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Full Length Research Paper

Expression of nitrogenase gene (*NIFH*) in roots and stems of rice, *Oryza sativa*, by endophytic nitrogen-fixing communities

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Putative endophytic nitrogen-fixing bacteria that actively express nitrogenase gene in rice were determined using reverse transcription-PCR (RT-PCR) technique. The amplified portion of dinitrogenase reductase genes (*nifH*) from mRNA of rice roots and stems were cloned and sequenced. Phylogenetic analysis of deduced amino acid sequences of isolated *nifH* clones (117 sequences) revealed that majority of these sequences, around 71.8% of all examined clones, formed a distinct and deeply branching assemblage. None of these sequences were closely related to any sequences from known diazotrophes extracted from GenBank/DDBJ databases (DNA data bank of Japan), although it gathered with *Geobacter sulfurreducens*. This novel set of strains was frequently recovered from stems of used cultivars more than the roots. The other expressed *nifH* sequences (around 28.2%) were either clustered with known members of division *Proteobacteria* or with different lineage of the known and unknown anaerobes and sulfur reducing bacteria. These results indicate that the active nitrogen-fixing community associated with rice was consisting mainly of uncultured organisms that are at least specific to rice ecosystem and yet uncharacterized.

Key words: *nifH* sequence, nitrogenase expression, phylogenetic tree, rice.

INTRODUCTION

Microbial communities are a main component of ecosystems that play critical roles in the biochemical transformations of elements including nitrogen fixation (Madigan et al., 2000). Therefore, nitrogen that is available to plants grown for many years without N fertilizers is considered to be due to biological fixation (Yoshida and Ancajas, 1973, and James, 2000). This process catalyzed by nitrogenase enzymes is essential for maintaining fertility in many ecosystems (Vitousek and Howarth, 1991). The ability to fix nitrogen is widely distributed among diverse groups of bacteria and archae, in different ecosystems. However, this distribution is non-random and depends on the habitat characteristics (Zehr et al., 2003).

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As the microorganisms in the ecosystems are not always culturable (Barer and Harwood, 1999), the DNA and more recently mRNA targeting techniques allow *in situ* analysis of microbial community structures and activities (Greer et al., 2001). PCR amplification and sequencing a segment of *nifH* gene from genomic DNA (*nif*-DNA) has been used to analyze the diazotroph community in different environments. It has been applied in marine (Zehr et al., 1998), soil (Widemer et al., 1999), termite gut (Ohkuma et al., 1999), fresh water (MacGregor et al., 2001) and roots of different rice cultivars (Ueda, 1995) and (Elbeltagy and Ando, 2005). But, *nif*-DNA only shows the communities that contain nitrogenase genes and does not indicate whether or not these nitrogenase genes are expressed and these communities are active and fix nitrogen. On the other hand, because of the tight relationship between nitrogenase activity and *nif*-mRNA transcription, studies on *nif*-mRNA can determine the community components involving in nitrogenase gene ex-

pression (Greer et al., 2001). In this regard, RT-PCR followed by comparative sequencing analysis of *nifH* mRNAs has been used to identify and evaluate the nitrogen-fixing bacteria in various natural habitat such as, Lake George (Zani et al., 2000), smooth cordgrass (Brown et al., 2003) and cyanobacterial mat (Omoregie et al., 2004).

In rice ecosystem, more N input, thus more cost, is needed to compensate its runoff during continuous irrigation, which also causes an adverse impact on the environment (Fischer, 2000). Therefore, it is important to define the natural N-fixing phylotypes that can operate and share in nitrogen input under flooded conditions of rice fields. Such effective diazotrophic phylotypes existing in rice ecosystem is not well characterized so far, specially, those living in rice stem. In this report, using *nifH*-specific reverse transcriptase (RT)-PCR technique, we aimed to determine the active phylotypes among the communities that can express nitrogenase genes in association with roots and stems of different rice types at the heading stage of growth.

MATERIALS AND METHODS

Sampling and surface sterilization

The cultivated rice varieties; *Oryza sativa* cv. Sprice and cv. Tetep (indica type) were used in this study. Sprice and Tetep rice are old cultivars grown for many years without fertilizers and used as fodder rice. These cultivars were grown under identical conditions in flooded rice field in the experimental farm of Japan International Research Center for Agricultural Sciences (JIRCAS) till they had reached the heading stage when they used for the experiments. Rice plants were carefully dug out from the wetland rice field, washed with tap water and separated into roots and stems. After removing the outer layer of the stems, the stems and roots were rinsed with deionized water, dried on towel and cut into small sections. All segments were then surface sterilized with ethanol 70% for 1 min and washed twice with sterilized distilled water, then used directly to extract RNA or kept on -80°C till use.

Extraction of RNA from rice roots and stems

Surface sterilized segments were transferred to tubes containing sterilized stainless steel beads (10 mm), then ground under liquid nitrogen using Bio-Medical Science apparatus (BMS-12 Ver.1.2-Japan) for 2 min. The ground powder was then subjected to RNA extraction using RNeasy Plant Mini Kit (QIAGEN) according the manufacturer's protocol. For complete removal of DNA from RNA, the RNase-Free DNase set (QIAGEN) was used to digest DNA according the protocol of the company. Pure RNA concentration was measured by SmatSpec Plus Spectrophotometer (BIO-RAD).

Reverse transcriptase and PCR amplification of *nifH* gene

Transcription of RNA was performed using transcriptase enzyme, and PCR amplification were carried out either by OneStep RT-PCR Kit (QIAGEN) with and without Q solution or by RETROscript (2-Step RT-PCR) kit (Ambion) according to company's protocol. A part of *nifH* gene of about 390 base pair was amplified by PCR techniques, the sequences of the used primers were; nH17K-F" (TAYGGNAASGGCGGTATCGGYAA) and nH139P-R" (TGGCATSGCRAARCCRCCGCAMACMACGTC), where Y represents C or T; R, A or G; S, C or G; N, A or C or G or T; M, A or C (Elbeltagy and Ando, 2005). The amplification was carried out with 35 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min), followed by one cycle at 72°C for 10 min. Two negative controls were conducted; the first is water instead of RNA and the second is RNA sample without reverse transcriptase enzyme (used for RNA transcription step) to test for DNA contamination. After amplification, RT-PCR products were run on the gel, stained with Ethidium Bromide to detect the bands. Specified bands were then excised from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN Science, USA).

Ligation and cloning of *nifH* gene into pGEM-T vector

The purified RT-PCR product was ligated into pGEM-T easy vectors using the 2 X rapid ligation buffer according to the instruction of the company (Promega, USA). The ligation products were introduced into the competent *Escherichia coli* JM109 to construct *nifH* library according to the technical manual (Promega).

The clones were selected at random from the *nifH* library and grew in LB medium (10 g, tryptone; 5 g, yeast extract and 5 g, NaCl/liter) containing 100 µg/ml ampicillin at 37°C overnight. The plasmids bearing *nifH* gene were extracted from grown *E. coli* using alkaline lysis method, then digested with the restriction enzyme *EcoR*I to confirm the presence of *nifH* insert.

Sequencing of *nifH* clones and phylogenetic analysis

Purified plasmids were sequenced using quick start kit and CEQ 8000 Genetic analysis system (Beckman Coulter Inc). Sequencing reaction mixture was prepared according the manufacturer's protocol using T7 primer. All resultant *nifH* sequences were carefully checked and corrected and biased sequences as well as the *nifH* segments corresponding to the sequencing primers were removed from all sequences prior to analysis. For phylogenetic analysis, more than 30 *nifH* sequences from different known diazotrophic bacteria and from various environmental sources were selected as reference nitrogen fixing bacteria using the FASTA search features of GenBank, EMBL, DNA Data Bank of Japan (DDBJ, Mishima, Japan). The nucleotide sequences were translated into amino acid to allow a better comparison, and the inferred amino acid was aligned using Clustal W program. The neighbor-joining method (Saitou and Nei, 1987) and the cluster W program were used to construct a rooted phylogenetic tree that compared with unrooted one. The *nifH* protein of *Anabaena* sp. was used as out-group.

Nucleotide sequence accession numbers

The *nifH* sequence segments that determined in this study have been deposited in GenBank under accession numbers AB208251 to AB-208365 and AB208367 to AB208425.

RESULTS AND DISCUSSION

nifH gene transcription and amplification

To study gene expression by the natural mixed communities existing in rice, pure RNA (free from DNA contamination) must be obtained from roots and stem of rice samples. Reverse transcription (RT) and PCR ampli-

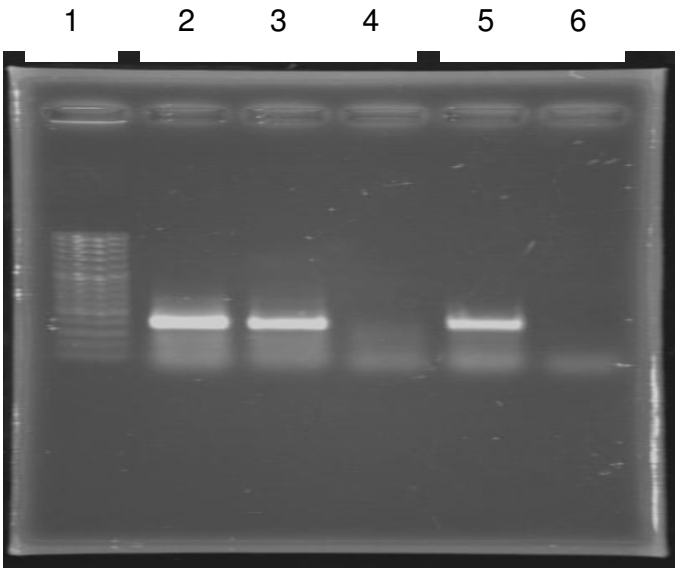

Figure 1. Reverse transcriptions-PCR of RNA extracted from rice roots; lane 1, size marker (0.1-2 kbp OneSTEP marker); lane 2, (5 µl of sprice RNA); lane 3, (3 µl of sprice RNA); lane 4, negative control (without RNA); lane 5, (3 µl of tetep RNA) and lane 6, negative control.

fication of *nifH* gene by the used primers yielded approximately 393 bp product from RNA and indicated expression of nitrogenase genes as visualized in a form of bands on agarose gel by electrophoresis (Figure 1). The negative control shown also in Figure 1 revealed no DNA contamination and subsequently confirmed that the RT-PCR results were from RNA.

Amino acid sequencing analysis of rice clones

A total of 117 *nifH* clones were recovered and sequenced from mRNA of roots and stems of cv. Tetep and cv. Sprice cultivars. The numbers of sequenced clones were 29 from root and 32 from stem of cv Sprice, and 37 from root and 19 from stem of cv. Tetep, as shown in Tables 1a and 1b, respectively.

Alignment of these sequences with those of different reference strains taken from Gene Bank resulted in three main *nifH* groups; 1, includes cluster A, B and C; 2, contain cluster X1 and X2 and 3, include cluster D (Figure 1). In this regard, it has been reported that *nifH* gene sequences generally cluster into four major groups designated as groups (1–4); group 1 includes *nifH* sequences from proteobacteria and cyanobacteria, group 2 contains sequences from some archaea and from second alternatives, group 3 has the sequences mostly from anaerobes, some sulfate reducers (delta-proteobacteria) and some green sulfur bacteria, and group 4 contains *nifH* like genes from archae and chlorophylide reductae genes (Chien and Zinder, 1996).

In view of this, the majority of amino acid sequences (71.8%) of this study (either from roots or stems) did not group within these four major clusters. However, they formed a distinct and deep cluster branching near the roots between current groups 2 (represented by A, B and C clusters) and 3 (cluster D), therefore designated as (group) X as shown in the phylogenetic rooted tree (Figure 2), and un-rooted tree (Figure 3). Sequences of this cluster were distributed in two sub-clusters; X1 and 2 (Figure 1). The closest known diazotroph sequences to cluster X1 in the amino acid identity were that of *Geobacter sulfurreducens* PCA with around 87.8% similarity. However, none of known strain sequences were gathered with cluster X 2.

This major cluster (84 out of 117 clones) was also detected in our previous study on *nifH* PCR clones generated from DNA of rice roots (cultivars, Koshihikari, Sprice and Nerica *5*), but with very low representation (only 4 out of 75 recovered clones) (Elbeltagy and Ando, 2005). Moreover, three clone sequences, H-RIC21 (D26304), H-RIC19 (D206302) and H-RIC14 (D26297) recovered from DNA of rice roots (cultivar Nipponbare) by Ueda et al. (1995) were also included in this cluster.

Apart from rice plant, the organisms belong to this cluster were also found in different habitats, like Neuse river estuary water (one clone, AF035511) (Affourtit et al., 2001), perennial grass roots, *Molinia coerulea,* grown in the edges of lake Neuchatel, Switzerland (one clone, AJ313279) (Hamelin et al., 2002) and the smooth cordgrass roots, *Spartina altrniflora,* grown in Atlantic coasts of North America (3 clones; AF216908, AF389739 and AF389789) (Lovell et al. 2000). For these studies, cluster X represented less than 10% of all recovered sequences (except of those of Hamelin et al., 2002) and most of them were detected from *nifH*-DNA. Interestingly, most of the sequences included in this uncommon cluster in all these studies (including the present study) were extracted from water or water logged grasses, revealing that bacteria of this cluster are at least specific to these aquatic environments.

On the other hand, around 18% of all recovered clone (21 sequences) were belonging to division of proteobacteria (group 1; including clusters A, B and C), most of them (15 clones) grouped with *Bradyrhizobium* sp. MAFF and *Methylocystis parvus* within subdivision alpha proteobacteria (cluster A) with a similarity of 90-97%. The symbiotic stranis, *Bradyrhizobium* sp. traditionally occurs in legume nodules, but its occurrence as endophyte in rice was also observed (Chaintreuil et al., 2000). Also, methanotrophic bacteria were found in submerged rice plants and their abilities to fix nitrogen were confirmed (Dianou and Adachi, 1999).

Among cluster 1, two clone sequences (Tet-ROR19 and Spr-ROR2) were grouped with subdivisions beta and two with subdivision gamma (Tet-STR10 and Tet-ROR3) (cluster B). These sequences showed similarity of 93-94% to *Azovibrio restrictus* and *Azotobacter chrococcum,* respectively. The remaining 2 clone sequences, Tet-

Table 1a. Similarities of amino acid sequences of *NifH* clones recovered from rice cultivar Sprice (L) to the closest sequences published in DNA Data Bank of Japan.

Rice type	No. of clones	Phylogenetic affiliation	Most similar organisms	Homology (%)
cv. Sprice root	5	α-proteobacteria	*Methylocystis parvus 93-3*	87-88.4
	1	α-proteobacteria	*Azospirillium brasilense*	91.5
	1	β and γ-proteobacteria	*Vibrio cincinnatiensis*	83.6
	1	β and γ-proteobacteria	*Azoarcus* sp.	92.9
	1	Anaerobes	Unidentified nitrogen fixing bacteria HD3-2	87.4
	1	Anaerobes	Unidentified nitrogen fixing bacteria CDN8	94.8
	1	Anaerobes	Uncultured procaryotes	89.8
	13	Cluster X1	Uncultured bacterium clone Qinflin-8	91.5-96.8
	5	Cluster X2	Uncultured bacterium clone NIS11-4	91.3-92.1
cv. Sprice Stem	8	Cluster X1	Uncultured bacterium Clone Qinglin-8	93-97
	24	Cluster X2	Uncultured bacterium clone NIS11-4	90.5-97

Table 1b. Similarities of amino acid sequences of *NifH* clones recovered from rice cultivar Tetep (L) to the closest sequences published in DNA Data Bank of Japan.

Rice type	No. of clones	Phylogenetic affiliation.	Most similar organisms	Homology (%)
cv. Tetep root	3	α-proteobacteria	*Methylocystis* sp., *Zymomonas mobilis*	87-90.7
	1	α-proteobacteria	*Azospirillium* sp	88.4
	2	α-proteobacteria	*Bradyrhizobium* sp	97.0
	1	β and γ-proteobacteria	*Azotobacter* sp., *Dechloromonas* sp.	94.0
	1	β and γ-proteobacteria	*Azoarcus* sp.	93.1
	1	δ-Proteobacteria	*Geobacter* sp.	87.8
	1	Anaerobes+anfH	*Clostridium* sp.	60.0
	1	Anaerobes+anfH	*Rhodopseudomonas* sp. *nifH2*	80.0
	2	Anaerobes+anfH	Uncultured clone	85.8-90.4
	3	δ-Proteobacteria	*Desulfovibrio* sp.	84.7-86.5
	11	Cluster X1	Uncultured clone	93.4-97.6
	10	Cluster X2	Uncultured clone	89.7-95.2
cv. Tetep stem	3	α-proteobacteria	*Methylocystis* sp., *Methylococcus* sp	97.0
	1	β and γ-proteobacteria	*Azotobacter* sp., *Dechloromonas* sp.	91.4
	2	Anaerobes+anfH	*Rhodopseudomonas* sp. *nifH2*	76.7
	1	Cluster X1	Uncultured clone	96.8
	12	Cluster X2	Uncultured clone	88.0-92.1

ROR16 and Spr-ROR33 were placed with *Heliobacterium* sp. group in a separate lineage within cluster 1 (cluster C) with a low identity (around 85.5%) and did not group with *Closteridium* sp. (group 3) although they are strict anaerobe, endospore-forming low GC bacteria. Placement of heliobacteria within cluster 1 was also shown by recent study of Amaglan et al. (2005). This group (heliobacteria) is widely distributed in rice fields and known to be active nitrogen-fixing bacteria either photosynthetically or in darkness (Amaglan et al., 2005).

In addition to group X and group 1, some clone sequences (12 clones) that represent around 10.2% of all recovered clones, were affiliated with group 3 (cluster D) (the anaerobes), most of them (10 clones) were from rice roots. These sequences were grouped with different lineage of the anaerobes. Some clones such as TetROR18 and TetROR47 were grouped with sulfate reducing bacteria, *Desulfovibrio gigas* and *Closteridium* sp. with similarity of around 85 and 60%, respectively. Other clones were similar to unknown environmental anaerobes, such as Spr-ROR13 that grouped with rice root clones H-RIC9 (D26292) and H-RIC5 (D26288) obtained by Ueda et al. (1995). Involvement of these bacteria in nitrogen fixation process in reduced sediments was proved, as *nifH* gene expression by sulfate reducing bacteria in marine sediments was confirmed (Steppe and Paerl, 2002). Previous DNA-based studies showed that the anaerobes (cluster 3) was dominant in some environments like Mono lake of northern California (Zehr et al., 2003), sea grass (Bagwell et al., 2002) and rice roots

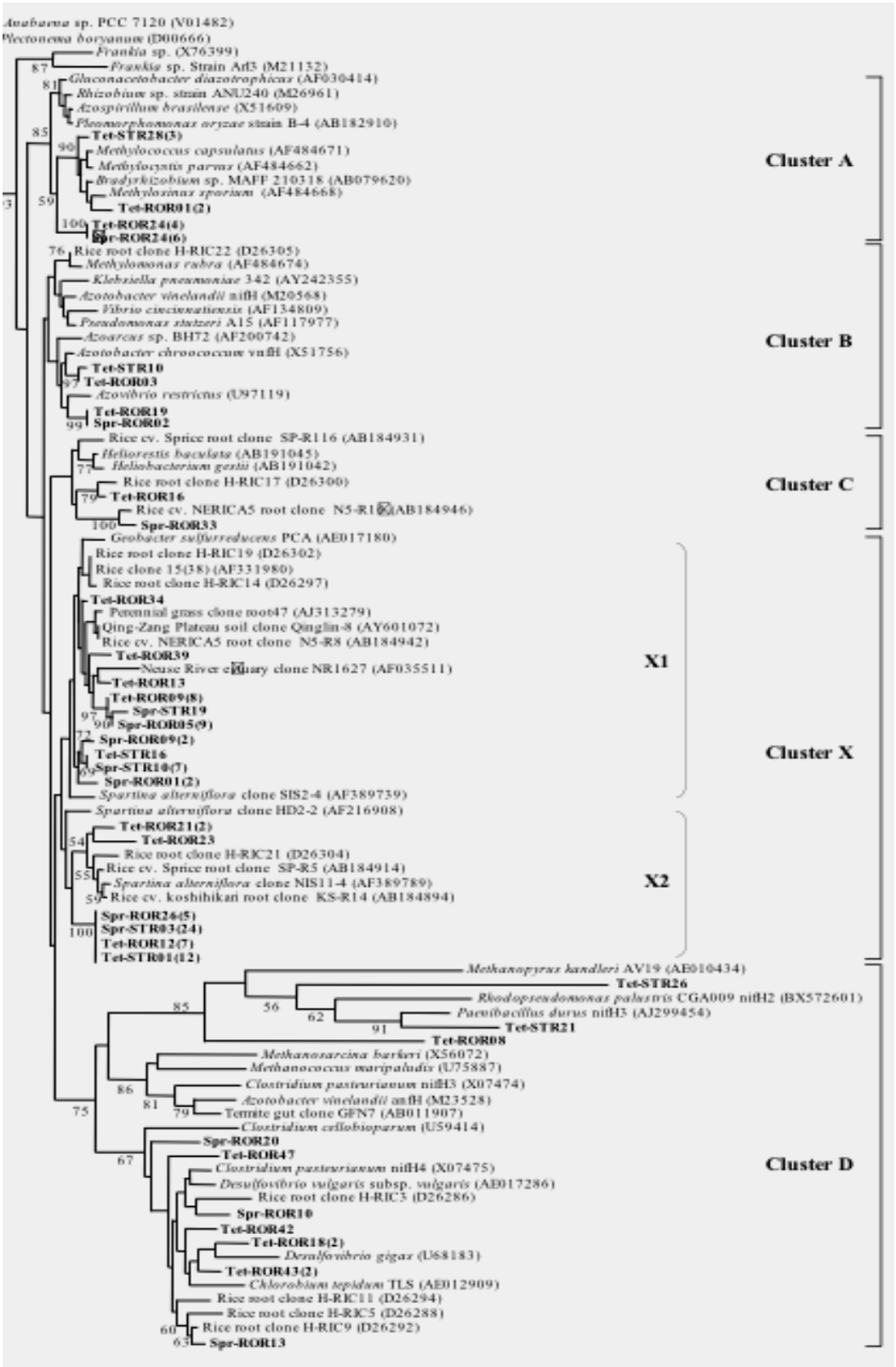

Figure 2. Phylogenetic rooted tree showing distribution of expressed *nifH* rice clusters. Numbers in parentheses represent the number of sequences that were 98% or more identical to each other. Tet.ROR and Tet-STR, are abbreviations of the clones recovered from cv. Tetep (root and stem), and Spr-ROR and Spr-STR from cv. Sprice (root and stem), respectively. Clusters A, B and C represent group I; cluster X represent group 2 and Cluster D represent group 3.

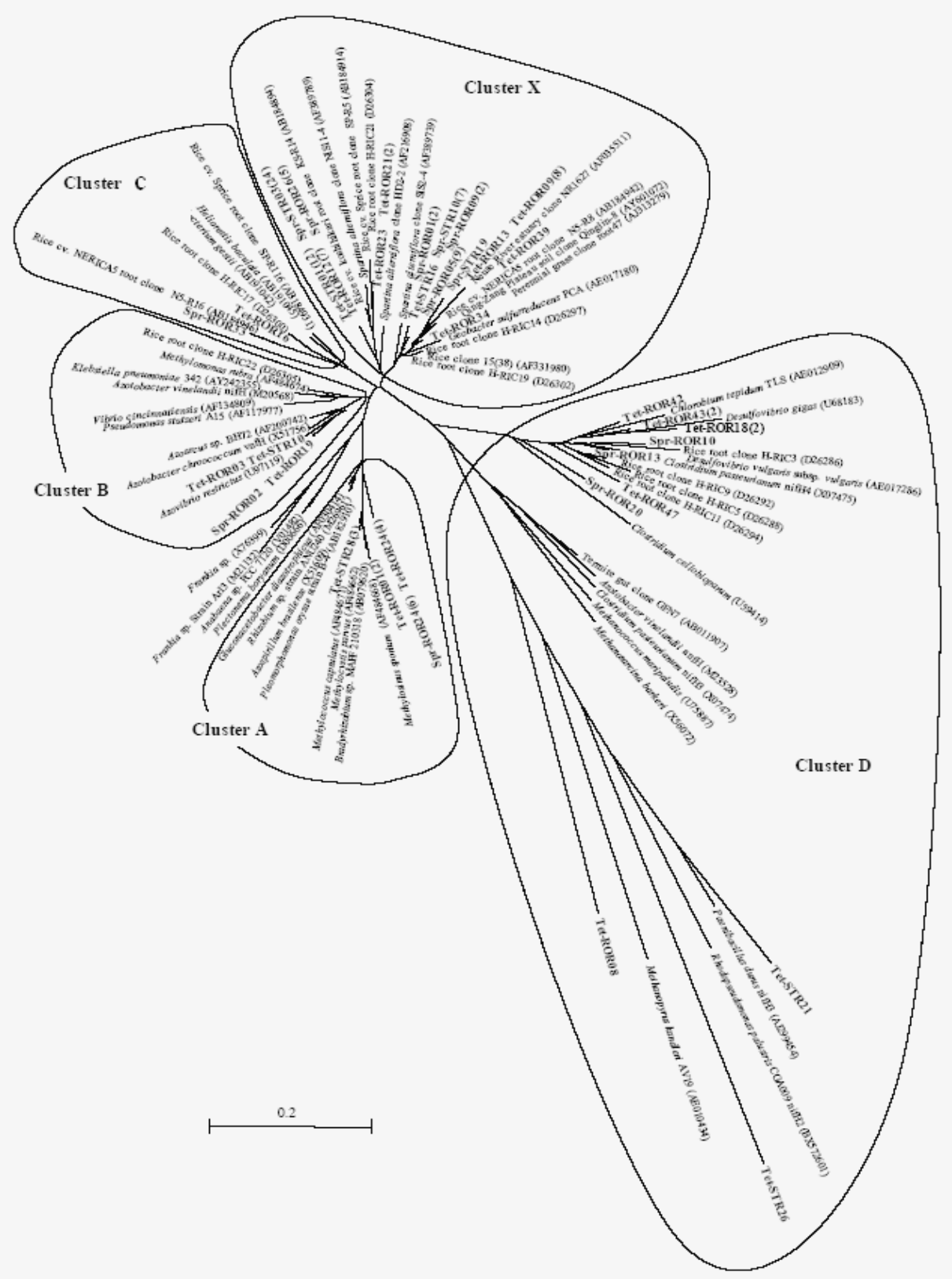

Figure 3. Un-rooted tree showing distribution of expressed *nifH* rice clusters.
Numbers in parentheses represent the number of sequences that were 98% or more identical to each other. Tet-ROR and Tet-STR, are abbreviations of the clones recovered from tetep (root and stem), and Spr-ROR and Spr-STR from cv. Sprice (root and stem), respectively.

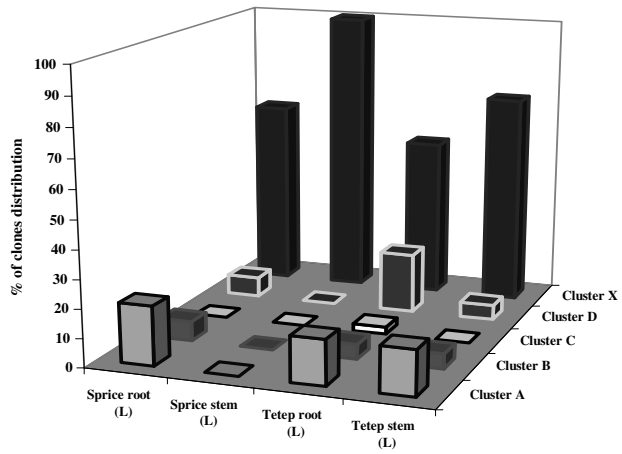

Figure 4. Distribution of detected clusters among roots and stems of rice (cv. Sprice and cv. Tetep).

(Elbeltagy and Ando, 2005) and absent in other environment like open ocean (Zehr et al., 2003). However, this study on RNA showed that members of this cluster were not the main nitrogen contributor to rice fields.

The diversity of nitrogen fixing bacteria residing in the stem is not documented so far. In this study, all clones sequences (100%) recovered from stem of cultivar Sprice and about 90% of those of cultivar Tetep were belonging to one group (group X), revealing limited genetic diversity of stem diazotrophes (Figure 4). Moreover, some sequences recovered from the roots showed 100% homology to those of stem when aligned with each other, illustrating that some nitrogen fixers were able to penetrate the roots and spread in the stems. Rice stem is considered, in some plant species, a preferred place for colonization by nitrogen fixing bacteria. In this regard, Elbeltagy et al. (2001) observed spreading of labeled *Herbaspirillum* sp. in the stem of wild rice more than those of the roots after one week of seed inoculation.

Use of uncultured molecular analysis to detect the occurrence of *nifH* genes in RNA of the tissues of non-legumes would give a clear picture on the active and predominant nitrogen-fixing bacteria living in the plant. This may aid in directing the research to the actually dominant N_2-fixer and thus, devising and optimizing nitrogen management strategies for sustainable low input farming systems in non-legume plants.

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