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Variation in the active diazotrophic community in rice paddy—*nifH* PCR-DGGE analysis of rhizosphere and bulk soil

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ABSTRACT

Biological nitrogen fixation (BNF) is an important source of nitrogen input in many natural ecosystems. The rice production today depends on large amounts of chemical nitrogen fertilizer, which is an environmental hazard in rice producing areas. Better exploitation of BNF is one way to reduce the use of chemical nitrogen fertilizer in the future. In this study the active diazotrophic community was investigated in nitrogen fertilized and un-fertilized rice field soils in Fujian Province, southeast China by PCR-DGGE of *nifH* mRNA, and the potential community by PCR-DGGE of the *nifH* gene. A total of 45 sequences representing 33 different sequence types were recovered from the DGGE gels. The retrieved cDNA sequences representing the active population of diazotrophs both in fertilized and un-fertilized soils dispersed throughout the *nifH* clades (alpha-, beta- and gamma *Proteobacteria*, *Firmicutes* and *Archaea*). Thirteen of the sequence types were most closely related to *Azoarcus* endophytes indicating widespread associations between heterotrophic nitrogen fixing bacteria and rice (*Oryza sativa*). The majority of the 13 sequence types were identified from the cDNA samples, showing that the *Azoarcus* might be an important active nitrogen fixing diazotroph in the paddy field. None of the sequence types were closely related to cyanobacteria, nevertheless previous studies from the same area had documented the presence of cyanobacteira in rice fields. The lack of identified cyanobacteria might be due to template discrimination in the PCR reactions, or low abundance of cyanobacteria compared to heterotrophic nitrogen fixing bacteria.

1. Introduction

Biological nitrogen fixation (BNF), the reduction of atmospheric nitrogen into bioavailable ammonium, constitutes an important source of nitrogen input in many natural ecosystems, both terrestrial and aquatic habitats. In terrestrial ecosystems, the estimated input of BNF averages 90–130 Tg per year (Kennedy and Islam, 2001) and in agricultural systems such as rice cultivation, the nitrogen input is essential for the fertility of the soil (Roger and Ladha, 1992; Ladha and Reddy, 2003). The capacity for nitrogen fixation is unique to certain groups within the domains *Bacteria* and

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Archaea (diazotrophs) (Young, 1992; Zehr et al., 2003). The *nifH* gene encoding the iron protein subunit of nitrogenase, is highly conserved among all diazotrophs and the phylogeny based on *nifH* genes has been shown largely to resemble the 16S rRNA phylogeny (Young, 1992, 1996; Zehr et al., 2003; Raymond et al., 2004) hence making *nifH* an ideal phylogenetic gene marker for investigation of potential nitrogen fixing organisms in natural environments. During the last few years successful amplification of the *nifH* gene have been achieved by polymerase chain reaction (PCR) to examine the diazotroph communities in various soil habitats (Rosado et al., 1998; Shaffer et al., 2000; Poly et al., 2001a; Bürgmann et al., 2004; Diallo et al., 2004; Yeager et al., 2004; Zhang et al., 2006b; Wakelin et al., 2007), and in association with plants (Ueda et al., 1995; Bürgmann et al., 2005; Knauth et al., 2005; Deslippe and Egger, 2006; Roesch et al., 2006; Soares et al., 2006; Zhang et al., 2006a). Although the *nifH* gene sequence is highly conserved among diazotrophs, PCR primers targeting slightly different fragments or designed with different degree of degeneracy has made it feasible to investigate certain groups or populations within the community. Most studies have focused on the diversity of the *nifH* gene pool by amplification of DNA directly extracted from the samples enquired, and those studies have given valuable information on the diversity of the potential nitrogen fixing population. However, novel molecular methods now give the opportunity to do research on the active diazotrophic community. Analyzing *nifH* mRNA extracted directly from the habitat of interest provides information about the diazotrophs actively expressing nitrogenase genes. This approach has recently been applied to the investigation of the active diazotropic community in aquatic environments (Bird et al., 2005; Church et al., 2005; Zehr et al., 2007), microbial mat (Steppe and Pearl, 2005), in rice roots (Ueda et al., 1995; Knauth et al., 2005) and soil samples (Burke et al., 2002; Brown et al., 2003; Bürgmann et al., 2005).

The paddy field is habitat for numerous groups of diazotrophs, which contribute significantly to the fertility of the soil (Kannaiyan et al., 1997; Engelhard et al., 2000; Hashem, 2001; Irisarri et al., 2001; Vaishampayan et al., 2001; Kennedy et al., 2004; Ariosa et al., 2005), and rice can get a notable proportion of its nitrogen requirement from the nitrogen fixed by the diazotrophs (Roger and Ladha, 1992; Roger, 1995; Ladha and Reddy, 2003). Despite extensive studies of biological nitrogen fixation in rice paddies, the present knowledge of diazotrophs in the rice field is predominantly based on cultivated organisms of cyanobacteria and members of the genera: *Azospirillum*, *Burkholderia*, and *Pseudomonas* (Choudhury and Kennedy, 2004; Kennedy et al., 2004). The traditional cultivation methods used to isolate these bacteria are usually highly selective, and often favor species that are not dominant in the natural habitat. Moreover, the diversity and presence of diazotrophs give no information on the active nitrogen fixing community.

In this study, the diversity of the potential nitrogen fixing community was investigated and compared with the active nitrogen fixing community by PCR amplification of the *nifH* fragment. DGGE was used to fingerprint the recoverable sequence diversity followed by sequencing of excised bands and classification by phylogenetic analysis.

Using this approach, the aim of the study was to detect potential changes in the *nifH* active diazotrophic community (i) between the rhizosphere and bulk soil, (ii) between fertilized and un-fertilized soils and, (iii) to monitor the changes as a function of day and night.

2. Materials and methods

2.1. Field characteristics and soil sampling

Soil was sampled from a paddy field in Yongtai County (25°39′N, 119°12′E), Fujian province, PR China. In Fujian province rice is harvested twice a year. The first growth season lasts from April until July, and the second from August to November. In March 2004, before the first growth season, a 1/15 ha paddy field was divided in two by a clay wall. Half of the field was fertilized according to local customs with N:C:P fertilizer and additional N in the form of urea (hereby referred to as fertilized field), while the other half received C:P fertilizer (hereby referred to as un-fertilized field). Chemical soil analysis was performed on soil samples collected 29 July 2004, before transplant of the second growth season (Table 1). The soil was a silt loam, and the heavy metal concentrations were found to be within the limits naturally occurring in soil; Cd 0.07 $\mu g\,g^{-1}$, Pb 30.7 $\mu g\,g^{-1}$, As 3.8 $\mu g\,g^{-1}$, and Hg 0.2 $\mu g\,g^{-1}$ (Alloway, 1995). The dry matter content in fertilized soil was 52%, and the pH 5.0, while the dry matter content of the un-fertilized soil was 46% and the pH 5.1. The pH of the surface water in both fields was 6.6.

Soil samples were collected on 27 September, during the tilling phase of the rice in the second growth season of 2004, at 12:00, and 24:00. At both times the temperature of the air, surface water, and soil surface was measured, and the light intensity was measured at 12:00; it was completely dark from about 20:30 (Table 2). Bulk soil was sampled from

Table 1 – The chemical components in the un-fertilized (UF) and fertilized (F) rice paddy soil before transplant the second growth season

	29th July	
	UF	F
Organic matter (%)	3.61	3.56
Total (%)		
N	0.21	0.23
K	1.47	1.32
P	0.03	0.03
Alkaline N ($mg\,kg^{-1}$)	299	549
Available ($mg\,kg^{-1}$)		
P	5.2	6.6
K	37	39
Trace elements ($mg\,kg^{-1}$)		
NH_4–N	61	67
NO_3–N	190	432
Exchangeable ($cmol\,kg^{-1}$)		
Ca	3.4	3.7
Mg	0.6	0.7

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Table 2 – Temperature and light intensity at the time of sampling

Temperature (°C)	Time, 12:00	Time, 24:00
Air	35	25
Surface water	30.7	25.6
Soil surface	29	26
Light intensity (lux)		
Above the rice plants	92000	0
Soil surface, shaded by the rice plants	13000	0

the surface soil (0–1 cm in depth) in a quadrate of approximately 10 cm × 10 cm at 3 randomly picked sites in the fertilized field, and pooled in a clean plastic bag. The soil was mixed and approximately 5 ml transferred to a 7 ml micro tube and submerged in liquid nitrogen until further handling (less than 36 h). The same procedure was repeated for surface soil from the un-fertilized field. Rhizosphere/root samples of 0–5 cm depth were taken from 3 randomly picked rice plants within each of the field types. Excess soil was carefully removed from the root and the 0–5 cm rhizosphere soil/root section was cut in pieces by a sterilized scissor and transferred to a sterile 7 ml micro tube and submerged in liquid nitrogen until further handling (less than 36 h).

2.2. RNA and DNA extraction

DNA and RNA were simultaneously extracted from the soil samples according to the protocol of (Hurt et al., 2001), with the following changes: 2 g from each of the three rhizosphere/root samples from un-fertilized soil was mixed in the same mortar. After graining with liquid nitrogen, the extraction process continued with 5 g of the root/soil/DNA/RNA solution. The same procedure was repeated for the fertilized rhizosphere samples. RNA and DNA were separated and rinsed using Qiagen RNA/DNA mini kit (Qiagen GmbH, Germany) and stored at −80 °C until further handling. cDNA was made from the RNA using cMaster RT (Eppendorf, Hamburg, Germany), in a two-step protocol as described below. DNA was extracted from batch cultures of the following microorganisms: cyanobacteria (*Nostoc* strain PCC7120, *Nostoc* strain PCC71102, *Anabaena variabilis* strain ATCC 29413, *Anabaena azollae* (Newton's isolate), and *Fisherella* sp.), *Methylocystis parvus* strain OBBP, *Methylosinus trichosporium* strain OB3b, *Rhizobium leguminosarum* biovar *trifolii*, and used as templates in optimization of the PCR conditions.

2.3. PCR

nifH primers with a broad specificity and applicable for environmental samples were tested. Included were the following primers: NifH1/NifH2 (Zehr and Reynolds, 1989) originally designed to target marine cyanobacteria and marine environmental diazotrophic communities, PolF/PolR and PolF/AQER (Poly et al., 2001a) initially designed for soil diazotrophs, and FGPH19/PolR (Simonet et al., 1991), where FGPH19 was originally designed as a *Frankia* specific primer. A two-step RT-PCR protocol was chosen for the RNA. The RT reaction was set up according to the manufacturer's protocol (Eppendorf, Germany), with a reaction temperature of 42 °C for 30 min. The cDNA was stored at −20 °C until further handling. A direct PCR approach with GC-clamp primers gave no products using the cDNA as template; therefore a nested PCR protocol was performed on the cDNA samples. In the first PCR reaction, a 370 base pair fragment of the *nifH* gene was amplified using the PCR primers PolF and PolR (Table 3) (Poly et al., 2001a). Each PCR reaction contained 1× PCR reaction buffer, 1× Q solution (Qiagen), 200 μm of each dNTP, 15 pmol of each primer, 1 unit Taq DNA polymerase (Qiagen), and 10 ng cDNA template. Amplification was performed in a Mastercycler® ep gradient (Eppendorf), with initial denaturation of 15 min at 95 °C, followed by 30 cycles of 1 min, 94 °C, 1 min, 55 °C, and 1 min, 72 °C, and a final extension of 10 min at 72 °C. PCR products were analyzed on a 2% agarose gel (SeaKem LE Agarose, Cambrex Bio Science Rockland, USA), stained with ethidium bromide, and visualized under UV light. In the second PCR, the same PCR mix and amplification protocol were used, but with the PCR primers PolFI/AQER-GC30 (Table 3), and 1 μl of the PCR products from first PCR as template. Products were checked for correct size at 2% agarose gel, and stored at −20 °C until analyzed on DGGE. The DNA was PCR amplified in a one step reaction with the primers PolFI/AQER-GC30 (Table 3) using the previously referred protocol, but with 40 cycles amplification. PCR amplification from cDNA and DNA was repeated three times and the different reactions were analyzed separately by denaturing gradient gel electrophoresis.

2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed on a Dcode system (BioRad, CA, USA), with 1 mm thick acrylamide–bisacrylamide (37.5:1) gels run in 0.5× TAE (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA) at 60 °C and 75 V. Gel solutions were made according to the manufacturer's instructions. The products were separated on an 8% (w/v) acrylamide–bisacrylamide gel with a 40–65%

Table 3 – Primers used in this study

Name	Sequence	Reference
PolF	TGC GAY CCS AAR GCB GAC TC	Poly et al. (2001a)
PolR	ATS GCC ATC ATY TCR CCG GA	Poly et al. (2001a)
PolFI	TGC GAI CCS AAI GCI GAC TC	Modifications this study
AQER	GAC GAT GTA GAT YTC CTG	Poly et al. (2001a)
AQER-GC30	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC GAC GAT GTA GAT YTC CTG	Modifications this study

Modified bases: I = Inosine, Y = CT, S = CG, R = AG, B = GCT.

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denaturing gradient, for 16 h. The gels were stained for 20 min in 1× SybrGold (Molecular probes, OR, USA) and visualized by UV transilluminator and recorded by typhoon scan. Bands appearing on the gels were excised and transferred to micro tubes containing 300 mM NaCl, 3 mM EDTA and 30 mM Tris–HCl, pH 7.6 (Rölleke et al., 1996). The bands were incubated at 4 °C over night for the DNA to disperse into the buffer. The DNA from the excised bands were then re-amplified and band purity was checked on DGGE, before sequencing.

2.5. *Sequencing and phylogenetic analyses*

Excised bands were PCR amplified and fluorescently labeled using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's manual, and sequenced in both directions using the Automatic ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems).

Sequences were assembled and corrected using Pregap4 and Gap4 of the Staden software package (Staden, 1996). The length of the sequences varied from 155 and 317 bp. The sequences were aligned using Clustal X software (Thompson et al., 1997) with manual amendments performed using Se-Al (Rambaut, 1996). A variant of the general time reversible model (Tavaré, 1986) with gamma distribution of rates (Yang, 1993) [SYM + G] was selected for data set by using MrAIC (Nylander, 2006) and PHYML (Guindon and Gascuel, 2003) with the AICc criterion. A phylogeny was estimated with Bayesian inference (Yang and Rannala, 1997) using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on a 7 processor cluster running GNU/Linux. Double analyses were performed starting from flat priors and a random tree, each running 4,000,000 generations of the Markov Chain (Monte Carlo) with eight separate chains and a chain temperature setting of 0.2. Sampling frequency was set to once every 1000 generations and the initial 25% of the samples were discarded (chain "burnin") before summarizing the results. The tree presented was based on graphical output from TreeView (Page, 1996).

3. Results

3.1. *PCR and DGGE optimization for the nifH primers*

nifH primers which have shown a broad specificity from former studies on environmental samples were tested (Table 3). The primer pairs were optimized with respect to different annealing temperatures and template concentrations of DNA extracted from different cultures of microorganism as well as from the paddy soil. Of the tested PCR primers, only the primer pair PolF/PolR gave one single PCR band of expected size from all the tested bacteria strains and the paddy soil DNA as visualized on agarose gel electrophoresis (data not shown). The other primer pairs either gave unspecific binding resulting in several amplicons, or failed in amplifying the *nifH* genes from some of the test organisms and/or environmental DNA (data not shown). However, the efficiency of the PCR amplification was reduced when a 40 bp GC clamp was attached to the forward PolF primer. Consequently, different lengths of the GC clamp (40 and 30 bp) as well as the position of the GC clamp at forward (NifH1, PolF) or reverse (PolR, AQER) PCR primers were tested. Moreover, replacing the fourfold degenerated oligonucleotides "N" of primer PolF with Inosine, to stabilize the melting temperature of the PCR product for DGGE analyses was investigated. The primer pair that gave a single PCR product of expected size, and good separation on DGGE gels was found to be PolFI/AQER-GC30 (Table 3). In this primer pair the degenerated nucleotides in the PolF primer was replaced with Inosine (PolFI), and a 30 bp GC clamp was attached to the reverse primer AQER (Table 3).

3.2. *DGGE analysis of the potential and active-diazotrophic community in rice paddy soil*

DNA extracted from bulk (surface soils) and rhizosphere soil at midday (12:00) and midnight (24:00) was successfully PCR amplified in a one step reaction with the primer pair PolFI/AQER-GC30. The generated products show stable and reproducible DGGE profiles (Fig. 1). The obtained DNA profiles gave several bands with an almost identical migration pattern.

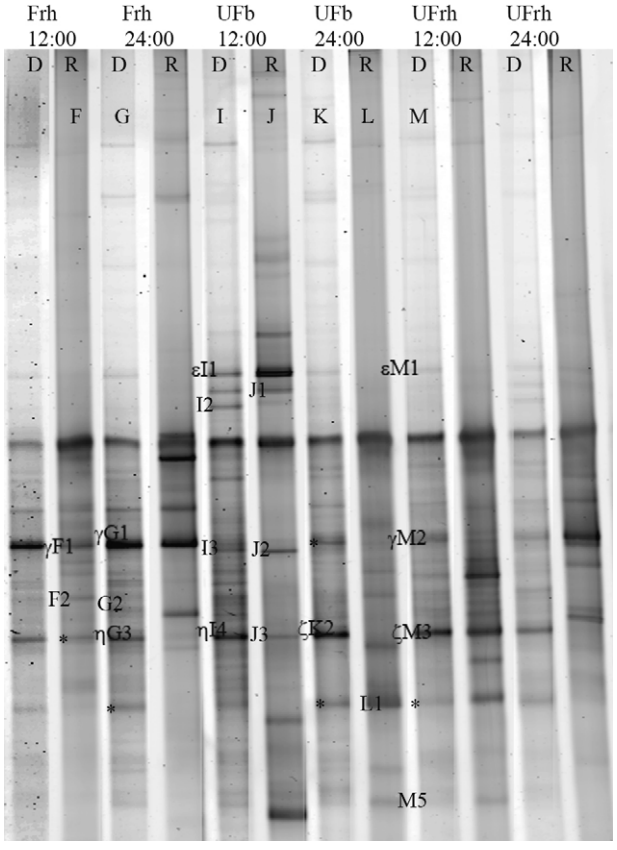

Fig. 1 – DGGE profiles of DNA (D) and c-DNA (R) from fertilized rhizosprere soil (Frh), un-fertilized bulk (UFb) and un-fertilized rhizosphere soil (UFrh) sampled at midday (12:00) and midnight (24:00). The bands indicated by letter (for the lane) and number (for the band) were excised and sequenced, while bands indicated by an asterisk were excised, but rejected from further analyses due to improper reamplification. Bands marked with the same symbols, had identical sequences.

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Table 4 – The most closely related described bacteria or clones to the sequences retrieved in this study, analyzed by NCBI BlastN

Band	No. of bases	Accession number	% Sequence identity	Source	Reference
‡2.3	303	AY231559	81	Uncultured nitrogen-fixing bacterium clone	Hurek et al. (unpublished)
‡7.2	302				
6.3	306	DQ520543	81	Uncultured bacterium clone *nifH* gene. Rhizosphere soil	Izquierdo et al. (unpublished)
F2	300				
1.2	225	DQ520307	79		
7.1	296	AF414618	88	Uncultured bacterium clone	Bagwell et al. (2002)
8.2	199	DQ480893	90	Uncultured bacterium clone	Izquierdo and Nuesslein (unpublished)
J2	212	DQ142736	95	Uncultured bacterium	Jasrotia and Ogram (unpublished)
J3	183	AY787580	92	Wastewater	Bowers (unpublished)
1.4	239	DQ776758	92	Diazotrophic rhizosphere community, Bt-transgenic white spruce	Lamarche and Hamelin (unpublished)
6.4	296	DQ776750	91		
α4.6	293	AF331989	95	Endophytic *Azoarcus* wild rice	Engelhard et al. (2000)
α5.2	304		95		
2.4	297		94		
5.1	274		94		
L1	304		93		
β3.2	293	AF331990	90		
β6.2	296		91		
γF1	309		93		
γG1	301		92		
γM2	317		93		
8.1	300	AF331988	92		
2.6	226	AY231522	91	*Azoarcus* kallar grass endophyte	Hurek et al. (2002)
δ3.3	302	AY231530	99		
δ6.1	297		99		
εI1	291		100		
εM1	293		100		
I2	309		98		
J1	314		97		
ζ1.3	288	AY231516	95		
ζ4.5	301		95		
ζK2	300		95		
ζM3	307		95		
6.5	303	AB184914	90	Diazotrophs associated with rice roots	Elbeltagy and Ando (unpublished)
M5	314	AB208251	84		
I3	309	AY231577	93	Root tissue, *Oryza longistaminata*	Hurek et al. (unpublished)
2.5	155	DQ426129	94	Uncultured endophytic bacterium clone associated with maize	Roesch et al. (unpublished)
4.3	290	DQ029203	86	*Azonexus caeni*	Quan et al. (2006)
ηG3	305	AE017282	91	*Methylococcus capsulatus* Bath	Ward et al. (2004)
ηI4	303		91		
1.6	297		91		
7.3	278		90		
G2	322	AY428644	91	*Rhizobium daejeonese*	Quan et al. (2005)
†1.5	295	AB217474	90	*Sphingomonas azotifigens*	Xie and Yokota (2006)
†8.3	295				

Bands marked with the same symbol had identical sequences.

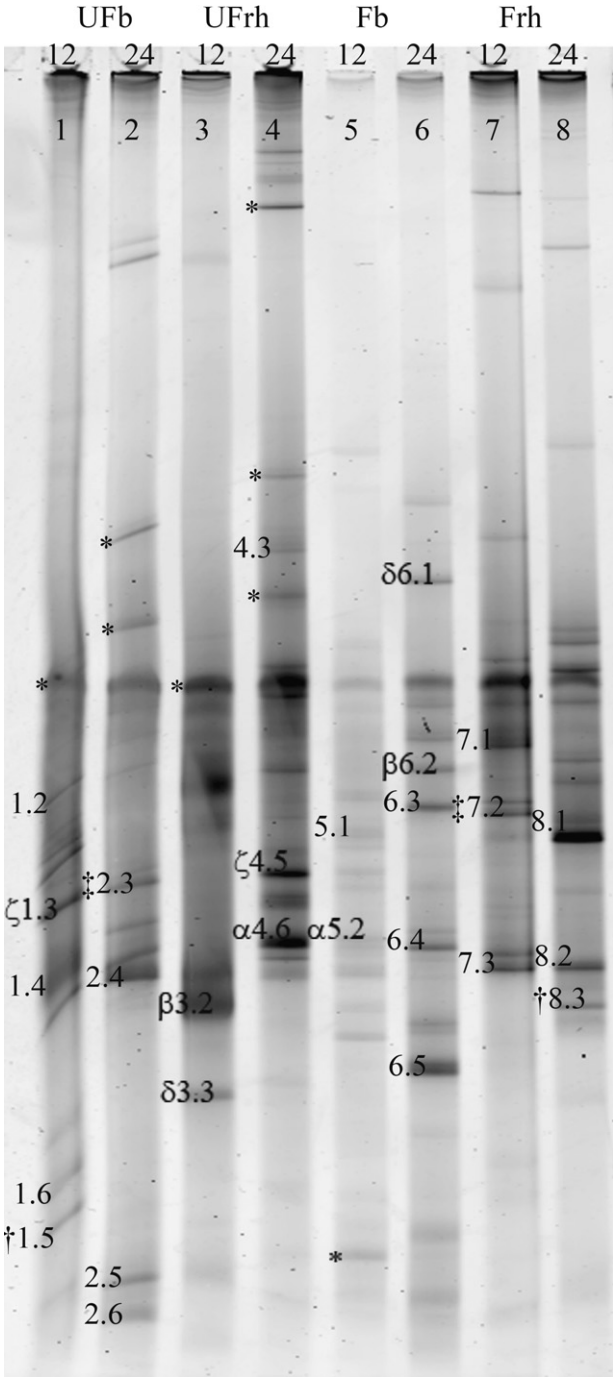

Fig. 2 – DGGE profiles of c-DNA from fertilized rhizosprere (Frh) and bulk soil (Fb), un-fertilized bulk (UFb) and rhizosphere soil (UFrh), sampled at midday (12:00) and midnight (24:00). The bands indicated by numbers (lane and band) were excised and sequenced, while bands indicated by an asterisk were excised, but rejected from further analyses due to improper reamplification. Bands marked with the same symbol, had identical sequences.

DGGE bands with the same migration position and identical sequences obtained from different soil types and sampling times were bands G1 (fertilized soil, rhizosphere 24:00) and M2 (un-fertilized soil, rhizosphere 12:00), G3 (fertilized soil, rhizosphere 24:00) and I4 (un-fertilized, bulk soil 12:00), band K2 (un-fertilized, bulk soil 24:00) and M3 (un-fertilized rhizosphere soil, 12:00), and bands I1 (un-fertilized, bulk soil 12:00) and M1 (un-fertilized soil, rhizosphere 12:00) (Fig. 1, and Table 4).

A nested PCR approach was required to obtain PCR products from the cDNA and in contrast to the DGGE profiles obtained from DNA, the profiles of the active population (RNA) were unique for all the different sampling parameters; fertilized vs. un-fertilized soil, bulk vs. rhizosphere soil, and day vs. night (Fig. 2). Only two of the sequenced bands had both identical migration patterns and sequences; band 4.6 and 5.2 (Fig. 2). A BlastN search in the NCBI database showed that these sequences had highest sequence similarity to *Azoarcus* AF331989 (95%) (Engelhard et al., 2000). However, band 4.6 originated from un-fertilized rhizosphere soil, night sampling, while 5.2 originated from fertilized bulk soil day sampling.

The DGGE profiles generated from cDNA gave small variations from one PCR reaction to another. This can be illustrated by comparing lane J (Fig. 1) with lane 1 (Fig. 2), and lane F (Fig. 1) with lane 7 (Fig. 2), which represent two different PCR amplifications from the same cDNA pool. The sequence of forward and reverse strand of the excised bands matched perfectly, and gave no indications of heteroduplex formation; nevertheless none of the bands from the two gels gave identical sequences (Table 4). Moreover, bands with different migration pattern but identical sequences were obtained. Those bands were respectively 1.5, 8.3; 3.2, 6.2; 2.3, 7.2; 1.3, 4.5 and 3.3, 6.1 (Fig. 2).

3.3. *Phylogenetic analysis*

In total, 45 DGGE bands were sequenced, where 12 originated from DNA; G1-G3, I1-I4, K1, M1-M3, and M5. The length of the sequences varied from 155 to 317 bp. A BlastN search revealed that the 45 sequences represented 33 different sequence types among which 13 sequence types (22 sequences) showed between 90 and 100% sequences identity to *Azoarcus,* either endophyte clones from wild rice (Engelhard et al., 2000) or Kallar grass (*Leptochloa fusca*) (Hurek et al., 2002) (Table 4). The Phylogenetic analysis showed that the retrieved sequences clustered within the alpha-, beta- and gamma *Proteobacteria*, and *Firmicutes* (Fig. 3).

4. Discussion

Rice paddies are known to hold an enriched community of cyanobacteria and heterotrophic nitrogen fixing bacteria. Many studies have been based on culture dependent approaches but in recent years applications of molecular methods have facilitated the study of individual population's and their specific activity (Ueda et al., 1995; Engelhard et al., 2000; Eller and Frenzel, 2001; Tan et al., 2001; Song et al., 2005; Xie and Yokota, 2006). In this study the potential and the active nitrogen fixing diazotrophic community was studied in rice field soil by *nifH* PCR-DGGE analysis.

Direct amplification of DNA for DGGE analysis with the primer pair PolFI/AQER-GC30, was successfully performed in this study. Nevertheless, a nested PCR approach was required

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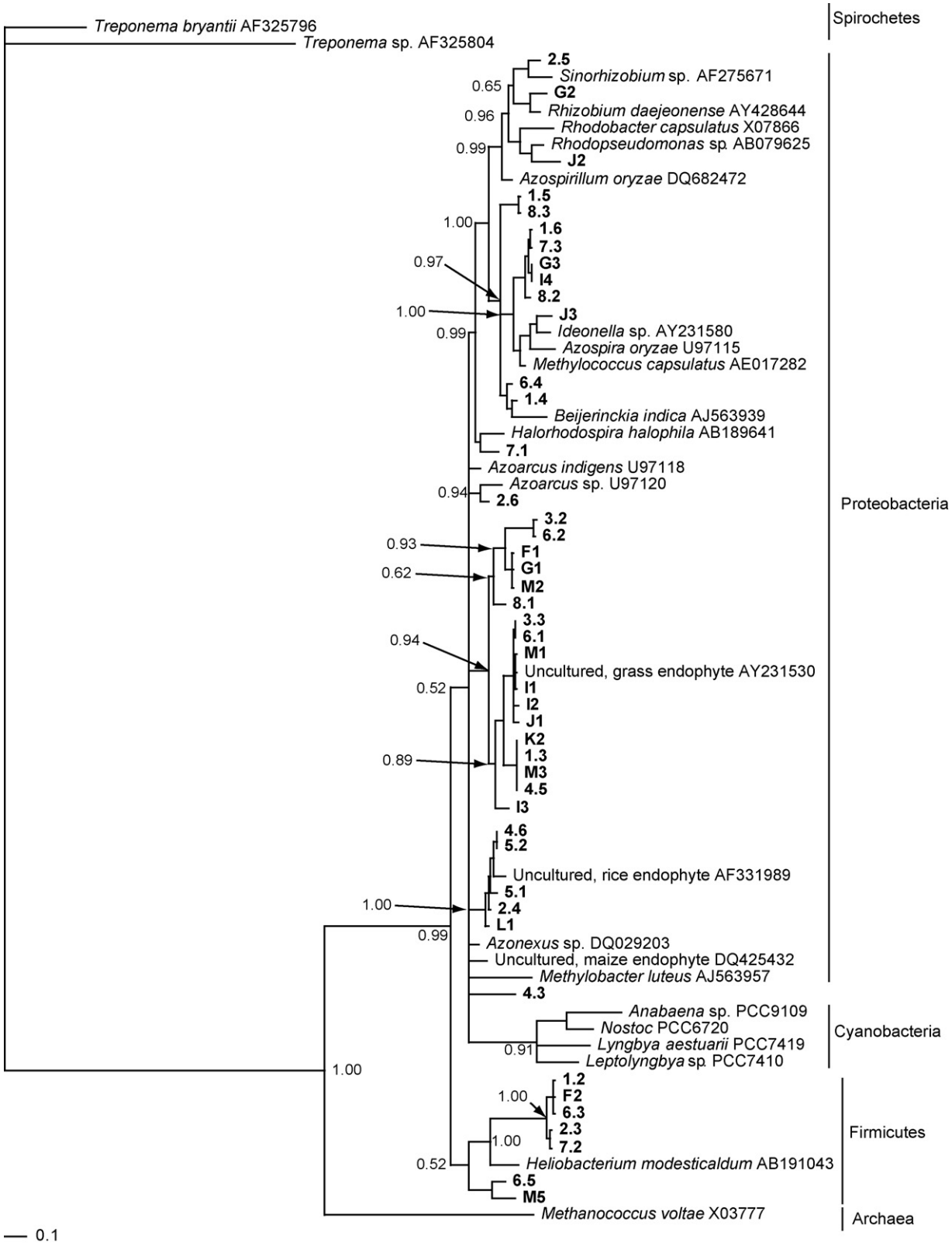

Fig. 3 – Phylogeny of the *nifH* DGGE bands from rice paddies analyzed in this study together with selected closely related clones and bacteria. The tree topology was estimated with Bayesian inference using MrBayes 3.1.2 and is a 50% majority rule consensus tree based on 6000 trees sampled from the Markov chain after "burning". It was rooted on the branch to *Treponema bryantii* (AF325796). Clade credibilities are indicated for the nodes. Accession numbers refer to GenBank and their names or habitats of isolation are given.

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to obtain PCR products from the cDNA which might be an indication for a low number of active diazotrophs in the soil sample. A low abundance of diazotrophs in the soil were also suggested as a possible explanation for the need of a nested PCR approach, when the diazotrophic community on DNA level under the legume trees *Balanites aegyptiaca* and *Acacia tortilis* ssp. *raddiana* were studied (Diallo et al., 2004). Use of nested PCR approach should be evaluated with caution since the PCR bias can increase due to preferential amplification (Suzuki and Giovannoni, 1996; Boon et al., 2002). However, previous studies have shown preferential amplification not to affect the analysis of the dominating species in a microbial community, and that minor abundant species, which will not be visible in a direct approach can be studied using an indirect approach (Heuer et al., 1997; Boon et al., 2002).

The relatively homogenous composition of *nifH* sequences observed from extracted DNA indicated a homogenous gene pool/potential diazotrophic community in the different soil types. This result is in accordance with other studies where the structure of the *nifH* gene pool has been shown to remain quite stable in the soil as a function of time (Shaffer et al., 2000; Poly et al., 2001b), even though the study of Diallo et al. (2004) demonstrated a significant difference in the diazotrophic pool as an effect of soil conditions (dry or rainy season) and sampling depth. The lack of observed variations between bulk and rhizosphere soil in the present study might be due to the farming practice. Before and after every growth season, the soil is thoroughly mixed, and straw ploughed into the soil, which result in a rather homogenous soil.

Consequently, an effect of root exudates on the bacterial community and in particular the diazotrophic population, which has been well studied and documented (Bürgmann et al., 2005; Knauth et al., 2005; Soares et al., 2006) could not be demonstrated in this ecosystem. The high numbers of sequence types related to *Azoarcus* found in the rhizosphere soil indicate a widespread association between rice and *Azoarcus*, and support previous findings (Reinhold-Hurek and Hurek, 1998; Hurek et al., 2002). *Azoarcus* is known to survive poorly outside the root (Reinhold-Hurek and Hurek, 1998), and the discovery of a high number of *Azoarcus* like sequences in the bulk soil was therefore quite surprising. These findings may, as mentioned above, be a result of the local farming practice which can create areas in the bulk soil where easy degradable nutrition in decaying plant material allows endophytes to grow. *Azoarcus* has also been found in association with fungi (Hurek et al., 1997), which one would expect to thrive in the rice field in general, and associated with the straws in particular.

Although it is well known that the application of N-fertilizer have a negative effect on nitrogen fixation activity in the rice field (Kyaw et al., 2005; Tanaka et al., 2006) recent investigations on the effect on the microbial population in the soil is contradictory (Knauth et al., 2005; Roesch et al., 2006). A remarkable stability in the rhizosphere diazotrophic population was observed after manipulation with the fertilization (Bagwell and Lovell, 2000; Piceno and Lovell, 2000). The results from this study show that even though a large difference in available nitrogen was observed between the fertilized and un-fertilized soils (Table 1), the structure of the potential nitrogen fixing community were not affected (Fig. 1). However, the DGGE profile of the active *nifH* transcribing community revealed a high diversity between the individual samples (Fig. 2). The variation observed in the DGGE profile between individual runs from the same PCR reaction might be due to preferential template amplification caused by a low abundance of the sequence types, or evenly abundant diazotrophic species (Felske et al., 1996; Suzuki and Giovannoni, 1996). Moreover, the presence of identical sequences of bands with different migration pattern is a common described bias associated with DGGE which is either due to co-migration (Ferris et al., 1996), or that dominant amplicons are generally dispersed in the gel (Nikolausz et al., 2005). Because of these DGGE biases it is not possible to rely solely on the DGGE gel profiles to observe population structure. The DGGE analyses have to be followed by sequencing and phylogenetic analysis to verify similarities and differences in population composition observed in the DGGE profiles.

Regardless that cyanobacteria are known as an abundant nitrogen fixing microorganism in the rice paddy (for review, see Vaishampayan et al., 2001), none of the sequence types resembled cyanobacteria. This was in contradiction to a previous study from the same province of China, using a PCR approach targeting 16S rDNA. In this study a total of 24 different cyanobacterial phylotypes were identified from bulk soil samples, representing 11 genera (Song et al., 2005). The absence of cyanobacteria from the present study is either due to bias caused by preferential amplification as discussed above, or that the cyanobacteria were present in such a low number that they were undetectable using the more universal *nifH* primers, compared to the cyanobacterial specific 16S rDNA primers used by Song et al., 2005. After all, the *nifH* primers used in the present study has been documented to target cyanobacteria from soil samples (Diallo et al., 2004), and did amplify cultured cyanobacteria used as control in this study. The cyanobacteria may contribute significantly to the total nitrogen fixation in the field even though they might not be dominating diazotrophs in the soil. It has recently been observed that the main contribution of nitrogen fixation from cyanobacteria in the rice field are from cyanobacteria epiphytically associated with the macrophyte *Chara vulgaris* (Ariosa et al., 2004).

5. Conclusion

In this study we found that the active diazotrophic community varied strongly between sites and time of sampling, however, the same sequence types were found at different times and soil types, and the majority of the sequences clustered with *Azoarcus* endophytes. This indicates that the endophytic association between *Azoarcus* like bacteria and rice plants seems to be widespread. Moreover, 11 of the 13 sequence types related to *Azoarcus* were identified from the cDNA samples; indicating that *Azoarcus* might be an important nitrogen fixing diazotroph in the paddy field. The lack of cyanobacteria-like sequences might indicate that heterotrophic diazotrophs are more abundant in the paddy field, both in bulk and rhizosphere soil, and more important contributors to biological nitrogen fixation than traditionally assumed. The DNA profiles were fairly stable between the different soils and thus shared

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the same genetic pool for a potential active diazotrophic community.

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Appendix A

The sequences have following accession numbering in GenBank database: 1.2, EF583561; 1.3, EF583562; 1.4, EF583563; 1.5, EF583564; 1.6, EF583565; 2.3, EF583566; 2.4, EF583567; 2.5, EF583568; 2.6, EF583569; 3.2, EF583570; 3.3, EF583571; 4.3, EF583572; 4.5, EF583574; 4.6, EF583575; 5.1, EF583576; 5.2, EF583577; 6.1, EF583578; 6.2, EF583579; 6.3, EF583580; 6.4, EF583581; 6.5, EF583582; 7.1, EF583583; 7.2, EF583584; 7.3, EF583585; 8.1, EF583586; 8.2, EF583587, 8.3, EF583588; F1; EF583589; F2, EF583590; G1, EF583591; G2, EF583592; G3, EF583593; I1, EF583594; I2, EF583595; I3, EF583596; I4, EF583597; J1, EF583598; J2, EF583599; J3, EF583600; K2, EF583601; L1, EF583602; M1, EF583603; M2, EF583604; M3, EF583605; M5, EF583606.

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