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Molecular Analysis of the Microbial Guild Fixing Nitrogen in Ricefield Soils in Missouri

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Abstract: Non-symbiotic diazotrophic microbes are important contributors to global N budgets in cereal crops. Knowledge of the biogeography of the organisms in this functional guild increases our understanding of biological N fixation in diverse locations and climates. Here, we describe the diazotrophic community in the previously unstudied, extensive ricefields of southeast Missouri, using restriction fragment length polymorphism (RFLP) analysis and sequencing of *nifH* gene clones. While nine RFLP patterns were observed in random *nifH* clones, these groups were not all supported by gene sequencing, suggesting that the RFLP of *nifH* genes alone is not suitable for describing diazotrophic guilds. Dozens of *nifH* clones from Missouri ricefield soils were sequenced and analyzed phylogenetically. The *nifH* genes detected were predominantly from *Geobacteraceae*, most closely related to *Geobacter* and *Geomonas* species. There were substantial clusters of *nifH* clones most closely related to Desulfovibrionales and other *Proteobacteria*. Many of the clones did not closely cluster with *nifH* sequences from known isolates or clades. No cyanobacterial or archaeal sequences were detected in the Missouri ricefield soils. The microbial guild fixing N appeared to be rich in anaerobes and lithotrophs. Organisms in *Geobacter* and *Geomonas* seem to be cosmopolitan, but endemism was evident, since *nifH* clones were recovered that formed clusters not previously reported from ricefields in other locations.

Keywords: diazotrophs; ecology; rice; soil

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1. Introduction

Rice is arguably the most important agricultural crop in the world, with more than 10% of the world's arable land occupied by rice, providing 20% of global human per capita energy and 15% of per capita protein [1]. Rice cultivation is important to the global ecosystem. Some 25% of total global methane emissions arise from rice paddies [2]. Microbial processes during rice cultivation influence soils and geochemical cycles in the field. There seems to a role for biological N fixation (BNF) in rice agroecologies [3]. Traditional wetland rice cultivation produces moderate but stable yields without N fertilization [4]. Modern high-yield rice cultivation requires N fertilization beyond the contribution from microbial activity. However, BNF can be an agronomically important fertilizer for rice production [5–7]. Non-symbiotic microorganisms contributed 24% of total N in cereal crops during a 50-year global budget assessment [8]. Given its limited nutrient runoff, BNF may be a sustainable contributor to improving crop yields [9,10]. The conditions of rice paddies are particularly appropriate for BNF since submerged soils maintain suitable oxygen tensions and the crop provides a ready supply of photosynthate. The structures of diazotroph communities that develop are key factors in regulating soil BNF in ricefields in diverse locations and climates. The current study examines the microbial community in a rice-growing area not previously investigated to better understand the biogeography of diazotrophs performing BNF in ricefields.

Previous studies have examined the non-symbiotic diazotrophic microbial community in the bulk soils of ricefields [11–15], including studies that used molecular techniques [16–20]. The sediments of rice paddies harbor a variety of diazotrophic microbes and methanotrophs

that can fix N [18,21,22]. Endophytic bacteria associated with rice roots that fix N have been detected, isolated, and characterized [22–24]. The description of the diazotrophic microbial community is commonly performed through an analysis of the Fe protein subunit of nitrogenase, which is encoded by the *nifH* gene. It has a well-conserved sequence, showing strong similarities among diverse diazotrophic organisms, including those containing the alternative nitrogenase [25–28]. *NifH* sequences are useful for demonstrating taxonomic relationships and characterizing complex diazotrophic communities in natural systems [26–31].

Nearly every study of diazotrophs in ricefields has been performed in Asia, with apparently only one significant study in US ricefields [32]. While equivalent environments might be expected to select for similar microbial communities, this often is not the case [33]. Certain microbes tend to be more cosmopolitan, while others are more endemic. Environments that seem equivalent often have subtle differences that influence microbial community structure, such as historical land use. The current study is the first focusing on diazotrophs in ricefields in the silty loam soil of southeast Missouri, an important region for US rice production.

2. Materials and Methods

2.1. Ricefields and Soil Sampling

The plots of Crowley silt loam at the Southeast Missouri State University facility were separated with individual levees, gates, and irrigation water supplies. The drill-seeded rice plots (450 seeds m^{-2}) were graded to 0.075% slope, with a field cultivator being used before planting with a 19 cm row spacing. Subplots were 1.3 m wide and 3.9 m long. All plots were planted in early May and treated with quinclorac + propanil herbicides to control weeds. Certain plots were flooded at the first tiller growth stage, and some were given a pre-flood and mid-season manure application of 200 kg N ha^{-1}.

Soils were sampled in bulk (approximately 0.5 kg) from the top 10 cm from random farm plots. Samples (4) were brought to the field station, where they were processed by hand to remove visible plant material and debris, homogenized, sieved with a 2-mm screen, aliquoted, and frozen on dry ice. Samples remained frozen during transport to Wichita State University and were stored at −80 °C prior to extraction.

2.2. Direct Extraction of DNA

Total community DNA was directly extracted from soils and cleaned using an extensive protocol for cell breakage, DNA extraction, and purification outlined previously, which was more effective than commercial kits for soils with high humic content [34]. Aliquots of soil (2 g) were suspended in 10 mL sterile centrifuge tubes with 5 mL of breakage buffer (100 mM Na_2SO_4, 1% [*w*/*v*] SDS, [pH 8.0]) and mixed with 1.5 g of sterile zirconia beads (0.1 mm dia). The samples were processed by bead beating using a vortex mixer at 20 °C for 3 cycles of 5 min on/5 min off to prevent overheating. Proteinase K was added (0.5 mL of fresh stock) to 100 µg mL^{-1} and the sample was incubated for 1 h at 37 °C with gentle agitation on a rotary shaker. Cell breakage was continued with six freeze–thaw cycles [35] of 3 min each in a dry ice/ethanol bath and an 80 °C water bath, with vortex mixing between cycles. An aliquot (900 µL) of 5 M NaCl was added to each sample along with 750 µL of 10% CTAB solution (hexadecyltrimethylammonium bromide; 0.75 M NaCl, 10 mM EDTA, 50 mM Tris [pH 8.0]) The homogenates were mixed well and incubated at 60 °C for 20 min without mixing. The remaining steps were performed at 4 °C or on ice. Soil particles and debris were removed by centrifugation at 3000× *g* for 5 min. The supernatant was transferred to a sterile centrifuge tube and the soil pellet washed with 2 mL of TE buffer (1 mM EDTA, 10 mM Tris [pH 7.6]). After centrifugation at 3000× *g* for 5 min, the supernatants were combined and brought to 10 mL with TE buffer. An equal volume of Tris-saturated phenol (pH 8.0) was used for extraction with centrifugation at 8000× *g* for 10 min. The aqueous phase was further extracted with an equal volume of 24:1 chloroform/isoamyl alcohol, followed by centrifugation at 8000× *g* for 10 min. An equal volume of isopropanol was added, and the DNA was precipitated overnight at −20 °C,

followed by centrifugation at 12,000× *g* for 20 min. The resulting pellet was washed with 2 mL of ice-cold 70% ethanol and dried in a vacuum desiccator.

An extensive purification scheme was used to remove contaminants from the DNA extracts that can inhibit PCR amplifications, as we found that commercial kits often were not sufficient in cleaning extracts from soils with high humic contents. The darkly colored dried pellets were rehydrated with 2 mL of TE buffer and dissolved at 60 °C for 30 min. The pH of the extract was adjusted to approximately pH 7.5. The DNA extract was applied to a small DEAE-cellulose column (DE52; Whatman, Buckinghamshire, UK) in a 5 mL syringe barrel, with a 2 mL bed volume, previously equilibrated with running buffer (10 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris [pH 7.5]). The column was washed with 20 mL of running buffer and the bound DNA released with elution buffer (10 mM $MgCl_2$, 500 mM NaCl, 10 mM Tris [pH 7.5]). The eluate was collected in a fresh sterile centrifuge tube and the DNA was precipitated with an equal volume of isopropanol overnight at –20 °C. DNA was pelleted at 12,000× *g* for 20 min, the was pellet washed with 70% ethanol, and then dried in a vacuum desiccator. The pellet was resuspended in 0.4 mL of pre-warmed (to 65 °C) TE buffer and further purified using commercially available silica gel spin columns (sequentially, the FastDNA SPIN Kit (BIO 101) and then the Wizard DNA Clean up Kit (Promega, Madison, WI, USA)), following the manufacturers' instructions.

2.3. PCR Amplification of nifH Genes

Degenerate primer sets were used to amplify *nifH* gene sequences, combining the forward primer of Ueda et al., 1995 [36] (UF; GCIWTYTAYGGIAARGGIGG) with the reverse primer of Zehr and McReynolds 1989 [29] (ZR; ADNGCCATCATYTCNCC) to generate approximately 464-bp amplicons. Each 100-µL reaction contained 1 µL of soil DNA extract, 1X buffer A (Fisher), nucleotide mixture, 0.2 µM of each primer, and 0.5 U of Taq polymerase. After an initial 5 min denaturation at 95 °C, the standard thermal profile for the subsequent 40 cycles was denaturation for 1 min at 92 °C, annealing for 1 min at 50 °C, and primer extension for 1 min at 72 °C, followed by a final 5 min extension at 72 °C. Products were examined by agarose gel (2%) electrophoresis and visualized by UV transillumination after staining with ethidium bromide. Images were captured using a Kodak gel documentation system equipped with an EDAS290 digital camera and Kodak 1D gel analysis software package v3.5.

2.4. Cloning, Fingerprinting, and Sequencing of PCR Amplicons

The products from *nifH* amplification were excised from the agarose gel and purified with the QiaQuick Gel Extraction Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The amplicons were then cloned into One-Shot *Escherichia coli* cells following the manufacturer's instructions using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Transformants were grown on LB plates supplemented with kanamycin (50 µg mL^{-1}) and coated with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution, following the manufacturer's instructions. White colonies were collected and maintained on LB medium with kanamycin. Plasmids were prepared using the Wizard Plus Miniprep DNA Purification System (Promega), following the manufacturer's instructions.

Purified plasmids were cut with EcoR1 to release the inserts, which were then gel-purified as described above. The tetrameric endonuclease Msp1 was used for restriction fragment length polymorphism analysis (RFLP), following the manufacturer's instructions, in a 20-µL reaction containing 1 µL of Msp1 (10 µg $µL^{-1}$), 13 µL of sterile deionized water, 2 µL of RE 10X buffer, 2 µL of acetylated BSA (10 µg mL^{-1}), and 2 µL of the plasmid, that was then incubated at 37 °C for 1.5 h. Fragments were separated by vertical PAGE (0.5 %) at 150 V for approximately 1.5 h. The gel was stained with ethidium bromide, visualized by transillumination, and analyzed using the Kodak 1D software package. Sequence data were obtained with the protocols and reagents that accompany the four-color PRISM BigDye kit designed for use in ABI automated DNA sequencing systems (model 373). Base calls were made using the ABI PRISM DNA Sequencing Analysis Software package (v. 3.3).

Sequences were trimmed, aligned using Muscle and known sequences from GenBank, and then trees were created using the most-likelihood method with the Kimura 2 parameter model in the Mega v7.0 software package [37]. The *nifH* sequences appear in GenBank with accession numbers PP711620-PP711683.

3. Results

3.1. Extraction and Fingerprinting

Sampling was focused on ricefield soils where free-living non-symbiotic diazotrophs were expected and therefore avoided sampling rhizospheres in heavily rooted areas and surficial cyanobacterial mats. An extensive protocol for cleaning the total community DNA extracts was found to be best for removing humic substances from the soils. For the current study, DNA extracts were made directly from ricefield soils and subjected to PCR amplification using *nifH*-specific primers. A *nifH* clone library was constructed and analyzed by RFLP and nucleotide sequencing. While a metagenomic approach would have yielded a more complete picture of the diazotrophic microbial community, those techniques were not available for this project.

When PCR amplification of *nifH* genes was performed on ricefield soil extracts using degenerate primers, the *nifH* amplicons were ~460 base pairs in length and suitable for cloning and sequencing. Plasmid libraries were generated in *E. coli* from the ricefield soil *nifH* amplicons. Previous studies of *nifH* amplicons have applied RFLP analyses (see Poly et al., 2001), being a straightforward and inexpensive technique. Amplicons were analyzed by RFLP using Msp1 digestion to segregate clones into groups. A summary of the RFLP patterns from representative clones are shown in Figure 1. Nine groups were identified by their RFLP patterns. Certain clones subjected to RFLP analyses were subsequently sequenced (v.i.). Unfortunately, it appears that some RFLP groups did not adequately describe the diversity of *nifH* sequences, while others were more consistent. For instance, RD 24 and 25 from Group 4 were widely separated on the phylogenetic tree (v.i.). In contrast, Group 8 appears to be consistently within the Desulfovibrionales. Variable consistency of RFLP patterns to their identification by sequence analysis may affect the interpretation of studies that rely solely on RFLP analyses of diazotrophic assemblages [38–43].

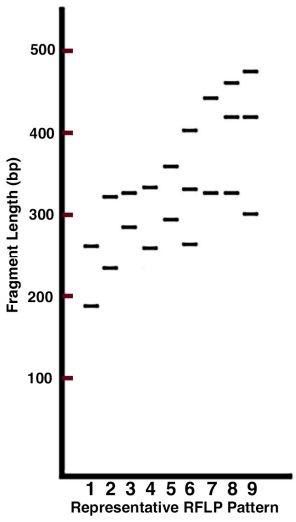

Figure 1. RFLP patterns of representative *nifH* amplicons from ricefield soil extracts. Groups of clones giving each pattern are as follows: 1: RG 10, 13, 14; 2: RG 15; 3: RG 5, 9, 11; 4: RD 24, 25; 5: RG 1; 6: RG 2, 3, 4, 6, 8, 12, 16; 7: RD 26; 8: RD 17, 18, 20, 21, 22, 23; 9: RD19.

3.2. Phylogenetic Analyses of nifH Clones

Cloned amplicons of *nifH* genes from ricefield soil extracts were sequenced and analyzed by comparison with known DNA sequences (Figure 2). Sequences from 64 *nifH* clones from the current study were included in the analysis. The sequence identity was mainly >90% to previously reported *nifH* genes. Missouri ricefield *nifH* clones often clustered with

nifH sequences from bacteria in *Geobacteraceae* within Desulfuromonales. Our clone library was particularly rich in *Geobacter* and *Geomonas* species. A substantial clade of *nifH* sequences was most closely related bacteria in Desulfovibrionales. This included a large cluster of *nifH* sequences that were not closely related to known isolates. A smaller cluster included representatives of other Gram-negative *Proteobacteria* such as *Methylomonas* species. Several of these ricefield *nifH* clones were not closely related to previously isolated organisms, most closely matching sequences only from uncultured bacteria, in some cases with only 80% sequence identity. A substantial number of clones did not closely match any known *nifH* sequences and formed separate clades within the Desulfovibrionales and *Geobacteraceae*. *NifH* sequences clustering within the cyanobacteria or archaea were not detected.

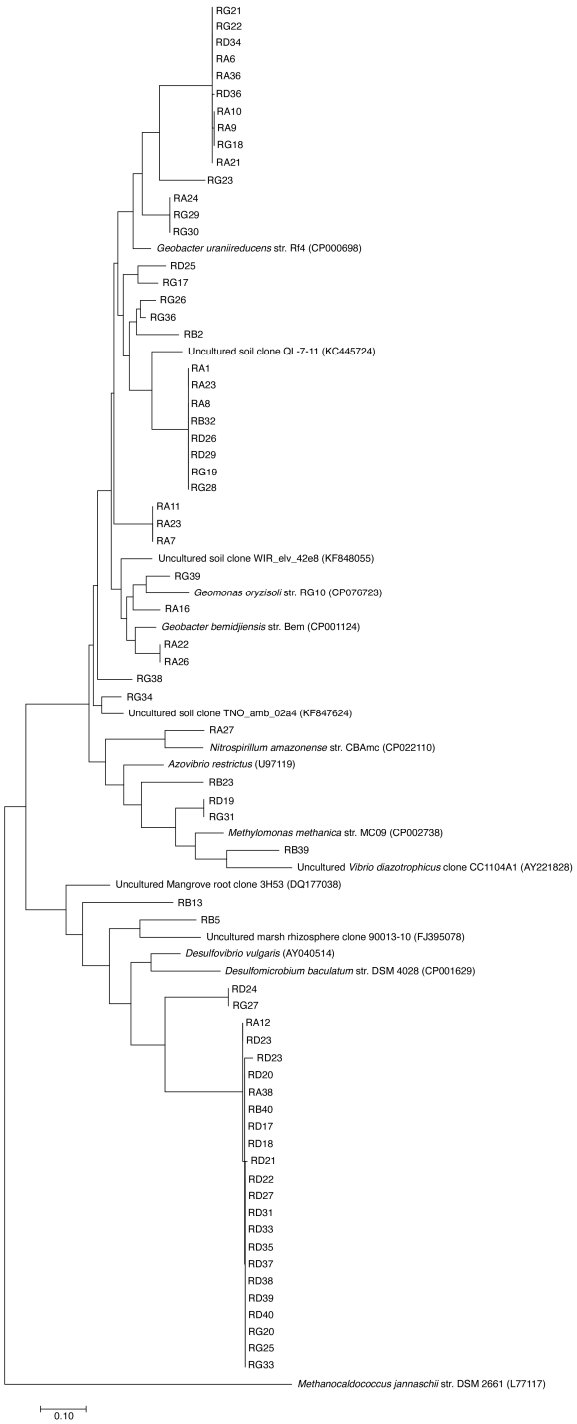

Figure 2. Maximum likelihood phylogenetic tree of *nifH* clones from ricefield soil extracts.

4. Discussion

Previous studies of ricefield diazotrophic microbial communities have reported a preponderance of *Anaeromyxobacter*, *Geobacter*, and *Geomonas* in bulk paddy soils [17,20,32,44–49]. Additionally, these studies and others have reported *Bradyrhizobium, Methylococcus, Pseudomonas, Rhizobium*, and cyanobacteria to be important contributors to the diazotrophic community in ricefield soils [22,50,51]. Diazotrophic microbes in the rhizosphere appear to differ from those in bulk paddy soils [16,21–23,49,52]. Root-associated diazotrophs appear to be rich in *Azoarcus*, with methanotrophs, *Enterobacter*, *Klebsiella*, and some *Geobacter*.

Nearly all of the *nifH* gene sequences obtained from direct extracts of Missouri rice paddy soils formed two main clusters of diazotrophs, most closely related to Desulfovibrionales and Desulfuromonales. Both are within the *Deltaproteobacteria*, a clade that is almost entirely organisms that can use sulfate and/or elemental sulfur as terminal oxidants for anaerobic respiration [53]. *Geobacter* and *Geomonas,* within the *Geobacteraceae*, are bacteria known to exhibit lithotrophic lifestyles, often using reduced minerals as energy sources. Clones within the Desulfovibrionales did not cluster with known *nifH* sequences from a particular genus. A smaller number of aerobic methanotrophs were detected as well, which may have been associated with the rhizosphere. Many of the clones were not closely related to cultivated or previously observed strains. Past molecular studies have widely found that a substantial proportion of the diazotrophs in ricefield soils were from groups not previously cultured [16–20]. This suggests that there may be significant endemism within the geographical distribution of ricefield diazotrophs. It is not surprising that cyanobacteria were not detected in the current study since sampling was limited to sediments and did not include mats or surficial waters. Similarly, the observation of typical endophytic strains such as *Azospirillum* was not expected since roots were not sampled directly.

It has been reported previously that diazotrophic guilds, such as those in *Spartina* grasses, can be quite stable and only exhibit changes over long periods of treatment [54–56]. This has been observed in rice paddies as well [17,50], although fertilization was shown to influence the community more quickly in another study [49]. It is interesting to note that rice stubble can act as a reservoir for diazotrophic microbes, including rice endophytes [57]. This may encourage the long-term stability of the diazotrophic guild in ricefield soils and thereby encourage endemism. These observations of stable communities demonstrate the potential for historical events to influence microbial diazotroph community structure. Microbes exhibit nonrandom distributions in nature, and the heterogeneity of their distribution can be observed at local and global scales [33,58,59]. The rate of colonization for different taxa may depend in part on nonrandom dispersal, survival during transport, and adaptation to a new environment. These considerations demonstrate the value of examining the communities from equivalent environments in different locales. Nearly all previous studies of ricefield diazotrophs were performed in Asia. The few studies in the Americas were performed on ricefields in Tennessee and in Uruguay [32,44]. Here, we have examined the diazotroph community in soils from a major region for rice cultivation in the US, namely, southeast Missouri. It seems that bacteria within *Geobacter* and *Geomonas* are relatively cosmopolitan diazotrophs in ricefields. However, there appears to be substantial endemism among the diazotrophs, as evidenced by the abundance of *nifH* genes that did not closely cluster with sequences reported from other locations.

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Conflicts of Interest: The authors declare no conflicts of interest.

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