Enrichment of Type I Methanotrophs with *nirS* Genes of Three Emergent Macrophytes in a Eutrophic Wetland in China

Ju-Mei Liu1,4, Zhi-Hua Bao2,3*, Wei-Wei Cao2, Jing-Jing Han2, Jun Zhao2, Zhen-Zhong Kang2, Li-Xin Wang2, and Ji Zhao2,3*

1College of Life Sciences, Inner Mongolia University, Hohhot, 010021, China; 2Ministry of Education Key Laboratory of Ecology and Resource Use of the Mongolian Plateau & Inner Mongolia Key Laboratory of Grassland Ecology, School of Ecology and Environment, Inner Mongolia University, Hohhot, 010021, China; 3Inner Mongolia Key Laboratory of Environmental Pollution Control & Waste Resource Reuse, Inner Mongolia University, Hohhot, 010021, China; and 4College of Chemistry and Environmental Engineering, Chongqing Key Laboratory of Environmental Materials & Remediation Technologies, Chongqing University of Arts and Sciences, Chongqing, 402160, China

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The *pmoA* gene, encoding particulate methane monoxygenase in methanotrophs, and *nirS* and *nirK* genes, encoding bacterial nitrite reductases, were examined in the root and rhizosphere sediment of three common emergent macrophytes (*Phragmites australis*, *Typha angustifolia*, and *Scirpus triqueter*) and unvegetated sediment from eutrophic Wuliangsuhai Lake in China. Sequencing analyses indicated that 334 out of 351 cloned *pmoA* sequences were phylogenetically the most closely related to type I methanotrophs (*Gammaproteobacteria*), and *Methylomonas denitrificans*-like organisms accounted for 44.4% of the total community. In addition, 244 out of 250 cloned *nirS* gene sequences belonged to type I methanotrophs, and 31.2% of *nirS* genes were the most closely related to paddy rice soil clone SP-2-12 in *Methylomonas* of the total community. Three genera of type I methanotrophs, *Methylomonas*, *Methylobacter*, and *Methylolylum*, were common in both *pmoA* and *nirS* clone libraries in each sample. A quantitative PCR (qPCR) analysis demonstrated that the copy numbers of the *nirS* and *nirK* genes were significantly higher in rhizosphere sediments than in unvegetated sediments in *P. australis* and *T. angustifolia* plants. In the same sample, the *nirS* gene copy number was significantly higher than that of *nirK*. Furthermore, type I methanotrophs were localized in the root tissues according to catalyzed reporter deposition-fluorescence in *situ* hybridization (CARD-FISH). Thus, *nirS*-carrying type I methanotrophs were enriched in macrophyte root and rhizosphere sediment and are expected to play important roles in carbon/nitrogen cycles in a eutrophic wetland.

**Key words**: emergent macrophytes, root-associated Type I methanotrophs, denitrifiers, eutrophic wetland, CARD-FISH
biofilms in membrane biofilm reactors. Stein and Klotz, (2011) inferred a genome inventory for the denitrifying pathway in methanotrophic isolates. Furthermore, the denitrification activity of individual type I methanotrophic isolates was reported by Kits et al. (2015a; 2015b). Thus, aerobic methanotrophs employ two processes for denitrification: (i) two strains (i.e. Methy lokomonas denitrificans strain FJG1 and Methy locibacter album strain BG8) perform direct denitrification under anoxic conditions in single cells (Kits et al., 2015a; 2015b); and (ii) aerobic methanotrophs and denitrifiers, which form consortia, also utilize methane as an external carbon source for denitrification via the aerobic methan e oxidation coupled to denitrification (AME-D) process (Zhu et al., 2016). In both cases, aerobic methano trophs appear to provide electron donors to the denitrifica tion reaction. Although type I methanotrophs have been detected as the dominant group on aquatic plant surfaces, the genetic potential for the denitrification of methanotrophs in natural wetlands/ecosystems has not yet been examined in detail.

Denitrification is a beneficial process in the eutrophica tion aquatic system. Conventional primers for nirS and nirK genes (encoding nitrite reductase enzymes) that have some limitations in detecting diverse nir/S/nirK-type environmental denitrifiers have been reported. The Methy lokomonas, Methy lobacter, Methy losarcina, Methylocibacter, and Methyloalam genera of Type I methanotrophs possess functional denitrification genes, including nirS or nirK (Stein and Klotz, 2011; Padilla et al., 2017). Methanotrophic denitrifiers from paddy soil have been detected using primers, including the primer set nirSC2F/nirSC2R (Wei et al., 2015). These primers are expected to promote the detection of methanotrophic denitrifiers in wetland ecosystems, including macrophytes.

Wuliangsuhai (WLSH) Lake is located near the city of Bayannur in the Inner Mongolia Autonomous Region in China. This lake is the largest freshwater lake in the Yellow River watershed; it is a rare, large-scale, shallow lake and grass wetland in this arid region. It has important ecological functions that maintain water resources, regulate the floods and droughts associated with an arid climate, and provide high biological diversity as a Ramsar site (Borruso et al., 2017). The lake recently became eutrophic after receiving large volumes of irrigation drainage water as well as municipal and industrial wastewater with high nitrogen and phosphorus contents from the Hetao Basin (Wu et al., 2017). Phragmites australis (common reed), Typha angustifolia (narrow leaf cattail), and Scirpus triqueter (bulrush) are the dominant macrophytes of WLSH (Duan et al., 2005). Many studies have examined microorganisms in their rhizosphere sediments, including the methylothroph- and heterothroph-mediated processes of carbon and other element cycles (Borruso et al., 2017). However, few studies have focused on the characteristics of methanotrophic communities in the roots of macrophytes or on denitrification by aerobic methanotrophs themselves in natural wetlands.

In the present study, we (i) examined the abundance and diversity of methanotrophs and denitrifying bacteria in the root zones of three emergent macrophytes, P. australis, T. angustifolia, and S. triqueter, as well as plant-free sediment from WLSH, a eutrophic wetland; (ii) identified which methanotroph groups had genetic potential for denitrification; and (iii) clarified the localization of methanotrophs in the root tissues of these three macrophytes.

Materials and Methods

Macrophyte roots, sediment, and water sampling and an analysis of physicochemical properties

Three plants each of P. australis (PA), T. angustifolia (TA), and S. triqueter (ST) were collected along with soil blocks from the littoral wetland of WLSH Lake (latitude 40°47′–41°03′N, longitude 108°43′–108°57′E) during the growing season on July 15th, 2015 (Fig. 1). Plant roots and rhizosphere sediments were sampled according to a previous study (Kimura, 2004). Briefly, after the plants with soil blocks were sampled, soil blocks including plants were divided vertically into two equal parts to collect the roots. Some of the exposed roots were carefully picked from the plants using sterilized forceps and placed into a 50-mL centrifuge tube containing sterile water. Rhizosphere sediment samples were obtained before and after sonication (5–10 min). After the rhizosphere sediment was removed, the root samples were transferred to new centrifuge tubes (50 mL) containing sterile water and centrifuged at 8,000×g at 4°C for 10 min. All samples were stored at −80°C for later molecular analyses.

Unvegetated sediment (S) was sampled in triplicate from depths of 10–20 cm along with water from non-vegetated areas (Fig. 1). Sediment cores were packed in sterile plastic bags and transported immediately to the laboratory in an ice-cooled box. Sediment samples were air-dried at room temperature and passed through a 2-mm sieve for the analysis of physicochemical properties, including pH, electrical conductivity (EC), total organic carbon (TOC), total nitrogen (TN), total phosphorus (TP), nitrate nitrogen (NO3−-N), and ammonium nitrogen (NH4+-N); the same properties were measured in water samples. Soil pH was assessed using a pH meter (HQ40D; Hach) in a 1:2.5 (w/v) suspension in ultrapure water. EC was measured in a soil-water suspension (1:2.5 (w/v)) using a conductivity meter (Leici DDS-307), and this was used to evaluate salinity (Wollenhaupt et al., 1986). TOC was measured by dichromate oxidation. TN was measured using the Kjeldahl method. TP was measured using the ammonium molybdate spectrophotometric method. NO3−-N content was assessed by UV spectrophotometry, and NH4+-N content was measured by indophenol blue colorimetry (Nelson and Sommers, 1982; Bao, 2000).

Nucleic acid extraction

Genomic DNA was extracted from 0.5–0.8 g of sediment or root samples using the Fast DNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer’s protocol. Regarding root samples, frozen tissues were ground into a powder using a mortar and pestle under liquid nitrogen before DNA extraction. DNA concentrations were quantified using a NanoPhotometer P-Class P-330C instrument (IMPLEN). Extracted DNA was immediately stored at −20°C.

Clone library construction and phylogenetic analysis

Clone libraries were constructed for roots and sediments using the primer sets A189F/mbo661R for the pmotA gene (Costello and Lidstrom, 1999) and nirSC2F/nirSC2R for the nirS gene (Wei et al., 2015) (Table S3). PCR amplification was performed with 50-μL mixtures in 0.2-mL tubes using a Mastercycler (Eppendorf). The reaction mixtures included 5.0 μL 10× PCR buffer (plus Mg2+), 4.0 μL 250 mM each dNTP, 0.4 μL 5 U μL−1 Ex Taq DNA polymerase (TaKaRa Biotech) plus 1.0 μL containing 10 mM of each primer and 1.0 μL bovine serum albumin (BSA; Amesco; 20 mg mL−1). BSA was added to reduce inhibition by humic substances (Kreader, 1996). Amplification was performed with an ini-
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Fig. 1. Sampling site diagram of three macrophytes and unvegetated sediment from the Wuliangsuhai wetland.

tial denaturation step at 95°C for 3 min followed by 35 cycles at
95°C for 1 min, 55°C for 1 min, and 72°C for 40 s (for \textit{pmoA}), or
at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 56°C
for 30 s, and 72°C for 30 s (for \textit{nirS}), with a final extension at
72°C for 10 min for both procedures. Regarding each plant spe‐
cies, three PCR products were mixed at equal molar ratios, and
these formed a composite PCR product. Composite PCR products
were then purified using a Gene JET PCR Purification Kit
(Promega) and ligated into the pGEM-T Easy Vector (Promega)
according to the manufacturer’s instructions. All clones were
sequenced by the Sanger method. Nucleic acid sequences were
translated with MEGA software, version 5.2.2 (Tamura
et al., 2011). After alignment, amino acid sequences were clustered into
operational taxonomic units (OTUs) at ≥91% amino acid identity
for the \textit{pmoA} gene (Heyer et al., 2002) or ≥90% amino acid iden‐
tity for the \textit{nirS} gene using mothur software (Schloss
et al., 2009).

Diversity indices (e.g. coverage, Chao1, ACE, Shannon, and
Simpson indices) were estimated by mothur. Representative
sequences for each OTU were selected by mothur software by
default, and these sequences were the most abundant within each
OTU. Phylogenetic analyses of the representative sequences for
each OTU were performed using MEGA with the neighbor-joining
method. Bootstrap values were based on 1,000 replications.

Quantification of \textit{pmoA}, \textit{nirS}, and \textit{nirK} genes

qPCR assays were performed in 96-well polypropylene plates on
a CFX Connect Optical Real-Time Detection System (Bio-Rad
Laboratories). Regarding each plant species, DNA samples from
three plants, serving as three biological replicates, were used for
qPCR of the target genes. Three technical replicates were run for
each DNA sample. qPCR reactions were performed in 20-μL reac‐
tion mixtures containing 10 μL 2x SYBR Premix Ex Taq (Takara
Biotech), 500 nM of each primer, and 10–20 ng DNA templates.
Blanks included sterile ultrapure water as the template instead of
extracted sample DNA. Details on the primers used and reaction
conditions for qPCR assays are provided in Table S3. We used the
methanotroph-specific primer set A189F/mb661R for the \textit{pmoA}
gene (Costello and Lidstrom, 1999), the denitrifying bacteria pri‐
mer set nirSC2F/nirSC2R for the \textit{nirS} gene (Wei et al., 2015), and
the primer set F1aCu/R3Cu for the \textit{nirK} gene (Hallin and
Lindgren, 1999). All primers were purchased from Sangon Bio‐
tech.

Standard curves for qPCR assays were generated as described
previously (Cai et al., 2016) and constructed using a 10-fold dilu‐
tion series of plasmids harboring the target gene. Plasmids were
extracted using the TIANprep Mini Plasmid Kit (Tiangen Biotech);
concentrations were measured using a NanoPhotometer P-Class
P-330C (IMPLEN) and used to calculate standard copy numbers.
Amplification efficiency ranged between 93.1 and 97.0% for
\textit{pmoA}, 94.0 and 96.3% for \textit{nirS}, and 90.2 and 93.2% \textit{nirK}. \(R^2\)
values for standard curves ranged between 0.996 and 1.000 for the
three genes. A melting curve analysis was used to confirm the spe‐
cific amplification of target genes and always showed a single
peak.

\textbf{CARD-FISH}

Catalyzed reporter deposition-fluorescence \textit{in situ} hybridization
(CARD-FISH) is a powerful tool in modern microbial ecology and
has strong signal sensitivity (Kubota, 2013). It has been used to
directly detect methanotrophs in paddy soil and field-grown rice
roots (Bao et al., 2014a; Cai et al., 2016). *M. koyamae* strain Fw12E-Y¹ (NCIMB14606) was used as a positive control. This strain was incubated in 1a medium at 30°C with 50% (v/v) methane in the headspace (Ogiso et al., 2012). *Thiobacillus thioxidans* (JCM 3867) was used as a negative control (Eller et al., 2001).

To detect type I methanotrophs in the root tissues of PA, TA, and ST, we used probes for *MY84* (3′-AGCCCGGACTGCTACC-5′) and *MY705* (3′-CTAGACTTCTGTGTCGTC-5′) (Eller et al., 2001). These probes were labeled with horseradish peroxidase at the 5′ end (Japan Bio Services). Plant roots were sectioned into 2-μm-thick pieces to visualize the location of type I methanotrophs. CARD-FISH analyses were performed as previously described (Bao et al., 2014a), with minor modifications. Briefly, endogenous peroxidases were inactivated with 1.2% H₂O₂ in methanol at room temperature (RT) for 60 min instead of 0.15% H₂O₂ in methanol for 30 min as described previously.

We used an epifluorescence microscope (Ci-L; Nikon) for microscopic observations and image acquisition. To visualize *M. koyamae* strain Fw12E-Y¹, we counterstained samples with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg mL⁻¹) at RT for 2 min. We viewed *MY84*+*MY705*-positive cells in the roots using a laser-scanning confocal microscope (LSM 710; Carl Zeiss) and ZEN 2012 software (Carl Zeiss).

**Statistical analysis**

We tested for significant differences in *pmoA*, *nirS*, and *nirK* gene numbers by a one-way ANOVA with the least significant difference (LSD) test. We used Pearson’s linear correlation to assess whether a correlation exists between *pmoA* and *nirS* gene copies. All statistical analyses were conducted using SPSS software, version 19.0 (IBM), and the significance level was *P* < 0.05. We applied the chi-squared test to examine the significance of differences in the proportion of OTU or bacteria taxa between PA plants and TA or ST plants. We used Canoco software, version 4.5 (Ithaca) for the principal component analysis (PCA).

**Nucleotide sequence accession numbers**

All cloned sequences were deposited in the GenBank (http://www.ncbi.nlm.nih.gov/BankIt/) nucleotide sequence database under accession numbers MG016967–MG017151 (roots), MG017152–MG017207 (unvegetated sediment), and MG017208–MG017213 (rhizosphere sediments) for the *pmoA* gene, and MG017217–MG016823 (roots), MG016724–MG016726, MG016728–MG016752, MG016754–MG016757, and MG016760 (unvegetated sediment), and MG016861–MG016966 (rhizosphere sediments) for the *nirS* gene.

**Results**

**Physicochemical properties of WLSH Lake**

Samples were collected from WLSH Lake in the Inner Mongolia Autonomous Region of China (Fig. 1). The properties of the water and sediment from WLSH Lake are shown in Table S1. The lake had saline-alkaline conditions, with the pH and EC of the water and sediment ranging between 9.0 and 9.3 and 1.35 and 6.25 ds m⁻¹, respectively. The high concentrations of TOC (up to 27.62 mg L⁻¹ in water and 0.19 mg L⁻¹ in water and 24.45 mg kg⁻¹ dry weight in sediment) and nitrate (up to 0.30 mg L⁻¹ in water and 2.60 mg kg⁻¹ dry weight in sediment), respectively, were found in the root zone of both PA (3.3–7.9%) and ST (0–11.9%), but not detected in the unvegetated sediment of PA (0%) (Fig. 3e).

The phylogenetic analysis revealed that the relative abundance of *Methylomonas* among all samples ranged between 23.3 and 82.4%. *Methylomonas* was clearly dominant over *Methylobacter* (1.6–23.3%) and *Methylomonas* (0–3.3%) (Fig. 3a). The relative abundance of *Methylococcus* in roots (1.8–6.4%) was lower than that in all sediments (including rhizosphere sediments and unvegetated sediment) (6.9–20.0%). Notably, the abundance of *Methylloglobulus* was higher in the root zone of PA (38.0–47.6%) than in those of TA (10.9–13.3%) and ST (0–1.5%) (Fig. 3c). In contrast, *Methylocystis* was mainly responsible for the abundance of type II methanotrophs in the root zones of both TA (3.3–7.9%) and ST (0–11.9%), but was not detected in the root zones of PA (0%) (Fig. 3e).

**Phylogenetic diversities of methanotrophs based on the pmoA gene**

Three hundred and fifty-one *pmoA* sequences were used to identify aerobic methanotroph diversity in the root zones of the PA, TA, and ST plants as well as plant-free sediment. Basic information regarding the *pmoA* clone library is also shown in Table S2. A phylogenetic analysis of all sequences revealed that the majority of sequences belonged to the type I methanotrophs of seven genera: *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylglobulus*, and *Methylococcus* (334 sequences, 95.2%), with type II methanotrophs accounting for a minority (17 sequences, 4.8%) (Fig. 2). Further analyses at finer levels showed that the relative abundance of *Methylomonas* among all samples ranged between 23.3 and 82.4%. *Methylomonas* was clearly dominant over *Methylobacter* (1.6–23.3%) and *Methylomonas* (0–3.3%) (Fig. 3a). The relative abundance of *Methylococcus* in roots (1.8–6.4%) was lower than that in all sediments (including rhizosphere sediments and unvegetated sediment) (6.9–20.0%). Notably, the abundance of *Methylloglobulus* was higher in the root zone of PA (38.0–47.6%) than in those of TA (10.9–13.3%) and ST (0–1.5%) (Fig. 3c). In contrast, *Methylocystis* was mainly responsible for the abundance of type II methanotrophs in the root zones of both TA (3.3–7.9%) and ST (0–11.9%), but was not detected in the root zones of PA (0%) (Fig. 3e).

The phylogenetic analysis revealed that the relative abundance of OTU STRS65 was higher in ST than in PA, TA, and unvegetated sediment. In contrast, OTU TAR109 was only absent in ST (Fig. 3b). These two OTUs were similar to *M. denitrificans* strain FJG1 (100% sequence identity) (WP036280011) and *Methylobacter luteus* (95% sequence identity) (WP027159170), respectively (Fig. 2). The relative abundance of OTU TAR107 among all samples ranged between 1.6 and 6.7%; however, it was absent in the rhizosphere sediment of PA. The relative abundance of OTU PAR101 was higher in PA (34.5–44.4%) than in TA and unvegetated sediment (5.5–14.3%). Moreover, the relative abundance of OTU PAR53 was higher in the four sediment samples (Fig. 3d). The representative sequences of these three OTUs showed higher levels of identity than those of *M. alcaliphilum* (99%) (WP01414702), *Methylloglobulus morosus* (96%) (WP023494957), and *Methylococcus capsulatus* (95%) (WP010961050) (Fig. 2). The representative sequence of OTU STR122 was closely related to *Methylocystis parvus* strain OBBP (AAA87219), with 96% identity; this sequence was detected in TA and ST, but not in PA (Fig. 2 and 3f).

**Relationship of phylogenetic diversities between methanotrophs and denitrifiers based on pmoA and nirS genes**

Two hundred and fifty nirS sequences were used to identify denitrifier diversity in the root zones of PA, TA, and ST plants as well as plant-free sediment. Basic information on the *nirS* clone library is also shown in Table S2. The phylogenetic analysis revealed that three OTUs (STR7, PAR53, and PAR37) were abundant in all samples in the *nirS* gene
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Fig. 2. Phylogenetic tree of representative OTUs (91% amino acid identity) based on translated pmoA gene sequences from roots and rhizosphere sediments of three plants and unvegetated sediment (PA, Phragmites australis; TA, Typha angustifolia; ST, Scirpus triqueter; R, root; RS, rhizosphere sediment; S, unvegetated sediment). The numbers in parentheses for each OTU indicate the number of clones out of the total number of clones. Gray shading indicates the main OTUs for roots and sediments.

To elucidate the relationship between methanotrophs and denitrifiers, we compared these two phylogenetic trees based on the pmoA and nirS genes. The results obtained showed that the three genera, Methylomonas, Methylobacter, and Methylovulum, were frequently detected in both the pmoA and nirS clone libraries (Fig. 5a and b).

We further calculated the relative abundance of the three most common genera in the pmoA and nirS clone libraries (Fig. 6a and b). Methylomonas (34.5–82.4% in the pmoA clone library, 57.1–100% in the nirS library) was predominant in the PA and ST root zone samples. In the TA root zone and unvegetated sediment samples, Methylomonas (23.3–58.2% in the pmoA clone library, 30.3–41.7% in the nirS library) and Methylobacter (12.7–25.0% in the pmoA clone library, 7.9–18.5% in the nirS library) were both dominant. In ST root zone samples, Methylobacter was less frequently detected in both clone libraries. Methylovulum (12.7–25.0% in the pmoA clone library, 7.9–18.5% in the nirS library) was detected at smaller numbers than Methylomonas and Methylobacter. Overall, clones of the pmoA and nirS genes belonging to Methylomonas, Methylobacter, and Methylovulum of type I methanotrophs were shared by the PA, TA, and ST plants and plant-free sediment. Thus, the relative abundance of Methylomonas slightly increased from sediment to rhizosphere sediment and root, whereas that of Methylobacter decreased.

The results of PCA clearly showed a tight cluster of community structures between root and rhizosphere sediment for the same plant, whereas distinct separation was observed among the different plant taxa. Differences among plant species were more obvious than those between microhabitats (roots versus the rhizosphere) (Fig. S1a and b).

Copy numbers of pmoA, nirS, and nirK genes

To estimate the population sizes of methanotrophs and denitrifying bacteria, we performed qPCR assays with the root and rhizosphere sediment of PA, TA, and ST as well as samples of unvegetated sediment. The copy numbers (×10^7 g^-1 dry weight) of the pmoA, nirS, and nirK genes in root and sediment samples of the three plant species were calculated, and were significantly higher in rhizosphere sediment than in unvegetated sediment, except for the pmoA gene in PA and TA plants (P<0.05) (Table 1). Moreover, in comparisons of both nir genes in the root zones of the three plant species and in unvegetated sediment, the copy numbers of the nirS gene were significantly higher than those of the nirK gene (P<0.05) (Table 1). Furthermore, Pearson’s linear correlation showed that the copy numbers of the pmoA gene positively correlated with those of the nirS gene among seven type samples (r=0.741, P<0.01, n=21). In addition, the copy numbers of both the pmoA and nirS genes...
Fig. 3. Phylogenetic compositions of pmoA gene clone libraries from roots and rhizosphere sediments of three plants and unvegetated sediments (PA, Phragmites australis; TA, Typha angustifolia; ST, Scirpus triqueter; RS, rhizosphere sediment; S, unvegetated sediment). (a, c, and e) Phylogenetic compositions at the genus level. (b, d, and f) Relative abundance of the main operational taxonomic units (OTUs) that occurred in the pmoA gene clone libraries. a and b: Methylomonas, Methylobacter and Methylovulum within type I methanotrophs, which were also frequently detected in the nirS gene clone libraries (Fig. 4); c and d: relative abundance of other type I methanotrophs, which were absent in the nirS gene clone libraries (Fig. 4); e and f: Type II methanotrophs, which were absent in the nirS gene clone libraries (Fig. 4). The abundance of each OTU (defined by ≥91% identity) corresponds to the data in Fig. 2. ** indicates a significant difference between PA plants and AT or ST plants at \(P<0.05\), calculated with the chi-squared test.

Fig. 4. Phylogenetic tree of representative OTUs (90% amino acid identity) based on translated nirS gene clone sequences from roots and rhizosphere sediments of three plants and the unvegetated sediments. The table lists the relative abundance of clones belonging to each OTU and the results of a BLAST search using representative sequences. The numbers in parentheses for each OTU indicate the number of clones out of the total clones. Gray shading indicates the main OTUs for roots and sediments.
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Fig. 5. Phylogenetic relationships between \textit{pmoA} and \textit{nirS} gene clones. Phylogenetic trees were constructed based on translated (a) \textit{pmoA} (91% amino acid identity) and (b) \textit{nirS} (90%) gene clone sequences from the roots and rhizosphere sediments of three plants and unvegetated sediment using the neighbor-joining method. The numbers in parentheses for each OTU indicate the number of clones out of the total clones. The accession numbers of the reference strains in the GenBank database are indicated in brackets. The bootstrap values (≥50%) from 1,000 replicates are indicated next to the branches.

Methylomonas (red), Methylobacter (green), and Methylovulum (blue) were commonly detected in both the \textit{pmoA} and \textit{nirS} gene clone libraries from all samples. Bold typeface indicates the main OTUs.

Fig. 6. Relative abundance of three common genera of \textit{Methylomonas}, \textit{Methylobacter}, and \textit{Methylovulum} based on \textit{pmoA} (A) and \textit{nirS} (B) gene clone libraries in roots and rhizosphere sediment of three plants and the unvegetated sediment. Bold typeface indicates the main OTUs.

in the roots were higher in PA than in ST and TA plants (Table 1). Combined with the sequencing analysis, these results suggest that the three emergent macrophytes acted selectively on methanotrophs and methanotrophic denitrifiers, particularly \textit{Methylomonas}, \textit{Methylobacter}, and \textit{Methylovulum} of the type I methanotrophs that were enriched in the root zone. Furthermore, the abundance, diversities, and community structures of both groups of microbes varied among plant species.
Localization of type I methanotrophs in roots of three plant species

To validate our probe, dye, and method, we used type I methanotroph strain *M. koyamae* Fw12E-Y\(^7\) (NCIMB14606) as a positive control. Clear signals showed that type I methanotrophs in 1a medium were successfully visualized by CARD-FISH (Fig. S2). These signals were not detected from strain *T. thiiooxidans*, which was used as a negative control (data not shown).

We applied CARD-FISH to the root tissues of the three plant species to identify the localization of type I methanotrophs. Using this method, signals for type I methanotrophs were clearly observed not only in the vascular cylinder (Fig. 7a, b, c, j, k, l, m, n, and o), but also around the aerenchyma (Fig. 7d, e, and f) of PA and TA root tissues. CARD-FISH signals were observed in epithelial cells in the vertical sections of ST root tissues (Fig. 7g, h, and i).

**Discussion**

Previous studies demonstrated that the abundance of *pmoA* differed among various genotypes of the rice plant and also that type I methanotrophs were abundant in the roots of rice plants, most likely due to root selection (Wu et al., 2009; Bao et al., 2014b). In addition, type I methanotrophs were found to be more abundant on the surfaces of aquatic plants than type II methanotrophs (Yoshida et al., 2014), which is consistent with the present results (Fig. 2 and Table 1). Moreover, many environmental factors, such as pH and the concentration of NH\(_4^+\), influence the community structure of methanotrophs. Molecular evidence has indicated that type I methanotrophs are predominant in the alkaline bodies of water and sediment (Deng et al., 2017). This phenomenon was attributed to the finding that some groups of type I methanotrophs were more likely to reside in saline-alkaline environments (Deng et al., 2017). A previous study suggested that an increase in NH\(_4^+\) exerted positive effects on type I members (Mohanty et al., 2006; Yang et al., 2016), whereas type II members were dominant under nitrogen-limited conditions (Graham et al., 1993). WLSH Lake, the location of this study, is a eutrophic alkaline wetland with rising salinity (Table S1), and, thus, may favor the predominance of type I methanotrophs in the root zone of wetland-grown macrophytes (Fig. 2).

*nirS* gene clones were frequently detected as *Methylomonas*, *Methylobacter*, and *Methylovulum* (Fig. 5a and b). These results support previous findings indicating that *M. denitrificans*, *M. methanica*, *M. lenta*, *M. koyamae*, *M. luteus*, and *M. miyakonense* possess functional denitrification genes, including *nirS* (Ren et al., 2000; Hoefman et al., 2014; Padilla et al., 2017). However, *Methyloglobulus*, *Methylococcus*, and *Methylmicrobium* were not detected with the *nirS* primer because these methanotrophs do not possess the *nirS* gene (Padilla et al., 2017) or they were present, but below the level of detection in plant roots.

A distinct separation was found in the community structures of denitrifying methanotrophs among the PA, TA, and ST plants based on PCA (Fig. S1), which is consistent with previous findings showing that plant species affect the microbial community structure in the rhizosphere (Berg and Smalla, 2009). Furthermore, the abundance of *nirS* genes showed that genetic potential was an effective indicator for potential denitrification activity in the roots, and the expression of these genes differed between wetland plant species (Hallin et al., 2015). These differences may be due to differences in root exudates, radial oxygen loss (ROL) rates, and oxygen availability among plant species, which may affect the rhizobacterial community composition and abundance (Calhoun and King, 1997; Wang et al., 2018).

Methane oxidation by aerobic methanotrophs generally occurs in aerobic environments; however, anaerobic and microaerobic conditions are required for denitrification. Rahalkar et al. (2009) reported that type I methanotrophs outnumbered type II methanotrophs in anoxic lake sediment by a factor of at least four. Type I methanotrophs were found to be more abundant than type II methanotrophs in the root tissues of rice plants, and this difference was more likely to be related to oxygen concentrations (Wu et al., 2009). Coincidently, the same finding was reported for the response of pure cultures of type I and II methanotrophs to oxygen concentrations in culture experiments (Graham et al., 1993). In the present study, signals from type I methanotrophs were visualized *in situ* in roots using CARD-FISH, as well as in the epidermal cell walls of *S. triqueter* and around the vascular cylinders of *P. australis* and *T. angustifolia* (Fig. 7). However, Armstrong et al. (2010) found that oxygen concentrations at the epidermal/hypodermal cylinder (2 kPa) were lower than those in the root center (>12 kPa) in emergent macrophytes, such as *P. australis*. Kits et al. (2015b) reported that methane-dependent *M. denitrificans* strain FJG1 simultaneously performed methane oxidation and denitrification under hypoxia (O\(_2\) concentration of 1.5%). Therefore, denitrification and CH\(_4\) oxidation may
Fig. 7. Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) detection of type I methanotrophs in roots of three macrophytes by confocal laser scanning microscopy. a–c, Cross-sections of the stele of a PA root; d–f, cross-sections of the aerenchyma of a PA root; g–i, vertical sections of the ST root; j–o, cross-sections of the stele of a TA root. a–i (×40); j–l (×20); m and n (×63); o (×100); a, d, g, and j: autofluorescence of the cell wall of roots (blue); b, e, h, and k: Alexa Fluor 488 of the Mγ705+Mγ84 probe (green); c, f, i, l, m, n, and o: their overlay. White arrows indicate the detected type I methanotrophs. (PA, Phragmites australis; TA, Typha angustifolia; ST, Scirpus triqueter).
mainly occur in the epidermis rather than in the vascular cylinder of the roots in type I methanotrophs. To elucidate the underlying mechanisms in more detail, a metagenomic and/or metaproteomic analysis of root-associated microorganisms is needed in order to clarify whether type I methanotrophs play an important role in methane oxidation and denitrification. Furthermore, it will be crucial to characterize the physiological activities of *Methylomonas*, *Methylobacter*, and *Methylovulum* isolates from wetland plants in the future.

In summary, we herein provide the first dataset for the abundance, diversity, and localization of root-associated methanotrophs and denitrifiers in three common emergent macrophytes (*P. australis*, *T. angustifolia*, and *S. triqueter*) in an important eutrophic wetland in northern China. The numbers of *nirS* and *nirK* gene copies were higher in rhizosphere sediment than in unvegetated sediment, suggesting that the presence of macrophytes increased the abundance of both groups of microbes over their levels in plant-free sediments. The abundance and diversities of both groups of microbes varied among plant species, with maximum numbers in *P. australis*, suggesting that macrophyte species had some influence on methanotroph numbers and diversity. The results of the present study emphasized that plant roots were more likely to be enriched with type I methanotrophic denitrifiers, including *Methylomonas*, *Methylobacter*, and *Methylovulum*, which most likely inhabit the epidermal cells, aerenchyma, and vascular bundles of root tissues of three emergent macrophytes due to root selection and environmental selection via excessive nitrogen input and saline-alkaline conditions. The present results suggest that root zone type I methanotrophic denitrifiers are of great importance for simultaneously mediating methane emission and nitrogen removal in vegetated wetlands.

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