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Soil diazotrophic community structure altered in rice crop rotated with mungbean or maize in Cai Lay district, Tien Giang province

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ABSTRACT

Nitrogen availability is often one of the limiting factors for intensive rice (Oryza sativa) cultivation. Rotation of rice with upland crops partially reduces nutrient depletion. This study was carried out at Cai Lay district, Tien Giang province with the aim to investigate the effect of alternating the rice crop with maize (Zea mays) and mungbean (Phaseolus aureus) in different rotational systems on the diazotrophic community in soil using pyrosequencing of the nifH genes. The results showed that the community structure of the diazotroph communities in soils was different when rice crop was alternated with either maize or mungbean crop than that in rice monoculture. The nifH sequences were highly diverse and assigned to eleven bacterial phyla and one Archaeal phylum. Proteobacteria and Firmicutes were the most common phyla carrying the nifH gene. The proportion of the Archaea, Betaproteobacteria, Cyanobacteria, Deltaproteobacteria, Firmicutes, Gammaproteobacteria and Nitrospira was significantly different among the treatments. The relative abundances of Anaeromyxobacter, and Geobacter were greater in rice-rice-rice than in the rice-mungbean-rice treatment while Heliobacterium and Desulfosporosinus were higher in the rice-mungbean-rice than those in the rice-rice-rice and rice-maize-rice treatment.

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1 INTRODUCTION

Nitrogen (N) availability is one of the most important factors determining rice crop yield, particularly for high-yielding varieties. The importance of cropping management practices that promote beneficial microbial communities (Larkin *et al.,* 2006; Xuan *et al.,* 2012) is increasingly being recognized as a means for enhancing sustainability in rice crop production by maintaining soil fertility and crop health. In rice ecosystems, paddy soils provide habitats for numerous diazotrophic bacteria and Archaea (Young, 1992; Zehr *et al.,* 2003). The diazotroph community in soils has been shown to be affected by the application of N, the type of compost, plant genotype as well as plant community (Mårtensson *et al.,* 2009; Pereira e Silva *et al.,* 2013).

Amplification of *nifH,* the gene encoding nitrogenase reductase subunit, has enabled further exploration of the composition of diazotroph communities

in natural habitats (Zehr *et al.,* 2003; Wartiainen *et al.,* 2008; Farnelid *et al.,* 2011). These have been found to be composed of taxa belonging to the Proteobacteria, Firmicutes, Cyanobacteria and Chlorobia (Moisander *et al.,* 2008; Mårtensson *et al.,* 2009). The impacts of different crop rotations on total soil bacterial communities in rice during two different cropping periods has been investigated by using 454 pyrosequencing of the16S rRNA (Xuan *et al.,* 2012). The results showed that rotation with alternative crops not only increased rice yield by up to 46%, but also changed the soil bacterial community structure in the presence of mungbean (*Phaseolus aureus*) or maize (*Zea mays*) crops compared with that found under a rice monoculture. In this current study, in-depth sequencing of the *nifH* gene was used to investigate the impact of rice rotation with either maize or mungbean on the soil diazotrophic community after rice grain harvest.

2 MATERIALS AND METHODS

2.1 Field site, experimental design and soil sampling procedure

A long-term rice crop rotation field experiment was established in 2001 in Cai Lay district, Tien Giang province, Vietnam (10º 34' N, 106º 00' E) within the collaboration of Can Tho University and Belgium (VLIR-IUC- R3-project). The field experiment was set up in a randomized block design with four replicates of each treatment. They consisted of (1) rice–rice–rice (RRR), (2) rice–maize–rice (RMR), and (3) rice–mungbean–rice (RMgR). Soil samples were collected from the plots in February one week after the rice crop was harvested. The soil samples from four replicates of each treatment were pooled together and represented for the treatment. The chemical composition of the soil samples was analyzed by Agrilab, Swedish University of Agricultural Sciences, Uppsala (Table 1).

2.2 DNA extraction and characterization of the *nifH* carrying community

All soil samples were homogenized and a 0.5 g of each subsample was used for DNA extraction according to Fantroussi *et al.* (1999), purified using the Wizard DNA clean-up kit (Promega, USA) and diluted to 0.5-0.8 ng μL^{-1} for the Polymerase chain reaction.

2.3 Nested PCR for N_2-fixing bacterial community

The N_2-fixing bacterial community was characterized using nested PCR, where the primer pair *nifH3* (ATRTTRTTNGCNGCRTA) and *nifH4* (TTYTAYGGNAARGGNGG) was used for the first round and primer pair PyrA*nifH2* (GCCTCCCTCGCGCCATCAGANDGCCATCA TYTCNCC) and PyrB*nifH1* (GCCTT-GCCAGCCCGCTCAG-tag-GAYCCNAARGCNGA) for the second round. The first PCR reaction was performed in a 50 µL reaction mixture containing 10 × buffer Y, 0.2 µMdNTPs, 1 µM *nifH3* and *nifH4*, 0.75 mM of $MgCl_2$, 0.03 U μL^{-1}ThermoRed DNA polymerase (SW förvaring, Sweden) and 12.5 ng of DNA extraction as a template. The first amplification program was performed with initial denaturing at 94°C for 3 min, followed by 25 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, and final extension at 72°C for 7 min. The second PCR reaction was performed in a 50 µL reaction mixture containing 10 × buffer Y, 0.2 µM dNTPs, 0.15 µM PyrB*nifH1* with different 8 bp tags representing each replicate and 0.15 µM PyrA*nifH2*, 0.75 mM of $MgCl_2$, 0.03 U μL^{-1} ThermoRed DNA polymerase (SW förvaring, Sweden) and 2 µL of PCR product from the first PCR reaction as a template. The second amplification program was performed with initial denaturing at 94°C for 3 min, followed by 25 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 30 s, and final extension at 72°C for 7 min. Samples were gel purified using a QIA Quick gel extraction kit (QIAGEN, Germany), quantified using Qubit (Invitrogen AB, USA) and used for pyrosequencing at a concentration of 10 ng μL^{-1} of each sample. Pyrosequencing was performed with a GS FLX sequencer (454 Life Sciences, Branford, USA) at the Royal Institute of Technology, Stockholm, Sweden.

2.4 Pyrosequencing analyses

The *nifH* sequences were processed using the Functional Gene Pipeline and Repository (FGPR, http://pyro.cme.msu.edu/). Using the FGPR Pyro Initial Process tool (http://fungene.cme.msu.edu), raw reads were sorted for each sample using the tags. Sequences passing initial quality controls were translated to amino acid sequences and frameshift corrected using the Ribosomal Database Project (RDP) FrameBot tool (http://fungene.cme.msu.edu/FunGenePipeline/framebot/form.spr).
Reads that shared less than 30% amino acid identity to the closest reference sequence were considered to be sequencing artifacts. These reads, along with those shorter than 60 amino acids in length, were filtered out. Protein reads passing FrameBot were aligned with HMMER (http://hmmer.org/) according to treatments using a model trained on the same set of representative sequences used. The amino acid sequences are available in GenBank under accession number ERP 001888. All *nifH*

sequences were clustered by complete linkage at 96% protein identity to calculate Shannon and Chao1 indices (http://fungene.cme.msu.edu/FunGenePipeline/).

The *nifH* clusters were classified using a subpackage from the RDP mcClust tool at a threshold of 50%. Sequences were classified to phyla and genera levels separately. Phyla detected at a relative abundance below 1.5% were grouped together and labeled collectively as 'minor groups'. Genera detected at a relative abundance below 0.05% were labeled with an asterisk.

2.5 Data analyses

The number of sequences belonging to each cluster in each treatment was used to create an environmental file. This file, in combination with the *nifH* phylogeny (data not shown), was analyzed in UniFrac (Lozupone *et al.,* 2007) by Jackknife cluster environment analysis and by lineage-specific analysis to determine lineages in the N_2-fixing bacterial community that contributed significantly to the observed differences among treatments.

The relative abundance of *nifH* phyla and nifH genera was calculated from the number of sequences in each phylum or genus divided by the total number of sequences in each treatment.

3 RESULTS AND DISCUSSION

In the study, a total of 25,370 *nifH* gene sequences including 6,799 unique protein sequences were identified in the three treatments (Table 1). The highest number of unique *nifH* sequences was 3,284 sequences and identified in soil from the RMR treatment and the lowest number was 2195 sequences and was identified in soil from the RMgR treatment.

In general, the results demonstrate that the rotation of rice with maize or mungbean influenced the phylogenetic composition and diversity of the diazotrophic community as shown by the Jackknife cluster environment analysis (Figure 1). Based on the total number of clusters, the Shannon and Chao1 richness (diversity) indices for the three treatments differed in the order: RMgR< RRR < RMR (Table 1). The differences of Shannon and Chao1 indices may be affected by the plants in these treatments.

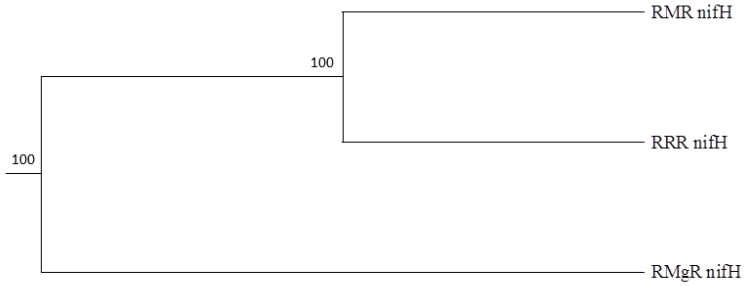

Fig. 1: Jackknife environment analysis showing phylogenetic differentiation of *nifH* communities in three different rice crop rotation treatments

The numbers indicate the frequency by which the nodes are supported by Jackknife analysis. Analysis was performed with respect to the abundance of the sequences in each cluster (weighted data)

Table 1: Chemical properties of soils, total number of sequences, number of unique sequences or number of clusters at 100% amino acid identity and number of clusters at 96% amino acid identity of the *nifH* gene in three rice crop rotation treatments

Treatment	Chemical properties of soils					Soil microbiological characteristics				
	pH	%C_{tot}	%N_{tot}	NH_4-N (mg/kg)	NO_3-N (mg/kg)	No. of sequences	No. of unique sequences	No. of clusters	Shannon index[1]	Chao1 index[2]
RRR	5,7	3,18	0,27	18,04	2,25	8717	2728	1275	4,98	1490
RMR	5,9	2,63	0,24	17,86	2,23	10282	3284	1416	5,14	1794
RMgR	5,9	2,55	0,29	20,23	2,53	6371	2195	1067	4,89	1180

[1 & 2] Shannon diversity and Chao1 richness estimation indices at 96% amino acid identity of nifH.

Gaby and Buckley (2011) analyzed the diversity of N_2-fixing bacteria in terrestrial and aquatic environments, and proposed that soils harbor a greater diversity compared with the marine environment. The number of unique *nifH* sequences and clusters found in the study was higher than those reported from other soils (Mao *et al.,* 2011; Pereira e Silva

et al., 2013) and from marine surface waters (Farnelid *et al.,* 2011). The findings concur with those reported by Orr *et al.* (2011) who showed that crop management regime affected the composition and diversity of both the total bacterial and the free-living N_2-fixing communities.

The *nifH* sequence clusters were classified in 11 bacterial phyla and one Archaeal phylum (Table 2). The major phyla Proteobacteria (Deltaproteobacteria, Betaproteobacteria) and Firmicutes represented 18% - 40% of all *nifH* sequences and 3% - 8% were assigned to Archaea, Gammaproteobacteria, Nitrospira and Spirochaetes (Figure 2). Lineage-specific analysis further identified the presence of clusters that were significantly different ($p<0,01$), in their abundance between the treatments and these belonged to Archaea, Betaproteobacteria, Cyanobacteria, Deltaproteobacteria, Firmicutes, Gammaproteobacteria and Nitrospira (Figure 1). Archaea has recently been reported to be involved in different biogeochemical processes, as major contributors to ammonia oxidation (Chen *et al.,* 2008) and methane emission (Singh *et al.,* 2012) in paddy soils. The high abundance of Archaea carrying the *nifH* gene in this study suggests its potential additional role in N_2-fixation.

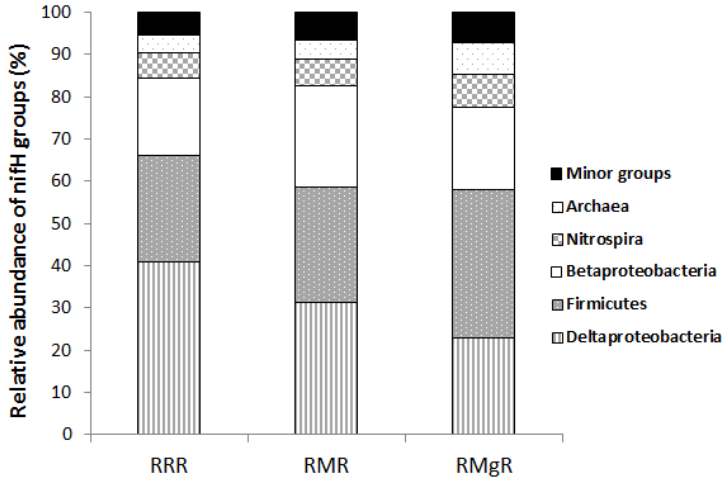

Fig. 2: Effect of different crop rotations on the relative abundance of different groups of *nifH* community in the treatments

The genera belonging to the N_2-fixing bacterial phyla assigned to the different *nifH* groups are summarized in Table 2. Those from most common phyla are *Anaeromyxobacter, Dechloromonas, Desulfosporosinus, Geobacter, Heliobacterium,* and *Methanosaeta* and have a high relative abundance in all treatments (Table 2). The relative abundances of *Anaeromyxobacter* and *Geobacter* were greater in RRR than in the RMgR treatment. These genera have been reported to be involved in degradation of possible contaminants (North *et al.,* 2004, Zhang *et al.,* 2012) and may have a role in intensive rice cultivation. The genera *Heliobacterium* and *Desulfosporosinus* contain spore-forming bacteria (Adams and Postgate, 1959; Kimble and Madigan 1992; Stackebrandt *et al.,*1997) and their relative abundances were higher in the RMgR and less in the RRR treatment suggesting their possible role as significant contributors to fixed nitrogen in the rice fields. Mårtensson *et al.* (2009) found *Azoarcus*, *Chlorobium, Desulfovibrio*, *Geobacter*, *Methylococcus, Methylocystis*, and *Rhizobium* to be abundant in rice paddy fields. These genera were also detected, but they were not abundant. This discrepancy can be explained by the differences in the seasonal dynamics as soil samples were collected at the active tillering phase of rice crop while this study's soil samples were collected at rice harvest in all treatments, therefore, the presence of the genus Rhizobium may be very few in the three treatments.

Table 2: The relative abundance of genera in three rice crop rotation treatments

Phylum	Genus	RRR	RMR	RMgR	Close match[1]
Proteobacteria-	*Azospirillum*	0,19	0,12	0,05	*Azospirillum* sp.
Alphaproteobacteria	*Gluconacetobacter*	0	*	0	*G. diazotrophicus*PAl 5
	Rhizobium	0,07	0,20	0	*Rhizobium* sp.
	Methylocella	*	*	0	*M. silvestris* BL2
	Methylocystis	0,39	0,16	0,06	*Methylocystis* sp. ATCC 49242
	Bradyrhizobium	*	*	*	*Bradyrhizobium* sp.
	Hyphomicrobium	0,15	0,30	*	*Hyphomicrobium* sp. MC1
	Magnetospirillum	0,05	*	0,05	*M. magneticum* AMB-1
	Rhodopseudomonas	*	*	*	*R. palustris* DX-1
	Zymomonas	0,07	0	0	*Z.mobilis*subsp. *mobilis* ATCC 10988
Proteobacteria-	*Burkholderia*	0,30	0,20	0,08	*Burkholderia*sp. Ch1-1
Betaproteobacteria	*Cupriavidus*	0,05	0	0	*C.taiwanensis* LMG 19424
	Azospira	0,15	0,13	0,74	*D.suillum* PS
	Azoarcus	*	*	0	*Azoarcus* sp. BH72
	Dechloromonas	11,02	15,78	10,27	*D.aromatica* RCB
	Leptothrix	0,81	1,41	0,25	*L.cholodnii* SP-6
	Polaromonas	4,27	4,74	1,19	P.*naphthalenivorans* CJ2
	Rubrivivax	*	0	0	*R.benzoatilyticus* JA2
	Sideroxydans	1,6	1,73	6,89	*S.lithotrophicus* ES-1
Proteobacteria-	*Desulfuromonas*	*	*	0	*D.acetoxidans* DSM 684
Deltaproteobacteria	*Geobacter*	17,39	10,10	5,78	*G.bemidjiensis*Bem
	Pelobacter	1,33	0,92	0,80	*P.carbinolicus* DSM 2380
	Anaeromyxobacter	16,32	14,19	9,62	*Anaeromyxobacter* sp. Fw109-5
	Desulfatibacillum	0,05	0,06	0,19	*D.alkenivorans* AK-01
	Desulfobacca	1,14	1,42	0,91	*D.acetoxidans* DSM 11109
	Desulfobacterium	0	0,05	*	*D.autotrophicum* HRM2
	Desulfobulbus	0,23	0,06	0,16	*D.propionicus* DSM 2032
	Desulfobacter	0,46	0,20	0,06	*D.postgatei* 2ac9
	Desulfomicrobium	*	0	0	*D.baculatum* DSM 4028
	Desulfovibrio	0,68	0,73	0,39	*Desulfovibrio* sp.
	Deltaproteobacteria	0,37	0,42	0,63	delta*proteobacterium* NaphS2
	Syntrophobacter	2,85	3,11	4,32	*S.fumaroxidans* MPOB
Proteobacteria- Epsilonproteobacteria	*Arcobacter*	0,05	0,01	0,09	*Arcobacter*sp. L
Proteobacteria-	*Acidithiobacillus*	0,33	0,50	1,43	*A.ferrivorans*
Gammaproteobacteria	*Methylomonas*	0,48	0,45	0,86	*M.methanica* MC09
	Methylococcus	0,22	0,18	0,11	*M.capsulatus* str. Bath
	Methylobacter	*	0,08	*	*M.tundripaludum* SV96
	Azotobacter	*	0,08	0,09	*A.vinelandii*
	Azotobacter	0	*	0	*A.vinelandii* DJ
	Pseudomonas	0,05	*	0	*P.stutzeri* A1501
	Allochromatium	0,09	0,18	0,14	*A.vinosum* DSM 180
	Brenneria	*	0	0	*Brenneria*sp. EniD312
	Halorhodospira	0	*	*	*H.halophila* BN9626
	Teredinibacter	*	0,13	*	*T.turnerae* T7901
	Thiocapsa	0,09	0,06	0,06	*T. marina* 5811
	Thiocystis	0	*	*	*T.violascens* DSM 198
	Thiorhodospira	0,16	0,19	0	*T.sibirica* ATCC 700588
Aquificales	*Thermocrinis*	0	0	*	*T.albus* DSM 14484
Bacteroidetes	*Azobacteroides*	0,06	*	0,05	*CandidatusAzobacteroidespseudo-trichonymphae*genomovar. CFP2
	Dysgonomonas	0	*	0,13	*D.gadei* ATCC BAA-286

Phylum	Genus	RRR	RMR	RMgR	Close match[1]
	Paludibacter	0,15	0,18	0,05	*P.propionicigenes* WB4
Chlorobi	*Chlorobium*	*	0	0	*C.phaeovibrioides* DSM 265
	Chloroherpeton	*	*	0,11	*C.thalassium* ATCC 35110
	Pelodictyon	0	0	*	*P.phaeoclathratiforme* BU-1
Chloroflexi	*Dehalococcoides*	0	*	*	*D.ethenogenes* 195
Cyanobacteria	*Anabaena*	0,29	0,12	0,49	*Anabaena* sp.
	Cyanothece	0,21	0,16	0,91	*Cyanothece* sp. PCC 7425
	Gloeothece	0	0	*	*Gloeothece* sp. KO68DGA
	Nostoc	*	*	*	*Nostoc* sp. PCC 7120
	Oscillatoria	*	*	*	*Oscillatoria*sp. PCC 6506
	Leptolyngbya	0,10	0,28	0,14	*L.boryana* IAM M-101
	Microcoleus	*	0	0	*M.chthonoplastes* PCC 7420
Archaea	*Methanosaeta*	4,07	4,63	7,27	*M.concilii* GP6
	Methanosarcina	0	0	*	*M.mazei* Go1
	Uncultured methanogenicarchaeon	*	0	*	Uncultured methanogenicarchaeon RC-I
Firmicutes	*Paenibacillus*	*	0	0	*P.durus* ATCC 35681
	Desulfitobacterium	0,11	0,06	0,08	*D.hafniense* DCB-2
	Heliobacterium	12,76	13,15	18,85	*H.chlorum* DSM 3682
	Clostridium	0,49	1,00	0,67	*C.acetobutylicum* ATCC 824
	Desulfosporosinus	11,57	12,88	15,32	*D.meridiei* DSM 13257
	Acetivibrio	*	*	0,00	*A.cellulolyticus* CD2
	Desulfotomaculum	*	*	*	*D.nigrificans* DSM 574
	Ethanoligenens	*	0	0	*E.harbinense* YUAN-3
	Syntrophobotulus	0,14	0,12	0,13	*S.glycolicus* DSM 8271
Fusobacteria	*Ilyobacter*	0	0	*	*I.polytropus* DSM 2926
Nitrospira	*Thermodesulfovibrio*	6,16	6,21	7,88	*T.yellowstonii* DSM 11347
Spirochaetes	*Spirochaeta*	1,60	2,52	1,52	*S.thermophila* DSM 6192
	Treponema	0,13	0,17	0,16	*T.primitia* ZAS-2
Verrucomicrobia	*Verrucomicrobiae*	0,11	0,12	0,14	*V. bacterium* DG1235
	Coraliomargarita	0,07	0,05	0	*C.akajimensis* DSM 45221
	Diplosphaera	0,27	0,2	0,6	*D.colitermitum* TAV2

[1] Close match based on the distance calculation between the sequence and each reference sequence from the Zehr's nifH ARB database

(*) Relative abundance of nifH genus was less than < 0,05%

4 CONCLUSIONS

The *nifH* sequences were highly diverse and assigned to 11 bacterial phyla and one Archaeal phylum. Proteobacteria and Firmicutes were the most common phyla carrying the *nifH* gene in the treatments; however, the proportion of the Archaea, Betaproteobacteria, Cyanobacteria, Deltaproteobacteria, Firmicutes, Gammaproteobacteria and Nitrospira was significantly different among the treatments. Genera from the most common phyla are *Anaeromyxobacter, Dechloromonas, Desulfosporosinus, Geobacter, Heliobacterium,* and *Methanosaeta.* They had a high relative abundance in all treatments. The relative abundances of *Anaeromyxobacter,* and *Geobacter* were greater in RRR than in RMgR treatment while *Heliobacterium* and *Desulfosporosinus* were higher in RMgR than those in RRR and RMR treatment. The study highlights the importance of studying N_2-fixing communities in a range of different rice cultivation systems to further understand their function, ideally in relation to *in situ* N_2-fixation and, to enhance biological N_2-fixation in rice ecosystems.

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