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Broad Distribution and Phylogeny of Anaerobic Endophytes of Cluster XIVa Clostridia in Plant Species Including Crops

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Endophytic clostridia present on various plants as obligate anaerobes were surveyed by terminal restriction fragment length polymorphism (TRFLP) analysis specific to the clostridial 16S rRNA gene. Endophytic clostridia were detected in 10 plant types: sugarcane, cultivated rice, corn, tobacco, soybean, bermuda grass, tall fescue, and three mangrove species. Phylogenetically, cluster XIVa clostridia were detected more frequently than cluster I clostridia in aerial parts. Isolation of clostridia from surface-sterilized sugarcane stem validated the TRFLP results. Plant-derived clostridia occupied two unique phylogenetic positions (groups I and II) within cluster XIVa. Most of cluster XIVa clostridia from other sources (e.g., human, animal, and insect intestines) were located outside these groups. Thus two unique groups of cluster XIVa clostridia are widely distributed in plants, including crops. In field-grown soybeans, TRFLP analysis revealed clostridia only in a non-nodulating mutant. Ribosomal intergenic spacer analysis (RISA) showed that the bacterial community in soybean shoot depended partly on the soybean nodulation genotype.

Key words: *Clostridium*, cluster XIVa, endophyte, sugarcane, soybean

Endophytes are microorganisms that can colonize living plant tissues without causing any apparent damage to the host[3,23,24,35]. Several diazotrophs have been isolated and characterized as nitrogen-fixing endophytes in plants, including *Gluconacetobacter diazotrophicus*[3,22], *Herbaspirillum* spp.[9,33,38,48], and *Azoarcus* sp.[19,31]. Novel anaerobic endophytic clostridia and their anaerobic nitrogen-fixing consortium (ANFICO) were recently isolated and characterized in grasses during attempt to overcome a problem with culturing nitrogen-fixing microbes[26]. ANFICO consists of nitrogen-fixing clostridia and diverse non-diazotrophic bacteria and expresses nitrogen-fixing activity associated with low oxygen tension and interspecies inducers[26].

Phylogenetic analysis indicated that the endophytic clostridia fell exclusively into clusters XIVa and I, as defined by Collins *et al.*[8], and were further subdivided into five groups[26]. Miyamoto *et al.*[27] developed a terminal restriction fragment length polymorphism (TRFLP) detection system specific for these clusters and groups of plant clostridia, and demonstrated that the group II clostridia in cluster XIVa[8] dominated the populations of diazotrophs and clostridia in a gramineous grass, *Miscanthus sinensis*. Inoculation examinations indicate that endophytic clostridia colonize plant tissues, alleviating the damage caused by salt stress[47] and potentially fixing nitrogen in the plant[34]. Thus, more attention should be paid to the endophytic clostridia in terms of their distribution, phylogeny, and function.

Recently, cluster XIVa clostridia have been detected and isolated from human faeces[12,18,28], animal intestines[2,20,21,25,44], termite guts[40,41], soil[7,17,45], and plants[26,27] using culture-independent and culture-dependent methods. However, the relationships between the phylogenetic positions and niches of those clostridia within cluster XIVa that are associated with plants and other environments remain to be resolved.

The aims of this study were (I) to survey how frequently endophytic clostridia reside in various plants, (II) to address whether cluster XIVa clostridia are dominant as plant clostridia in species other than the gramineous grass *Miscanthus sinensis*, and (III) to elucidate the phylogenetic positions of cluster XIVa clostridia from plants. Thus, we collected 67 plant samples derived from 12 plant species, examined the existence of their clostridial endophytes by TRFLP analysis targeting the 16S rRNA gene, and constructed a phylogenetic tree based on 16S rRNA gene sequences of cluster XIVa clostridia derived from several sources, including the clostridial isolates in the present study.

Materials and Methods

Bacterial strains, media, cultivation, and counting

Clostridium sp. strains Sukashi-1, Kas107-2, Kas104-4, and Kas106-4 were used as standard strains of plant clostridia[26]. They were grown anaerobically in Viande-Levure (VL) medium at 30°C, as described previously[34]. Clostridial cultures were decimally diluted with sterile saline. The numbers of cells in the solutions were counted microscopically, as described previously[27]. Rennie medium supplemented with rice-extract (RMR)[26] was used to isolate clostridia.

Sample collection and plant cultivation

Cultivated crops or natural vegetation were surveyed for endophytic clostridia. The 67 plant samples included six families and 12 plant species (Table 1). Sugarcane, bermuda grass, and tall fescue

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Table 1. Plants used in this study

Family Plant[a]	Tissue[b]	Place[c]	Date
Gramineous			
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	L, S, SE, R	T	Oct 10, 2003
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	L, S, SE, R	T	Mar 2, 2004
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	SE	T	May 13, 2004
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	SO, SE, R	T	June 15, 2004
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	L, S, SE, R	T	July 23, 2004
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	L, S, SE, R	T	Aug 26, 2004
Sugarcane *Saccharum* hybrid sp. cv. NiF-8	S	T	Oct 14, 2004
Rice *Oryza sativa* cv. Koshihikari	L, S	F	Aug 13, 2004
Rice *Oryza sativa* cv. Sasanishiki	L, S, R	K	Aug 17, 2004
Corn *Zea mays*	L, S	F	Aug 13, 2004
Bermuda grass *Cynodon dactylon*	S, R	T	Mar 18, 2005
Tall fescue *Festuca arundinacea*	S, R	T	Mar 18, 2005
Solanaceous			
Tabaco *Nicotiana tabacum*	L, S	F	Aug 13, 2004
Leguminous			
Soybean *Glycine max.* cv. Enrei[d]	L, S, R	K	Aug 17, 2004
Soybean *Glycine max.* cv. Enrei[d]	R(2)	K	Aug 31, 2004
Soybean *Glycine max.* line En1282[e]	L, S, R	K	Aug 17, 2004
Rhizophoraceae			
Mangrove plant *Kandelia candel*	L, A(2), B	I	Jan 27, 2005
Mangrove plant *Rhizophora stylosa*	L, A(2), R, B, X	I	Jan 27, 2005
Mangrove plant *Bruguiera gymnorrhiza*	L, A(2), R, B, X	I	Jan 27, 2005
Vernenaceae			
Mangrove plant *Avicennia marina*	L, A, B	I	Jan 27, 2005
Sonneratiaceae			
Mangrove plant *Sonneratia* sp.	L, A, R, B, X, BU	I	Jan 27, 2005

[a] Plant names are expressed as the English, Genus, and Species names. Cultivars (cv.) and line are shown for sugarcane, rice, and soybean. [b] Tissue names are abbreviated as follows: L, leaf; S, stem; SE, seed stem; R, root; SO, shoot; A, aerial root; B, branch; X, xylem; BU, bud. Seed stem means progenitor sugarcane stem for propagation. Aerial root is a differentiated organ specific for mangrove plants. Numbers in parentheses are numbers of sample. [c] Sampling places are abbreviated as follows: T, a greenhouse in Tohoku University, Katahira-campus, Aoba-ku, Sendai, Miyagi, Japan; F, a farmer's field in Kitakata, Fukushima, Japan; K, the Kashimadai field station of Tohoku University, Kashimadai, Miyagi, Japan; I, the seashore of Iriomote island, Okinawa, Japan. [d] A soybean cultivar that nodulates with rhizobia. [e] A non-nodulating soybean mutant line derived from soybean cultivar Enrei[10].

were re-cultivated in a greenhouse (Katahira Campus, Tohoku University, Sendai, Japan) and then sampled. Sugarcane stems from an experimental field (Crops Lab, Miyako Branch, Okinawa Prefectural Agricultural Experiment Station, Japan) were transplanted into sterile vermiculite. The 20-day-old sugarcane plantlets with progenitor stems were then replanted into a commercial soil mixture. Bermuda grass was collected from natural stands at Kurume, Fukuoka, Japan, on 30 September 1997. Tall fescue was collected from natural stands at Fuji, Shizuoka, Japan, on 3 August 2004. These grasses had been cultivated in a mixture of Kuroboku soil and vermiculite at Mayekawa Mfg. Co. (Shizuoka, Japan). The grasses were cultivated in the greenhouse of Tohoku University as described for sugarcane.

Terminal restriction fragment length polymorphism (TRFLP) analysis

Plant materials were carefully washed with tap water and separated into parts (Table 1), such as leaf, stem, shoot, and root. Plant samples were cut with sterilized scissors, and the surface was peeled off with a sterilized razor to prevent contamination by epiphytic microorganisms. The resultant plant tissues were then macerated thoroughly in liquid nitrogen using a sterilized pestle and mortar, and stored at −80°C until DNA extraction.

DNA manipulations were performed using standard techniques[37]. TRFLP was carried out as described previously[27]. DNA was isolated directly from the plant tissues and clostrdial culture for DNA sequencing by use of a FastDNA kit and a FastDNA Spin kit for soil (BioSystems, Carlsbad, CA, USA) according to the manufacturer's instructions[27]. Primer sets C142f/C1090r and Cy5-C142f/C796r[27] were used to amplify the 16S rRNA gene of plant clostridia. The PCR product was purified by polyethylene glycol precipitation[27] and then digested with either 10 U of *Hae*III or *Msp*I at 37°C for 5 h. Aliquots (2 μL) of digested PCR products (terminal restriction fragments; TRFs) were electrophoresed in ALFexpress II DNA Analyzer (Amersham Pharmacia Biotech, New Jersey, USA).

Isolation of clostridial strains from sugarcane

Clostridia were isolated by using a method for selecting spore-forming anaerobes[6]. Segments (5 cm long) of sugarcane stems were strongly surface-sterilized with 70% ethanol for 10 s and 1% NaOCl for 5 min, macerated, and then inoculated into RMR semi-solid medium[27]. Full-growth cultures were heated at 70°C for 10 min and treated with 50% ethanol for 45 min, and then inoculated on RMR and VL agar plates as described previously[26,27]. An acetylene reduction assay in RMR semi-solid medium was conducted to evaluate nitrogen fixation by bacterial cultures[9].

DNA sequencing and phylogenetic analysis

A 16S rRNA gene (1,081–1,222 bp) and a partial *nifH* gene (380 bp) of the clostridial isolates were amplified and sequenced as previously described[34]. A sequence similarity search was performed via BLASTN[1] at the DNA Database of Japan (DDBJ). The phylogenetic analysis was performed using the CLUSTAL W program[39]. The phylogenetic tree was constructed by the neighbor-joining method[36] with 1,000 bootstrap replicates using default parameters. NJ Plot software[30] was used to display and analyze the tree.

Ribosomal intergenic spacer analysis (RISA)

The ribosomal intergenic spacer analysis (RISA) was carried out using modifications previously reported by Ikeda *et al.*[13,15]. Briefly, DNA was directly extracted using a FastDNA Spin kit for soil (Applied Biosystem, Carlsbad, CA, USA) with bead-beating to disrupt the microbial cells. The primer set was ITSF/ITSReub, which targeted the end of the 16S rRNA gene and the beginning of the 23S rRNA gene for bacterial RISA[5]. The PCR temperature program and gel electrophoresis were as previously described[15]. MM-CFTFL 50–1000 MapMarker (BioVentures, Inc. Murfreesboro, TN, USA) was used as a size marker. Following gel electrophoresis, digital images were obtained using a fluorescent scanner (FLA-2000, Fuji Photo Film, Tokyo, Japan). The cloning and sequencing of amplicons from the RISA profiles were carried out according to the method of Ikeda *et al.*[14]. All sequence data were aligned to a public database (DDBJ) using the BLASTN subroutine[1]. Sequence matches were considered significant when the score was >50.

Nucleotide sequence accession numbers

We deposited the DNA sequences in this work into DDBJ under the following accession numbers: AB275139–AB275142 (16S rRNA gene), AB279996–AB279999 (*nifH* gene), and AB276076–AB276082 (ribosomal intergenic spacer).

Results

Efficiency of clostridial detection

The efficiency of DNA extraction and sensitivity of semi-nested PCR for the TRFLP analysis were examined using known concentrations of four representatives of group I, II, IV, and V clostridia (Fig. 1)[26]. The presence of 10^3 to 10^4 cells in the extraction tube could be clearly visualized at approximately 650 bp, which corresponds to the size of the amplicon of the clostridial 16S rRNA gene obtained using semi-nested PCR primers[27]. It is likely that the PCR-based detection of group IV and V clostridia was slightly sensitive compared with that of group I and II clostridia. One hundred milligrams of plant material was introduced into the DNA extraction tube. Thus, this result indicated that endophytic clostridia were detected at rates of at least 10^4 to 10^5 cells g^{-1} plant fresh weight by semi-nested PCR for TRFLP analysis.

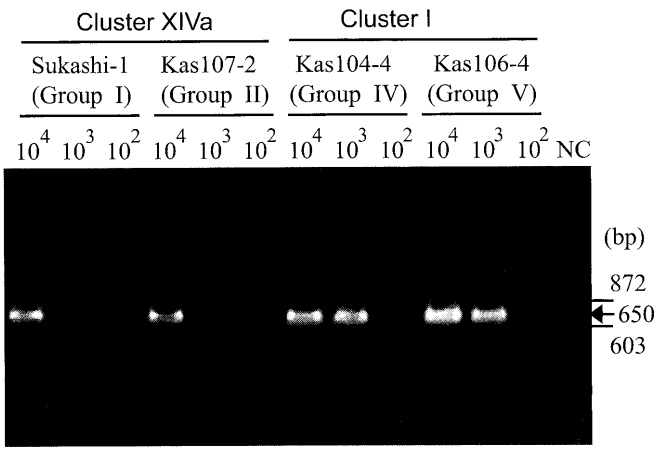

Fig. 1. Amplification of the 16S rRNA gene by semi-nested PCR using DNA extracted from free-living cells of *Clostridium* sp. Sukashi-1 (Group I), Kas107-2 (Group II), Kas104-4 (Group IV), and Kas106-4 (Group V). Values above lanes are cell number equivalents (10^2, 10^3, and 10^4) in the DNA extraction tube. Cell numbers were adjusted by direct counting under a microscope before DNA extraction. NC, negative control (PCR amplification without DNA template). The arrow shows the expected size of the PCR amplicon (650 bp).

Detection of endophytic clostridia by TRFLP analysis

Figure 2A is an electrogram of the TRFLP profiles of the *Msp*I-digested 16S rRNA gene amplified from DNA extracted from sugarcane stems on 2 March 2004 (Table 2). Two peaks appeared, corresponding to groups I and II of cluster XIVa clostridia[27], whereas no peak of cluster I clostridia appeared at all (Fig. 2A). The result of *Hae*III digestion was identical to that of *Msp*I-digestion (data not shown). The sample was therefore scored as having groups I and II of cluster XIVa clostridia.

When we analyzed all 67 plant samples from 12 plant species (Table 1) using TRFLP, clostridia of cluster XIVa or cluster I were detected in 26 samples derived from 10 plant species (*Saccharum* sp., *Oryza sativa*, *Zea mays*, *Cynodon dactylon*, *Festuca arundinacea*, *Nicotiana tabacum*, *Glycine*

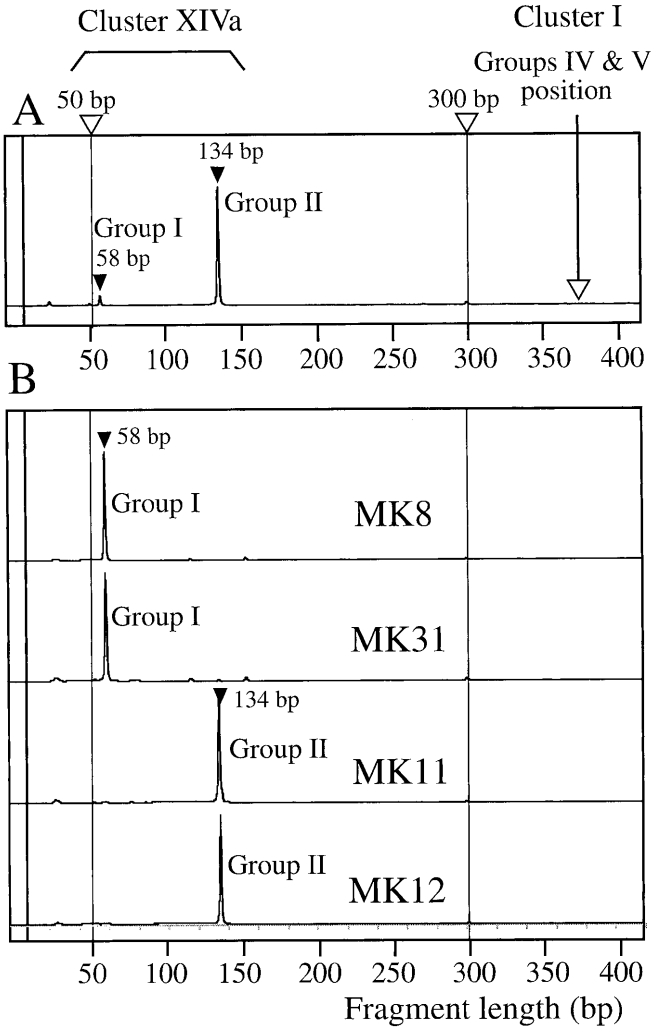

Fig. 2. TRFLP profiles of the *Msp*I-digested 16S rRNA gene amplified from DNA extract (A) and clostridial isolates (B) from sugarcane stems. The stems of sugarcane sampled on 2 March 2004 were used simultaneously for direct DNA extraction (A) and clostridial isolation (B). Clostridial groups I and II in cluster XIVa were identified by TRF sizes 58 and 134 bp[27]. Arrowheads indicate positions of internal size standards (50 and 300 bp).

Table 2. Detection of clostridia in various plants by TRFLP analysis targeting the 16S rRNA gene using DNA directly extracted from plant tissues

Family Plant[a]	Tissues	Date	Cluster XIVa[b] (Groups I and II)[c]	Cluster I[b] (Groups IV and V)[c]
Gramineous				
Sugarcane, *Saccharum* hybrid sp.	L	Oct 10, 2003	I	—[d]
Sugarcane, *Saccharum* hybrid sp.	L	Jul 23, 2004	I, II	—
Sugarcane, *Saccharum* hybrid sp.	S	Oct10, 2003	I	—
Sugarcane, *Saccharum* hybrid sp.	S	Mar 2, 2004	I, II	—
Sugarcane, *Saccharum* hybrid sp.	S	Jul 23, 2004	—	IV, V
Sugarcane, *Saccharum* hybrid sp.	S	Aug 26, 2004	II	—
Sugarcane, *Saccharum* hybrid sp.	S	Oct 14, 2004	I, II	—
Sugarcane, *Saccharum* hybrid sp.	SE	Jun 15, 2004	—	IV, V
Sugarcane, *Saccharum* hybrid sp.	SE	Jul 23, 2004	I, II	IV, V
Sugarcane, *Saccharum* hybrid sp.	SE	Aug 26, 2004	—	V
Sugarcane, *Saccharum* hybrid sp.	R	Jun 15, 2004	II	V
Rice, *Oryza sativa* cv. Koshihikari	L	Aug 13, 2004	II	—
Rice, *Oryza sativa* cv. Sasanishiki	L	Aug 17, 2004	II	—
Rice, *Oryza sativa* cv. Sasanishiki	S	Aug 17, 2004	I, II	V
Rice, *Oryza sativa* cv. Sasanishiki	R	Aug 17, 2004	I, II	IV, V
Corn, *Zea mays*	L	Aug 13, 2004	I, II	—
Corn, *Zea mays*	S	Aug 13, 2004	I	—
Bermuda grass, *Cynodon dactylon*	R	Mar 18, 2005	II	V
Tall fescue, *Festuca arundinacea*	S	Mar 18, 2005	—	V
Solanaceous				
Tabaco, *Nicotiana tabacum*	S	Aug 13, 2004	I, II	—
Leguminous				
Soybean, *Glycine max.* line En1282[e]	S	Aug 17, 2004	II	V
Soybean, *Glycine max.* line En1282[e]	R	Aug 17, 2004	II	—
Rhizophoraceae				
Mangrove plant, *Kandelia candel*	A	Jan 27, 2005	I	—
Mangrove plant, *Bruguiera gymnorrhiza*	R	Jan 27, 2005	I	—
Vernenaceae				
Mangrove plant, *Avicennia marina*	L	Jan 27, 2005	I	—
Mangrove plant, *Avicennia marina*	B	Jan 27, 2005	I	—

[a] Plant names are expressed as the English, Genus and Species names. Cultivars (cv.) and line are shown for rice and soybean. Sugarcane cultivar is NiF-8. Among 67 plant samples (Table 1), clostridia were detected in 26 samples. [b] Tissue names are abbreviated as follows: L, leaf; S, stem; SE, seed stem; R, root; A, aerial root; B, branch. Seed stem means progenitor sugarcane stem for propagation. Aerial root is a differentiated organ specific for mangrove plants. [b] Cluster XIVa and I as defined by Collins *et al.*[8]. [c] Groups I, II, IV, and V are based on phylogenetic analysis of the 16S rRNA gene of clostridia[26]. [d] "—" indicates that a clostridial TRF signal was not detected. [e] A non-nodulating plant mutant line derived from soybean cultivar Enrei[10].

max, *Kandelia candel*, *Bruguiera gymnorrhiza*, and *Avicennia marina*) (Table 2). In *G. max* (soybean), clostridia were detected exclusively in the non-nodulating line En1282 (Table 1, Table 2).

Incidence of cluster XIVa clostridia in plants

In a previous study[27], the group II clostridia in cluster XIVa[8] dominated the populations of diazotrophs and clostridia in the gramineous grass *Miscanthus sinensis*. Therefore, we calculated their incidence above the detection limit of approximately 10^4 to 10^5 cells g^{-1} fresh weight, as described above. Among the 26 samples in which clostridia had been detected, cluster XIVa clostridia were found in 22 samples (85%) from nine plant species, whereas cluster I clostridia were found in 10 samples (38%) from five plant species (Table 2). Thus, the overall incidence of cluster XIVa clostridia (85%) in all tissues tested was double that of cluster I clostridia (38%). In particular, among 17 samples of plant aerial parts, cluster XIVa clostridia were detected in 15 samples (88%) from six plant species, whereas cluster I clostridia were detected in four samples (24%) derived from three grass plant species and non-nodulating soybeans (Table 2). Thus, the cluster XIVa clostridia (88%) were detected more than three times as frequently as cluster I clostridia (24%) in the aerial parts of plants. These results suggest that cluster XIVa clostridia dominated the clostridial communities in these plants, in the same way as in the grass *M. sinensis*, and that they preferentially reside in the aerial parts, whereas the distribution of cluster I clostridia may be restricted mainly to grasses. In sugarcane, cluster XIVa clostridia were detected in spring (March), summer (June, July, and August), and autumn (October) (Table 2), whereas cluster I clostridia were more likely to be detected during the summer (June, July, and August) (Table 2).

Endophytic viability of cluster XIVa clostridia in sugarcane

To examine the viability of the cluster XIVa clostridia from plants, we isolated MK8, MK11, MK12, and MK31 from the strongly surface-sterilized sugarcane stem sampled on 2 March 2004 (Table 2). When total DNA prepared from each of the four isolates was subjected to TRFLP analysis, signals of cluster XIVa clostridia were detected on *Msp*I-

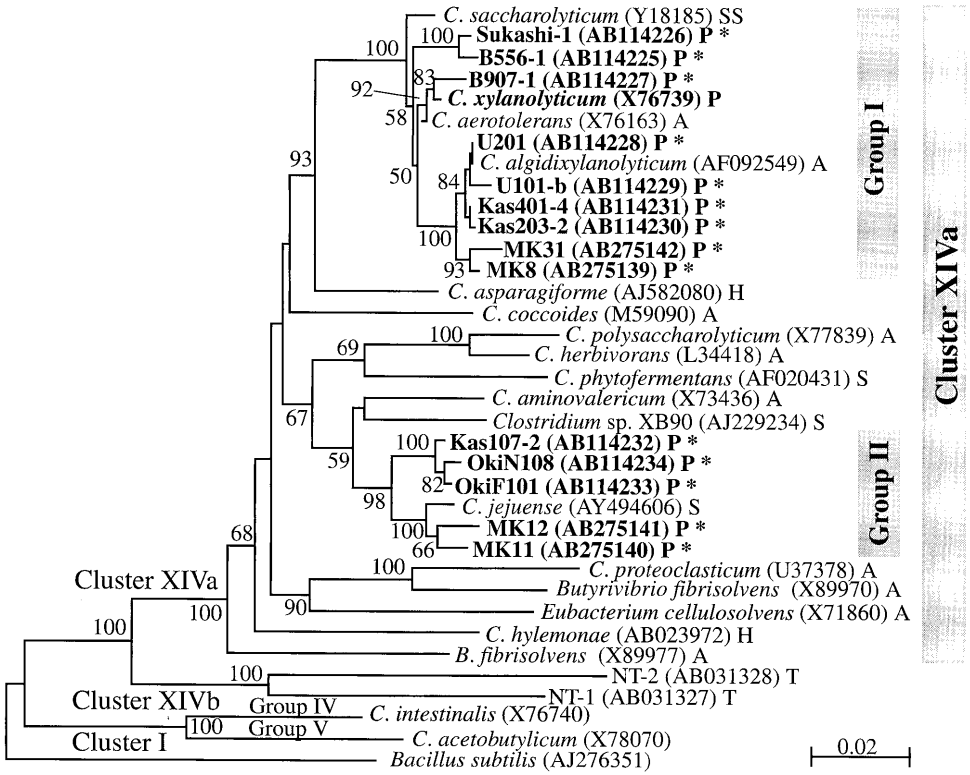

Fig. 3. Phylogenic tree of plant-derived culturable clostridia, with other representatives of culturable cluster XIVa clostridia, based on 16S rRNA gene sequences. The tree was constructed by the neighbor-joining method on the basis of more than 1,080 nt using 16S rRNA gene sequences of culturable clostridial isolates. Numbers at the nodes are bootstrap values, expressed as percentages of 1000 replications. Bootstrap values above 50% are shown for each node. Clusters XIVa, XIVb, and I are subdivisions of *Clostridium* and its neighbors, as defined by Collins *et al.*[8]. Groups I, II, IV, and V are based on phylogenetic analysis of the 16S rRNA gene of plant clostridia[26]. Plant-derived clostridia are shown in bold. Asterisks indicate nitrogen-fixing clostridia. Accession numbers of the 16S rRNA gene are shown in parentheses. Sources of clostridia are abbreviated as follows: P, plant; A, animal intestines, rumens, or raw lamb meat; SS, sewage sludge; H, human feces; S, soil; and T, termite guts. *Clostridium intestinalis* and *C. acetobutylicum* were used as representative sequences of group IV and V clostridia[26]. *Bacillus subtilis* was used as an outgroup sequence. The bar indicates the distance corresponding to a two-nucleotide change per 100 nucleotide positions.

digestion (Fig. 2B). Isolates MK8 and MK31 produced a TRF peak (58 bp) corresponding to group I, whereas isolates MK11 and MK12 showed another TRF peak (134 bp) corresponding to group II (Fig. 2B). The TRFLP profile of DNA directly extracted from sugarcane stems (Fig. 2A) was explained by these TRF peaks (Fig. 2B). When the full sequences of the 16S rRNA genes of these four isolates were determined, they indeed fell into group I or II of cluster XIVa clostridia (Fig. 3). The 16S rRNA genes were highly homologous (98%) among strains MK31, MK8 and *C. algidixylanolyticum* in group I. The 16S rRNA gene sequences of strains MK11, MK12 and *C. jejuense* also showed 97–98% homology in group II. However, the homology between these two groups was low (approximately 91%). These results confirm that cluster XIVa clostridia reside endophytically, at least in sugarcane stems, and support the validity of a TRFLP analysis for plant clostridia.

Acetylene-reducing activity of the isolates MK8, MK11, MK12, and MK31 was positively detected in semi-solid RMR cultures under anaerobic conditions (0.2–20 μmol C_2H_4 produced h^{-1} $tube^{-1}$). In addition, these isolates also carried *nifH* genes (AB279996–AB279999); functionally important residues, such as cysteine residues for iron-sulfur clusters, are conserved in the amino acid sequences encoded by these genes. The results indicate that these new clostridial isolates from sugarcane were nitrogen-fixing.

Phylogenetic analysis of cluster XIVa clostridia

The members of cluster XIVa include phenotypically and phylogenetically heterogeneous microorganisms, such as non-spore-forming cocci and aerotolerant rods[4]. In the last decade, cluster XIVa clostridia have been found in various environments (human feces, animal intestines, rumens, termite guts, soil, and plants), as described in the Introduction. Because most of these niches are relevant to animals and plants, one can expect that cluster XIVa clostridia might have adapted to lifestyles of symbiosis with higher organisms.

Thus, we performed a phylogenetic analysis of cluster XIVa clostridia by using a data set of the 16S rRNA gene sequences (1,201 nt) of cultivated clostridia that had originated from various sources (Fig. 3). The clostridia that appeared to have originated from plant materials were concentrated into two phylogenetic positions, which roughly corresponded to those of groups I and II, as described previously[26].

Among the 13 strains of group I, 10 were derived from plants: strains Sukashi-1, U201, U101-b, Kas401-4 and Kas203-2 from a wild grass (*M. sinensis*); strains B556-1 and B907-1 from wild rice[26]; strains MK31 and MK8 from sug-

arcane in the present study; and *Clostridium xylanolyticum* ATCC49623^T from wood chips[32]. The group II clostridia formed a compact cluster that included five plant-derived strains (Kas107-2 from *M. sinensis* and OkiN108, OkiF101, MK12, and MK11 from sugarcane), together with *Clostridium jejuense* strain HY-35-12^T from Korean soil[17].

Most of the cluster XIVa clostridia derived from animal and human intestines and soil were phylogenetically located outside groups I and II (Fig. 3). This was also true when a phylogenetic tree was constructed by using an additional data set of 16S rRNA gene clone sequences (617 nt) of cluster XIVa clostridia determined by culture-independent methods (data not shown).

RISA of soybean endophytes

When we performed a TRFLP analysis of nodulating and non-nodulating soybean plants cultivated under identical field conditions (Table 1), clostridia were detected only in the non-nodulating soybean mutant (*G. max* line En1282) (Table 2), not in nodulating soybean (*G. max* cv. Enrei). This result suggests that rhizobial nodulation or the nodulation genotype in host plant affects colonization by endophytic clostridia. Accordingly, we applied RISA to the shoots of soybean plants. Comparison of the RISA profiles of the nodulating and non-nodulating soybeans revealed several differences in the leaf and stem. Nodulating soybeans showed specific bands (bands A, B, C in Fig. 4), whose ITS sequences were homologous to those of *Micrococcus* sp. and *Ehrlichia* sp. (Table 3). The non-nodulating soybean mutant EN1282 showed specific bands (bands D, E, and F in Fig. 4), whose sequences were homologous to those of *Acetobacter* sp., *Ehrlichia* sp., and a rice phyllosphere bacterium, respectively (Table 3). These results suggested that the soybean nodulation genotype partly affects plant bacterial communities other than clostridia.

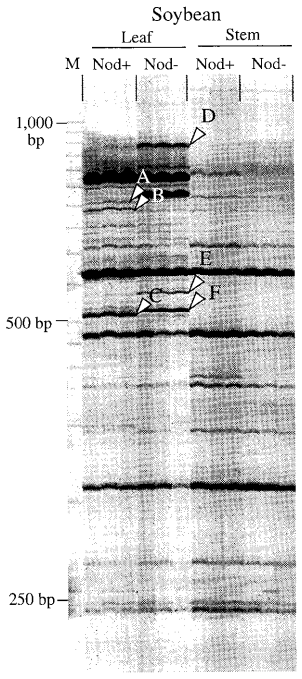

Fig. 4. RISA profiles showing microbial community structure in leaves and stems of soybean. In each treatment, three independent PCR products were applied using a single DNA template preparation. Leaves and stems of nodulating (Nod+) soybean (*Glycine max* cv. Enrei) and non-nodulating (Nod−) soybean (*Glycine max* line En1282) cultivated in an experimental field at Tohoku University. Leftmost numbers indicate marker fragment lengths. Upper case letters correspond to the clones shown in Table 3.

Discussion

Distribution of clostridia in plants

Previously, macerated dilutions of surface-sterilized *M. sinensis* were cultivated in a semi-solid medium and the clostridial populations evaluated by TRFLP analysis and most-probable-number (MPN) calculations[27]. In the present study, we applied a TRFLP analysis directly to DNA preparations extracted from plants. The direct TRFLP system allowed us to analyze large sets of samples efficiently with a detection threshold of around 10^4 to 10^5 bacterial cells g^{-1} plant fresh weight. Analysis of clostridial isolates from sugarcane supported the validity of this TRFLP analysis for plant clostridia (Fig. 2).

In our previous study[26], endophytic clostridia were isolated and characterized from nine plant species belonging to two families. The species were *O. sativa* (cultivated rice), *Oryza officinalis* (wild rice), *Oryza nivara* (wild rice), *Oryza ridleyi* (wild rice), *Oryza rufipogon* (wild rice), *M. sinensis* and *Saccharum spontaneum* (grasses), *Saccharum* hybrid sp. (sugarcane) of the Gramineae family, and *Polygonum sachalinense* of the Polygonaceae family. Therefore, in the present study, endophytic clostridia were newly detected in an additional eight plant species (*Z. mays*, *C. dactylon*, *F. arundinacea*, *N. tabacum*, *G. max*, *K. candel*, *B. gymnorrhiza*, and *A. marina*), which belong to four additional families (Solanaceae, Leguminosae, Rhizophoraceae, and Verbenaceae) (Table 2). These results indicate that endophytic clostridia are distributed in a wide range of taxonomic groups of plant species. In particular, it is surprising that agricultural crops such as rice, corn, tobacco, and soybean carried endophytic clostridia, just as did natural grasses.

Phylogeny of cluster XIVa clostridia in plants

The results of the TRFLP analysis indicated that groups I and II of cluster XIVa clostridia were frequently detected in the various plants tested (Table 2). Phylogenetic analysis revealed that the phylogenetic positions were generally correlated with the habitat of clostridia within cluster XIVa (Fig. 3). The broad distribution of groups I and II of cluster XIVa clostridia in diverse plants, as shown here, suggests that these groups might adapt to plant associations, although we can not specify the evolutionary history with higher organisms based only on the phylogenetic tree (Fig. 3).

Group I includes *C. xylanolyticum* from wood chips[32], *C. aerotolerans* from rumens[42], *Clostridium algidixylanolyticum* from raw lamb meat[4], and *C. saccharolyticum* from a methanogenic cellulose-enrichment culture of sewage sludge[29]. The first three strains are all able to degrade xylan, whereas *C. saccharolyticum* uses cellobiose. Because xylan is a component of the plant cell wall, it is possible that plant-deived group I clostridia have a capacity to degrade plant cell walls (e.g., in the form of xylanase) that allows them to sur-

Table 3. Organisms in soybean plants estimated by RISA[a]

Band[b]	Length (bp)	Closest relative	Blast		Putative classification
			Score	Expect	
A	635	*Micrococcus luteus*	84	$6e^{-13}$	*Actinobacteria; Micrococcaceae*
B	635	*Micrococcus luteus*	84	$6e^{-13}$	*Actinobacteria; Micrococcaceae*
C	472	*Ehrlichia chaffeensis*	341	$1e^{-90}$	*Alphaproteobacteria; Anaplasmataceae*
D*	817	*Acetobacter pasteurianus*	147	$6e^{-32}$	*Alphaproteobacteria; Acetobacteraceae*
E**	497	*Ehrlichia* sp.	80	$7e^{-12}$	*Alphaproteobacteria; Anaplasmataceae*
F**	470	Rice phyllosphere bacterium	216	$5e^{-53}$	*Actinobacteria; Micrococcaceae*

[a] Accession numbers of DNA sequence and closest relatives are AB276076 & AB088763 for band A, AB276077 & AB088763 for band B, AB276078 & AF000721 for band C, AB276079 & AM049398 for band D, AB276080 & AF000722 for band E, and AB276081 & AY485405 for band F, respectively. [b] The band name corresponds to that of the RISA profile in Fig. 5. The sequence was confirmed with two (*) or more than three (**) independent clones.

vive in these environments.

Functions of plant clostridia

Inoculation experiments with *Clostridium* sp. Kas 201-1 and Kas 107-1, which belong to cluster I, indicate that the bacterium colonizes plant tissue[47] and potentially fixes nitrogen in the plant[34]. Assays of acetylene reduction and the *nifH* gene showed that MK8, MK11, MK12, and MK31 newly isolated from sugarcane stems were nitrogen-fixing cluster XIVa clostridia (Fig. 3). The present study also showed that cluster XIVa clostridia were more widely distributed in various plants than were cluster I clostridia. Therefore, it would be worthwhile to reevaluate the nitrogen fixation in plants by cluster XIVa clostridia.

The presence of endophytic bacteria and fungi often increases salt-stress tolerance[43,47]. In particular, grass plants inoculated with clostridia show more tolerance than uninoculated plants[47]. Because mangrove plants and bermuda grass inhabit seashores and can adapt to high-salt environments, we collected and analyzed these plants. Group I clostridia were detected in only two samples (Table 2) among the 26 samples of mangrove plants (Table 1), although clostridia were found in the roots of Bermuda grass (Table 2). These results suggest that clostridia do not abundantly reside in, or endow salt-stress tolerance upon, naturally occurring mangrove plants.

Effect of soybean nodulation genotypes on bacterial community

Soybean is a legume crop symbiotically associated with rhizobia, such as *Bradyrhizobium japonicum*, which forms root nodules to fix nitrogen[16]. Non-nodulating soybean mutants have been constructed by chemical mutagenesis and cannot be infected by rhizobia[10,11,46]. In field-grown soybeans, clostridia were detected only in the non-nodulating mutant by TRFLP analysis (Tables 1 and 2). The results of RISA also showed that the community structure of endophytic bacteria partly differed among soybean nodulation genotypes, even in the same field environment (Fig. 4, Table 3). These results are interesting, because rhizobial nodulation or nodulation genotypes affected the community structure of endophytic bacteria.

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References

1) Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2) Attwood, G.T., K. Reilly, and B.K. Patel. 1996. *Clostridium proteoclasticum* sp. nov., a novel proteolytic bacterium from the bovine rumen. Int. J. Syst. Bacteriol. **46**:753–758.
3) Baldani, J.I., and V.L. Baldani. 2005. History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience. An. Acad. Bras. Cienc. **77**:549–579.
4) Broda, D.M., D.H. Saul, R.G. Bell, and D.R. Musgrave. 2000. *Clostridium algidixylanolyticum* sp. nov., a psychrotolerant, xylan-degrading, spore-forming bacterium. Int. J. Syst. Bacteriol. **50**:623–631.
5) Cardinale, M., L. Brusetti, P. Quatrini, S. Borin, A.M. Puglia, A. Rizzi, E. Zanardini, C. Sorlini, C. Corselli, and D. Daffonchio. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl. Environ. Microbiol. **70**:6147–6156.
6) Cato, E.P., W.L. George, and S.M. Finegold. 1986. Genus *Clostridium*, p. 1141–1200. *In* P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*, Vol. 2. Williams and Wilkins, Baltimore, USA.
7) Chin, K.J., D. Hahn, U. Hengstmann, W. Liesack, and P.H. Janssen. 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. **65**:5042–5049.
8) Collins, M.D., P.A. Lawson, A. Willems, J.J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J.A.E. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. **44**:812–826.
9) Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui, and K. Minamisawa. 2001. Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. **67**:5285–5293.
10) Francisco, P.B. JR., and S. Akao. 1993. Autoregulation and nitrate inhibition of nodule formation in soybean cv. Enrei and its nodulation mutants. J. Exp. Bot. **44**:547–553.
11) Gwata, E.T., D.S. Wofford, P.L. Pfahler, and K.J. Boote. 2003. Pollen morphology and in vitro germination characteristics of nodulating and nonnodulating soybean (*Glycine max* L.) genotypes. Theor. Appl.

Genet. **106**:837–839.
12) Hayashi, H., M. Sakamoto, M. Kitahara, and Y. Benno. 2003. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. Microbiol. Immunol. **47**:557–570.
13) Ikeda, S., D.M. Roberts, K.N. Watanabe, and N. Ytow. 2004. Microbial community analyses using a simple, rapid detection method for DNA fingerprints with a fluorescence scanner. J. Biosci. Bioeng. **98**:500–503.
14) Ikeda, S., T. Fujimura, and N. Ytow. 2005. Potential application of ribosomal intergenic spacer analysis to the microbial community analysis of agronomic products. J. Agric. Food. Chem. **53**:5604–5611.
15) Ikeda, S., S. Fuji, T. Sato, N. Ytow, H. Ezura, and K. Minamisawa. 2006. Community analysis of seed-associated microbes in forage crops using culture-independent methods. Microbes Environ. **21**:112–121.
16) Ito, N., M. Itakura, S. Eda, K. Saeki, H. Oomori, T. Yokoyama, T. Kaneko, S. Tabata, T. Oowada, S. Tajima, T. Uchiumi, E. Masai, M. Tsuda, H. Mitsui, and K. Minamisawa. 2006. Global gene expression in *Bradyrhizobium japonicum* cultured with vanillin, vanillate, 4-hydroxybenzoate, and protocatechuate. Microbes Environ. **21**:240–250.
17) Jeong, H., H. Yi, Y. Sekiguchi, M. Muramatsu, Y. Kamagata, and J. Chun. 2004. *Clostridium jejuense* sp. nov., isolated from soil. Int J Syst. Evol. Microbiol. **54**:1465–1468.
18) Kitahara, M., F. Takamine, T. Imamura, and Y. Benno. 2000. Assignment of *Eubacterium* sp. VPI 12708 and related strains with high bile acid 7alpha-dehydroxylating activity to *Clostridium scindens* and proposal of *Clostridium hylemonae* sp. nov., isolated from human faeces. Int. J. Syst. Evol. Microbiol. **50 Pt 3**:971–978.
19) Krause, A., A. Ramakumar, D. Bartels, *et al.* 2006. Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus* sp. strain BH72. Nature Biotech. **24**:1385–1391.
20) Krause, D.O., B.P. Dalrymple, W.J. Smith, R.I. Mackie, and C.S. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. Microbiology **145**:1797–1807.
21) Lan, P.T., M. Sakamoto, and Y. Benno. 2004. Effects of two probiotic *Lactobacillus* strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by molecular analysis of 16S rRNA genes. Microbiol. Immunol. **48**:917–929.
22) Madhaiyan, M., V.S. Saravanan, D.B. Jovi, H. Lee, R. Thenmozhi, K. Hari, and T. Sa. 2004. Occurrence of *Gluconacetobacter diazotrophicus* in tropical and subtropical plants of Western Ghats, India. Microbiol. Res. **159**:233–243.
23) Mano, H., F. Tanaka, A. Watanabe, H. Kaga, S. Okunishi and H. Morisaki. 2006. Culturable surface and endophytic bacterial flora of the maturing seeds of rice plants (*Oryza sativa*) cultivated in a paddy field. Microbes Environ. **21**:86–100.
24) Mano, H., F. Tanaka, C. Nakamura, H. Kaga and H. Morisaki. 2007. Culturable endophytic bacterial flora of the maturing leaves and roots of rice plants (*Oryza sativa*) cultivated in a paddy field. Microbes Environ. **22**:175–185.
25) Minamida, K., I.N. Sujaya, A. Tamura, N. Shigematsu, T. Sone, A. Yokota, K. Asano, Y. Benno, and F. Tomita. 2004. The effects of di-D-fructofuranose-1,2':2,3'-dianhydride (DFA III) administration on human intestinal microbiota. J. Biosci. Bioeng. **98**:244–250.
26) Minamisawa, K., K. Nishioka, T. Miyaki, B. Ye, T. Miyamoto, M. You, A. Saito, M. Saito, W.L. Barraquio, N. Teaumroong, T. Sein, and T. Sato. 2004. Anaerobic nitrogen-fixing consortia consisting of clostridia isolated from gramineous plants. Appl. Environ. Microbiol. **70**:3096–3102.
27) Miyamoto, T., M. Kawahara, and K. Minamisawa. 2004. Novel endophytic nitrogen-fixing clostridia from the grass *Miscanthus sinensis* as revealed by terminal restriction fragment length polymorphism analysis. Appl. Environ. Microbiol. **70**:6580–6586.
28) Mohan, R., P. Namsolleck, P.A. Lawson, M. Osterhoff, M.D. Collins, C.A. Alpert, and M. Blaut. 2006. *Clostridium asparagiforme* sp. nov., isolated from a human faecal sample. Syst. Appl. Microbiol. **29**:292–299.
29) Murray, W.D., A.W. Khan, and L. van den Berg. 1982. *Clostridium saccharolyticum* sp. nov., a saccharolytic species from sewage sludge. Int. J. Syst. Bacteriol. **54**:1465–1468.
30) Perriere, G., and M. Gouy. 1996. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie. **78**:364–369.
31) Reinhold-Hurek, B., T. Hurek, M. Gillis, B. Oste, M. Vancanneyt, K. Kersters, and J. De Ley. 1993. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with root of Kallar grass (*Leptochloa fusca* (L.) Kunth) and description of two species *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. **43**:574–584.
32) Rogers, G.M., and A.A.W. Baecker. 1991. *Clostridium xylanolyticum* sp. nov., an anaerobic xylanolytic bacterium from decayed *Pinus patula* wood chip. Int. J. Syst. Bacteriol. **37**:102–105.
33) Rothballer, M., M. Schmid, I. Klein, A. Gattinger, S. Grundmann, and A. Hartmann. 2006. *Herbaspirillum hiltneri* sp. nov., isolated from surface-sterilized wheat roots. Int. J. Syst. Evol. Microbiol. **56**:1341–1348.
34) Saito, A., and K. Minamisawa. 2006. Evaluation of the nitrogen-fixing ability of endophytic clostridia based on acetylene reduction and reverse transcription-PCR targeting the *nifH* transcript and ribosomal RNA. Microbes Environ. **21**:23–35.
35) Saito, A., S. Ikeda, H. Ezura and K. Minamisawa. 2007. Microbial community analysis of the phytosphere using culture-independent methodologies. Microbes Environ. **22**:93–105.
36) Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406–425.
37) Sambrook, J., and D.W. Russell. 2001. *Molecular Cloning: a Laboratory Manual*, Vol. 1 and Vol. 2 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.
38) Tanaka, K., T. Shimizu, M. Zakria, J. Njoloma, Y. Saeki, M. Sakai, T. Yamakawa, K. Minamisawa and S. Akao. 2006. Incorporation of a DNA sequence encoding green fluorescent protein (GFP) into endophytic diazotroph from sugarcane and sweet potato and the colonizing ability of these bacteria in brassica oleracea. Microbes Environ. **21**:122–128.
39) Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.
40) Thongaram, T., Y. Hongoh, S. Kosono, M. Ohkuma, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo. 2005. Comparison of bacterial communities in the alkaline gut segment among various species of higher termites. Extremophiles **9**:229–238.
41) Tokuda, G., I. Yamaoka, and H. Noda. 2000. Localization of symbiotic clostridia in the mixed segment of the termite *Nasutitermes takasagoensis* (Shiraki). Appl. Environ. Microbiol. **66**:2199–2207.
42) van Gylswyk. N.O., and J.J.T.K. van der Toorn. 1987. *Clostridium aerotolerans* sp. nov., a xylanolytic bacterium from corn stover and from the rumina of sheep fed corn stover. Int. J. Syst. Bacteriol. **37**:102–105.
43) Waller, F., B. Achatz, H. Baltruschat, *et al.* 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc. Natl. Acad. Sci. **102**:13386–13391.
44) Wang, X., S.P. Heazlewood, D.O. Krause, and T.H. Florin. 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. **95**:508–520.
45) Weber, S., S. Stubner, and R. Conrad. 2001. Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl. Environ. Microbiol. **67**:1318–1327.
46) Xie, Z.P., C. Staehelin, H. Vierheilig, A. Wiemken, S. Jabbouri, W.J. Broughton, R. Vogeli-Lange, and T. Boller. 1995. Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiol. **108**:1519–1525.
47) Ye, B., A. Saito, and K. Minamisawa. 2005. Effect of inoculation with anaerobic nitrogen-fixing consortium on salt tolerance of *Miscanthus sinensis*. Soil Sci. Plant Nutr. **51**:243–249.
48) Zakria, M., J. Njoloma, Y. Saeki and S. Akao. 2007. Colonization and nitrogen-fixing ability of *Herbaspirillum* sp. strain B501 *gfp*1 and assessment of its growth-promoting ability in cultivated rice. Microbes Environ. **22**:197–206.