



## Phylogenetic analysis of *nifH* gene sequences from nitrogen-fixing endophytic bacteria associated with the roots of three rice varieties

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### Abstract

The community structure of nitrogen-fixing endophytic bacteria associated with roots of three varieties, *Oryza sativa* cv. Sprice and cv. Koshihikari and line NERICA 5 (an interspecific hybrid between *O. sativa* and *O. glaberrima*) was analyzed using culture-independent molecular techniques. A segment of *nifH* gene was amplified from crude rice root DNA, cloned to construct *nifH* library and sequenced. About 25 clone sequences from each rice root were recovered and their amino acid sequences were compared with those published in DNA Data Bank of Japan (DDBJ). Majority of *nifH* sequences (79%) of the clones recovered from cv. Sprice were similar to those of unknown and known nitrogen-fixing anaerobes forming cluster (cluster C) distantly related to other nitrogen-fixing bacteria. Moreover, no clone sequences belonging to  $\beta$ - and  $\gamma$ -subdivisions of Proteobacteria (cluster B) were recovered, revealing limited *nifH* diversity for cv. Sprice root associated community. Although the clone sequences recovered from both cv. Koshihikari and NERICA 5 were belonging to same clusters and dominated mainly by genera belonging to  $\beta$ - and  $\gamma$ -subdivisions of Proteobacteria (cluster B), the composition and occupancy of each cluster were relatively different. However, the clones recovered from line NERICA 5 roots were more enriched with sequences belonging to  $\alpha$ -subdivision of Proteobacteria (cluster A) than those of vs. Koshihikari and Sprice. This variability may reveal the impact of plant genotype on community structure of root-associated diazotrophs.

**Key words:** Nitrogen-fixing endophytic bacteria, *nifH* sequences, phylogeny, rice.

### Introduction

Nitrogen that is available to rice in flooded soil even in fields that have been planted for many years without fertilizer application is considered to be due to biological nitrogen fixation<sup>1</sup>. In support of this view, <sup>15</sup>N and N balance studies showed that certain Asian rice varieties receive about 20% of their nitrogen requirements from biological sources<sup>2</sup>.

Recent reports showed that endophytic nitrogen-fixing bacteria colonizes a broad spectrum of plant species and plant parts including annual and perennial ones<sup>3,4</sup>. Isolation and identification of various diazotrophic endophytes that might be responsible for N-gain is important for agricultural application as well as understanding ecosystem process<sup>5</sup>. However, bacteria isolated from natural environments using current culture techniques represent only a small portion of total number of bacteria observed by microscopy, as around 0.1-10% of bacterial cells in soil are cultivable in currently used media<sup>6</sup>. Moreover, the uncultured microorganisms constitute the majority among those existing in different environments<sup>7,8</sup>.

Molecular techniques showed a more accurate image of the total bacterial diversity including cultured and uncultured bacteria. Such approaches may be applied to functional genes, such as dinitrogen fixation genes<sup>9</sup>. The *nifH* gene was widely used to detect nitrogen-fixing bacteria, as it encodes for the dinitrogenase reductase, a key enzyme in the nitrogen fixing process<sup>10</sup>. Also it has a highly conserved regions as well as a great divergence in other regions and it can, therefore, be used to evaluate the phylogenetic relationships among nitrogen-fixing bacteria of several groups<sup>11</sup>.

Rice shows a remarkable diversity because of its long history of cultivation and selection under various climatic and biotic environments in geographic diverse area<sup>12</sup>. Thus, the natural bacterial communities associated with rice would have a quite diverse array of organisms. In the current report, we aimed to use a cultivation-independent technique to characterize and compare the cultured and uncultured bacterial communities associated with three rice cultivars grown under same conditions by direct amplification of the *nifH* gene from DNA of rice roots and analyzing the clone sequences recovered from cDNA library.

### Materials and Methods

**Sampling, surface sterilization and DNA extraction:** Two varieties of cultivated rice, *Oryza sativa* cv. Koshihikari and cv. Sprice, and the cross line NERICA 5, New Rice for Africa, were used in this study. Koshihikari cultivar is regularly cultivated in Japan and used as edible rice, Sprice is an old cultivar grown for many years without fertilizer and used now as fodder rice and NERICA 5 is an interspecific cross line derived from Asian rice (*O. sativa*) and African rice (*O. glaberrima*)<sup>13,14</sup> and used as edible rice in West Africa. These three types were grown under identical conditions in flooded soil in the experimental farm of Japan International Research Center for Agricultural Sciences (JIRCAS) till they had reached the heading stage when they were used for the experiments. Rice plants were dug out from the wetland rice field. The roots were washed with tap water to remove the attached soil and cut into small sections. The segments were then surface sterilized with ethanol 70% for 1 min.

and washed twice with sterilized distilled water. For DNA extraction, the sterilized segments were frozen with liquid nitrogen and ground to a fine powder with mortar and pestle. DNA was extracted using DNeasy Plant Mini Kit (QIAGEN Science, USA) and visualized by gel electrophoreses.

**PCR amplification, ligation and cloning of *nifH* gene:** 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” (TGGCATSGCRAARCCRCGCAMACMACGTC), 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 (Yuhashi and Minamisawa, K., Tohoku University, personal communication). The 50  $\mu$ l reaction mixture contained 25  $\mu$ l (1.25 unit) of premix *Taq* (Takara Bio. Co., LTD, Japan), 3  $\mu$ l template (<1 $\mu$ g), 0.2-1  $\mu$ M final concentration of nH17K-F primer and same for nH139P-R primer, then the mixture was filled up to 50  $\mu$ l with sterilized distilled water. The amplification program was as follow: 1 min. at 94°C for one cycle, 1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C for 30 cycle and final chain extension (one cycle) for 2 min. at 72°C. Negative control (water instead of DNA) showed no amplification.

The PCR products of *nifH* gene were visualized by electrophoresis on ethidium bromide-stained 1.0% agarose gel, then purified using GFX-PCR DNA and Gel Band purification kit (Amersham Pharmacia Biotech Europe GmbH., Freiburg, Germany). After purification, PCR products of the *nifH* gene were run on low melting agarose gel electrophoreses. The specific bands were then excised from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN Science, USA). The purified PCR product was ligated into pGEM-T easy vectors using the 2X rapid ligation buffer according to the instruction of the company (Promega, USA). The ligation products were cloned into the competent *Escherichia coli* JM109 to construct *nifH* library according to the technical manual (Promega).

The clones that showed no  $\beta$ -galactosidase activity 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  $\mu$ g/ml ampicillin at 37°C overnight. The plasmids bearing *nifH* gene were extracted from grown *E. coli* cells using alkaline lysis method<sup>15</sup>, then digested with the restriction enzyme *EcoRI* 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 manufacture’s protocol using T7 primer. All resultant *nifH* DNA sequences were carefully checked and corrected and biased sequences (longer or shorter than the amplified fragment) 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 blast 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 acids were aligned using Clustal W program. The

neighbor-joining method and the cluster W program were used to construct a phylogenetic tree<sup>16</sup> and *nifH* protein of the Cyanobacteria was used as out group.

**Nucleotide sequence accession numbers:** The *nifH* sequence segments determined in this study have been deposited in GenBank under accession numbers AB184886-ABB184911 for vs. Koshihikari, AB184912-AB184935 for vs. Sprice and AB184936-AB184960 for line NERICA 5.

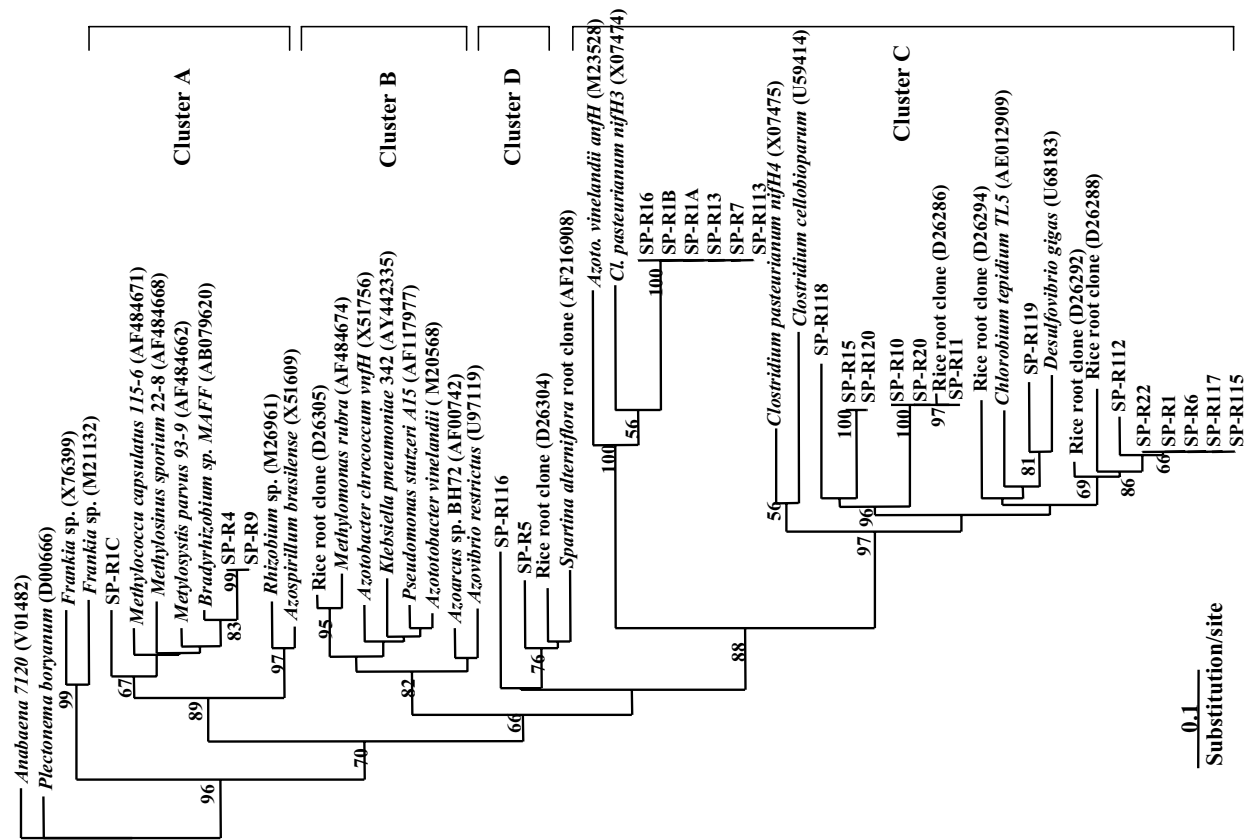
## Results and Discussion

A fragment of *nifH* genes of distantly related nitrogen-fixing bacteria could be successfully amplified from DNA extracted directly from the roots of the three examined rice types. However, sequences from diazotrophic bacteria belonging to *Cyanobacteria*, *Archae* and *Frankia* were not detected, a total of 24, 26 and 25 cloned *nifH* sequences of diverse nitrogen-fixing bacteria were recovered from the roots of cv. Sprice, cv. Koshihikari and line NERICA 5, respectively.

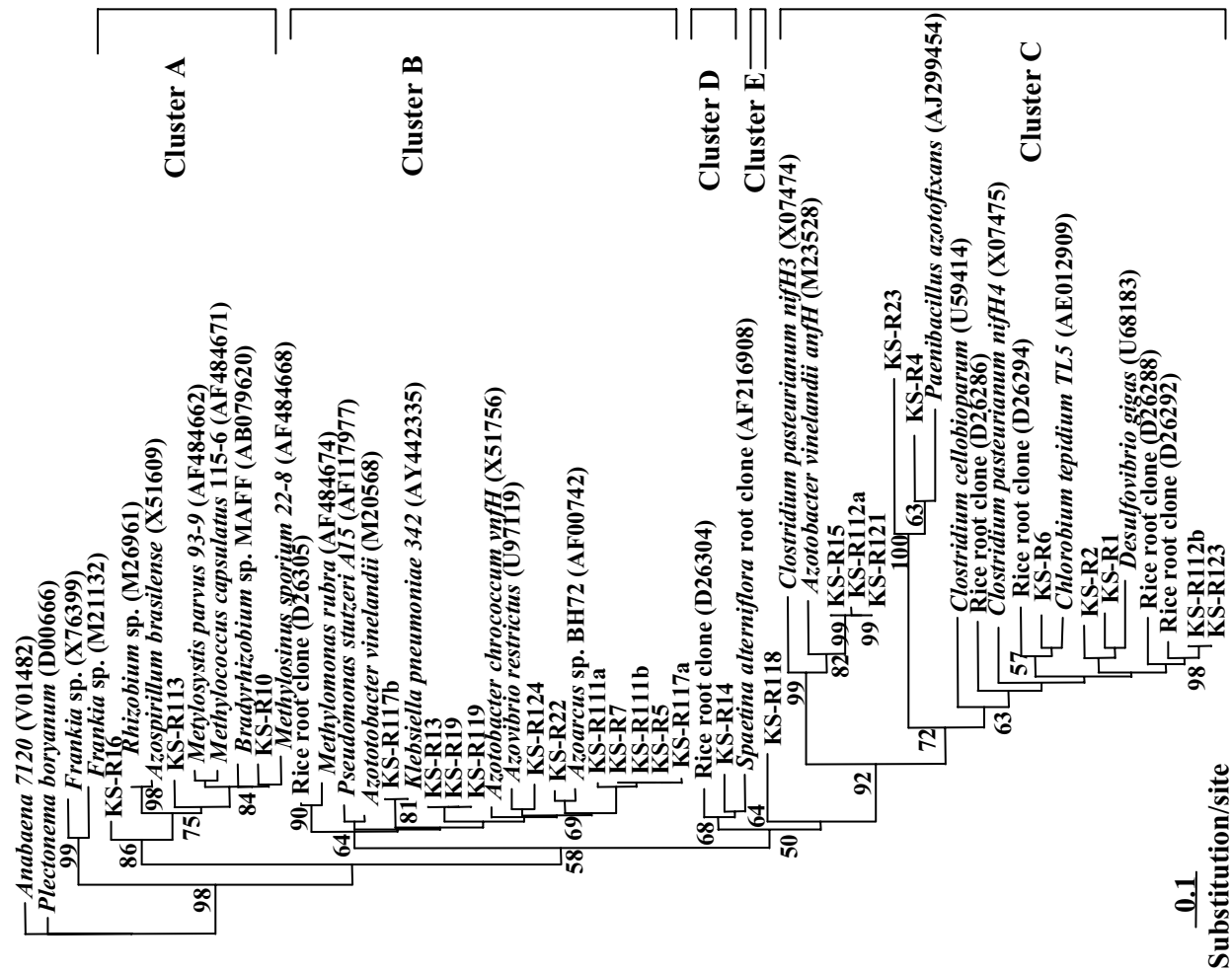
**Clone sequences of cv. Sprice:** The phylogenetic position of partial *nifH* sequences recovered from cv. Sprice roots (Fig. 1) revealed assemblage of 3 clusters: cluster A is poorly represented and composed of two clone sequences (SP-R4 and SP-R9) of high similarities (>95%) 96.9 and 97.7 %, respectively with *Bradyrhizobium* sp. and one clone sequence (SP-R1C) of substantial similarity (93%) to *Methylocystis* sp. and all fell under  $\alpha$ -subdivision of Proteobacteria. Similarly, cluster D composed of only two clone sequences (SP-R116 and SP-R5) without known close relatives from diazotrophes, but gathered with the clone sequences recovered from the roots of *Spartina alterniflora* (AF216908) and rice (D26304).

In contrast, the clone sequence of cluster C was dominant and accounts for 79% of analyzed sequences. Although the ‘Sprice’ clone sequences of this cluster affiliated with anaerobic and  $\delta$ -Proteobacteria, it divided into two sub-clusters. It appears that the first included *nifH* sequences homologous to *nifH3* of *Clostridium pasteurianum* and the second included sequences related to other *Clostridium*, *Desulfovibrio gigas* and unknown anaerobes. It was noticeable that the putative *nifH* sequences similar to those belonging to  $\beta$ - and  $\gamma$ -proteobacterial groups were not recovered from cv. Sprice roots. Because of the absence of these groups as well as the high dominance of the sequence belonging to the anaerobic cluster, the diversity of the *nifH* gene pool in Sprice cultivar was considered to be limited as compared to those recovered from the roots of *O. sativa* cv. Nihonbare by Ueda et al.<sup>17</sup>. It was consistent with the results obtained by Hamelin et al.<sup>9</sup> where limited *nifH* gene diversity was found in the bacterial community associated with the perennial grass *Molinia coerulea*.

**Clone sequences of cv. Koshihikari:** The phylogenetic position of *nifH* amino acid sequences recovered from Koshihikari cultivar (Fig. 2) showed more diverse assemblage, as the recovered sequences were spread among five clusters A, B, C, D and E. Cluster A represented only by 3 clone sequences belonging to known members of  $\alpha$ -Proteobacteria, two clones, KS-R10 showed high similarity (96%) and KS-R113 showed substantial similarity (91%) with those of *Methylocystis* sp., and clone KS-R16 showed



**Figure 1.** Phylogenetic position of amino acid sequencing of *nifH* clones recovered from cv. Sprice root as constructed by NJ method. Reference accession numbers are indicated after bacterial name. The percentage of 1000 bootstrap more than 50% are indicated on the left.



**Figure 2.** Phylogenetic position of amino acid sequencing of *nifH* clones recovered from cv. Koshihikari root as constructed by NJ method. Reference accession numbers are indicated after bacterial name. The percentage of 1000 bootstrap more than 50% are indicated on the left.

low similarity (86%) with *Azospirillum* sp. sequence. Cluster B was dominant and accounts for 42.3% of all sequences recovered and comprised clone sequences of known relatives from cultured diazotrophes belonging to subdivisions  $\beta$ - and  $\gamma$ -Proteobacteria. The clone sequences of this cluster (11 sequences) showed substantial similarities with *nifH* sequences of *Azotobacter* (with average of 92.3% for 7 clones) and *Azoarcus* (with average of 93.5% for 3 clones) and one clone sequence of high similarity with *Klebsiella* (98.5%). Cluster D contained only one clone sequence (KS-R14) without known close sequences of diazotrophes but gathered with those recovered from the roots of *Spartina alterniflora* (AF216908) and rice root clone (D26304). Cluster E is made up of one clone (KS-R118) representing a monophyletic group without close relatives unknown or known diazotrophes and representing unknown cluster. In this regard, it has been found that most of the sequence recovered from rhizosphere of *Spartina alterniflora* formed well-supported monophyletic group that showed no close relatives with known diazotrophes<sup>18</sup>.

Cluster C accounts for 38.4% of all analyzed sequences and comprised of *nifH* sequences from distantly related diazotrophes showing highly divergent lineages (Fig. 2). Most of these sequences are similar to those of anaerobes including known and unknown anaerobes, sulphate reducing and photosynthetic green sulphur anaerobes. In addition, the three sequences, KS-R15, KS-R112a and KS-R121 having a low similarity (averaged 86%) with *Azotobacter anfh* and the two sequences KS-R4 and KS-R23 of a very low similarity (averaged 75.4%) with *Paenibacillus* sp. (Gram positive anaerobes,  $\delta$ -Proteobacteria) affiliated under this cluster. The last two clone sequences were not recovered from either 'Sprice' or NERICA roots and characterized by their long branch length.

This diverse assemblage fell into this cluster was supported by the analysis of *nifH* diversity reported by Zehr et al.<sup>19</sup> who combined distantly related diazotrophes such as gram positive,  $\delta$ -Proteobacteria, green sulphur bacteria and Archae under one cluster and found that the diazotrophes represented by *nifH* within this cluster are mostly strict anaerobes. This cluster includes known diazotrophes like *Chlorobium*, *Clostridium*, *Desulfovibrio* and *Methanosarcina*, and characterized by deep bifurcations and long branch length.

**Clone sequence of line NERICA 5:** The phylogenetic tree of analyzed clone sequences recovered from NERICA 5 roots shown in Fig. 3 revealed 5 clusters (A, B, C, D and E). They are similar to those of 'Koshihikari' but different in percentage of occupancy distribution among the clusters. Unlike the poor representation of cluster A in the community structure of Sprice and Koshihikari cultivars, it accounts for 28% among all clone sequences of line NERICA 5. Moreover, it was enriched with sequences of high similarity (averaged 95%) with methanotrophes such as *Methylocystis* sp. and *Methylosinus* sp. Cluster B consists of clone sequences (8 clones) of known relatives from diazotrophes belonging to  $\beta$ - and  $\gamma$ -Proteobacteria and accounts for 32% of all examined sequences. It was occupied mainly by clone sequences (7 out of 9) of remarkable similarity (94%) with *Azoarcus* and *Azotobacter* genera. The other 2 sequences N5-R3 and N5-R17 showed high similarity with *Klebsiella* sp. (98.5%) and *Methylomonas* sp. (96.2%), respectively. Cluster E is made of

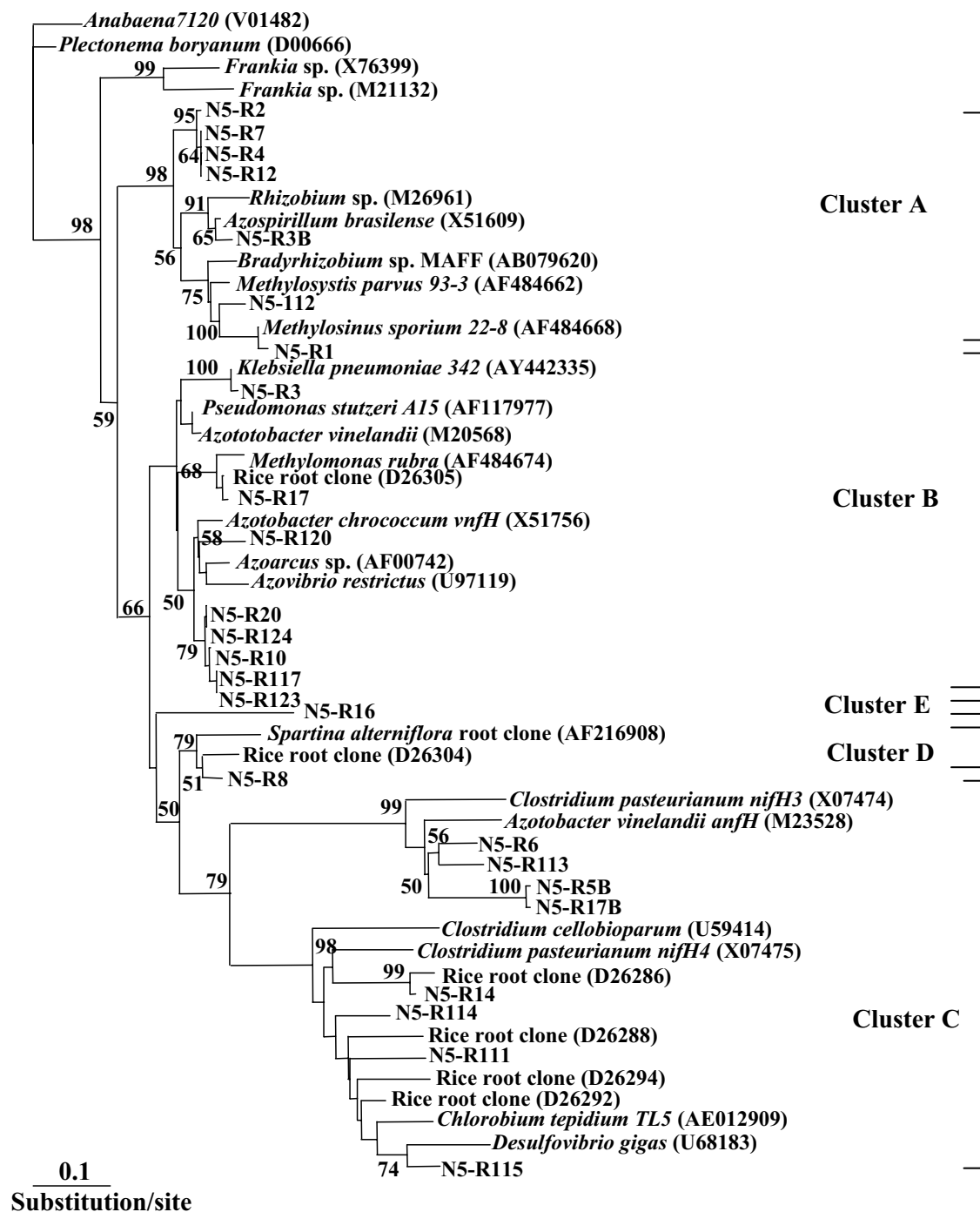
one clone, N5-R16 without close relatives from the diazotrophes, as the sequence of *Pseudomonas* sp. was the closest one with similarity of 81.6% representing unknown cluster. Cluster D is also represented by one clone, N5-R8 without close relatives of cultured diazotrophes but gathered with clone sequences recovered from rhizosphere of *Spartina alterniflora* (AF 216908) and rice clones D26304 and D26302. Cluster C accounts for 32% (similar to cluster B) of all analyzed sequences and includes anaerobes, sulphur reducing, green sulphur anaerobes, anaerobes from environmental samples with no close relatives from cultured one as well as *Azotobacter* with alternative nitrogenase (*anfH*). Some clone sequences (N5-R5B and N5-R17B) showed low similarity (82.4 and 82.3%) with those of *Clostridium pasteurianum nifH3*, respectively and clone N5-R111 revealed 85.5% similarity to those of *Clorobium tepidum TLS*. Similarly, clone N5-R6 and N5-R113 revealed low similarity (87 and 85.5%) to those of *Azotobacter anfh*, respectively.

Some other clones showed no close relatives from cultured diazotrophes, such as N5-R115 showed 89.8% similarity with those of uncultured clone SC10 (AF414632) recovered from seagrass that gathered with anaerobes as reported by Bagwell et al.<sup>20</sup> and clone N5-R14, showed high similarity 98.2% with uncultured clone H-RIC 12 recovered from rice<sup>17</sup>.

Although the three rice types used in this study were raised under identical flooded condition in JIRCAS experimental farm, a distinguishable assemblage of diazotrophic bacteria were found associated with their roots (Fig. 4). This might show the impact of plant genotypes variation on the composition of *nif* gene pool. Similarly, Briones et al.<sup>21</sup> demonstrated an impact of rice genotype on microbial diversity of root associated nitrifying bacteria of *O. sativa* at cultivar level. In addition, the T-RFLP patterns of prevalent *nifH* genes were dependent on the plant species (*O. sativa* and *O. longstaminata*) grown under controlled conditions in phytotron, however, the influence of environmental condition on population structure of diazotrophes was greater than those of plant species<sup>22</sup>.

It was noticeable that the clone sequences similar to those of known cultured or unknown uncultured anaerobes were frequently recovered from all examined cultivars. Such anaerobic cluster has been observed among diazotrophic community recovered by cloning-sequencing method from the rhizosphere of *Molinia coerulea*<sup>9</sup>, marine plant (*Spartina alterniflora*)<sup>18</sup> and seagrass<sup>20</sup>. In addition, a set of clostridia was isolated from the stem of different gramineous plants<sup>23</sup>. In this regard, the saturation of root zone with water during the growth season of flooded rice, as well as the respiration of root cells and rhizospheric microorganisms would favor anoxic microenvironment that can stimulate the growth of anaerobes associated with roots. Even in the aerial part, clostridia were found to be dwelling in anoxic microzones produced by consortia consisting of aerobic and nitrogen-fixing anaerobic clostridia<sup>23</sup>, however, the community structure of diazotrophes associated with upland rice remains to be examined for presence of such anaerobic cluster.

Although the genera *Azospirillum* sp. was widely found to be associated with grass<sup>24</sup>, only one clone (N5-R3B) out of 75 clone sequence was recovered in this study, also, *Herbaspirillum* sp. which is widely isolated from a wide range of gramineous plants including rice<sup>25</sup> was not recovered from the examined cultivars. This observation has also been reported by Tan et al.<sup>20</sup>



**Figure 3.** Phylogenetic position of amino acid sequencing of *nifH* clones recovered from line NERICA 5 root as constructed by NJ method. Reference accession numbers are indicated after bacterial name. The percentage of 1000 bootstrap more than 50% are indicated on the left.

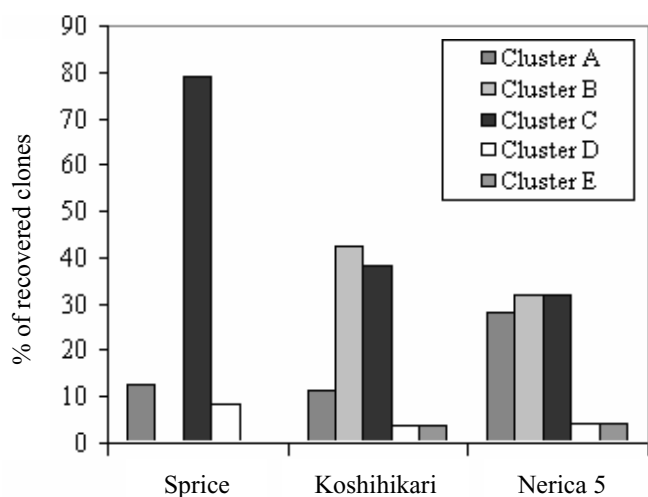
who concluded that *Azospirillum* sp. might not be among the prevalent diazotrophes associated with rice roots when unculturable strains are taken into account. It underlines that some diazotrophes, although able to grow on culture media, may have no impact in the natural environment. It is possible that these genera were present, but with only low relative abundance and therefore were not detected.

The phylogenetic analysis of *nifH* sequences obtained in this study showed that nitrogen-fixing bacteria were associated with roots. We are currently studying the impact of the root associated

diazotrophes on the plant by extracting RNA and subsequently detecting the expression of *nifH* inside the root of rice plant.

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**Figure 4.** Comparison of phylogenetic clusters recovered from each rice type ( $\alpha$ -Proteobacteria = Cluster A;  $\beta$ - and  $\gamma$ -Proteobacteria = Cluster B; Anaerobes+anfH+  $\delta$ -Proteobacteria = Cluster C; Environmental clones = Cluster D; Monophyletic clones = Cluster E ).

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