Analysis of Diversity of Diazotrophic Bacteria Associated with the Rhizosphere of a Tropical Arbor, *Melastoma malabathricum* L.

ATSUYA SATO¹, TOSHIHIRO WATANABE², YUSUKE UNNO³, ERY PURNOMO⁴, MITSURU OSAKI^{1,2}, and TAKURO SHINANO^{3*}

¹Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kitaku, Sapporo 060–8589, Japan;

²Research Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kitaku, Sapporo 060–8589, Japan;

³National Agricultural Research Center for Hokkaido Region (NARCH), Hitsujigaoka 1, Toyohiraku, Sapporo 062–8555, Japan; and ⁴Center for Tropical Adverse Soil Studies Natural Resource and Environmental Management Lambung Mangkurat University, PO Box 1028 Banjarbaru 70714, South Kalimantan, Indonesia

(Received December 8, 2008—Accepted February 4, 2009—Published online March 10, 2009)

The diversity of diazotrophic bacteria in the rhizosphere of *Melastoma malabathricum* L. was investigated by cloning-sequencing of the *nifH* gene directly amplified from DNA extracted from soil. Samples were obtained from the rhizosphere and bulk soil of *M. malabathricum* growing in three different soil types (acid sulfate, peat and sandy clay soils) located very close to each other in south Kalimantan, Indonesia. Six clone libraries were constructed, generated from bulk and rhizosphere soil samples, and 300 *nifH* clones were produced, then assembled into 29 operational taxonomic units (OTUs) based on percent identity values. Our results suggested that *nifH* gene diversity is mainly dependent on soil properties, and did not differ remarkably between the rhizosphere and bulk soil of *M. malabathricum* except in acid sulfate soil. In acid sulfate soil, as the Shannon diversity index was lower in rhizosphere than in bulk soil, it is suggested that particular bacterial species might accumulate in the rhizosphere.

Key words: nitrogen-fixing bacteria, rhizosphere, *Melastoma malabathricum* L., diversity

The levels of nitrogen (N) and other minerals (phosphorus, calcium and magnesium) in acid soils (pH<4) are generally very low and insufficient for the growth of most plants (20). *Melastoma malabathricum* L. is a tropical woody plant known as an aluminum-accumulator. This plant is able to adapt to very nutrient-poor environments such as acid soils (22, 23, 36) and is widely distributed in acid sulfate soil areas in central and south Kalimantan, Indonesia. As soil nutrient levels are quite low, the ability to acquire N must also be considered. We have found that *M. malabathricum* continued to grow at low N-levels in hydroponic cultures with significant increases in dry weight and N content, though other plants could not grow (data not shown). It has been considered that microorganisms inhabiting the rhizosphere of *M. malabathricum* may help with plant growth by supplying biologically-fixed nitrogen (BFN). Several candidate bacteria have been evaluated (13); however, they were isolated from the *M. malabathricum* rhizosphere, and the report did not describe the diazotrophic diversity and evaluate their contribution to N acquisition of *M. malabathricum* in an actual nutrient-poor soil.

Diazotrophic bacteria are known to directly and indirectly affect plant growth: directly through a substantial contribution of BNF to N acquisition of the plant, and indirectly through the synthesis and export of organic substances like phytohormones that enhance root growth (10). These two plant growth-promoting effects must be considered separately. The free-living and plant-associated diazotrophic bacteria are ubiquitous in soil, but there is little understanding of

their diversity and contribution to N input. This led to our interest in whether any diazotrophic bacterial distribution associated with the rhizosphere of *M. malabathricum* is unique. We hypothesize that *M. malabathricum* growing in different soils forms an environment-dependent plant-micro-organism relationship and develop different diazotrophic bacterial communities. Comparing the diazotrophic bacterial populations of three soil types may help elucidate their role in the acquisition of N through the rhizosphere of *M. malabathricum* grown in nutrient-poor soils, such as acid sulfate soil.

Nitrogenase catalyzes BNF and all known nitrogen-fixing organisms are prokaryotes belonging to the bacterial and archaeal domains (25). However, details on the functional diversity of microorganisms in natural environments are still unclear, because more than 99% of microbial species are refractory to cultivation in the laboratory (3). Recently, several molecular biological methods have been used to describe the structure of diazotrophic bacteria in natural environments (28); such methods that do not use cultivation techniques avoid misinterpretations of the composition of diazotrophic populations. The *nifH* gene encodes the iron-protein component of nitrogenase, the enzyme necessary for BNF. Since most diazotrophic organisms have *nifH*, the gene is a useful molecular marker for nitrogen-fixing organisms. Several degenerate primers were designed for PCR-amplification of *nifH*, based on highly conserved regions of a *nifH* amino acid sequence (24, 39).

Studies have revealed the diversity of the *nifH* gene in various environments using molecular ecological tools such as clone libraries (6, 12, 27, 33), denaturing gradient gel electrophoresis (DGGE) (8, 19), (terminal)-restriction fragment

* Corresponding author. E-mail: shinano@affrc.go.jp; Tel: +81–11–857–9243; Fax: +81–11–857–9243.

length polymorphism (RFLP) (9, 31); and a *nifH* gene-specific macroarray (30). Some studies have focused on the *nifH* gene pool in the rhizosphere (6, 9, 12, 19, 27, 31, 33). These studies have demonstrated that molecular ecological techniques are useful for describing *nifH* diversity in the rhizosphere of plants. However, there is limited understanding of the diazotrophic population associated with the rhizosphere of pioneer plants like *M. malabathricum*. The aim of the present study is to describe the diversity of diazotrophic bacteria in the rhizosphere of *M. malabathricum* grown in three soil types (acid sulfate, peat and sandy clay soils) in south Kalimantan, Indonesia by using molecular ecological techniques as a basis for future microbial applications to agriculture in nutrient-poor soils.

Materials and Methods

Sampling site

Rhizosphere soil was collected from *M. malabathricum* growing naturally in three soil types in the suburbs of Banjarbaru, in south Kalimantan, Indonesia. The three types were classified as acid sulfate, peat and sandy clay soils, respectively, and three plots were examined for each type. All specimens of *M. malabathricum* sampled were less than 1 m in height. *M. malabathricum* was the dominant plant at all sampling sites, and there were few other plant species especially in the acid sulfate soil. However, *M. malabathricum* grew well at all three sites. Material was collected from one plant per plot for each soil type.

Preparation of the rhizosphere soil

The root system of *M. malabathricum* plants was dug up and cut into pieces (10 cm long) to obtain *ca.* 0.5 g of rhizosphere soil. To prevent endophytic contamination of the soil sample, root cross-sections were covered with sterilized paraffin. The roots collected from the same plot were mixed and then immersed in a 50-mL conical polyethylene tube containing 40 mL of sterile phosphate-buffered saline (PBS) for 20 min to remove loosely attached soil; this detached soil fraction was termed "bulk soil". After 20 min, roots were transferred to a new 50-mL conical polyethylene tube containing 40 mL of sterile PBS. The bulk soil was recovered by centrifugation for 30 min (6,000×g, at 4°C). The tube containing roots with closely adhering soil was shaken for 15 min (180 rpm) to remove soil. After the roots were removed from the tube, the soil fraction was recovered by centrifugation for 30 min (6,000×g, at 4°C) and termed "rhizosphere soil". Each soil sample was stored at -20°C until DNA extraction.

DNA extraction from soil

DNA was extracted from the rhizosphere soil of *M. malabathricum* and the bulk soil (0.5 g of each sample) from each plot using ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the protocol provided by the manufacturer. DNA concentrations were measured with a NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE, USA).

PCR amplification and clone library-based analysis of *nifH* genes

The *nifH* sequences were amplified using nested PCR to increase sensitivity. The primers used were originally developed by Poly *et al.* (24). The first PCR was performed with the forward primer FGPH19 (5'-TACGGCAARGGTGGNATHG-3') and reverse primer PolR (5'-ATSGCCATCATYNTCRCCGGA-3'). The second PCR was performed with PolF (5'-TGCGAYCCSAARGCB-GACTC-3') and AQER (5'-GACGATGTAGATYTCCTG-3'). The final PCR cocktails contained 2 µL of 10×PCR buffer (Mg²⁺-free), 1.5 mM of MgCl₂, 0.5 µM of each degenerate oligonucleotide primer, 200 µM of each deoxynucleoside triphosphate, 8 ng of bovine serum albumin and 1 U of *Ex Taq*TM (Takara, Otsu, Japan)

DNA polymerase. For the first PCR, 10 ng of the extracted DNA was amplified in Takara Thermal Cycler Dice (Takara). For the second PCR, 1 µL of the first PCR product was used as a template. Each mixture was adjusted to a final volume of 20 µL with sterile MilliQ water. The reaction profile comprised 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C for the first PCR and at 48°C for the second PCR, and primer extension at 72°C for 2 min, with a final extension at 72°C for 5 min. The presence of PCR products was determined by analyzing 5 µL of product on 2% agarose gels, and staining with SYBR Green I (Cambrex, East Rutherford, NJ, USA). PCR products (*ca.* 340 bp) were purified with a MinElute PCR Purification Kit (Qiagen, Tokyo, Japan) and cloned into the pGEM-T easy cloning vector (Promega, Madison, WI, USA), according to the manufacturer's instructions. Each clone library was constituted of three replications constructed from PCR products amplified from individually extracted DNAs. Six libraries were constructed for rhizosphere and bulk soils of the three soil types, and >50 clones per library were randomly selected to determine the *nifH* gene sequence.

Sequencing and phylogenetic analysis

Plasmids were prepared with the QIAprep Miniprep Kit (Qiagen) using the manufacturer's protocol. The cloned fragment was sequenced with a CEQ DTCS-quick start Kit (Beckman Coulter, Fullerton, CA, USA) and a CEQ 8000 sequencing system (Beckman Coulter) using the T7 forward primer.

Phylogenetically unusual *nifH* sequences were checked for chimeric characteristics by comparing with the GenBank database (DNA Databank of Japan, Shizuoka, Japan) using the blastn program from BLAST search (2) with full-length and partial sequences, and chimeric *nifH* sequences were omitted from later analysis.

Analyzing the diversity of the *nifH* gene in rhizospheres of *M. malabathricum* in three soil types

A *nifH* database based on all the sequences from each *nifH* clone library was made. Each read was regarded as a query sequence, and sequence identity was computed by a reciprocal blast search of our *nifH* database using the blastn program 2.2.18 (2). A reciprocal pairwise comparison of two reads yields two sequence identity values. If both values exceeded a certain criterion, the two reads were considered homologous and assembled into one cluster. Any cluster that included >2 reads was defined as an operational taxonomic unit (OTU). The criterion for this definition was 85–100% of the sequence identity value. The number of reads grouped into all OTUs and the number of OTUs at different sequence identity levels are shown in Fig. 1. The criterion for defining an OTU was 94% of the sequence identity value and only matches as defined above were considered for further analysis. The validity of and grounds for this criterion are mentioned in "Results and Discussion". Reads composing one OTU had the characteristics of *nifH* libraries, from which these reads were derived (*e.g.*, bulk and rhizosphere soils of

Fig. 1. The number of reads grouped into all operational taxonomic units (OTUs) (●) and the number of OTUs (■) at different percent identity levels. The number of reads grouped into all OTUs was represented by the summation of reads assembled into all OTUs.

Table 1. Data matrix of the information on the derivation and number of reads in each soil type

Soil types ^a	OTU name																												
	S1	S2	S3	S4	S5	S6	S7	P1	P2	P3	P4	P5	P6	P7	P8	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14
AR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	3	22	2	12	4
AB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	3	3	3	2	4	6	3	2	2	1	0	0	0
PR	0	0	0	0	10	0	0	0	0	14	1	2	5	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PB	0	0	0	0	7	0	0	2	2	22	2	0	4	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SR	2	0	2	2	28	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SB	8	2	0	0	29	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

^a AR, rhizosphere soil from acid sulfate soil; AB, bulk soil from acid sulfate soil; PR, rhizosphere soil from peat soil; PB, bulk soil from peat soil; SR, rhizosphere soil from sandy clay soil; SB, bulk soil from sandy clay soil.

Fig. 2. Heatmap and dendrograms showing the similarity of *nifH* clusters (shown on x-axis) and those of six different soil types (bulk and rhizosphere soil samples of *M. malabathricum* in acid sulfate, peat and sandy clay soils, respectively) (on y-axis). Each column shows the number of OTUs according to a color scale (shown at the upper right). The dendrogram was constructed using the package pvclust (<http://www.is.titech.ac.jp/~shimo/prog/pvclust/>), available from The R 2.7.1 program (method.dist=correlation; method.hclust=average; 10,000 bootstrap replications). Clusters with $P<0.05$ are highlighted by rectangles, and are strongly supported by the data.

M. malabathricum in acid sulfate, peat and sandy clay soils, respectively). Each OTU was characterized using information on derivation and the number of reads, and a data matrix for all OTUs was constructed from these two parameters (Table 1). The similarities between both OTUs and soil samples were shown by dendrograms using The R (<http://www.R-project.org>) based on this data matrix (Fig. 2).

Statistical analysis of nifH gene clone library

Coverage (*C*) was calculated to verify whether the *nifH* gene clone library was large enough to reflect the actual *nifH* gene pool: $C=1-(n1/N)$ where *n1* is the number of clones that occurred once and *N* is the total number of clones (5). The Shannon diversity index (*H*) (14) was calculated using the software EstimateS 8.0.0 (<http://viceroy.eeb.uconn.edu/estimates>).

Nucleotide sequence accession numbers

All sequences were deposited in DDBJ under accession numbers AB471014 to AB 471313.

Results and Discussion

Soil properties

The aim of this study was to describe the diversity of diazotrophic bacteria in the rhizosphere of *M. malabathricum*. To reduce the effect of differences in climate among sampling sites, the three soil types (acid sulfate, peat and sandy clay soils) were located very close together, thus they can be categorized within the same type of climate and geographic location (Table 2). We checked the properties of the three soils (Table 2). The soils were characterized by soil pH (H₂O), available P and inorganic N. The acid sulfate soil had a relatively low pH (H₂O), as well as low levels of P and inorganic N. The peat had a relatively low pH (H₂O), and high levels of available P and inorganic N. The sandy clay had a relatively high pH (H₂O), low available P content and small amount of inorganic N. Thus, among the three types, the acid sulfate soil was considered a low-pH and nutrient-poor soil. Nutrient poor soils may have more developed plant-microorganism

Table 2. Chemical properties of acid sulfate, peat and sandy clay soils

Soil type	Sampling site		pH (H ₂ O)	P Bray I (mg kg ⁻¹)	NH ₄ (mg kg ⁻¹)	NO ₃ (mg kg ⁻¹)
Acid sulfate	03°26'24"S	114°50'50.7"E	3.00–3.22	0.83±0.00	144±66	2.2±3.1
Peat	03°26'42.6"S	114°39'57.4"E	3.10–3.75	32.22±7.06	271±261	2.0±2.2
Sandy clay	03°26'22.2"S	114°50'57.4"E	4.43–4.75	0.87±0.48	111±24	5.7±6.0

* The value of P Bray I, NH₄ and NO₃ are means±SDs (n=3).

relationships than other soils. Therefore, these three types of soil were considered suitable for investigating whether the diversity of diazotrophic bacteria associated with the rhizosphere of *M. malabathricum* was affected by the properties of soil.

Soil sampling

There is extensive heterogeneity of microorganisms in natural environments; especially in soil (1). To obtain accurate information on the diversity of diazotrophic bacteria, the method of soil sampling used is vital. Several recent reports have insisted that at least 4–6 plant samples are required to retrieve diazotroph-related genes from rhizosphere soils (6, 9, 19). We sampled the root systems of *M. malabathricum* from three individual plants in each soil type. We also sampled a large number (>10) of root pieces (*ca.* 10 cm) from the entire root system of a plant, and then mixed them. Thus, although the number of plants sampled was less than in other reports, the rhizosphere soil was sampled not from one root but from the root system, and then pooled. Thus we consider that DNA extracted from this pool represents DNA of the rhizosphere of the entire root system of *M. malabathricum*.

PCR amplification targeting the *nifH* gene

Kuske *et al.* (17) demonstrated that the amount of soil template DNA used to initiate PCRs was critical to the detection of target microorganisms. Therefore, the amounts of template DNA for PCR amplification were unified among samples from each soil type.

For PCR, two major *nifH*-specific primer sets have been used in recent studies. One is the “P-primer” designed by Poly *et al.* (24), and the other, the “Z-primer” designed by Zehr *et al.* (39). In this study, we used P-primer for amplification of the *nifH* gene. When the diversity of the *nifH* pool in a complex environmental sample was assessed by RT-PCR-DGGE, a more detailed fingerprint was detected with Z-primer than with P-primer, due to the presence of mismatches at nondegenerate positions (8). PCR even with P-primer detected a sufficient fraction of the diversity of the *nifH* gene pool among each soil type tested (Fig. 2). Thus our result was meaningful enough to describe the diazotrophic diversity in the rhizosphere of *M. malabathricum*, despite the limitations of P-primer.

Nested PCR is useful for detecting low copy-number genes in environmental samples. First, we tried to retrieve the *nifH* gene fragments from soil samples by PCR only with primers PolF and AQER, but could not (data not shown). As direct amplification failed, to increase sensitivity, the *nifH* gene fragments were retrieved by nested PCR. Demba Diallo *et al.* (7) explained that direct amplification failed using PolF and AQER in the first PCR due to quite a low *nifH* copy

number compared with the other *nifH* gene pools in the sample. Another possible explanation is contamination, by inhibiting substances (*e.g.*, humic acids), of the DNA extracted from soil samples (38). However, in the present study, the purity of extracted DNA was checked photometrically (based on spectra and the A260/230 ratio) and the level of contamination seemed low (data not shown).

Diversity of the *nifH* gene in the rhizosphere of *M. malabathricum* in three soil types

Although there have been many attempts to describe the diversity of *nifH* gene pools in environmental samples (6, 7, 9, 12, 19, 30, 31, 33), few studies have simultaneously displayed both qualitative and quantitative differences in *nifH* gene composition (18, 26, 27, 32). Phylogenetic trees have been frequently used to describe visually the microbial diversity in diverse environments. However, in this study, we tried to illustrate visually the difference in diversity of the *nifH* gene among soil types. Therefore, we assembled *nifH* reads into OTUs based on homology, characterized each OTU from the composition of reads, and analyzed the diversity of *nifH* in samples using a data matrix (Table 1).

The criterion for distinguishing OTUs must be determined prior to analysis. In the case of the 16S rRNA gene, sequences with >97% identity are typically assigned to the same species, though this criterion is controversial. On the other hand, the cut-off value, adjusted for some ubiquitous single copy protein-coding marker genes (such as *AtpD*, *CyrB*, *Hsp70*, *RecA*, *RpoB* and *TufA*), was defined as 94% identical at the nucleotide level, and was roughly comparable to the 97% cut-off commonly used for rRNA (34). Furthermore, Lovell *et al.* (19) adopted 94% identity in nucleotide sequence to distinguish OTUs based on *nifH* gene sequences, and distinguished 29 OTUs (disregarding OTUs with only one read) from 256 *nifH* reads. The validity of this cut-off value for *nifH* sequences from the *M. malabathricum* root system in the present study was verified by comparing the number of reads and the number of OTUs from 85 to 100% of the cut-off value (Fig. 1). For analyzing diversity, 300 reads were sequenced and assembled into only one OTU at a cut-off value of up to 86%, and 232 reads were assembled into 29 OTUs at a cut-off value of 94%. When the cut-off value was increased further (up to 98%), the number of reads decreased and the number of assembled OTUs increased. We defined an OTU as including >2 reads, therefore the 68 reads that were not clustered with other reads at the 94% cut-off value were excluded from the construction of the data matrix, and not used in the subsequent analysis of diversity.

The similarity of OTUs and soil type, according to the composition of *nifH* reads, were demonstrated through heat-mapping by combining two dendrograms (Fig. 2). At $P<0.05$,

the 29 OTUs were divided into two clusters. The pattern of distribution of OTUs differed between the three soil types, with fourteen *nifH* clusters (A1–A14) in the acid sulfate soil, eight clusters (P1–P8) in the peat soil, and seven clusters (S1–S7) in the sandy clay soil. The most dominant cluster was A11 in acid sulfate soil, P3 in peat and S5 in sandy clay, respectively. Furthermore, the most closely related organism found in the blastn search was *Bradyrhizobium* sp. IRBG228 (ca. 90% identity) for A11, *Beijerinckia indica* (ca. 85% identity) for P3 and *Mesorhizobium loti* (ca. 93% identity) for S5. Though lateral gene transfer has been speculated for the nitrogenase gene, *nifH* is considered a useful molecular marker for detecting and identifying diazotrophic organisms, because the phylogeny of the *nifH* gene generally corresponds to that of the 16S rRNA gene (40). Therefore, phylogenetic information on the dominant OTU within each soil type may be useful for inference of function in each soil type. S5 was the only common OTU not found in acid sulfate soil but present in both sandy clay and peat. The pattern of distribution of OTUs differed with soil type. However, a difference between rhizosphere and bulk soils was only found in the acid sulfate soil sample, while the pattern of distribution of OTUs did not differ in the peat and sandy clay soil. There was no OTU existing in all soil samples (i.e. “generalists”). In contrast, there were some OTUs that existed in only one soil sample (i.e. “specialists”). Considering the ubiquitous distribution of free-living diazotrophic bacteria, the lack of generalists among the three soil samples is not compelling. However, in low-nutrient and acid soils, the diversity of the *nifH* gene might be strongly dependent on soil properties.

Scale of the *nifH* clone library

In this study, the clone libraries were constructed from the *nifH* gene fragments retrieved from rhizosphere and bulk soils of *M. malabathricum*. Although the accuracy of *nifH* diversity from the clone library is dependent on the number of *nifH* reads (i.e. the scale of the *nifH* clone library), obtaining a large number of *nifH* reads is a time-consuming and expensive process. Therefore, we checked whether the scale of the *nifH* clone libraries reflected the number of reads sufficiently to assess the diversity of the *nifH* gene in natural environments by using *C* of the libraries (5). At a 94% cut-off value, the range of *C* was 78–92% (Table 3). Coelho *et al.* (6) compared the diversity of *nifH* gene pools in the rhizosphere of two cultivars of sorghum (*Sorghum bicolor*) treated with contrasting N levels. Coelho *et al.* (6) obtained 56–69 reads from each library, and the *C* of each *nifH* clone library had a range of 91.8–98.3%. Though the scale of *nifH* clones in our libraries was similar to their report (we obtained 50 reads from each *nifH* clone library), our *C* was relatively low. The reason for this difference is the criterion used for distinguishing OTUs. The criterion for the same clone at the genus level, was determined by the blastn top hit identification (including <94% identity) (6). On the other hand, in the present study, only clones with >94% identity were regarded as the same clone, even if the blastn top hit indicated the same genus. Therefore, our criterion for distinguishing a clone that appeared only once was stricter than that of Coelho *et al.* (6). According to the criterion defined by Coelho *et al.* (6), our *C* was in the range of 88–98%, in good

Table 3. Shannon diversity index and Coverage index (*C*) of each *nifH* clone library

Soil types	Fraction	Shannon diversity index	Coverage (%)
Acid sulfate	Rhizosphere	1.81	92
	Bulk	2.84	76
Peat	Rhizosphere	2.78	72
	Bulk	2.33	82
Sandy clay	Rhizosphere	2.82	78
	Bulk	2.05	84

The Shannon diversity index (*H*) and *C* were calculated from 50 reads from each *nifH* library based on 94% identity as the criterion for regarding an OTU as the same.

agreement with their results. We concluded that the *nifH* libraries were sufficiently large to describe the diversity of *nifH* gene pools in the overall root system of *M. malabathricum*.

The effects of the *M. malabathricum* rhizosphere

In this study, we investigated the diversity of diazotrophic bacteria in the habitat around the root of *M. malabathricum* in three soil types. Several plant species are known to release mucilage composed of detached cells and root exudates, and containing carbohydrates, organic acids, amino acids, vitamins and other compounds, into rhizosphere soil. Up to 20% of photosynthetically fixed carbon was released into the soil during the vegetative period of spring wheat (15). The amount and/or composition of root exudates released into the rhizosphere varies with plant species, growth stage and other environmental factors (15, 16). In hydroponic culture, *M. malabathricum* is one plant species that can exude large amounts of mucilage from its roots (37). It is also known that the activity and biomass of microorganisms are greater in rhizosphere soil than in bulk soil. Bacterial densities, metabolic signatures and genetic structures are also modified by the addition of artificial root exudates to bulk soil (4). Diazotrophic bacteria, which utilize root exudates for their growth, might accumulate in the rhizosphere of plants grown in low-nutrient conditions (11). However, in this study, the pattern of distribution of the *nifH* gene did not differ significantly between the rhizosphere and bulk soils in peat and sandy clay. There was a large difference in diazotrophic bacterial diversity with soil type. In peat and sandy clay soils, *H* indicated that the diversity of *nifH* was higher in rhizosphere than in bulk soil (Table 3). It seems that root exudates did not affect the accumulation of *nifH* diversity in the rhizosphere. In contrast, in acid sulfate soil, *H* was lower in the rhizosphere than bulk soil (Table 3). These results indicated that particular bacterial species might accumulate in the rhizosphere of *M. malabathricum* in acid sulfate soil.

Usefulness of clone libraries and further study

Molecular biological techniques such as PCR-DGGE and RFLP have been used to compare differences in diazotrophic diversity, based on fingerprint patterns (7, 8, 19, 31). These techniques are suitable for distinguishing existing microbial community structures and provide the advantage of high sample throughput. However, they are unsuited to determining the relative abundance of *nifH* gene phylotypes and to

sequencing individual *nifH* genes constituting a community. On the other hand, by using clone libraries, *nifH* gene sequences can be determined, revealing the composition of *nifH* gene pools. Real-time quantitative PCR (qPCR) has been used to determine the abundance of the *nifH* gene in the oceanic environment and soil (18, 35). The information obtained from analyzing clone libraries in this study will be utilized to design specific primer(s) for each respective *nifH* phylotype with qPCR.

Conclusion

This study revealed the diversity of the *nifH* gene associated with *M. malabathricum* roots in three soil types. Our results suggested that the diversity and phylogenetic composition of *nifH* were dependent on soil properties, and not remarkably different between rhizosphere and bulk soils in peat and sandy clay. However, in acid sulfate soil the diversity of the *nifH* gene shifted between rhizosphere and bulk soils. The *nifH* gene sequences were amplified not from RNA but from DNA extracted directly from soil, so the functions and expression of *nifH* in soil could not be determined. Furthermore, this information does not clarify the role of these diazotrophic bacteria in the supply of N to *M. malabathricum*. Diazotrophic bacteria may accumulate, depending on their environment, and their role in the acquisition of N for *M. malabathricum* may also differ among soil types. Furthermore, the co-culture of diazotrophic bacteria and non-diazotrophic bacteria was reported to enhance or induce nitrogen-fixing activity (21, 29). So we need to pay attention to microbial interactions between diazotrophic bacteria and other indigenous bacteria in the rhizosphere of *M. malabathricum*. Further study of the role of diazotrophic bacteria in the rhizosphere of *M. malabathricum* is progressing. It is also necessary to clarify the relationship between *nifH* gene expression and root exudates, and to compare the diversity of the *nifH* gene in the rhizosphere of different plant species.

Acknowledgements

This project was partly supported by eDNA project, Ministry of Agriculture, Forestry and Fisheries, Japan.

References

- Alexander, M. 1961. Introduction to Soil Microbiology. John Wiley & Sons, Inc., New York, USA.
- Altschul, S.F., T.L. Madden, A.A. Schäffer, 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.
- Amman, R.L., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59:143–169.
- Baudoin, E., E. Benizri, and A. Guckert. 2003. Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biol. Biochem.* 35:1183–1192.
- Chelius, M.K., and E.W. Triplett. 2001. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb. Ecol.* 41:252–263.
- Coelho, M.R.R., M. de Vos, N.P. Carneiro, I.E. Marriel, E. Paiva, and L. Seldin. 2008. Diversity of *nifH* gene pools in the rhizosphere of two cultivars of sorghum (*Sorghum bicolor*) treated with contrasting levels of nitrogen fertilizer. *FEMS Microbiol. Lett.* 279:15–22.
- Demba Diallo, M., A. Willems, N. Vloemans, and *et al.* 2004. Polymerase chain reaction denaturing gradient gel electrophoresis analysis of the N₂-fixing bacterial diversity in soil under *Acacia tortilis* ssp. *raddiana* and *Balanites aegyptiaca* in the dryland part of Senegal. *Environ. Microbiol.* 6:400–415.
- Demba Diallo, M., B. Reinhold-Hurek, and T. Hurek. 2008. Evaluation of PCR primers for universal *nifH* gene targeting and for assessment of transcribed *nifH* pools in roots of *Oryza longistaminata* with and without low nitrogen input. *FEMS Microbiol. Ecol.* 65:220–228.
- Deslippe, J.R., and K.N. Egger. 2006. Molecular diversity of *nifH* genes from bacteria associated with high arctic dwarf shrubs. *Microb. Ecol.* 51:516–525.
- Dobbelaere, S., J. Vanderleyden, and Y. Okon. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit. Rev. Plant Sci.* 22:107–149.
- Döbereiner, J., and F.O. Pedrosa. 1987. Nitrogen-Fixing Bacteria in Nonleguminous Crop Plants. Science Tech, Inc., Madison, WI, USA.
- Hamelin, J., N. Fromin, S. Tarnawski, S. Teyssier-Cuvelle, and M. Aragno. 2002. *nifH* gene diversity in the bacterial community associated with the rhizosphere of *Molinia caerulea*, an oligonitrophilic perennial grass. *Environ. Microbiol.* 4:477–481.
- Hashidoko, Y., Y. Gotou, M. Osaki, E. Purnomo, L.H. Suwido, and S. Tahara. 2006. Characterization and ecological role of free-living nitrogen-fixing bacteria isolated from the rhizoplane of *Melastoma malabathricum* inhabiting acidic plain lands in Kalimantan. *Tropics* 15:365–369.
- Hayek, L.A.C., and M.A. Buzas. 1997. Surveying Natural Populations. Columbia University Press, New York, USA.
- Hütsch, B.W., J. Augustin, and W. Merbach. 2002. Plant rhizodeposition—an important source for carbon turnover in soils. *J. Plant Nutr. Soil Sci.* 165:397–407.
- Kuiper, I., L.V. Kravchenko, G.V. Bloembergen, and B.J.J. Lugtenberg. 2002. *Pseudomonas putida* strain PCL1444, selected for efficient root colonization and naphthalene degradation, effectively utilizes root exudate components. *Mol. Plant-Microbe Interact.* 15:734–741.
- Kuske, C.R., K.L. Banton, D.L. Adorada, P.C. Stark, K.K. Hill, and P.J. Jackson. 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Appl. Environ. Microbiol.* 64:2463–2472.
- Langlois, R.J., D. Hümmel, and J. LaRoche. 2008. Abundances and distributions of the dominant *nifH* phylotypes in the Northern Atlantic Ocean. *Appl. Environ. Microbiol.* 74:1922–1931.
- Lovell, C.R., P.V. Decker, C.E. Bagwell, S. Thompson, and G.Y. Matsui. 2008. Analysis of a diverse assemblage of diazotrophic bacteria from *Spartina alterniflora* using DGGE and clone library screening. *J. Microbiol. Methods* 73:160–171.
- Marschner, H. 1995. Mineral Nutrition of Higher Plants. Academic Press, London, UK.
- Minamisawa, M., K. Nishioka, T. Miyaki, and *et al.* 2004. Anaerobic nitrogen-fixing consortia consisting of clostridia isolated from gramineous plants. *Appl. Environ. Microbiol.* 70:3096–3102.
- Osaki, M., M. Matsumoto, T. Watanabe, T. Kawamukai, T. Shinano, T. Nuyim, C. Nilnond, and T. Tadano. 1998. Strategies for adaptation of plants grown in adverse soils, p. 537–546. In N.E. Bassam, R.K. Behl, and B. Prochnow (ed.), Sustainable Agriculture for Food, Energy, and Industry. James & James Ltd., London, UK.
- Osaki, M., T. Watanabe, T. Ishizawa, C. Nilnond, T. Nuyim, C. Sittibush, and T. Tadano. 1998. Nutritional characteristics in leaves of native plants grown in acid sulfate, peat, sandy podzolic, and saline soils distributed in Peninsular Thailand. *Plant Soil* 201:175–182.
- Poly, F., L.J. Monrozier, and R. Bally. 2001. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res. Microbiol.* 152:95–103.
- Raymond, J., J.L. Siefert, C.R. Staples, and R.E. Blankenship. 2004. The natural history of nitrogen fixation. *Mol. Biol. Evol.* 21:541–554.
- Reiter, B., H. Bürgmann, K. Burg, and A. Sessitsch. 2003. Endophytic *nifH* gene diversity in African sweet potato. *Can. J. Microbiol.* 49:549–555.
- Roesch, L.F.W., F.A.O. Camargo, F.M. Bento, and E.W. Triplett. 2008. Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. *Plant Soil* 302:91–104.
- 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.
29. Shrestha, A., K. Toyota, M. Okazaki, Y. Suga, M.A. Quevedo, A.B. Loreto, and A.A. Mariscal. 2007. Enhancement of nitrogen-fixing activity of *Enterobacteriaceae* strains isolated from sago palm (*Metroxylon sagu*) by microbial interaction with non-nitrogen fixers. *Microbes Environ.* 22:59–70.
 30. Steward, G.F., B.D. Jenkins, B.B. Ward, and J.P. Zehr. 2004. Development and testing of a DNA microarray to assess nitrogenase (*nifH*) gene diversity. *Appl. Environ. Microbiol.* 70:1455–1465.
 31. Tan, Z., T. Hurek, and B. Reinhold-Hurek. 2003. Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in roots of rice. *Environ. Microbiol.* 5:1009–1015.
 32. Terakado-Tonooka, J., Y. Ohwaki, H. Yamakawa, F. Tanaka, T. Yoneyama, and S. Fujihara. 2008. Expressed *nifH* genes of endophytic bacteria detected in field-grown sweet potatoes (*Ipomoea batatas* L.). *Microbes Environ.* 23:89–93.
 33. Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi. 1995. Remarkable N₂-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *J. Bacteriol.* 177:1414–1417.
 34. Venter, J.C., K. Remington, J.F. Heidelberg, and *et al.* 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304:66–74.
 35. Wallenstein, M.D., and R.J. Vitgalys. 2005. Quantitative analyses of nitrogen cycling genes in soils. *Pedobiologia* 49:665–672.
 36. Watanabe, T., M. Osaki, T. Yoshihara, and T. Tadano. 1998. Distribution and chemical speciation of aluminum in the Al accumulator plant, *Melastoma malabathricum* L. *Plant Soil* 201:165–173.
 37. Watanabe, T., S. Misawa, S. Hiradate, and M. Osaki. 2008. Characterization of root mucilage from *Melastoma malabathricum*, with emphasis on its roles in aluminum accumulation. *New Phytol.* 178:581–589.
 38. Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63:3741–3751.
 39. Zehr, J.P., and L.A. McReynolds. 1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl. Environ. Microbiol.* 55:2522–2526.
 40. Zehr, J.P., B.D. Jenkins, S.M. Short, and G.F. Steward. 2003. Nitrogenase gene diversity and microbial community structure: A cross-system comparison. *Environ. Microbiol.* 5:539–554.