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ORIGINAL ARTICLE

T4-type bacteriophage communities estimated from the major capsid genes (*g23*) in manganese nodules in Japanese paddy fields

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Abstract

The present study compared the capsid gene (*g23*) of T4-type bacteriophages (phages) in Mn nodules with those in the plow layer soil and subsoils of two Japanese paddy fields by applying the primers MZIA1bis and MZIA6 to DNA extracts from the nodules and soils. The deduced amino acid sequences of the *g23* genes in the Mn nodules were similar to those in the plow layer soil and in the subsoils. This result indicated that similar T4-type phage communities developed at these sites and that the diversity of T4-type phage communities was wide enough to cover those in the plow layer soil and in the subsoils. The majority of *g23* clones formed several clusters with the clones and phages obtained from far-apart paddy fields, and the sequences of two clones were completely identical to a phage and a clone from other paddy fields at the nucleotide or amino acid level, indicating horizontal transfer of *g23* genes between those paddy fields. A clone with a long nucleotide residue (686 bp) and a distribution remote from the other clones in the phylogenetic tree indicated that there were many uncharacterized, novel *g23* genes in the paddy fields.

Key words: bacteriophage community, *g23* gene, manganese nodule, paddy field, T4-type phage.

INTRODUCTION

The paddy field is a unique agro-ecosystem, where the soil is maintained under flooded conditions during most periods of rice cultivation. Flooding the field brings about a reduction in the plow layer soil, and Mn^{2+} ions produced in the plow layer are leached downwards by water percolation and accumulate at a specific soil depth in the subsoil, namely at the Mn illuvial horizon. These ions are ultimately oxidized to Mn oxides after the field is drained with the formation of spot-like manganiferrous mottles (Kyuma 2004). In a previous study, we revealed that the bacterial communities in Mn nodules in Japanese paddy fields are different from those in the plow layer soil and subsoils and there is a selective proliferation of bacteria relating to Mn reduction (Cahyani *et al.* 2007).

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Viruses are the most abundant biological entities on earth and many studies indicate the ecological importance of viruses in controlling microbial communities in the environment (Weinbauer 2004). Most viruses in aquatic environments, including the floodwaters of paddy fields are estimated to be bacteriophages (phages) (Nakayama *et al.* 2007a,b; Wommack and Colwell 2000), among which tailed phages dominate (Ackermann 2003; Breitbart *et al.* 2002, 2004). Tailed phages are classified as *Caudovirales*, and *Caudovirales* consists of three families, *Myoviridae, Siphoviridae* and *Podoviridae* (Fauquet *et al.* 2005). T4-type phages, important members of the *Myoviridae* family, are widely distributed in marine and soil environments (Filée *et al.* 2005; Fujii *et al.* 2008; Jia *et al.* 2007; Nakayama *et al.* 2008).

As phages cannot survive without bacterial hosts and the host specificity of phages is generally very strict, phage communities in Mn nodules are assumed to be different from those in the plow layer soil and in the subsoil (Cahyani *et al.* 2007). The present study aimed at evaluating the effect of a difference in bacterial communities on T4-type phage communities at various sites using a comparison of major capsid gene (*g23*) sequences.

MATERIALS AND METHODS

Manganese nodules

Manganese illuvial horizons were collected from two Japanese paddy fields that were located in the Kojima Bay reclamation area, Okayama Prefecture, on 16 March 2006 (Site A was at 34°34′N, 133°51′E and Site B was at 34°35′N, 133°54′E). In addition, subsoil samples apart from the Mn illuvial horizons were taken from the same fields as the reference samples. The soil properties at Sites A and B were as follows: (1) plow layer soils: total C content 16 and 17.1 g kg^{-1}; total N content 1.4 and 1.4 g kg^{-1}; pH (H_2O) 6.6 and 6.4; cation exchange capacity (CEC) 20.1 and 18.8 $cmol_c\ kg^{-1}$, respectively, (2) subsoils: total C content 5.6 and 12.8 g kg^{-1}; total N content 0.5 and 1.2 g kg^{-1}; pH (H_2O) 6.8 and 6.6; CEC 17.4 and 18.5 $cmol_c\ kg^{-1}$, respectively (Cahyani *et al*. 2007). In the laboratory, large and firm Mn nodules (several millimeters in diameter) were collected carefully with tweezers by breaking soil blocks into small pieces. The nodules were immersed into sterilized distilled water and sonicated for 1 min to dislodge any clinging soil particles from the nodules. This washing was repeated several times to obtain clean Mn nodules. The nodules were stored at –80°C until use. The Mn nodules from sites A and site B contained 31 and 42 g Mn kg^{-1} of soil and 22 and 38 g Fe kg^{-1} of soil, respectively (Cahyani *et al*. 2007).

DNA extraction and polymerase chain reaction amplification

The present study used identical DNA extracts from the Mn nodules, the plow layer soil and the subsoils to our previous study for a direct comparison between bacterial and phage communities (Cahyani *et al*. 2007). Collection of the Mn nodules and the DNA extraction method were described in detail in Cahyani *et al*. (2007).

DNA was extracted using a FastDNA SPIN Kit for Soil (BIO 101; Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocols. The DNA was eluted from the binding matrix with 100 μL of TE buffer (10 mmol L^{-1} Tris–HCl [pH 8.0], 1 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA]) and stored at –20°C. Three replicate DNA extractions were carried out for each sample.

The capsid genes (*g23*) of the T4-type phages were amplified with primers MZIA1bis and MZIA6 (Filée *et al*. 2005). The primers were without GC clamp because Fujii *et al*. (2008) observed better polymerase chain reaction (PCR) amplification without GC clamp than with it for soil DNA samples. The PCR was carried out using a TaKaRa PCR Thermal Cycles Model TP 240 (TaKaRa, Tokyo, Japan). The total volume of the reaction mixture was 50 μL and contained 0.4 μL of each primer (50 μmol L^{-1} of each), 5 μL of 2.5 mmol L^{-1} dNTP mixture, 5 μL of 10× Ex Taq buffer (20 mmol L^{-1} Mg^{2+}; TaKaRa), 1 μL of 0.1% bovine serum albumin (TaKaRa), 0.5 μL of Ex Taq DNA polymerase (TaKaRa), 1 μL of environmental DNA template and 36.7 μL of milli-Q water. The cycle conditions for the PCR amplification were as follows: one cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and one cycle of final extension at 72°C for 5 min. Three replicate DNA extracts from each sample were subjected to PCR amplification. The PCR products were purified using a QIA quick PCR Purification Kit (Qiagen, Tokyo, Japan).

Denaturing gradient gel electrophoresis analysis, direct sequencing and sequencing after cloning

The denaturing gradient gel electrophoresis (DGGE) was carried out by loading the PCR products onto an 80 g L^{-1} polyacrylamide gel with denaturing gradient ranging from 20 to 60% in an electrophoresis cell D-code System (Bio-Rad Laboratories, Hercules, CA, USA) with 1× TAE buffer (40 mmol L^{-1} Tris, 20 mmol L^{-1} acetic acid and 1 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA] at pH 8.0). The electrophoresis conditions for the DGGE analysis followed the study of Fujii *et al*. (2008) by running at 60°C and 150 V for 15 h. Visualization of the bands was achieved by staining with SYBR Green I nucleic acid gel stain (BMA, Rockland, ME, USA).

All recognizable DGGE bands were excised and amplified again with the same primers. As direct sequencing failed to be applied for the excised DGGE bands, cloning was carried out on those bands before sequencing. The cloning procedures were the same as those described in Cahyani *et al*. (2003). One positive clone from each transformation was chosen for sequence from the white colonies and checked for the correct insertion of the *g23* fragment by PCR using the primers MZIA1bis and MZIA6. The mobility of the insert was checked by DGGE from a comparison with the mobility of the excised DGGE band.

The isolated plasmids were used as templates for sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions; M13 primer RV and M13 primer M4 were used. After ethanol/EDTA precipitation, sequencing was run using an ABI 3130 Genetic Analyzer (Applied Biosystems).

Sequence analysis

Nucleotide sequences obtained from the DGGE band clones were translated to deduced amino acid sequences

using the EMBOSS Transeq program on the European Bioinformatics Institute website (http://www.ebi.ac.uk/emboss/transeq/index.html). Translated amino acid sequences were compared with the database of the National Center for Biotechnology Information ([NCBI] http://www.ncbi.nlm.nib.gov/) using a BLAST search.

Alignments of *g23* fragments in the present study were compared with those of T-evens, PseudoT-evens, SchizoT-evens, ExoT-evens and marine clones (Filée *et al*. 2005) to examine whether they were soil specific from their distribution in the phylogenetic tree. A phylogenetic tree of *g23* fragments was constructed that included the clones obtained in the present study and clones and phages from other paddy fields (Fujii *et al*. 2008; Jia *et al*. 2007; Nakayama *et al*. 2008). Alignment comparisons with other phylogenetic trees were carried out using Clustal 1.81 (Thompson *et al*. 1997). A neighbor-joining tree was constructed using Molecular Evolutionary Genetic Analysis software (MEGA 3.0) (Kumar *et al*. 2004) with 1000-fold bootstrap support. DNA sequences of partial *g23* genes were deposited in the DNA Data Bank of Japan ([DDBJ] http://www.ddbj.nig.ac.jp/Welcome-e.html) under accession numbers AB368379–AB368422.

RESULTS AND DISCUSSION

Denaturing gradient gel electrophoresis patterns of the *g23* fragments

There was no significant variation in the DGGE patterns among replicates, and the reproducibilities of both the PCR amplification and the DGGE separation were judged to be satisfactory for every sample (data not shown). In total, 14, 9 and 21 different bands were identified from the Mn nodules (from Sites A and B), the reference plow layer soil (from Site A) and the reference subsoils (from Sites A and B), respectively, based on the band positions in the gel (Fig. 1). There were several bands that were located at the same positions between the lanes of the Mn nodules, the plow layer soil and the subsoils. For example, bands PA6 and SB2, bands MnA2, MnB3, SA6 and SB4, bands MnB4, SA7 and SB5, bands MnA5, MnB5, SA8 and SB6, and bands SA10 and SB8 (Fig. 1).

There tended to be fewer DGGE bands in the Mn nodules than in the respective reference soils in Fig. 1; this result was in accordance with the finding of fewer bands in Mn nodules than in the reference soil samples for DGGE patterns of bacterial communities (Cahyani *et al*. 2007). However, this finding did not necessarily indicate reduced diversity of T4-type phages in Mn nodules because Fujii *et al*. (2008) observed the same mobility of *g23* fragments with different sequences and lengths. The DGGE band patterns of *g23* fragments cannot be reliably used to evaluate the diversity of the *g23* assembly. Therefore, we used DGGE in the present study not to evaluate the diversity of the phage communities but rather to obtain different *g23* clones efficiently because Jia *et al*. (2007) observed very low recovery of *g23* fragments in their clone libraries that were constructed from surface soil and rice straw samples with only 5.3–8.0% of clones containing correct *g23* inserts.

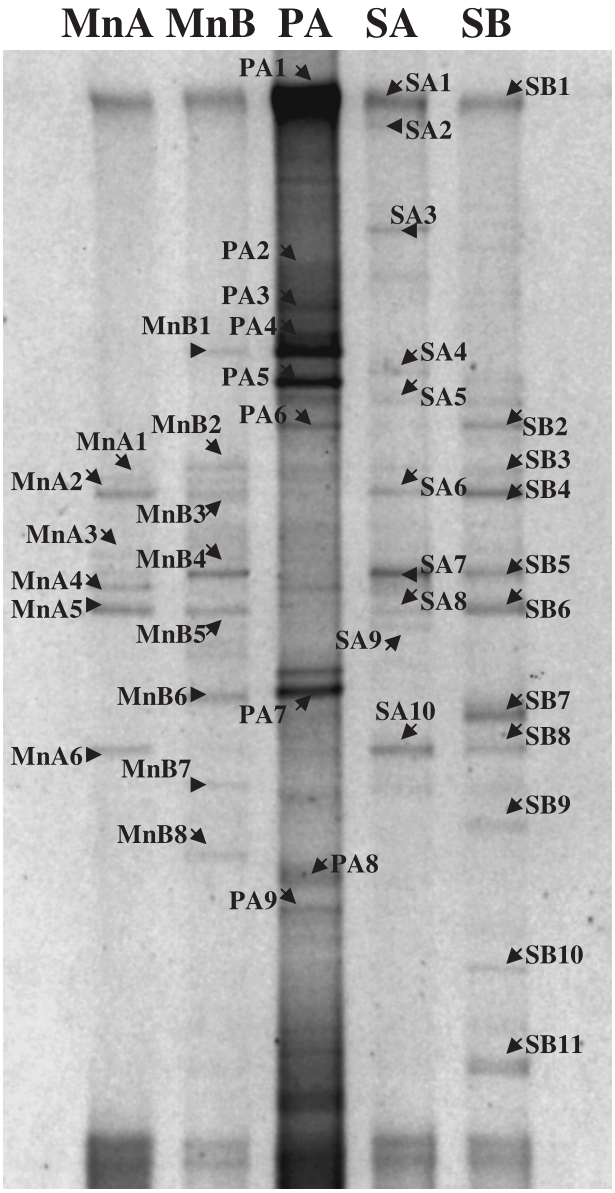

Figure 1 Denaturing gradient gel electrophoresis pattern of the *g23* gene fragments in the Mn nodules and reference soil samples from paddy field soils. MnA, Mn nodules at site A; MnB, Mn nodules at site B; PA, plow layer soil at site A; SA, subsoil at site A; SB, subsoil at site B.

Sequences of the *g23* fragments

All DGGE bands were successfully sequenced and aligned according to deduced amino acid sequences. As all of the bands had *g23*-specific conserved regions (amino acid sequences from 111 to 127 and from 261 to 304 in the coliphage T4 sequence; Parker *et al*. 1984), they were identified as *g23* clones. The number of deduced amino acid residues ranged from 124 to 172 for Mn nodules, from 116 to 147 for the plow layer soil and from 124 to 229 (124–160 except for a clone with 229 amino acid residue) for the subsoils. There was no marked difference in the distribution of *g23* fragment lengths between the Mn nodules and the reference soils. To the best of outr knowledge, the length of the 229 amino acid residue (SB7) is the longest ever recorded among phages and clones. The longest amino acid residue in the DDBJ database is 197 from *Aeromonas salmonicida* phage 44RR2.8t (NP932516) and enterobacterial phage RB49 (NP891732) belonging to PseudoT-evens. This finding might indicate that there are very novel *g23* genes in the soil environment.

Bands SA4 and SA5 had the same sequence at the nucleotide level, whereas bands MnB8 and SA10 and bands PA1 and SB10 had the same sequences at the amino acid level. However, the mobilities differed from each other between the pairs (Fig. 1), particularly between bands PA1 and SB10 in which only one nucleotide was different in a 383 nucleotide sequence.

Closest relatives of the *g23* fragments

The identity between the clones in the present study ranged from 28% (MnB3 and SB7) to 100% (SA4 and SA5) among all clones, from 42% (MnA4 and MnB6) to 99% (MnA6 and MnB8) among clones from the Mn nodules, and from 29% (SA3 and SB7) to 100% (SA4 and SA5) among clones from the references soils.

According to a BLAST search within the NCBI for the closest relatives at the amino acid level, all close relatives were clones or phages obtained from Japanese paddy fields with identities ranging from 54 to 100%, except for clones SB9 and SB11, which showed 63 and 61% identity, respectively, with clone 3758 (AAZ17592) obtained from the marine environment (Filée *et al*. 2005; Fujii *et al*. 2008; Jia *et al*. 2007; Nakayama *et al*. 2008). The lowest identity of 54% was observed for SB2, followed by SA3 (57%) and SB7 (58%). In contrast, MnA5 (AB368383) and SA8 (AB368409) showed 100% identity with *Novosphingobium* phage N-AfCm0615-3 (AB331966) and soil clone OmCm-Jl27-7 (AB300013) at the amino acid level and nucleotide level, respectively. The nucleotide sequence differed by only one nucleotide from each other in the total 371 bp between clones MnA5 and N-AfCm0615-3.

As the paddy fields in the present study were remote from those from which N-AfCm0615-3 (34°48′N, 137°30′E) and OmCm-Jl27-7 (39°49′N, 140°50′E) were obtained, this finding might indicate horizontal transfer of *g23* genes between these paddy fields. Nakayama *et al*. (2008) reported a similar phenomena and found that the *g23* sequences obtained from several phages were identical to those from clones in a paddy field far away.

Phylogenetic analysis of the *g23* clones in the Mn nodules

As shown in Fig. 2, the clones in the present study formed independent clusters from the clusters of enterophages and marine clones. No clone in the present study fell into the clusters of enterophage and marine members, and no enterophages or marine clones belonged to the clusters of soil clones in the present study, except for marine clone 3758. This result coincided with previous findings showing that *g23* sequences in paddy fields are unique and different from those of marine origins (Fujii *et al*. 2008; Jia *et al*. 2007; Nakayama *et al*. 2008).

From the detection of many specific *g23* sequences in the clones retrieved from a Japanese paddy field, Fujii *et al*. (2008) grouped soil-specific *g23* sequences into six novel groups, namely Paddy Groups I to VI. Figure 3 compared the sequences of *g23* amino acid residues found in the present study with those found in other paddy fields (Jia *et al*. 2007; Fujii *et al*. 2008; Nakayama *et al*. 2008). Many clones in the present study could be grouped into Paddy Groups I, II, III, V and VI; there were no clones belonging to Paddy Group IV. In addition, some clones in the present study formed independent clusters within the tree (designated as ungrouped), which indicated that they might be uncharacterized, novel *g23* genes specific to the paddy fields in our study. In particular, clone SB7, with the longest amino acid residue, was completely isolated from any clusters in Fig. 3, and it was estimated to be very novel in sequence.

All clusters to which the clones from the Mn nodules were distributed also included clones from the reference soils, which indicated that the T4-type phage communities in the Mn nodules were not phylogenetically different from those in the plow layer soil and in the subsoils. This result was in contrast to the result found for bacterial communities in Mn nodules, estimated from DGGE and sequencing analyses, in which the bacterial communities in the Mn nodules were different from those in the reference soils. Communities in the Mn nodules were dominated by bacteria related to Mn reduction (Cahyani *et al*. 2007). As phages cannot survive without bacterial hosts and the host specificity of phages is generally very strict, the unexpected similarity of the phage communities in the Mn nodules to

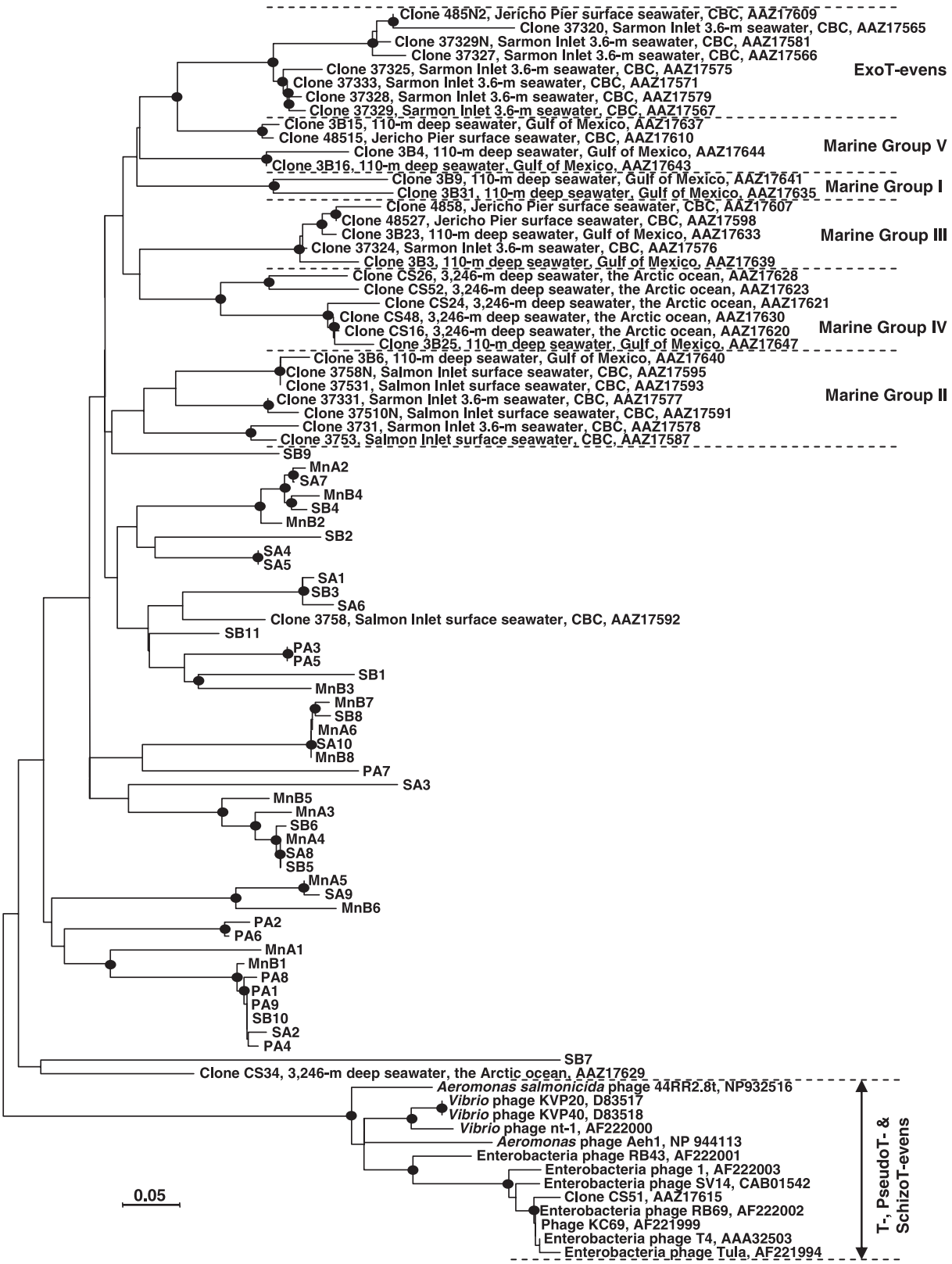

Figure 2 Neighbor-joining phylogenetic tree showing the relationships of *g23* amino acid sequences among clones in the present study, marine clones and enterophages (Filée *et al*. 2005). Filled circles indicate internal nodes with at least 50% bootstrap support. CBC, British Columbia. The scale bar represents the abundance of amino acid substitutions per residue.

Figure 3 Neighbor-joining phylogenetic tree of *g23* sequences showing the relationships of *g23* amino acid sequences among clones in the present study and phages and clones in other paddy fields (Fujii *et al*. 2008; Jia *et al*. 2007; Nakayama *et al*. 2008). Filled circles indicate internal nodes with at least 50% bootstrap support. The first letter of the isolated phage (N- and S-) indicates *Novosphingobium* and *Sphingomonas* hosts, respectively. The scale bar represents the abundance of amino acid substitutions per residue.

those in the reference soils is interesting, but rationally unexplainable at present.

In conclusion, the present study revealed that the T4-type phage communities estimated from *g23* sequences were similar to each other in the Mn nodules, plow layer soil and subsoils. This result was in contrast to the result for the bacterial communities, in which the communities in Mn nodules differed from those in the plow layer soil and subsoils. A clone with a very long nucleotide residue and with a distribution remote from any other soil clones and phages in the phylogenetic tree indicated that there are still many uncharacterized, novel *g23* sequences (T4-type phages) in paddy fields. In addition, the finding of a completely identical *g23* sequence to that obtained from a paddy field far away indicated horizontal transfer of *g23* genes between distant paddy fields.

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