RESEARCH ARTICLE

Molecular evidence of the existence of anaerobic ammonia oxidation bacteria in the gut of polychaete (*Neanthes glandicincta*)

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Abstract: *Neanthes* are one of the most important groups of polychaete in coastal sediments, which play an important role on the nutrient cycling in coastal sediments. Here we report on the existence of anammox bacteria in the gut of polychaete *Neanthes glandicincta* based on the analysis of 16S rRNA gene and fluorescence *in situ* hybridization (FISH). Three distinct clusters of anammox bacteria are found in different gut sections of *N. glandicincta*, and one of them is considered as a novel, gut specific anammox bacteria after comparing with the anammox bacteria recovered from surrounding pre-digested sediment. The uniform axial distribution of anammox bacteria in different gut sections of *N. glandicincta* is also found in present study. These results extend our knowledge of microbial ecology of anammox bacteria in the natural environments.

Keywords: anammox bacteria, gut, *Neanthes glandicincta*, phylogenetic diversity, axial distribution

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1. Introduction

Anaerobic ammonia oxidation (anammox) is a newly discovered pathway of the microbial nitrogen cycle that allows ammonia to be oxidized by nitrite or nitrate under anoxic conditions[1,2]. Owing to the distinct metabolism, anammox bacteria received considerable attention by many fields. In the natural ecosystem, anammox is a key process in the global nitrogen cycle[3–6], which may account for 30 to 70% of oceanic N_2 production from a global context[7,8]. The now well-established significance of anammox in the natural environment has emerged from a combination of ^{15}N-based tracer studies, analysis of ladderane lipid biomarkers, fluorescent *in situ* hybridization (FISH) and phylogenetic and quantitative PCR analysis of 16S rRNA and functional genes[9–15]. To date, anammox has been documented in marine, coastal and estuarine sediments[16–20], anoxic basins[3,4], oxygen minimum zones (OMZs) of West Africa[21], Chile[22] and Peru[23], mangroves[24,25], sea-ice[26], soils[7,27–31], freshwater[32–34]. Recently presence and activity of anammox bacteria was also confirmed at deep-sea hydrothermal vent[35,36]. Although anammox process is widely distributed in different natural environments, this process is so far linked to one group of organisms, which are monophyletic members of the phylum *Planctomycetes*[9,37], including *Candidatus* Scalindua[38], *Candidatus* Brocadia[37], *Candidatus* Anammoxoglobus[39], *Candidatus* Jettenia[40], and

Candidatus Kuenenia[41]. Interestingly, most of the available anammox 16S rRNA sequences from marine and estuarine environments are all related to *Candidatus* 'Scalindua sp.'[42]. However, there is no investigation to indicate the presence of anammox bacteria in the intestinal tracts, where low oxygen and abundant nitrogen species provide good condition for growth of anammox bacteria.

Neanthes are one of the most important groups of polychaete in coastal sediments. Many species show a particular preference for littoral and supralittoral areas in association with decaying vegetation including mangroves, the strand zone on beaches and inland waters such as riverbanks and sinkholes (subterranean waters); some species can tolerate highly polluted waters[43,44]. The small polychaete *Neanthes glandicincta* is one of the most important polychaete in Mai Po Inner Deep Bay Ramsar Site of Hong Kong[45,46]. Previous study has pointed out *Neanthes glandicincta* inhibited abundant microbes in the gut, and these intestinal microbes show obvious axial distribution in different gut sections. The difference in microbial community structure in the gut sections reflects the physicochemical conditions in the particular habits[47]. In the present study, we analyzed the anammox bacteria diversity in different gut sections and digested sediment of *N. glandicincta* in detail using 16S rRNA gene clone libraries. Based on the clone libraries, we compare the community structure of anammox bacteria in the digested sediment and the gut of *N. glandicincta*, and characterize the distribution of anammox bacteria along the gut axis. The existence of anammox bacteria in different gut sections and digested sediment of *N. glandicincta* were also confirmed by fluorescence *in situ* hybridization (FISH) using specific oligonucleotide probe of anammox bacteria.

2. Materials and Methods

2.1 Sampling and Preparation

Specimens of *N. glandicincta* were collected from Mai Po wetland of Hong Kong on May 2007. To preserve the vigor of *N. glandicincta*, samples were collected with the ambient sediment back to laboratory. In order to remove the epidermal microbes, 5 fresh samples were washed with sterile seawaters (5 times), sterile waters (5 times), and carefully dissected aseptically into three parts, including anterior, middle, and posterior sections for further experiments. For checking whether our washing is sufficient to remove attached microorganisms from the surface of a polychaete, parts of washed samples were sent to do scanning electron microscopy (SEM) according to the protocols of Bright and Sorgo[48].

2.2 DNA Extraction

Different section of *N. glandicincta* gut was transferred into individual 1.5 mL Eppendorf tube. Total genomic DNA was extracted from the samples by the DNEasy tissue kit, using a modified DNEasy tissue extraction protocol (Qiagen, Valencia, CA) to ensure maximal lysis the bacteria. Following overnight digestion of tissue samples with proteinase K (20 µL, 600 mAU/mL) and ATL tissue lysis buffer (Qiagen), the samples were centrifuged, and DNA was isolated from the supernatant. Briefly, the supernatant was incubated with a guanidium isothiocyanate-based buffer (AL buffer) for 10 min at 70°C before transferred into a spin column. Following washes with AW1 buffer and AW2 buffer, the DNA was eluted in Tris chloride elution buffer (AE buffer). The pellet formed from centrifugation of the lysed tissue was subjected to an extraction using the DNEasy protocol for gram-positive bacteria to ensure recovery of any possibly recalcitrant gram-positive bacteria. Briefly, the pellet was re-suspended in a lysozyme buffer (20 mM Tris Cl [pH 8.0], 2 mM EDTA, 1.2% Triton X-100) and incubated at 85°C for 10 min to inactivate the proteinase K, and 3.6 mg of lysozyme (Sigma, St. Louis, MO) was added to the re-suspended pellet and incubated 45 min at 37°C. Proteinase K (25 µL, 600 mAU/mL) and AL buffer were added to the lysate and incubated at 70°C for 30 min. Following the addition of 200 µL of ethanol, the lysate was applied to a spin column, washed with AW1 and AW2 buffers and eluted in elution buffer (AE buffer) as described above. DNA from both extractions was pooled and stored at 4°C prior to use for PCR[49]. Total DNA of digested sediment sample was extracted using the Master Soil DNA Extract Kit according to the manufacturer's instruction.

2.3 PCR Amplification and Cloning

In order to amplify 16S rRNA gene of anammox bacteria, the primer set were selected as primer Brod541F (5'-GAGCACGTAGGTGGGTTTGT-3')-Amx820R (5'-AAAACCCCTCTACTTAGTGCCC-3'), which shows the highest specificity for detecting anammox bacteria from natural environment in all the available anammox specific primers[10]. Polymerase chain reaction (PCR) mixtures (final volume of 50 µL) containing 1

μL of DNA extract consisted in (as final concentration) 1 × DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.125 mM of each dATP, dCTP, dGTP and dTTP, 0.1 mM of each primer, 1.25 U of Taq DNA polymerase (Promega, Charbonnières, France). PCR cycles were performed as follows: 95°C for 5 min followed by 30 cycles at 95°C for 45 sec, 60°C for 1 min, and at 72°C for 1 min and a final extension period of 10 min at 72°C. PCR products were visualized on 1.0% (w/v) agarose gel stained with ethidium bromide.

Clone libraries of 16S rRNA genes for anammox bacteria were created from the PCR products as described as previous study[10]. Briefly, the PCR amplified products were purified using a Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and cloned into the PMD18 T-vector (Takala, Japan). The insertion of an appropriate-sized DNA fragment was determined by PCR amplification with the primer set M13F and M13R.

2.4 Sequencing and Phylogenetic Analysis

All right inserted clones were selected and subjected to sequencing using M13F and M13R. The sequences were determined by using an ABI 3100 genetic analyzer (Applied Biosystems, FosterCity, CA) with BigDye. Sequences were subjected to the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST)[50] and the Ribosomal Database Project II[51] to determine the levels of similarity with other 16S rRNA genes. Partial 16S rRNA gene sequences were manually compiled and aligned using the CLUSTAL W package[52]. Phylogenetic trees were constructed by MEGA 4.0 software with the neighbor-joining method[53]. Bootstrap resampling analysis for 1,000 replicates was performed to estimate the confidence of the tree topologies.

2.5 Fluorescence *In Situ* Hybridization

To confirm that the 16S rRNA sequences originated from the *N. glandicincta* gut, whole-cell in situ hybridizations were performed on different gut section and digested sediment by using the *Planctomycetes* specific probe Pla46 and anammox specific probes Amx-0820-a-A-22[9] (Invitrogen, Hong Kong). Sample preparations were followed the methods of Bright and Sorgo[48] and Schmid *et al.*[41]. In briefly, *N. glandicinta* were fixed for 3 hrs in 4% paraformaldehyde of 1 × PBS buffer (130 mM NaCl, 10 mM Na_2HPO_4, pH 7.2, 0.2 μm pore size filter sterilized) at 4°C, then rinsed twice with 1 × PBS and transfer to 1:1 (PBS: 100% ethanol) for incubation by Steedman's polyester wax. Different gut sections were cut (6 to 8 μm thick with a microtome) from wax-embedded species. After wax and tissue autofluorescence removal, gut section can be used for further FISH experiment. Fixed samples were dehydrated in an ethanol series (70, 80, 90, 95, and 100%) followed by three washes in sterile waters. Approximately 0.5 mL of preheated hybridization solution was placed on each sample, and 100 ng of hydrolyzed, fluor-labeled polynucleotide probe was subsequently added. Hybridization mixtures were incubated 90 min in at 46°C. After incubation, the dish was removed and placed in 50 mL sterile water for 3 times washes. The samples were subsequently rinsed in sterile water. Image acquisition was done with a confocal scanning laser microscopy (LSM 510). For digested sediment sample, the experiment procedure was the similar protocols as above.

2.6 Statistical Analysis of Clone libraries

The Shannon diversity index (*H*) and Simpson's Index (1-λ) have been used to measure biodiversity. The advantage of these indexes is that they take into account the number of species and the evenness of the community. The rarefaction analysis was used to compare the number of species found in different regions[54]. In present study, the Shannon diversity index and Simpson's Index were calculated according to methods described by Mullins *et al.*[55] and Hunter[56], and rarefaction analysis was carried out by using the Analytic Rarefaction software (version 1.2; S.M. Holland, University of Georgia, Athens, GA, USA). LIBSHUFF statistics were applied to determine the significance of the differences between the clone libraries based on available sequence data[57].

2.7 Nucleotide Sequence Accession Numbers

The sequences obtained in this study were submitted to the GenBank database and assigned accession no. KX029339–KX029460.

3. Results

3.1 SEM for the Surface of Polychaete *N. glandicincta*

SEM experiment was carried out for checking whether there are any attached microorganisms from the surface of *N. glandicincta* after the washing procedures. Figure 1 showed that there are no any microorganisms

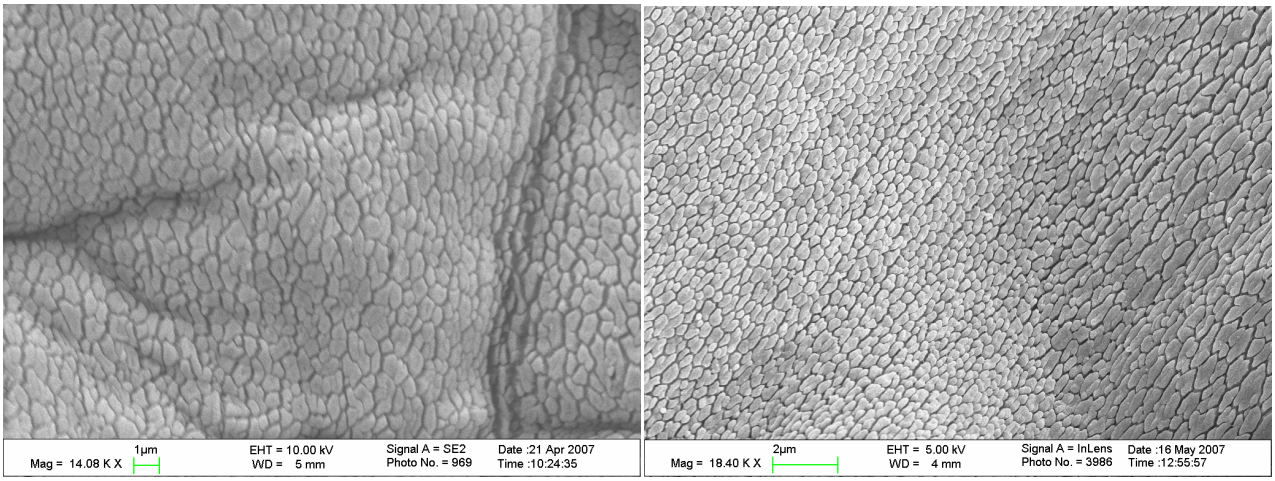


Figure 1. SEM results for the surface of *N. glandicincta* after washing procedures.

associated on the surface of *N. glandicincta* after the washing procedures, which indicates all the bacteria clones obtained from *N. glandicincta* are really existed in the gut of research animals.

3.2 Clone Library and Phylogenetic Analysis

16S rRNA gene clone libraries of anammox bacteria in different gut sections of polychaete *N. glandicincta* were constructed through PCR amplification using the anammox specific primer set (Brod541F-Amx820R). In each gut sections, bacterial clones were randomly selected for sequencing, and these 16S rRNA gene sequences were divided into different unique phylotypes which were arbitrarily defined using the criterion of >98% sequence identity. In order to check whether the clone number of the individual clone libraries was sufficient to cover the majority of the anammox bacteria in each section of animal body and predigested sediment, rarefaction analysis were carried out based on phylotypes and selected clone numbers. The rarefaction analysis curves of each clone library were showed in Figure 2, where the slope of each curve indicated the selected clone number was sufficient for describing the most of anammox bacteria in each library (Figure 2). Table 1 summarized the details of clone and phylotypes numbers in each clone library in present study. 25 clones were divided into 4 different phylotypes from the Anterior section, while in the Middle and Posterior section, 4 and 5 different phylotypes were obtained from 32 and 31 clone sequences, respectively. The clone library of anammox bacteria in predigested sediment of *N. glandicincta* was also obtained in parallel study, and 6 different phylotypes were recovered from 29 clone sequences (Table 1). From the results of Shannone Index and Simpson's Index, it is clearly that Posterior section has the highest diversity in the gut of *N. glandicincta*, followed by the Middle and Anterior gut section. However, compared with the predigested sediment, the predigested sediment has the highest value, where Shannon Index and Simpson's Index are 1.75 and 0.86, respectively. The distribution of anammox bacteria was not evenly among the three section; evenness values were generally lower than 0.5 except for the bacteria in the Posterior section (Table 1). The coverage index in each clone library also provided evidence that the sampling size (clone number) was covered most of anammox bacteria in each library since the all the coverage indexes were more than 0.9 (Table 1).

In all clone libraries, including different gut sections

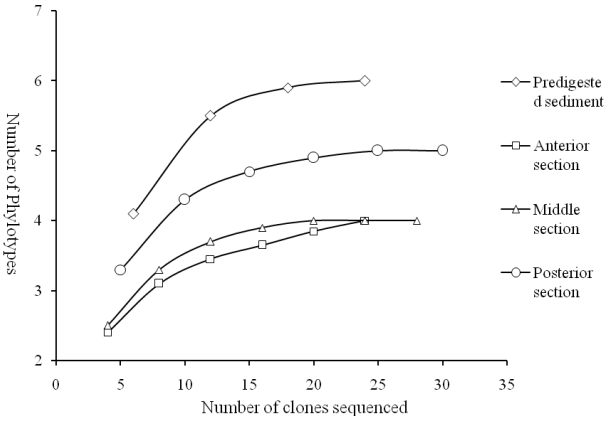


Figure 2. Rarefaction analysis of the clone libraries from the three different sections of intestinal tract of *N. glandicincta* and their predigested sediment. The expected number of phylotypes was calculated using cut-off values of sequence similarity for the species (>97%).

Table 1. Phylotype, diversity, coverage, and evenness of the anammox bacteria in different gut sections of *N. glandicincta* and their predigested sediment

Samples	Screen clones	Phylotype[a]	Shannon Index [b]	1-λ [c]	Coverage	Evenness [d]
Predigested sediment	29	6	1.75	0.86	0.96	0.52
Anterior section	25	4	1.21	0.64	0.92	0.37
Middle section	32	4	1.24	0.67	0.97	0.36
Posterior section	31	5	1.56	0.80	0.93	0.63

[a] Number of phylotype (based on > 98% sequence identity)
[b] Shannon Index(H')[58].
[c] The Simpson's Index of Diversity was calculated with the formula $1-\lambda=\sum_i[Ni(ni-1)]/[N(N-1)]$, where *Ni* is the individual number of OTU (*i*), and *N* is their total number.
[d] Evenness ($J=H'/H_{max}$)[58].

and predigested sediment, most of the 16S rRNA gene sequences exhibited >93% nucleotide identity to the Candidatus *Scalindua* group (Candidatus *Scalindua brodae* AY254883.1, Candidatus *Scalindua sorokinii* AY257181.1, and Candidatus *Scalindua wagneri* AY-254882.1), which was consistent with previously published results[42] in that the available anammox 16S rRNA gene sequences from marine and estuarine environments were all related to Candidatus *Scalindua* species (Figure 2). However, from the phylogenetic analysis, anammox bacteria clones recovered from the gut of *N. glandicincta* were divided into 3 clusters, including Cluster 1, Cluster 2 and Cluster 4; and anammox bacteria detected from the digested sediment were divided into Cluster 1, Cluster 2 and Cluster 3, respectively. The result showed that Cluster 4 and Cluster 3 were novel phylogenetic groups for the gut of *N. glandicincta* and predigested sediment, respectively (Figure 3).

The relative clone frequencies of anammox bacteria phylogenetic groups were calculated in each clone library (Figure 4). In Anterior and Middle section, Cluster 1 was the major phylogenetic group, which held more than 50% in their clone libraries; while Cluster 2, as the major anammox bacteria, took up 53% and 59% of clone number in Posterior section and predigested sediment. The gut specific anammox bacteria group — Cluster 4 was taken up 20 to 29% bacterial clone in the whole gut of *N. glandicincta*, and sediment specific anammox bacteria group — Cluster 3 was only taken up 14% bacterial clone in the predigested sediment (Figure 4).

3.3 The Existence of Anammox Bacteria in the Gut of *N. glandicincta* by Fluorescence *In Situ* Hybridization

The presence of anammox bacteria in the gut was examined and confirmed with FISH using the anammox-specific probe (Amx-0820-a-A-22 a) labeled with Cy3. All contents from different gut sections were detected positive for the visible fluorescence signal of anammox though the intensities were different from different gut sections (Figure 5A). The existence of anammox bacteria in predigested sediment was also confirmed by the same the anammox-specific probe, where the cell density of anammox bacteria was higher than that in the gut contents (Figure 5B). These results not only indicated the existence of anammox bacteria in the gut of *N. glandicincta* and their predigested sediment, but also indicated the different cell density of anammox bacteria in different gut sections and the predigested sediment.

3.4 Comparison of 16S rRNA Gene These in Different Gut Section of *N. glandicincta* and Their Predigested Sediment

To determine the significance of the differences between the clone libraries based on available sequence data, LIBSHUFF statistics were applied (Table 2). The *P* value ($P< 0.0043$) showed that there are no significant differences among the anammox bacteria clone libraries obtained from different gut sections, except when the clone library of Posterior section compared to the clone library of Middle section. However, there are significant differences between the clone library of predigested sediment and clone libraries of different gut sections of *N. glandicincta*, where all the P values are below the critical *p*-value of 0.0043.

4. Discussion

The gastrointestinal environment is usually known as a fermentor under oxygen-limited environments where the host animal and microorganisms compete for available nutrients[59], and the microbial community

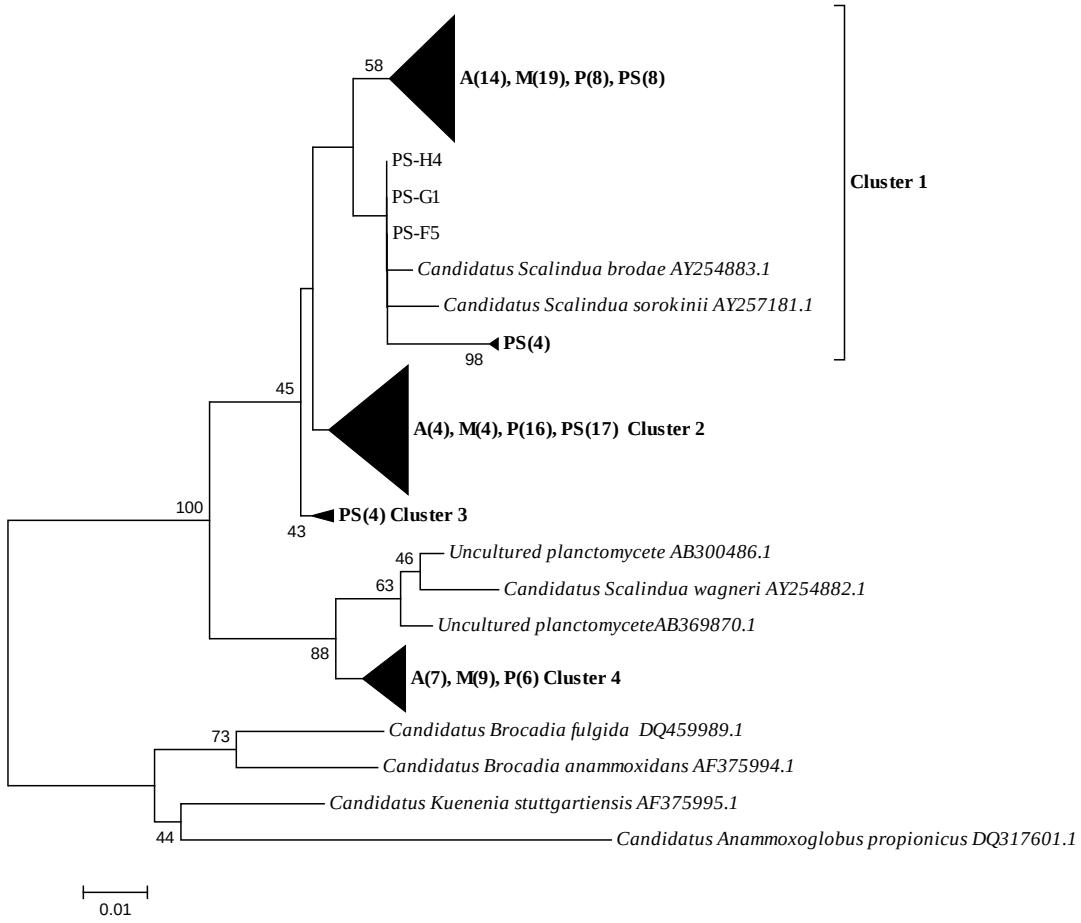


Figure 3. Neighbor-joining phylogenetic tree of anammox bacteria associated with different gut section of *Neanthes glandicincta* and their predigested sediment. Analyses are based on about 300 bps of aligned nucleotide sequences. Numerical values accompanying branch nodes reflect branching confidence based on a bootstrap of 1000 replicate re-samplings of the data. The horizontal scale bar corresponds to 0.01 substitutions per nucleotide position. A: Anterior section, M: Middle section, P: Posterior section, PS: Predigested sediment.

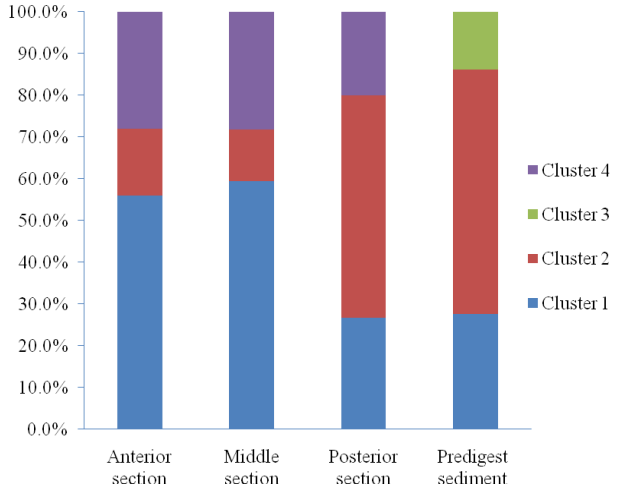
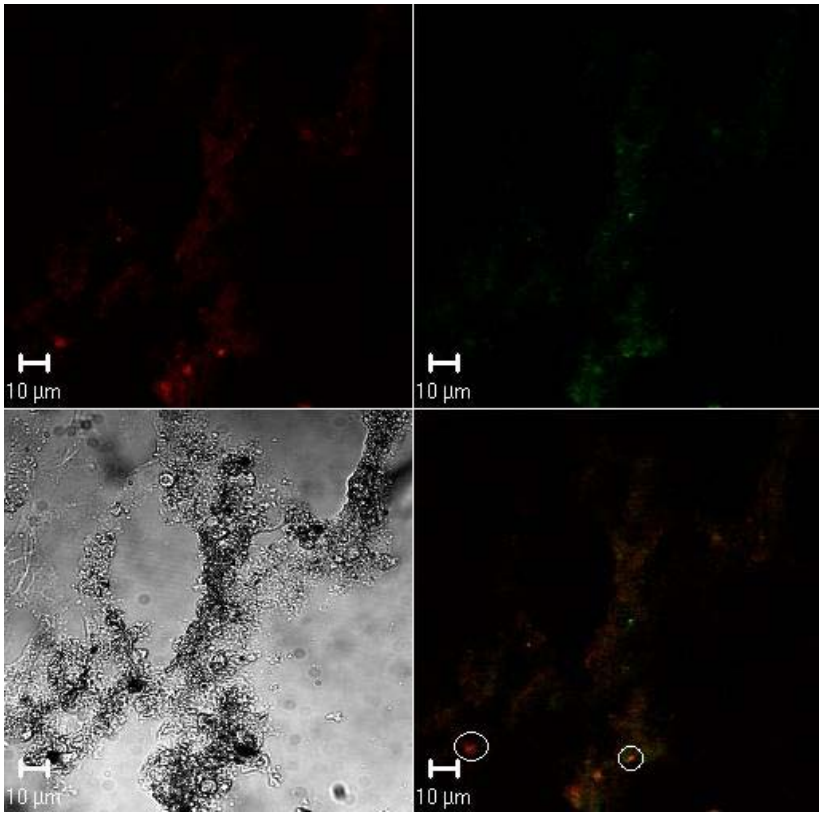


Figure 4. Relative clone frequencies in the major phylogenetic groups of anammox bacteria in the clone libraries of different intestinal tracts of *Neanthes glandicincta* and their predigested sediment

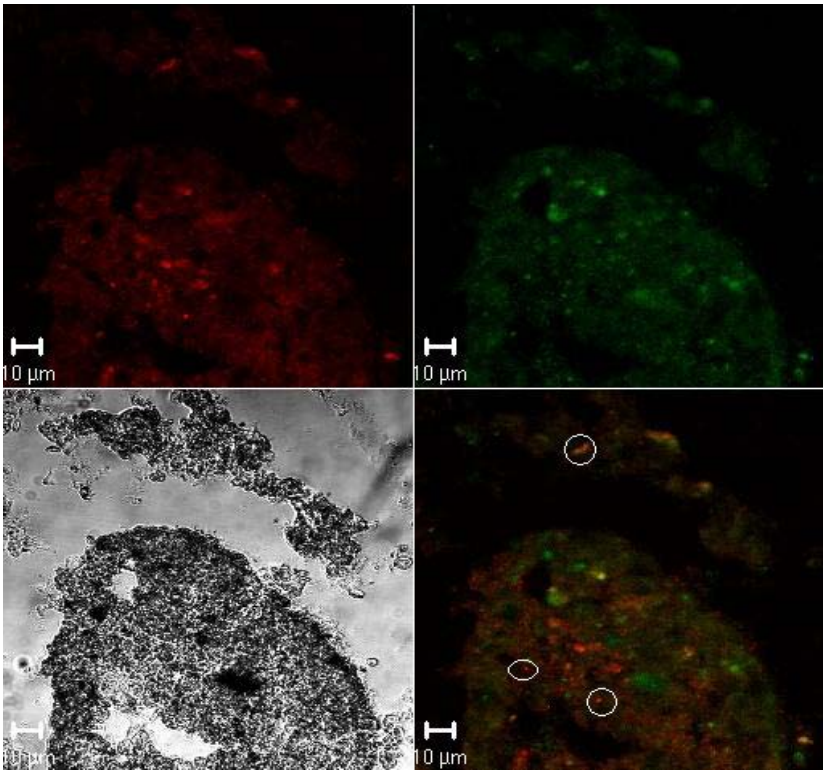
Table 2. *P* values from LIBSHUFF by comparing different clone libraries [a]

Homologous library X	Homologous library Y			
	Predigested sediment	Anterior section	Middle section	Posterior section
Predigested sediment	-	0.001*	0.001*	0.001*
Anterior section	0.001*	-	0.782	0.854
Middle section	0.001*	0.572	-	0.152
Posterior section	0.001*	0.009	0.004*	-

[a] When comparing multiple libraries, a LIBSHUFF *p*-value $\leqslant$ the critical value for any individual pairwise comparison insures that at least one of the libraries is different with $P = 0.05$. This critical value is calculated from the relationship: $P = 1-(1-a)^{k(k-1)}$, where p is the experiment-wise p-value of 0.05, a is the critical p-value, and k is the number of libraries. In present study, a LIBSHUFF comparison of four libraries yields $a = 0.0043$. For each pairwise comparison, if the lower of the two P-values calculated by LIBSHUFF is *less than or equal to* the critical P-value, the result indicates a significant difference in the composition of the communities sampled by each library.



(A)



(B)

Figure 5. Confocal laser micrographs of anammox bacteria in different gut sections contents of *N. glandicincta* and their predigested sediment by FISH analysis using the probes: S-*-Amx-0820 -A-18 (labeled with Cy3, *red*) and probe Eub338 (labeled with FAM, *green*). **(A)**: gut contents; **(B)**: predigested sediment.

structure in intestinal tract is quietly complex due to high diversity of bacteria and archaea, which included most of the phylogenetic phyla of microbes[60,61]. In 2003, the first indications for the presence of planctomycetes in the intestinal tract of soil-feeding termites were provided by a 16S-rRNA-based investigation of the gut microbiota in the hindgut of *Cubitermes ugandensis*[62], and recently one new study also reported that the hindgut of soil-feeding *Cubitermes* species contains the highest densities of planctomycetes ever reported for natural environments, and distributions of planctomycetes were deepened on niche heterogeneity of intestinal tracts of termites[63]. However, until now all the planctomycetes found in the gastrointestinal environment were far away from the anammox bacteria based on the phylogenetic analysis.

In present study, results of 16S rRNA gene sequences analysis and FISH provide evidences the existence of anammox bacteria in the whole gut of polychaete *N. glandicincta*, which further proof the conclusion that anammox bacteria are ubiquitous in natural environment.

Based on the phylogenetic of 16S rRNA gene sequences, at least 3 different phylotypes of anammox bacteria existed in the gut of *N. glandicincta* though all these anammox bacteria phylotypes are belonged to the genus of "Scalindua" group. However, compared with the surround environment -predigested sediment of *N. glandicincta*, only two phylotypes were similar with that of predigested sediments, and the third phylotype of anammox bacteria found in the gut is unique, which are more close to Candidatus *Scalindua wagneri* (AY254882.1). Combining the results of SEM, where it shows no sequences in clone libraries have originated from microorganisms attached to the surface of the animal, it is clear that Cluster 4 anammox bacteria are specific for the gut of polychaete *N. glandicincta*, while other anammox bacteria are similar with that of predigested sediment. The result indicates that the part of anammox bacteria in the gut of polychaete might come from the environment through the digestion; however, the novel anammox group might also exist in the gut of *N. glandicincta*, which might indicate the niche heterogeneity determines anammox bacteria community structure.

Furthermore, the axial distributions of anammox bacteria in different gut sections of *N. glandicincta* were also investigated in present study. The results show that anammox were quite unanimous in different gut section, and no specific gut section anammox bacteria were found. Comparison of 16S rRNA gene with these in different gut section of *N. glandicincta* also shows that there are no significance differences among the three gut sections, while there are significance differences of anammox bacteria 16S rRNA gene between the gut of *N. glandicincta* and their predigested sediment. However, composition of different phylogenetic group are quite different, might indicate the gradient change of physicochemical characteristic in the gut of polychaete.

In conclusion, this study firstly provides evidences for the existence of anammox bacteria in the gut of polychaete *N. glandicincta*, and one novel anammox bacteria are found, which is closely related to the species *Candidatus* Scalindua wagneri (AY254882). However, the activity and function of these novel anammox bacteria are still unclear, and further experiments are needed to carry out in order to understanding the actions of anammox bacteria in the gut of polychaete and whether their existence is related to the nutrition utilize of animal.

Conflict of Interest and Funding

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