. 2008)
		Ana-hzo2R	ACCTCTTCWGCAGGTGCAT			
	CL2	hzocl2aF1	GGTTGYCACACAAGGC	525	48°C	(Schmid et al. 2008)
		hzocl2aR2	ATATTCACCATGYTTCCAG			
<i>nirS</i>	ScnirS	ScnirS372F	TGTAGCCAGCATTGTAGCGT	473	59°C	(Lam et al. 2009)
		ScnirS845R	TCAAGCCAGACCCATTGCT			
	AnnirS	AnnirS379F	TCTATCGTTGCATCGCATT	442	51°C	(Li et al. 2011b)
		AnnirS821R	GGATGGGTCTTGATAAACA			
<i>hzsA</i>	HZSL	hzsA_526F	TAYTTTGAAGGDGACTGG	1331	54°C	(Harhangi et al. 2012)
		hzsA_1857R	AAABGGYGAATCATARTGGC			
	HZSS	hzsA_1597F	WTYGGKTATCARTATGTAG	260	54°C	
		hzsA_1857R	AAABGGYGAATCATARTGGC			

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 701 **Table 2** Copy numbers of 16S rRNA, *hzo* and *hzsA* genes

Sample	Gene copy no. per gram (dry weight)		
	16S rRNA gene	<i>hzo</i> gene (Ratio*)	<i>hzsA</i> gene (Ratio*)
WW	2.51×10 ⁶	1.11×10 ⁶ (2.26)	1.14×10 ⁶ (2.20)
MP	1.69×10 ⁶	1.21×10 ⁵ (13.97)	1.67×10 ⁵ (10.12)
SCS	2.02×10 ⁶	2.29×10 ⁵ (8.82)	1.26×10 ⁶ (1.60)

702 Ratio*: compared by 16S rRNA gene copy numbers

Figure Captions

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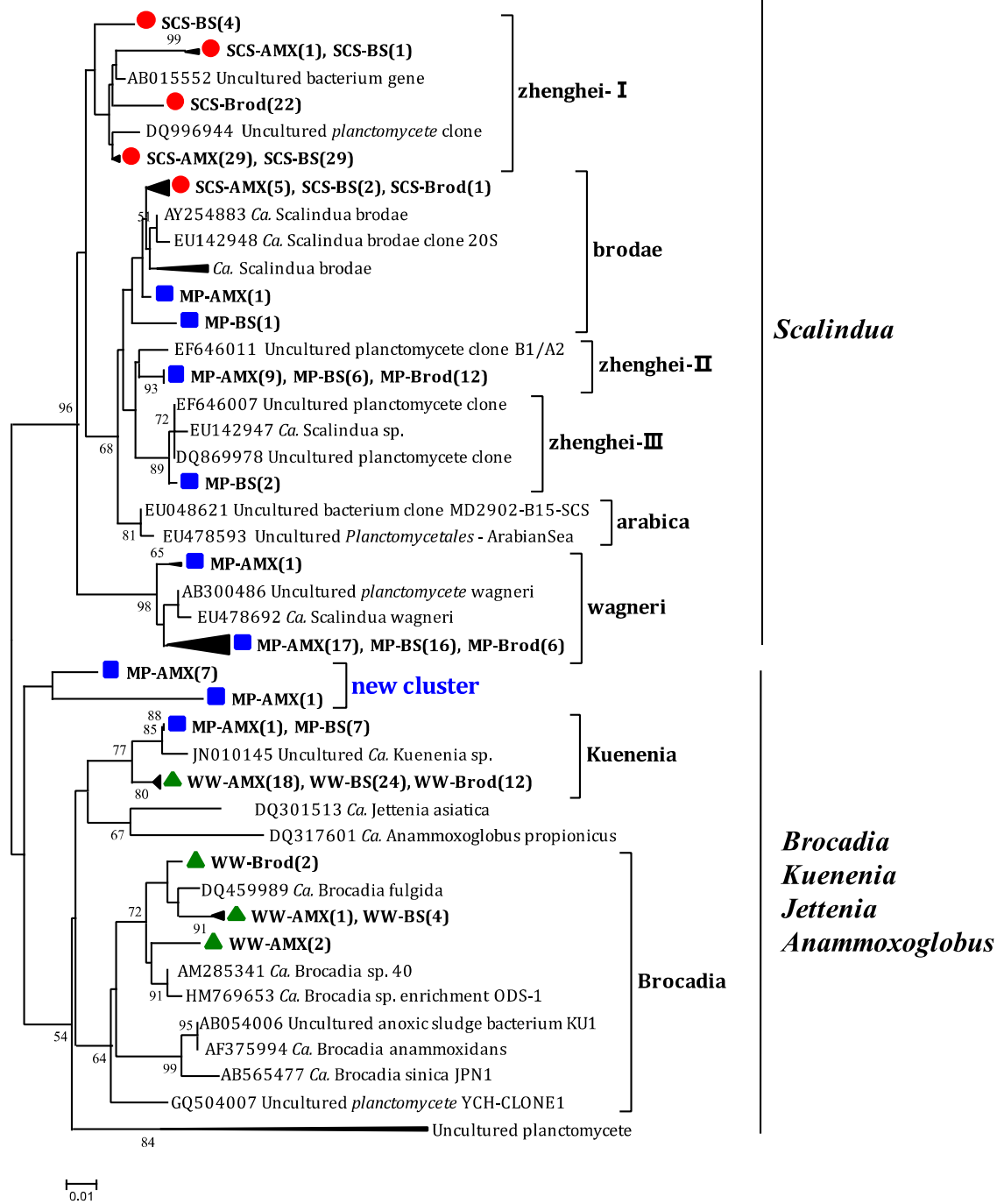
Figure 1 Phylogenetic tree constructed with distance and neighbor-joining method from an alignment of PCR amplified (a) 16S rRNA gene sequences, (b) HZO protein sequences, (c) NirS protein sequences, (d) HzsA protein sequences (with primer set HZSL) and (e) HzsA protein sequences (with primer set HZSS) along with their closely related sequences from GenBank. WW (Green): wastewater treatment plant; SCS (red): the South China Sea; MP (blue): Mai Po Nature Reserve. Primer sets used were placed behind the sample name and numbers refer to how many clones retrieved. The numbers at the nodes are percentages that indicate the levels of bootstrap support based on 1000 resampled data sets (only values greater than 50% are shown). Branch lengths correspond to sequence differences as indicated by the scale bar.

Figure 2 CCA ordination plots for the first two principal dimensions of the relationship between the distribution of anammox species and clusters with environmental parameters of WW, MP and SCS used in this study. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors). (Abbreviations are the same as in Figure 1)

Table S1 Physiochemical characteristics of samples used in this study

Table S2 Diversity and richness indices from the three samples based on sequences of anammox 16S rRNA gene, HZO, NirS and HzsA protein

Figure S1 PCoA analyses with 100 replicates Jackknife supporting test of anammox (a) 16S rRNA gene sequences, (b) HZO protein sequences, (c) NirS protein sequences and (d, e) HzsA protein sequences in WW, MP and the SCS on the basis of the online software UniFrac.



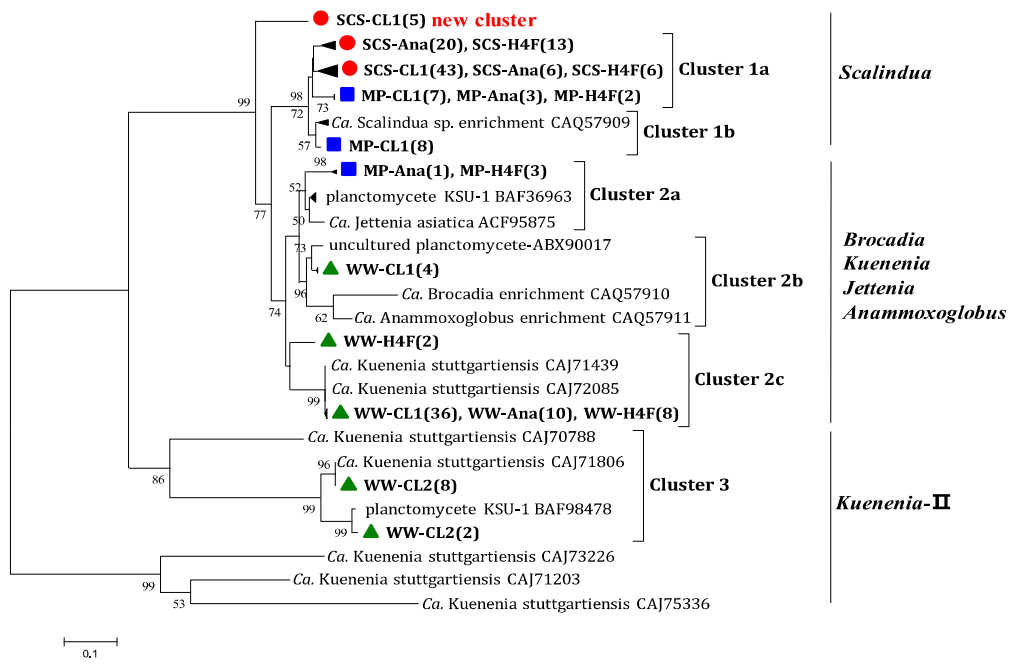
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Figure 1a

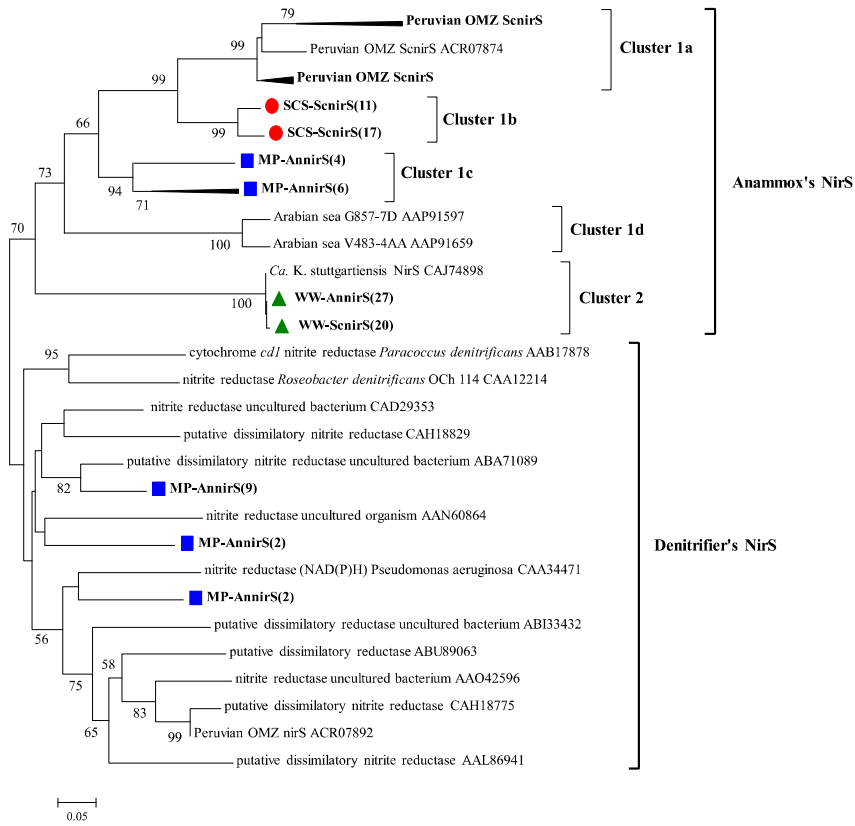


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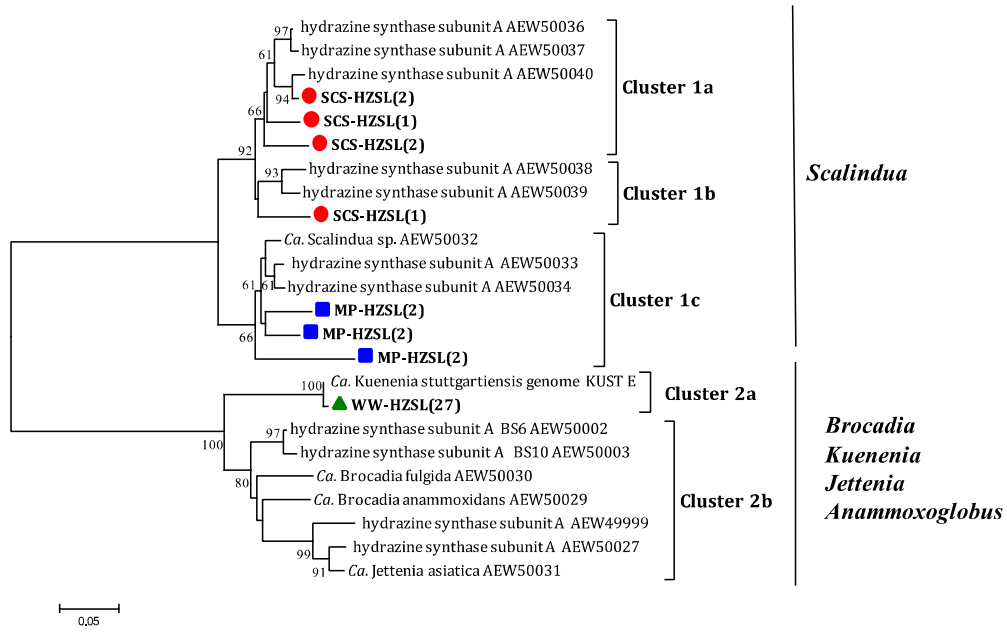
Figure 1b



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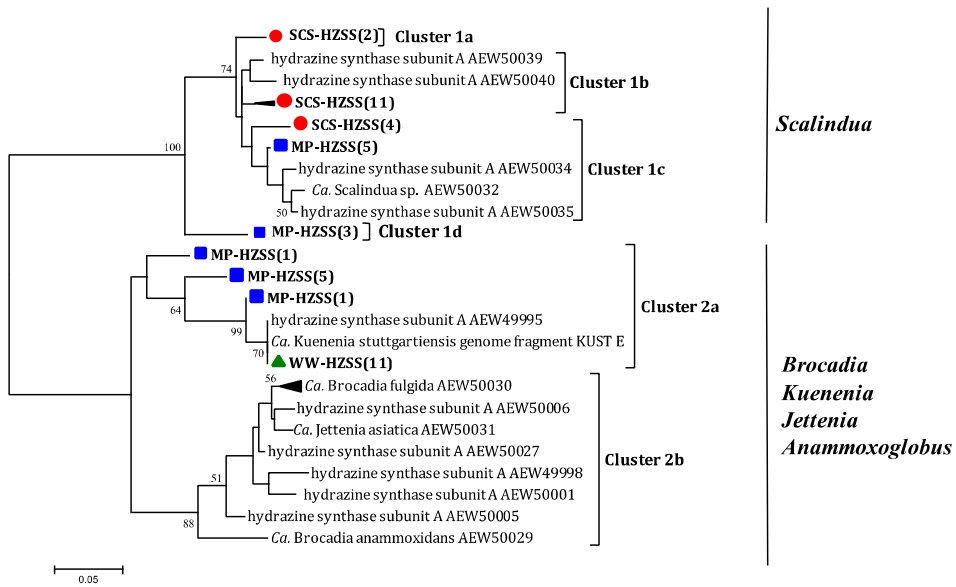
Figure 1c



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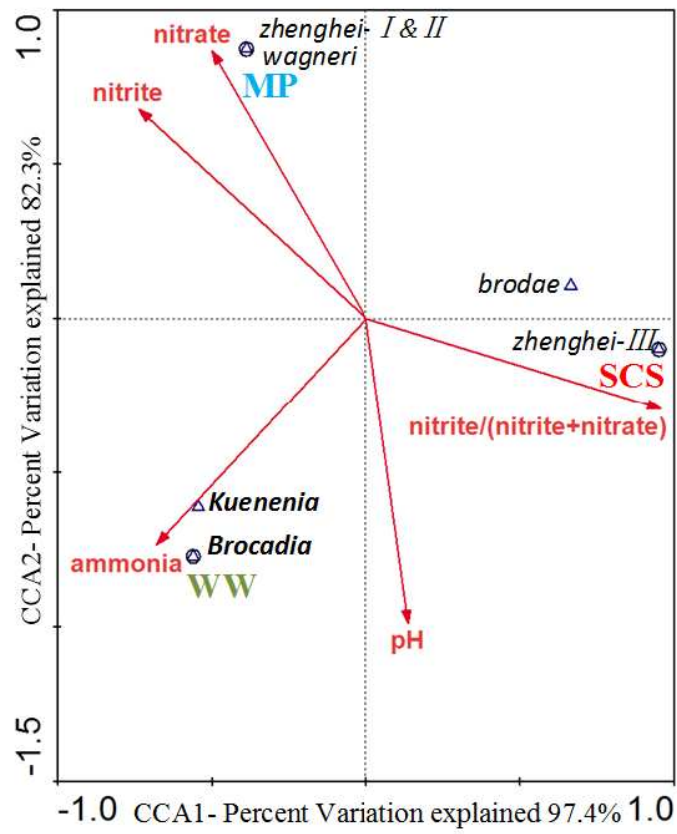
Figure 1d



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Figure 1e



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Figure 2