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Switchgrass (*Panicum virgatum* L.) is a perennial C_4 grass native to North America that is being developed as a feedstock for cellulosic ethanol production. Industrial nitrogen fertilizers enhance switchgrass biomass production but add to production and environmental costs. A potential sustainable alternative source of nitrogen is biological nitrogen fixation. As a step in this direction, we studied the diversity of nitrogen-fixing bacteria (NFB) associated with native switchgrass plants from the tallgrass prairie of northern Oklahoma (United States), using a culture-independent approach. DNA sequences from the nitrogenase structural gene, *nifH*, revealed over 20 putative diazotrophs from the alpha-, beta-, delta-, and gammaproteobacteria and the firmicutes associated with roots and shoots of switchgrass. Alphaproteobacteria, especially rhizobia, predominated. Sequences derived from *nifH* RNA indicated expression of this gene in several bacteria of the alpha-, beta-, delta-, and gammaproteobacterial groups associated with roots. Prominent among these were *Rhizobium* and *Methylobacterium* species of the alphaproteobacteria, *Burkholderia* and *Azoarcus* species of the betaproteobacteria, and *Desulfuromonas* and *Geobacter* species of the deltaproteobacteria.

Switchgrass (*Panicum virgatum* L.) is a warm-season C_4 grass that is native to the tallgrass prairies of North America, and it has been targeted for development as a bioenergy crop by the U.S. Department of Energy (1). Its features, such as perenniality, adaptation to diverse edaphic conditions, wide geographic distribution, growth on acidic soils, and mutualistic associations with soil microorganisms, make it an attractive choice for cultivation on marginal lands that are poorly suited to food crops (2–4).

Although switchgrass is thrifty in its use of N to produce biomass compared to other crops (2, 5), addition of N fertilizer enhances growth and generally is needed to maintain switchgrass productivity over multiple years (6, 7). However, application of N fertilizer increases production costs, reduces the energy balance of biomass production, and can be harmful to the environment through release of N-containing gases to the atmosphere and soluble N compounds to groundwater, which can lead to eutrophication (8). Biological nitrogen fixation has the potential to reduce the use and negative consequences of industrial N fertilizer and to put the nascent biofuels industry on a more sustainable path. Biological nitrogen fixation has been demonstrated in association with switchgrass (9, 10). However, to the best of our knowledge, the bacteria responsible for such activity are unknown, as is the diversity of potentially nitrogen-fixing bacteria associated with this species.

Nitrogen-fixing bacteria (NFB) associate with many grasses, including maize (11–13), rice (14), sugarcane (15), and *Miscanthus* species (16). These diazotrophs are endophytic, living between plant cells, and/or epiphytic, living on the surface of plant organs, and most do not elicit plant defense responses (17). However, the extent to which diazotrophic bacteria contribute to the N economy of grasses remains unclear.

Most soil microbes remain uncultured and largely uncharacterized, in part because appropriate culture conditions have not been found. Therefore, we used a culture-independent approach, based on PCR amplification and sequencing of *nifH* DNA (18, 19), to assess the diversity of potential diazotrophs associated with switchgrass. *nifH* has been used by many researchers to study total and functional diversity of bacteria in various environments (15, 20–22), and phylogenies based on *nifH* and 16S rRNA generally are congruent (23, 24). Additionally, PCR amplification of cDNA derived from *nifH* RNA was used to determine whether any bacteria express this gene in association with switchgrass. The results of this work indicate diverse and abundant NFB associated with switchgrass and active expression of the *nifH* nitrogen fixation gene in some of these bacteria.

MATERIALS AND METHODS

Plant material. Switchgrass (*Panicum virgatum* L.) plants were harvested from separate locations in the tallgrass prairie (Oklahoma, USA) in April and July 2010. GPS coordinates of the sampling sites were between 36°38′38″N to 36°48′48″N latitude and 96°10′26″W to 96°26′23″W longitude (see Table S1 in the supplemental material). Plants were uprooted, and loosely attached soil was removed from roots by vigorous shaking. Roots and shoots were rinsed with tap water at the collection site to remove soil particles and were transferred to the laboratory on dry ice. Samples were stored at −80°C within 24 h of collection.

Nucleic acid extraction. Shoot and root pieces were pulverized in liquid nitrogen using a cryo-mill (6870 freezer mill; SPEX SamplePrep, USA). DNA was extracted from 0.5 g pulverized tissue using a modified cetyltrimethylammonium bromide (CTAB) method (25). Total RNA was extracted from 100 mg of pulverized tissue using an RNeasy plant minikit (Qiagen, USA) by following the manufacturer's instructions. Genomic DNA was removed by DNaseI treatment (Turbo DNase; Ambion, USA), followed by column purification of RNA using an RNeasy MinElute cleanup kit (Qiagen, USA). RNA was quantified using a Nanodrop spec-

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trophotometer (ND-100; NanoDrop Technologies, Willington, DE) and evaluated for purity with a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Checks for genomic DNA contamination of RNA were performed using *nifH* primers (mentioned below), 16S rRNA gene 27F (26), and 1492R primers (27).

PCR conditions for *nifH* amplification. A nested-PCR approach was used to amplify *nifH* gene fragments as described previously (28). Two degenerate primers, *nifH* 3 (5′-ATR TTR TTN GCN GCR TA-3′) and *nifH* 4 (5′-TTY TAY GGN AAR GGN GG-3′), were used to amplify an ~460-bp region. One microliter of the resultant PCR product was used as the template to amplify an ~362-bp region using degenerate primers *nifH* 1 (5′-TGY GAY CCN AAR GCN GA-3′) and *nifH* 2 (5′-AND GCC ATC ATY TCN CC-3′). All sample manipulations were performed under laminar flow to avoid airborne contamination. PCRs were initiated with denaturation at 95°C (5 min); 30 cycles of denaturation at 95°C (30 s), annealing at 55°C (1 min), and extension at 72°C (1 min); and a final extension at 72°C (10 min). Reverse transcription-PCR (RT-PCR) was done as described by Zani et al. (28), and cDNA synthesis was carried out using 1 μg of DNase-treated total RNA with primer *nifH* 3 and SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, USA). Nested PCR was performed with the *nifH* 3 and *nifH* 4 primer combination, followed by the *nifH* 1 and *nifH* 2 combination. No-RT (i.e., no cDNA) controls were included for all RNA samples analyzed to check for genomic DNA contamination.

nifH amplification products were separated by electrophoresis through 2.0% agarose gels and visualized with Sybr green. Amplified fragments were excised from gels and purified using a Promega gel extraction kit (Promega, USA) and then cloned into the pGEM-T Easy vector (Promega, USA). Bacterial transformation was done using Z-competent cells (Zymo Research, USA). Forty-eight white colonies per sample were picked, and colonies were grown in 96-well plates.

***nifH* sequencing and phylogenetic analysis.** Recombinant pGEM-T plasmids containing *nifH* fragments were sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA). DNA sequences were edited to remove vector sequences using GeneDoc (29), and identical sequences were grouped into operational taxonomic units (OTUs), using the FastGroupII tool (30) with the method of percent sequence identity with gaps of 100% identity. DNA sequences were translated into protein amino acid sequences using BioEdit software (31). Protein sequences were subjected to BLAST analysis (32) against GenBank, and *nifH* protein sequences from cultivated bacteria that showed the closest match to query sequences were included for phylogenetic analysis. OTUs representing 4 or more sequences were subjected to phylogenetic analysis. The MAFFT web interface was used for alignment (33).

Phylogenetic trees were constructed using MEGA (ver. 5.0) with the maximum likelihood method based on a Poisson correction model (34). The bootstrap consensus tree was produced from 1,000 replicates, and *Methanothermococcus okinawensis* NifH (accession no. NZ_AEDA01000001) was used as an outgroup.

Data analysis. *nifH* sequences from root DNA, root RNA (cDNA), and shoot DNA of individual plants were subjected to rarefaction analysis, and Shannon-Weaver and Simpson diversity indices were calculated using PAST statistical software (35). The percentage of total sequence diversity captured in sequenced clones of each organ for each plant was estimated by Good's method using the formula $[1 - (n/N)] \times 100$, where n is the number of clones appearing only once in a library and N is the total number of clones sequenced (36). Venn diagrams were drawn using Excel (Microsoft).

Accession numbers. Representative sequences from each OTU were deposited in GenBank under accession numbers KF541058 to KF541088, and the protein accession numbers are AIE38835 to AIE38865.

RESULTS

Diversity of *nifH* sequences. A total of 28 clone libraries were produced from *nifH* PCR amplicons derived from DNA of roots and shoots separately from 10 independent plants and RNA from roots of 8 of these plants. Up to 48 clones were sequenced from each library, yielding a total of 1,087 high-quality *nifH* sequences. Based upon shared sequence identity, these sequences formed 52 distinct OTUs, which were tentatively associated with known species based on the highest sequence similarity (Table 1).

Estimations of species coverage, diversity, and dominance were calculated for each library separately (Table 2). Good's coverage averaged over 90% for root DNA libraries, over 95% for root RNA, and over 98% for shoot DNA libraries, indicating that the majority of *nifH*-containing and *nifH*-expressing bacteria associated with switchgrass plants were represented in the sequences obtained. Based on the number of OTUs represented by sequences in each library, bacteria containing *nifH* were significantly more diverse in roots than in shoots ($P = 0.0145$) of switchgrass plants, with an average of approximately 10 and 6 OTUs per plant, respectively (Table 2). About one-third of *nifH*-containing bacteria were found to express the gene in association with switchgrass roots (Table 1). Simpson's and Shannon's indices supported these conclusions about *nifH* bacterial diversity in the different sample types (Table 2). Overall dominance of one or a few OTUs was highest in root RNA, followed by shoot DNA and root DNA (Table 2). In three of the eight plants analyzed for RNA, only a single OTU was represented in the *nifH* sequences (i.e., dominance = 1).

Seven OTUs were common to all sample types, while 22 were specific to roots (DNA plus RNA), and 10 OTUs were specific to shoots (Fig. 1).

OTUs were matched to the most closely related known nitrogen-fixing species based on *nifH* sequence comparisons using the BLAST algorithm. A total of 23 OTUs representing 704 sequences appeared to derive from alphaproteobacteria, 9 OTUs (177 sequences) from deltaproteobacteria, 8 from betaproteobacteria (146 sequences), 6 from gammaproteobacteria (37 sequences), 2 from cyanobacteria (3 sequences), and 1 OTU each from *Firmicutes* (15 sequences) and *Verrucomicrobia* (3 sequences) (Table 1 and Fig. 2).

***nifH* diversity and expression in roots.** Among the *nifH* sequences derived from root-associated DNA, more than 65% originated from alphaproteobacteria, 15% from betaproteobacteria, and 12% from deltaproteobacteria. The largest number of sequences in a single OTU (OTU-8; 81 sequences) were most similar (95 to 99% identity) to *nifH* from *Sphingomonas azotifigens* (accession no. BAE71134). Approximately one-third of all sequences (33%) were affiliated with various *Bradyrhizobium* species and distributed into 3 OTUs (1, 2, and 10) (Table 1 and Fig. 3). At the individual plant level, OTUs 1, 2, 4, 5, 8, and 10 (all alphaproteobacteria) were present in 7 to 8 out of 10 plants (see Table S2 in the supplemental material).

Among the *nifH* sequences derived from root-associated RNA, 58% matched sequences from alphaproteobacteria, 20% from betaproteobacteria, and 19% from deltaproteobacteria (Table 1 and Fig. 2). Over one-third of transcribed *nifH* sequences (88) fell into OTU-14, which is affiliated with *Rhizobium helanshanense* (ADP37388; 95 to 100% identity) or *Sinorhizobium meliloti* (CCH40450), while 38 sequences fell into OTU-12, affiliated with *Methylobacterium nodulans* (AAQ82902). Five OTUs contained sequences derived from root RNA but not root DNA (OTU-11, affiliated with *Rubrivivax gelatinosus*; OTU-13, affiliated with *Amorphomonas oryzae*; OTU-14, affiliated with *Rhizobium helanshanense/S. meliloti*; OTU-21, affiliated with *Desulfuromonas ace-*

TABLE 1 Phylotypes of *nifH* associated with switchgrass

Phylotype	Closest relative	Accession no.	Similarity (%)	No. of sequences per sample type				Taxonomic description
				Root		Shoot		
				DNA	RNA	DNA	Total	
OTU-01	*Bradyrhizobium* sp. strain BTAi 1	BAC07281	98–100	35	0	15	50	*Alphaproteobacteria*
OTU-02	*Bradyrhizobium* sp. strain MAFF 210318	BAC07283	94–98	46	2	71	119	*Alphaproteobacteria*
OTU-03	*Mesorhizobium loti*	BAF95636	100	0	0	4	4	*Alphaproteobacteria*
OTU-04	*Methylocystis* sp. strain LW2	AAK97418	97–98	24	2	8	34	*Alphaproteobacteria*
OTU-05	*Burkholderia* sp. strain WSM3937	ABX80637	93–98	58	17	1	76	*Betaproteobacteria*
OTU-06	*Burkholderia* sp. strain JPY105	AFM28513	97	7	7	3	17	*Betaproteobacteria*
OTU-07	*Burkholderia* sp. strain PTK47	AAU85620	96–100	3	0	20	23	*Betaproteobacteria*
OTU-08	*Sphingomonas azotifigens*	BAE71134	95–99	81	0	55	136	*Alphaproteobacteria*
OTU-09	*Novosphingobium* sp. strain Rr 2-17	ZP_10360790	98–99	29	0	0	29	*Alphaproteobacteria*
OTU-10	*Bradyrhizobium japonicum*	ACT67985	92–98	71	0	2	73	*Alphaproteobacteria*
OTU-11	*Rubrivivax gelatinosus*	BAE15985	90–94	0	5	0	5	*Betaproteobacteria*
OTU-12	*Methylobacterium nodulans*	AAQ82902	94–97	6	38	4	48	*Alphaproteobacteria*
OTU-13	*Amorphomonas oryzae*	BAF48338	96	0	1	7	8	*Alphaproteobacteria*
OTU-14	*Rhizobium helanshanense*	ADP37388	95–100	0	88	40	128	*Alphaproteobacteria*
OTU-15	*Sinorhizobium* sp. strain SCAU224	AFH96094	98	3	0	1	4	*Alphaproteobacteria*
OTU-16	*Azospirillum lipoferum*	ABG88868	96–100	3	2	22	27	*Alphaproteobacteria*
OTU-17	*Methylomonas* sp. strain MG30	CCH22595	93	5	0	0	5	*Gammaproteobacteria*
OTU-18	*Pseudomonas stutzeri*	CAC03734	97	7	4	0	11	*Gammaproteobacteria*
OTU-19	*Klebsiella* sp. strain AL060224_04	ACM68399	99–100	0	0	18	18	*Gammaproteobacteria*
OTU-20	*Azoarcus* sp. strain BH72	YP_932042	96–98	1	21	0	22	*Betaproteobacteria*
OTU-21	*Desulfuromonas acetoxidans*	ZP_01312343	94–99	0	32	31	63	*Deltaproteobacteria*
OTU-22	*Anaeromyxobacter* sp. strain Fw109-5	YP_001380211	93–97	12	0	15	27	*Deltaproteobacteria*
OTU-23	*Geobacter uraniireducens* Rf4	YP_001229952	92	1	0	5	6	*Deltaproteobacteria*
OTU-24	*Geobacter* sp. strain M21	YP_003021955	93–96	4	3	0	7	*Deltaproteobacteria*
OTU-25	*Geobacter* sp. strain M21	YP_003021955	97–98	35	15	5	55	*Deltaproteobacteria*
OTU-26	*Geobacter uraniireducens* Rf4	YP_001229952	95–97	3	0	6	9	*Deltaproteobacteria*
OTU-27	*Anaeromyxobacter* sp. strain Fw109-5	YP_001380211	88–91	0	5	2	7	*Deltaproteobacteria*
OTU-28	*Desulfotomaculum gibsoniae*	ZP_09101754	93–95	0	0	15	15	*Firmicutes*
OTU-29	*Geobacter* sp. strain M21	YP_003021955	89–92	13	0	0	13	*Deltaproteobacteria*
OTU-30	*Desulfovibrio magneticus* RS-1	YP_002953433	89	4	0	0	4	*Deltaproteobacteria*
OTU-31	*Syntrophobacter fumaroxidans*	YP_845148	90–93	0	0	16	16	*Deltaproteobacteria*
OTU-32	*Methylocystis* sp. strain LW5	AAK97419	98	2	0	0	2	*Alphaproteobacteria*
OTU-33	*Coraliomargarita akajimensis*	YP_003550022	89	1	0	1	2	*Verrucomicrobia*
OTU-34	*Cupriavidus* sp. strain pp2.75	ADM25241	92	1	0	0	1	*Betaproteobacteria*
OTU-35	*Rhizobium* sp. strain SMF 466_6	CCN27451	98	0	0	2	2	*Alphaproteobacteria*
OTU-36	*Paenibacillus graminis*	BAH23271	94	0	0	2	2	*Firmicutes*
OTU-37	*Rhizobium* sp. strain SMF 466_6	CCN27451	92	0	2	0	2	*Alphaproteobacteria*
OTU-38	*Nostoc* sp. strain PCC 7120	NP_484917	99	0	0	2	2	*Cyanobacteria*
OTU-39	*Calothrix* sp. strain LEGE 06100	AGG40738	97	0	0	1	1	*Cyanobacteria*
OTU-40	*Ectothiorhodospira haloalkaliphila*	ABN10975	88	1	0	0	1	*Gammaproteobacteria*
OTU-41	*Desulfatibacillum alkenivorans*	YP_002430688	93	1	0	0	1	*Deltaproteobacteria*
OTU-42	*Bradyrhizobium* sp. strain cmy11	AEP33459	96	1	0	0	1	*Alphaproteobacteria*
OTU-43	*Rhizobium* sp. strain SMF 466_6	CCN27451	97	0	0	1	1	*Alphaproteobacteria*
OTU-44	*Cupriavidus* sp. strain pp2.75	ADM25241	98	1	0	0	1	*Betaproteobacteria*
OTU-45	*Dechloromonas aromatica*	YP_284634	98	1	0	0	1	*Betaproteobacteria*
OTU-46	*Ectothiorhodospira haloalkaliphila*	ABN10975	90	1	0	0	1	*Gammaproteobacteria*
OTU-47	*Heliorestis baculata*	BAD80875	88	0	1	0	1	*Firmicutes*
OTU-48	*Mesorhizobium tianshanense*	CAR57837	96	0	0	1	1	*Alphaproteobacteria*
OTU-49	*Methylocystis echinoides*	AAO49390	89	1	0	0	1	*Alphaproteobacteria*
OTU-50	*Pelobacter carbinolicus*	YP_006717849	93	0	0	1	1	*Deltaproteobacteria*
OTU-51	*Geobacter bemidjiensis*	YP_002138883	95	1	0	0	1	*Deltaproteobacteria*
OTU-52	*Halorhodospira halophila*	ABN10970	94	0	1	0	1	*Gammaproteobacteria*

toxidans; and OTU-27, affiliated with *Bradyrhizobium japonicum*), indicating measurable *nifH* gene expression in several apparently low-abundance root bacteria (Table 1 and Fig. 3). Overall, 3 OTUs contained sequences derived from root DNA and RNA, 14 OTUs contained sequences from root DNA but not RNA, and 5 OTUs contained sequences from root RNA but not DNA (Table 1 and Fig. 1). At the individual plant level, OTU-4 contained *nifH* RNA sequences from the most plants (only 4 of 8 plants), indicating that no single *nifH*-expressing species was common to all switchgrass plants (see Table S2 in the supplemental material).

TABLE 2 *nifH* diversity and coverage estimates for root DNA and RNA and shoot DNA

DNA/RNA type and parameter	Value for plant no.: E-01	E-04	E-06	E-08	E-13	E-18	E-28	E-31	E-36	E-40	Avg
Root DNA											
No. of taxa	11	12	14	15	8	10	5	5	9	9	9.8
No. of clones	48	48	48	44	48	46	46	44	44	48	46.4
Good's estimator (%)	89.58	83.33	93.75	79.55	95.83	93.48	97.83	93.18	93.18	95.83	91.55
Dominance	0.201	0.237	0.133	0.137	0.248	0.158	0.306	0.717	0.185	0.170	0.249
1 − Simpson diversity index	0.799	0.763	0.867	0.863	0.752	0.842	0.694	0.283	0.815	0.829	0.750
Shannon's diversity index	1.897	1.8	2.311	2.266	1.647	2.002	1.333	0.622	1.86	1.929	1.766
Evenness	0.606	0.50411	0.721	0.643	0.649	0.740	0.759	0.372	0.711	0.765	0.646
Root RNA											
No. of taxa	5	6	3		1	1		3	1	6	3.25
No. of clones	41	31	32		33	25		19	32	34	30.87
Good's estimator (%)	97.56	93.33	100.00		100.00	100.00		100.00	100.00	100.00	98.86
Dominance	0.327	0.611	0.639		1	1		0.424	1	0.279	0.659
1 − Simpson diversity index	0.673	0.389	0.361		0	0		0.576	0	0.721	0.340
Shannon's diversity index	1.271	0.884	0.656		0	0		0.972	0	1.492	0.659
Evenness	0.713	0.404	0.643		1	1		0.882	1	0.741	0.798
Shoot DNA											
No. of taxa	3	4	5	3	6	7	8	9	8	9	6.2
No. of clones	38	38	44	41	47	32	31	42	28	35	37.6
Good's estimator (%)	100.00	97.37	95.45	100.00	100.00	90.63	93.55	97.62	89.29	91.43	95.53
Dominance	0.367	0.540	0.371	0.411	0.231	0.320	0.213	0.194	0.212	0.211	0.307
1 − Simpson diversity index	0.633	0.459	0.629	0.589	0.769	0.679	0.787	0.806	0.788	0.789	0.693
Shannon's diversity index	1.043	0.872	1.155	0.967	1.579	1.437	1.769	1.874	1.753	1.827	1.428
Evenness	0.946	0.598	0.635	0.877	0.809	0.601	0.733	0.724	0.722	0.691	0.733

***nifH* sequences from shoot bacteria.** BLAST results indicated that 63% of sequences derived from shoot-associated DNA were affiliated with alphaproteobacteria and 20% with deltaproteobacteria. Sequences affiliated with firmicutes were associated only with shoots (OTU-28) (Fig. 2). Eighteen sequences affiliated with gammaproteobacteria (*Klebsiella* species accession no. ACM68399) were represented by OTU-19 (Table 1 and Fig. 3). OTU-2 contained the largest number (71 sequences) of *nifH* sequences from shoots, which were affiliated with an uncultured bacterium related to *Bradyrhizobium* sp. strain MAFF 210318 (94 to 98% identity). Ten OTUs were unique to shoots (OTUs 3, 19, 28, 31, 35, 36, 38, 39, 48, and 50; Fig. 1), among them sequences affiliated with *Mesorhizobium loti* (OTU-3; 100% identity) and with *Syntrophobacter fumaroxidans* (OTU-31; 90 to 93% identity) (Table 1 and Fig. 3). Half or more of the plants contained *nifH* DNA sequences affiliated with *Bradyrhizobium* sp. strain BTAi 1 (OTU-1), *Burkholderia* sp. strain PTK47 (OTU-7), and *Sphingomonas azotifigens* (OTU-8). A small number of *nifH* sequences from shoots were affiliated with cyanobacteria (Table 1 and Fig. 3).

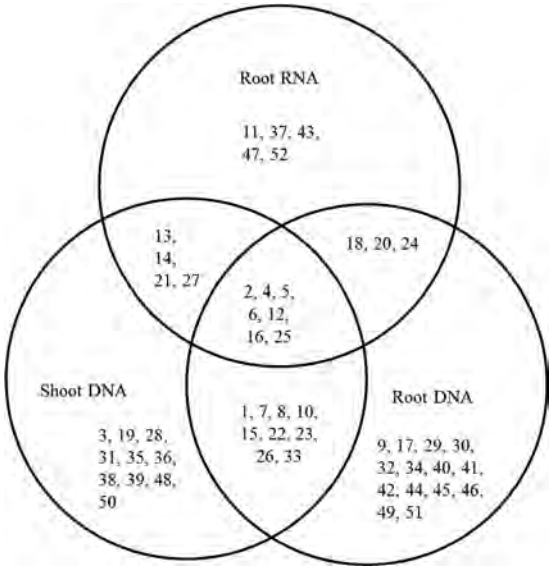

FIG 1 Venn diagram showing the number of shared and unique OTUs among the three sample types (root DNA, root RNA, and shoot DNA). Numbers indicated in the diagram are OTU identity numbers (details of each OTU are given in Table 1).

DISCUSSION

We used a culture-independent, *nifH* sequence-based approach to survey the diversity of potential nitrogen-fixing bacteria associated with switchgrass. To our knowledge, this is the first study of its kind for switchgrass, and as such it lays a foundation for future work on the isolation and characterization of associative diazotrophic bacteria from switchgrass and their potential use as a nitrogen source for this biofuel crop.

Over 1,000 *nifH* PCR amplicons derived from root and shoot DNA and root RNA of 10 plants were cloned and sequenced in this study. Roots of switchgrass plants harbored a greater diversity of *nifH*-containing bacteria than did shoots (Table 2). Similar results have been obtained for maize (37). However, only a small fraction of root-associated *nifH*-containing bacteria expressed this gene

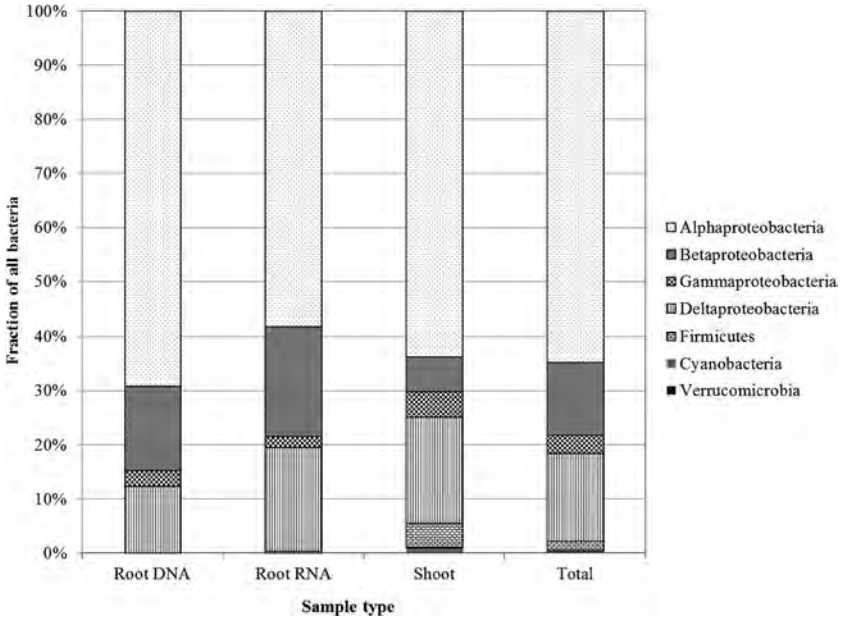

FIG 2 Class-level composition of *nifH* clone libraries. Percent abundances per clone library from root RNA (242 sequences), root DNA (451 sequences), shoot DNA (397 sequences), and overall sequences (1,060) are shown.

(Table 2). Similar results have been found in sugarcane, spruce (15), and rice (14). Shannon's diversity indices for *nifH* transcripts associated with individual plants ranged from 0.142 to 1.245 for sugar cane, and for one spruce tree it was 0.184 (15), similar to what was found here for switchgrass.

Alphaproteobacteria were the main source of *nifH* sequences in both roots and shoots, accounting for 62% of all DNA and RNA sequences combined. Within this group, 58% of the sequences were related to *nifH* in bacteria from the order *Rhizobiales* and were present in all sample types. More specifically, these sequences were affiliated with the genera *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, and *Sinorhizobium*, all of which contain species that are known to fix nitrogen in symbioses with legumes (38–45).

Based on the frequency of *nifH* recovered from DNA, *Bradyrhizobium* was the dominant genus associated with switchgrass roots and shoots, with 22% (243 sequences) of all clones distributed in 3 OTUs (Table 1). OTU-10 was closely related to *Bradyrhizobium japonicum* sequences. *B. japonicum* is a functionally diverse species (38), and some strains can fix nitrogen under free-living conditions (46). *Bradyrhizobium* sp. strain MAFF210318, affiliated with OTU-2, is a nonphotosynthetic bacterium that can fix nitrogen under free-living conditions (47). *Bradyrhizobium* sp. strain BTAi 1, affiliated with phylotype 1, is a photosynthetic rhizobium that also can fix nitrogen under free-living conditions (8, 48). *Bradyrhizobium* species have been found as endophytes in rice (49) and sugarcane (15, 50). Despite the abundance of *Bradyrhizobium* bacteria associated with switchgrass, however, the paucity of *Bradyrhizobium*-like *nifH* sequences derived from root RNA suggests that these bacteria fix little or no nitrogen in association with native switchgrass.

Evidence was obtained for *nifH* gene expression in a variety of bacterial genera associated with switchgrass, including affiliates of *Rhizobium/S. meliloti* (OTU-14), *Methylobacterium* (OTU-12), *Desulfuromonas* (OTU-21), *Burkholderia* (OTU-5 and OTU-6), *Azoarcus* (OTU-20), *Geobacter* (OTU-25), *Bradyrhizobium* (OTU-2, OTU-24, and OTU-27), *Rubrivivax* (OTU-11), *Pseudomonas* (OTU-18), *Methylocystis* (OTU-4), *Azospirillum* (OTU-16), and *Amorphomonas* (OTU-13), in descending order of prevalence based on numbers of sequenced clones. Half of these genera belong to the alphaproteobacteria, while the remainder partitioned into the beta-, gamma-, and deltaproteobacteria. *Rhizobium helanshanense/S. meliloti* (ADP37388/CCH40450)-affiliated sequences (alphaproteobacteria) were prominent among root *nifH* RNA, accounting for 36% of all root RNA-derived sequences (Table 1). However, only one sequence was detected from root DNA, and many (44 sequences) were identified from shoot DNA. OTU-14 showed over 97% identity with sequences from *S. meliloti* and various *Rhizobium* species, such as *Rhizobium* sp. strain CCNWSX0878 (accession no. AEB96238), *R. yanglingense* (AFD62623), and *R. undicola* (AEP04095). These results indicate active *nifH* gene expression in a very small population of specific rhizobia, a conclusion that warrants confirmation in future work designed to identify the best potential nitrogen-fixing bacteria for switchgrass.

Practical application of nitrogen-fixing bacteria for N supply to switchgrass will depend not only on active nitrogenase genes but also on the ability of the plant to host a sufficiently large population of the bacteria. Given the low number of rhizobial *nifH* DNA sequences that match *nifH* RNA sequences from switchgrass roots, the latter would seem to be an impediment to the development of effective nitrogen fixation in the roots of switchgrass.

A large number of *nifH* clones (38 in OTU-12) derived from root RNA were affiliated with *Methylobacterium nodulans* ORS 2060 (AAQ82902), another alphaproteobacteria able to form nitrogen-fixing nodules on legumes (51), as well as *Desulfuromonas acetoxidans* (32 in OTU-21) and *Azoarcus* sp. strain BH72 (21 in OTU-20). Here, *nifH* expression for these three bacteria outweighed their DNA abundance in the samples investigated.

Within the betaproteobacterial group, *Burkholderia* species ap-

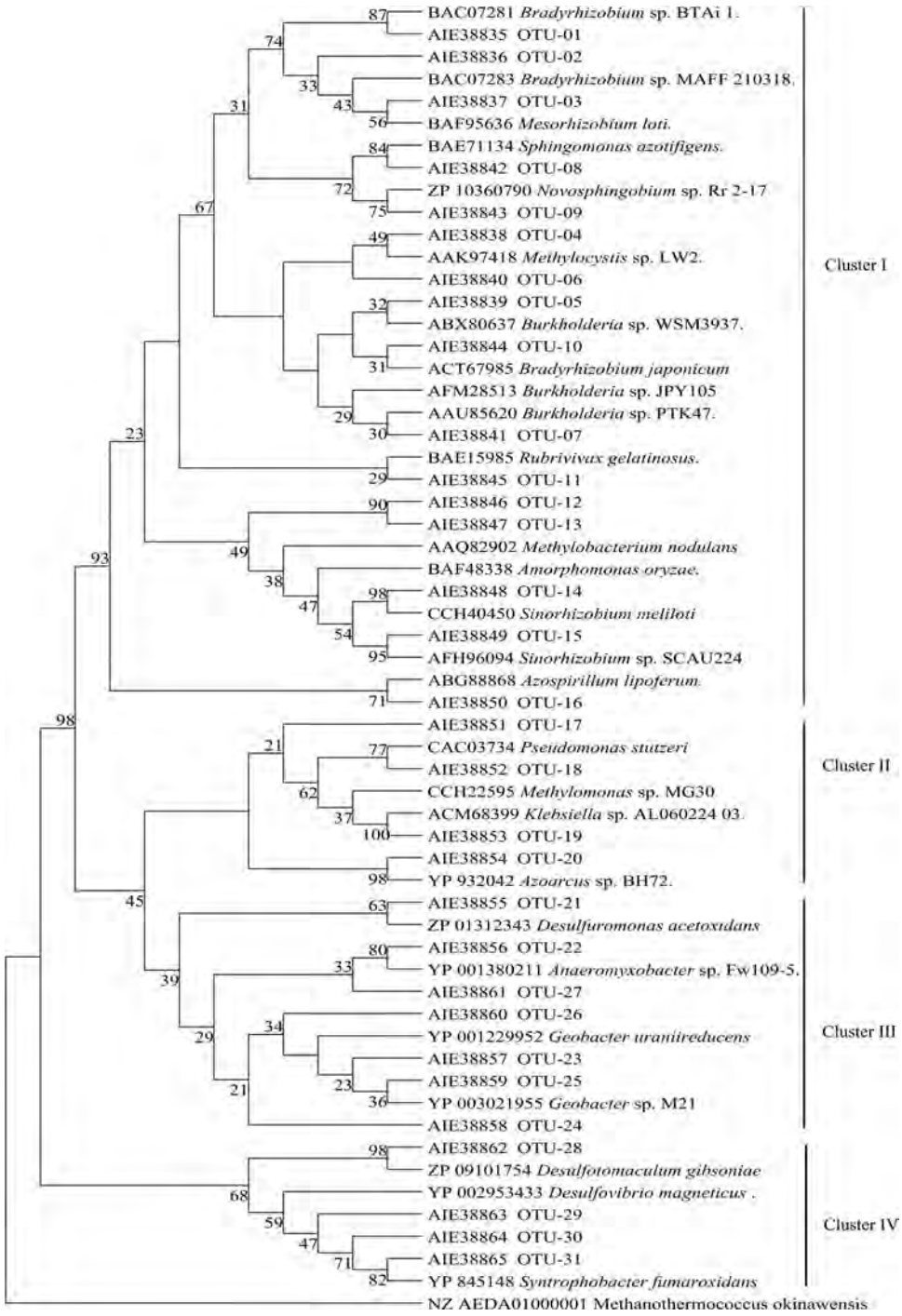

FIG 3 Phylogeny and composition of *nifH* phylotypes representing four or more sequences. OTUs from this study are represented by OTU number and the associated protein accession number. The evolutionary history was inferred by using the maximum likelihood method based on the Poisson correction model. The bootstrap consensus tree was inferred from 1,000 replicates. There were a total of 113 positions in the final data set, and analyses were conducted in MEGA5. Sequences from known bacteria are indicated by name and NCBI protein accession numbers. This tree was rooted with the *nifH* gene from the archaeon *Methanothermococcus okinawensis*.

peared to be the source of some *nifH* sequences. *Burkholderia* species have been found to nodulate and fix nitrogen in mimosoid legumes like *Mimosa pigra* (52), and some are known to associate with maize and sugarcane (53, 54). Expressed *nifH* sequences also affiliated with the betaproteobacterium *Azoarcus* sp. strain BH72 (YP_932042), which was isolated from Kallar grass and can colonize rice roots and express high levels of *nifH* transcripts there (55, 56).

Finally, the deltaproteobacteria *nifH* transcripts identified in switchgrass roots were affiliated with *Geobacter* sp. strain M21 and

D. acetoxidans (ZP_01312343). *Geobacter nifH* transcripts have been found previously in roots and stems of rice (22, 57).

We are aware that taxonomic inferences based on single genes, such as *nifH*, can be complicated by horizontal gene transfer. For this reason, the tentative assignments of bacterial genus and species associated with switchgrass made here should be substantiated by further work with isolated, cultured bacteria in the future. However, an independent, 16S rRNA gene-based metagenomic study of bacteria associated with switchgrass from the same sites in Oklahoma, harvested at different times of year, identified 14 of the 19 genera found in the present study (S. R. Chaluvadi and J. L. Bennetzen, personal communication).

In summary, we have identified some of the natural diversity of diazotrophic bacteria associated with switchgrass from the Oklahoma prairie. Evidence was presented that *nifH* genes of many bacterial species from the alpha-, beta-, gamma-, and deltaproteobacterial groups are expressed in switchgrass roots. Prominent among these were *Rhizobium* and *Methylobacterium* species of the alphaproteobacteria, *Burkholderia* and *Azoarcus* species of the betaproteobacteria, and *Desulfuromonas* and *Geobacter* species of the deltaproteobacteria. This work provides a basis for future work on the isolation, culture, and functional characterization of nitrogen-fixing endophytes of switchgrass and their possible use as nitrogen sources for cultivated switchgrass.

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REFERENCES

1. **Bouton J.** 2008. Improvement of switchgrass as a bioenergy crop, p 295–308. *In* Vermerris W (ed), Genetic improvement of bioenergy crops. Spinger Science and Business Media, New York, NY.
2. **Parrish DJ, Fike JH.** 2005. The biology and agronomy of switchgrass for biofuels. Crit. Rev. Plant Sci. **24:**423–459. http://dx.doi.org/10.1080/07352680500316433.
3. **Parrish DJ, Fike JH.** 2009. Selecting, establishing, and managing switchgrass (*Panicum virgatum*) for biofuels. Methods Mol. Biol. **581:**27–40. http://dx.doi.org/10.1007/978-1-60761-214-8_2.
4. **Ghimire SR, Charlton ND, Bell JD, Krishnamurthy YL, Craven KD.** 2011. Biodiversity of fungal endophyte communities inhabiting switchgrass (*Panicum virgatum* L.) growing in the native tallgrass prairie of northern Oklahoma. Fungal Divers. **47:**19–27. http://dx.doi.org/10.1007/s13225-010-0085-6.
5. **Lemus R, Parrish DJ, Wolf DD.** 2009. Nutrient uptake by alamo switchgrass used as an energy crop. BioEnergy Res. **2:**37–50. http://dx.doi.org/10.1007/s12155-009-9032-3.
6. **Yang JD, Worley E, Wang MY, Lahner B, Salt DE, Saha M, Udvardi M.** 2009. Natural variation for nutrient use and remobilization efficiencies in switchgrass. BioEnergy Res. **2:**257–266. http://dx.doi.org/10.1007/s12155-009-9055-9.
7. **Guretzky JA, Biermacher JT, Cook BJ, Kering MK, Mosali J.** 2010. Switchgrass for forage and bioenergy: harvest and nitrogen rate effects on biomass yields and nutrient composition. Plant Soil **339:**69–81. http://dx.doi.org/10.1007/s11104-010-0376-4.
8. **Spiertz JHJ.** 2010. Nitrogen, sustainable agriculture and food security. A review. Agron. Sustain. Dev. **30:**43–55. http://dx.doi.org/10.1051/agro:2008064.
9. **Tjepkema JD, Burris RH.** 1976. Nitrogenase activity associated with some Wisconsin prairie grasses. Plant Soil **45:**81–94. http://dx.doi.org/10.1007/BF00011131.
10. **Tjepkema J.** 1975. Nitrogenase activity in the rhizosphere of *Panicum virgatum*. Soil Biol. Biochem. **7:**179–180. http://dx.doi.org/10.1016/0038-0717(75)90016-4.
11. **Montañez A, Abreu C, Gill PR, Hardarson G, Sicardi M.** 2008. Biological nitrogen fixation in maize (*Zea mays* L.) by 15N isotope-dilution and identification of associated culturable diazotrophs. Biol. Fertil. Soils **45:**253–263. http://dx.doi.org/10.1007/s00374-008-0322-2.
12. **Gutiérrez-Zamora ML, Martínez-Romero E.** 2001. Natural endophytic association between Rhizobium etli and maize (Zea mays L.). J. Biotechnol. **91:**117–126. http://dx.doi.org/10.1016/S0168-1656(01)00332-7.
13. **Rosenblueth M, Martínez-Romero E.** 2004. *Rhizobium etli* maize populations and their competitiveness for root colonization. Arch. Microbiol. **181:**337–344. http://dx.doi.org/10.1007/s00203-004-0661-9.
14. **Sessitsch A, Hardoim P, Doering J, Weilharter A, Krause A, Woyke T, Mitter B, Hauberg-Lotte L, Friedrich F, Rahalkar M, Hurek T, Sarkar A, Bodrossy L, van Overbeek L, Brar D, van Elsas JD, Reinhold-Hurek B.** 2012. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol. Plant Microbe Interact. **25:**28–36. http://dx.doi.org/10.1094/MPMI-08-11-0204.
15. **Burbano CS, Liu Y, Rosner KL, Reis VM, Caballero-Mellado J, Reinhold-Hurek B, Hurek T.** 2011. Predominant nifH transcript phylotypes related to *Rhizobium rosettiformans* in field-grown sugarcane plants and in Norway spruce. Environ. Microbiol. Rep. **3:**383–389. http://dx.doi.org/10.1111/j.1758-2229.2010.00238.x.
16. **Kirchhof G, Reis VM, Baldani JI, Eckert B, Dobereiner J, Hartmann A.** 1997. Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. Plant Soil **194:**45–55. http://dx.doi.org/10.1023/A:1004217904546.
17. **Hurek T, Handley LL, Reinhold-Hurek B, Piche Y.** 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol. Plant Microbe Interact. **15:**233–242. http://dx.doi.org/10.1094/MPMI.2002.15.3.233.
18. **Zehr JP, Mellon MT, Hiorns WD.** 1997. Phylogeny of cyanobacterial *nif*H genes: evolutionary implications and potential applications to natural assemblages. Microbiology **143:**1443–1450. http://dx.doi.org/10.1099/00221287-143-4-1443.
19. **Hurek T, Reinhold-Hurek B.** 2006. Molecular ecology of N_2-fixing microbes associated with graminaceous plants: hidden activities of unknown bacteria, p 173–198. *In* Werner D, Newton WE (ed), Nitrogen fixation in agriculture, forestry, ecology, and the environment. Springer, New York, NY.
20. **Zehr JP, Mellon M, Braun S, Litaker W, Steppe T, Paerl HW.** 1995. Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. Appl. Environ. Microbiol. **61:**2527–2532.
21. **Lema KA, Willis BL, Bourne DG.** 2012. Corals form characteristic associations with symbiotic nitrogen-fixing bacteria. Appl. Environ. Microbiol. **78:**3136–3144. http://dx.doi.org/10.1128/AEM.07800-11.
22. **Martensson L, Diez B, Wartiainen I, Zheng W, El-Shehawy R, Rasmussen U.** 2009. Diazotrophic diversity, nifH gene expression and nitrogenase activity in a rice paddy field in Fujian, China. Plant Soil **325:**207–218. http://dx.doi.org/10.1007/s11104-009-9970-8.
23. **Young JPW.** 1992. Phylogenetic classification of nitrogen-fixing organisms, p 43–86. *In* Stacy G, Burris RH, Evans HJ (ed), Biological nitrogen fixation. Chapman and Hall, New York, NY.
24. **Young JPW.** 1993. Molecular phylogeny of rhizobia and their relatives, p 587–592. *In* Palacios R, Mora J, Newton W (ed), New horizons in nitrogen fixation, vol 17. Springer, Amsterdam, Netherlands.
25. **Murray MG, Thompson WF.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8:**4321–4325. http://dx.doi.org/10.1093/nar/8.19.4321.
26. **Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:**7843–7853. http://dx.doi.org/10.1093/nar/17.19.7843.
27. **Weisburg WG, Barns SM, Pelletier DA, Lane DJ.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
28. **Zani S, Mellon MT, Collier JL, Zehr JP.** 2000. Expression of *nifH* genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. Appl. Environ. Microbiol. **66:**3119–3124. http://dx.doi.org/10.1128/AEM.66.7.3119-3124.2000.
29. **Nicholas KB, Nicholas HB, Jr, Deerfield DW, II.** 1997. GeneDoc: analysis and visualization of genetic variation. EMBNEW News **4:**14.
30. **Yu Y, Breitbart M, McNairnie P, Rohwer F.** 2006. FastGroupII: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. BMC Bioinformatics **7:**57. http://dx.doi.org/10.1186/1471-2105-7-57.
31. **Hall TA.** 1999. BioEdit: a user-friendly biological sequence alignment

editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
32. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
33. **Katoh K, Toh H.** 2010. Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics **26:**1899–1900. http://dx.doi.org/10.1093/bioinformatics/btq224.
34. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
35. **Hammer Ø, Harper DAT, Ryan PD.** 2001. Past: paleontological statistics software package for education and data analysis. Palaeontol. Electronica **4:**9.
36. **Good IJ.** 1953. The population frequencies of species and the estimation of population parameters. Biometrika **40:**237–264.
37. **Roesch LFW, Camargo FAO, Bento FM, Triplett EW.** 2008. Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. Plant Soil **302:**91–104. http://dx.doi.org/10.1007/s11104-007-9458-3.
38. **Sachs JL, Kembel SW, Lau AH, Simms EL.** 2009. *In situ* phylogenetic structure and diversity of wild *Bradyrhizobium* communities. Appl. Environ. Microbiol. **75:**4727–4735. http://dx.doi.org/10.1128/AEM.00667-09.
39. **Ahmad MJ, Muhammad K, Amer J.** 2011. Short communication: screening of *Mesorhizobium ciceri* isolates from rainfed area for improvement in growth and nodulation of chickpea seedlings under controlled conditions. Soil Environ. **30:**160–165.
40. **Batista JS, Hungria M, Barcellos FG, Ferreira MC, Mendes IC.** 2007. Variability in *Bradyrhizobium japonicum* and *B. elkanii* seven years after introduction of both the exotic microsymbiont and the soybean host in a cerrados soil. Microb. Ecol. **53:**270–284. http://dx.doi.org/10.1007/s00248-006-9149-2.
41. **Dakora FD, Joseph CM, Phillips DA.** 1993. Alfalfa (*Medicago sativa* L.) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. Plant Physiol. **101:**819–824.
42. **Gangwar S, Dubey M.** 2012. Chickpea (*Cicer arietinum* L.) root nodulation and yield as affected by micronutrients application and *Rhizobium* inoculation. Crop. Res. **44:**37–41.
43. **Guimaraes AP, Morais RF, de Urquiaga S, Boddey RM, Alves BJR.** 2008. *Bradyrhizobium* strain and the 15N natural abundance quantification of biological N2 fixation in soybean. Sci. Agric. **65:**516–524. http://dx.doi.org/10.1590/S0103-90162008000500011.
44. **Laranjo M, Young JPW, Oliveira S.** 2012. Multilocus sequence analysis reveals multiple symbiovars within *Mesorhizobium* species. Syst. Appl. Microbiol. **35:**359–367. http://dx.doi.org/10.1016/j.syapm.2012.06.002.
45. **Jourand P, Renier A, Rapior S, de Faria SM, Prin Y, Galiana A, Giraud E, Dreyfus B.** 2005. Role of methylotrophy during symbiosis between *Methylobacterium nodulans* and *Crotalaria podocarpa*. Mol. Plant Microbe Interact. **18:**1061–1068. http://dx.doi.org/10.1094/MPMI-18-1061.
46. **Chaintreuil C, Giraud E, Prin Y, Lorquin J, Bâ A, Gillis M, de Lajudie P, Dreyfus B.** 2000. Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. Appl. Environ. Microbiol. **66:**5437–5447. http://dx.doi.org/10.1128/AEM.66.12.5437-5447.2000.
47. **Cantera JJL, Kawasaki H, Seki T.** 2004. The nitrogen-fixing gene (nifH) of *Rhodopseudomonas palustris*: a case of lateral gene transfer? Microbiology **150:**2237–2246. http://dx.doi.org/10.1099/mic.0.26940-0.
48. **Molouba F, Lorquin J, Willems A, Hoste B, Giraud E, Dreyfus B, Gillis M, de Lajudie P, Masson-Boivin C.** 1999. Photosynthetic bradyrhizobia from *Aeschynomene* spp. are specific to stem-nodulated species and form a separate 16S ribosomal DNA restriction fragment length polymorphism group. Appl. Environ. Microbiol. **65:**3084–3094.
49. **Tan Z, Hurek T, Vinuesa P, Muller P, Ladha JK, Reinhold-Hurek B.** 2001. Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S-23S ribosomal DNA intergenic spacer-targeted PCR. Appl. Environ. Microbiol. **67:**3655–3664. http://dx.doi.org/10.1128/AEM.67.8.3655-3664.2001.
50. **Thaweenut N, Hachisuka Y, Ando S, Yanagisawa S, Yoneyama T.** 2011. Two seasons' study on nifH gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of *nif*H genes similar to those of rhizobia. Plant Soil **338:**435–449. http://dx.doi.org/10.1007/s11104-010-0557-1.
51. **Jourand P, Giraud E, Béna G, Sy A, Willems A, Gillis M, Dreyfus B, de Lajudie P.** 2004. *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. Int. J. Syst. Evol. Microbiol. **54:**2269–2273. http://dx.doi.org/10.1099/ijs.0.02902-0.
52. **Chen W-M, James EK, Chou J-H, Sheu S-Y, Yang S-Z, Sprent JI.** 2005. β-Rhizobia from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. New Phytol. **168:**661–675. http://dx.doi.org/10.1111/j.1469-8137.2005.01533.x.
53. **Perin L, Martínez-Aguilar L, Castro-González R, Estrada-De Los Santos P, Cabellos-Avelar T, Guedes HV, Reis VM, Caballero-Mellado J.** 2006. Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Appl. Environ. Microbiol. **72:**3103–3110. http://dx.doi.org/10.1128/AEM.72.5.3103-3110.2006.
54. **Estrada P, Mavingui P, Cournoyer B, Fontaine F, Balandreau J, Caballero-Mellado J.** 2002. A N_2-fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico. Can. J. Microbiol. **48:**285–294. http://dx.doi.org/10.1139/w02-023.
55. **Hurek T, Reinhold-Hurek B, Van Montagu M, Kellenberger E.** 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. **176:**1913–1923.
56. **Egener T, Hurek T, Reinhold-Hurek B.** 1999. Endophytic expression of nif genes of *Azoarcus* sp. strain BH72 in rice roots. Mol. Plant Microbe Interact. **12:**813–819. http://dx.doi.org/10.1094/MPMI.1999.12.9.813.
57. **Elbeltagy A, Ando Y.** 2008. Expression of nitrogenase gene (*nif*H) in roots and stems of rice, Oryza sativa, by endophytic nitrogen-fixing communities. Afr. J. Biotechnol. **7:**1950–1957.