ECOLOGY OF NITROGEN-FIXING BACTERIA ASSOCIATED WITH MISCANTHUS

BY

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THESIS

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Abstract

Sustainability of is one of the biggest concerns for bioenergy crops, such as *Miscanthus*. Nitrogen is the most expensive and most limiting nutrient in agricultural systems, so it is necessary to develop strategies to reduce anthropogenic inputs of nitrogen fertilizer. Diazotrophs associated with non-leguminous grasses have great potential to contribute biologically fixed nitrogen as a sustainable N source for bioenergy grasses. There are previous few studies investigating diazotroph community composition associated with *Miscanthus*. The following thesis investigated two important aspects of *Miscanthus*-associated N-fixing bacteria with both culture-based methods and molecular approaches: *i.* confirming the presence diazotrophs associated with *Miscanthus* and evaluating their potential to provide nitrogen to the plant; *ii.* examining how environmental factors may influence the diazotroph communities. In order to address the objectives, both rhizomes and rhizosphere samples were collected from native *Miscanthus* in Taiwan and agricultural samples from Illinois. Thirty-two diazotroph strains were isolated from native *Miscanthus* rhizomes, and most of them were *Gamma-proteobacteria*. The taxonomic classification of the strains was similar to diazotroph strains previously isolated from agricultural *Miscanthus*. In addition, the nitrogen fixation potential was confirmed for these strains through detection of nitrogenase genes and assays to detect nitrogenase activity. Molecular approaches were introduced to investigate if the biological nitrogen fixation (BNF) process could be carried out by diverse bacterial groups besides the isolated taxa. Diazotrophs from 57 genera were found in native *Miscanthus* rhizomes, while diazotrophs from 73 genera were found in the rhizosphere. Molecular approaches also enable us to compare diazotrophs from different conditions. I first compared the endorhizosphere diazotrophs of native and agricultural *Miscanthus*, and the results indicated that the native *Miscanthus* rhizomes harbored more diverse diazotroph communities. I then investigated both total bacterial communities and diazotroph communities from the rhizosphere and endorhizosphere of native *Miscanthus* with deep-sequencing methods. The result showed that total bacteria communities showed strong response to plant niches, while diazotroph bacteria did not. For both total bacterial and diazotroph communities, the *Miscanthus* rhizosphere soil harbored communities with higher richness and biodiversity than

Miscanthus rhizome tissues. Other factors, such as geographic distribution and soil edaphic factors also influenced the diversity and community structure of both total bacterial and diazotroph communities. Understanding community composition of diazotrophs associated with *Miscanthus* and the environmental factors that govern the diazotroph community may facilitate the agricultural management to effectively utilize beneficial microbes for sustainable crop production.

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Introduction

1.1 Sustainability

Concerns about climate change and energy security have heightened importance of developing sustainable, renewable energy sources (43, 117). One possible solution for global energy supply and the reduction of greenhouse gas emission is to use bioenergy (117). Biomass is considered a promising sustainable energy source compared with conventional energy sources such as fossil fuel and petroleum (12). Modern bioenergy has great potential to contribute to future sustainable energy systems for both developing and industrialized countries (51, 53, 125, 143). Hence, significant research effort has been devoted to the feasibility of replacing conventional energy systems with bio-energy ones (140).

Biofuels such as ethanol are derived from plant biomass and are a more environmentally friendly energy source than fossil fuels. Biofuels are generally considered to be carbon-neutral or even carbon-negative, which means that the carbon emitted while burning biofuel is equal to or less than the carbon sequestered during the plant growth (90). For instance, the ethanol blended vehicle fuel can significantly decrease the greenhouse emission (43).

In order to meet the requirement of sustainable production, other aspects such as maintenance of soil fertility and biofuel feedstock yield need to be considered for biofuel production. Sustainability has been defined as "using methods, systems and materials that won't deplete resources or harm natural cycles." (31)

The United States used 3.4 billion gallon of ethanol for blended fuel in 2004, and almost all of the ethanol is produced from corn (43). However, it is fossil fuel that provides the necessary energy for corn production as well as the fermentation and distillation processes. As a result, the energy output of corn ethanol is 29% less than the fossil energy input. In addition, corn production demands more fertility and pesticides than other crops, leading to a more serious soil erosion problem. Moreover, corn is also an important food and forage source (100). Increasing the scale of corn ethanol production will cause the price-raising of corn-related food (77). Thus, it is not a sustainable approach to meet the ethanol need based only on corn ethanol (101). Development of biofuel feedstock for

conversion of lignocellulose biomass to ethanol and other hydrocarbons is the focus of recent work (120).

1.2 Bioenergy and biofuel feedstock candidates

Due to the disadvantages of corn ethanol, researchers are investigating lignocellulosic feedstocks as potential biofuel crops (122). This second generation of biofuel feedstocks are derived from cellulosic plant material, providing abundant cheap and non-food resources that can be used for biofuel production (97). Typical second generation of biofuel feedstocks include short rotation coppice (polar and willow), perennial grasses (*Miscanthus*, switchgrass) and plant residuals from the wood industry. Studies have shown that these 2nd generation biofuel feedstocks have several advantages over the traditional corn ethanol. The 2nd generation biofuel feedstocks are usually carbon neutral or even carbon negative in terms of their impact to CO_2 concentration. Additionally, they do not compete with food supply and require less fossil energy input (97). Because of those advantages mentioned above, cellulosic ethanol has greater potential as a sustainable energy source. The US Department of Energy has set a goal of replacing 30% of gasoline consumption with lignocellulosic biofuel by 2030 (61).

Currently, several 2nd generation energy crop candidates are under investigation, including giant reed, switchgrass, and *Miscanthus* (80). Among those candidates, *Miscanthus* is particularly promising due to its high biomass production capability, nutrient use efficiency and the low energy requirement for management. *Miscanthus* is a genus of perennial C_4 grass that has great potential for producing lignocellulosic biomass. The genetic origin of *Miscanthus* is in East Asia and can be divided into 4 sections, "*Kariyasua*", "*Diandra*", "*Thiarrhena*", and "*EuMiscanthus*" (1, 78). One high-yielding genotype, *Miscanthus × giganteus,* is widely used in trials for biofuel feedstock in Europe. It is also the main potential biofuel feedstock under investigation in the US. *M. × giganteus* is a hybrid of *Miscanthus sacchariflorus* and *Miscanthus sinensis*. It is a self-sterile triploid hybrid, and can reproduce only by rhizome transplanting and *in vitro* culture (80). Reported yields of *M. × giganteus* range from 7 to 38 Mg/ha/yr over a variety of climate and fertility conditions (34, 104, 140). Long-term studies indicate that *Miscanthus* can maintain high yields for

more than 10 years (25). A number of ecological and economical benefits of using *Miscanthus* as a biofuel feedstock have been reported. *Miscanthus* can be grown on a wide range of soil types, including sandy soil. As a perennial grass, limited soil management (tillage) is needed for growing *Miscanthus*, which reduces soil erosion. Moreover, the *Miscanthus* rhizome system allows nutrients to be translocated and stored in the below-ground plant tissues during harvesting season, thus *Miscanthus* requires low nutrient inputs (4). Utilizing *Miscanthus*' high nutrient using efficiency will help to minimize the fertility input and to optimize the soil management.

1.3 How Miscanthus responds to nitrogen input

In order to produce cost-effective biofuels, an ideal biofuel feedstock should provide high-energy output for little energy. Hence, one desired character for biofuel feedstock is high nutrient use efficiency. In addition, this is also a key character for sustainable feedstock production. Whereas a number of environmental factors need to be taken into consideration in terms of sustainable biofuel feedstock production, in this study, we only focus on biological nitrogen inputs for *Miscanthus*.

Nitrogen (N) is typically the most limiting nutrient for plant growth, and inputs of N fertilizer account for a major portion of fossil fuel use in agricultural systems (132). In addition, applying nitrogen fertilizer will also lead to several environmental and ecological problems. For example, excess nitrogen fertilizer that is not taken up by plants may be leached into surface waters in the form of nitrate, which leads to eutrophication and water quality issue in receiving waters (44). Annual zones of hypoxia in coastal waters of the Gulf of Mexico result from decay of algal blooms fueled by nutrient pollution in the Mississippi River, and the leached nitrate from the agricultural areas in the Mississippi River basin is a main contributor to these coastal water quality issues (64). Other problems such as greenhouse gas emission (in the form of nitrous oxide) can also result from nitrogen inputs (134).

For all the reasons listed above, efforts to reduce nitrogen inputs would favorably improve the sustainability of *Miscanthus* production. Worldwide researchers have carried out field experiments for *Miscanthus* growth at different locations and climate conditions. A

review study conducted by Heaton implies that *Miscanthus x giganteus* shows a significant positive response to nitrogen fertilization (57). The result is representative because it is based on 21 peer-review articles and 174 representative observations, which are almost all the available records at the time of the study. However, this conclusion is based on a p-value of 0.07, which does not offer very strong support to demonstrate that *Miscanthus* yield responds positively to nitrogen.

On the other hand, several short and long term field experiments indicate that the amount of nitrogen fertilizer applied has no effect on *Miscanthus* yields. Schwarz and co-workers conducted a cultivation experiment for *Miscanthus × giganteus* for three years in Australia (116). Different amount of nitrogen ranging from 0 to 180 Kg/ha were applied annually to the field. The results showed that a high yield (22 t/ha) of 3-year old *Miscanthus × giganteus* can be established despite a relative low nitrogen addition. Several other similar short-term studies also have similar results, demonstrating that high biomass yield can be achieved at a relatively low nitrogen level (32, 41, 46, 79). In addition, nitrogen use efficiency is significantly higher if no nitrogen fertilizer applied (32).

Similar results were observed from a long-term experiment. Christian and coworkers carried out a *Miscanthus* cultivation trial for 14 years on a silty clay loam soil in Southern England (25). They investigated the sustainability of *Miscanthus* biomass production over a relatively long time scale at different N levels. Three treatments with different nitrogen fertilizer amounts were introduced in this study: 0 kg ha^{-1} yr^{-1} (control), 60 kg ha^{-1} yr^{-1} (N60), and 120 kg ha^{-1} yr^{-1} (N120). The *Miscanthus* yields from all nitrogen treatments increased for the first six years, followed by a stable yield of 14 to 17 t ha^{-1} yr^{-1} for 8 years. The yield for all treatments decreased to about 10 t ha^{-1} yr^{-1} at the 14^{th} year. Their results suggested no significant difference among N treatments, thus they concluded that *Miscanthus × giganteus* yield did not respond to N inputs.

The N balance and nutrient efficiency experiments revealed that besides fertilizer and aerial deposition, other sources contributed to the nitrogen content of *Miscanthus*. Christian and coworkers conducted an N balance experiment for the 14-year trial. They considered fertilizer and aerial deposition as nitrogen inputs, while crop biomass removed and leaching were considered as nitrogen outputs. The N balance data indicated that quantity of the outputs was larger than the inputs (25).

One possible source of N is biological nitrogen fixation carried out by diazotrophs. Diazotrophs have been known to associate with many non-legume grasses, including maize, sugarcane, rice and wheat (68). In addition, diazotroph communities have been demonstrated to contribute to the plant nitrogen for some non-legumes (15). It is possible that the diazotrophs contribute to the sustainability and high nutrient efficiency observed on the growth of *Miscanthus*. Therefore, it is necessary for us to investigate if the association of nitrogen-fixing bacteria with *Miscanthus* is part of the reason *Miscanthus* yield does not respond significantly to N fertilizer. Up to this point, a few studies have reported diazotrophs colonizing the plant tissue and rhizosphere soil of *Miscanthus* (35, 40, 71, 138), however, in order to harness the potential of this beneficial interaction, the environmental and ecological factors that govern the *Miscanthus*-associated diazotroph community must also be understood. Consequently, it is vital for us to investigate the diazotroph communities associated with *Miscanthus*, and identify their ecological drivers. Optimizing biological N fixation will enable agronomic practices that effectively utilize beneficial microbes for sustainable crop production.

1.4 Nitrogen fixation and diazotrophs

Biological nitrogen fixation is a process that involves transforming atmospheric N_2 to biologically available ammonium. The energetic cost of fixing nitrogen from atmospheric dinitrogen gas is relatively high compared with utilization of available nitrate or ammonium (133). Gutschick (52) calculated that it costs a diazotroph 8-12 g of glucose to fix 1 g of nitrogen, not including the construction or maintenance costs of any specialized structures. Under optimal conditions, the energy consumed during dinitrogen reduction can be expressed as (121):

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i.$$

Because of the metabolic expense of this reaction, nitrogen fixation is tightly regulated, and is inhibited by the present of available nitrate or ammonium. Diazotrophs have a competitive advantage when available nitrogen is limiting.

Nitrogen fixation is carried out by the nitrogenase enzyme - a multi-component enzyme - which normally consists of an iron (Fe) protein and molybdenum iron (MoFe)

protein (63). The *nifHDK* genes encode these subunits of the nitrogenase enzyme. It is not yet clear if the phylogeny of *nifD*, *nifH* and *nifK* is always congruent with each other (141). Previous studies showed that phylogenetic trees constructed for the *nifH* and 16S rRNA genes based on the same lineages generally show consistent phylogenetic patterns Thus far, sequencing studies surveying diazotroph diversity have focused on primarily on the *nifH* gene rather than on the *nifD* and *nifK* genes, which leads to relatively few sequences available for *nifD* and *nifK* genes (141). Hence, the *nifH* gene has been developed as a phylogenetic marker for diazotrophs, providing greater phylogenetic resolution among closely related strains than rRNA genes (141).

1.5 Nitrogen fixation in non-legumes

The ecological and economic importance of nitrogen fixation has long been recognized in *Rhizobium*-legume symbioses. Rhizobia associated with legumes cooperate with their host plant to develop root nodules. In the root nodules, the bacteria infect, inhabit, and construct a co-metabolic system with the legumes, so as to form a well-developed symbiosis. Rhizobia inside root nodules fix nitrogen into ammonium and export it to the leguminous plants (82). In return, the diazotrophs benefit from root exudates and nutrients. This process is highly efficient and can provide a significant proportion of plant nitrogen. Diazotrophs can fix all the nitrogen needs for grain legumes, such as peanuts, soybean and alfalfa (76).

Wild non-leguminous grasses can grow in nitrogen-deficient areas, indicating that diazotrophs may also associate with those plants. Nitrogen-fixing bacteria associated with gramineous plants were first isolated from the rhizosphere soil of sugarcane grown in tropical soil (38). Since then, many nitrogen-fixing bacteria have been isolated from other grasses, such as maize, wheat, and rice (68). A diverse variety of diazotrophs and non-diazotrophs have been isolated from rhizosphere and endorhizosphere of gramineous plants. A number of isolated diazotrophs have already been identified as *Acetobacter*, *Azoarcus*, *Herbaspirillum* and *Klebsiella* (67, 94).

Differences have been observed in the N-fixing bacteria associated with legumes and non-legumes. First, the N-fixing bacteria associated with legumes and non-legumes belong to different phylogenetic groups. Most legume-associated diazotrophs belong to

Alphaproteobacteria (Genus *Rhizobium, Mesorhizobium, Sinorhizobium* etc.), while those diazotrophs detected from non-legumes are more phylogenetically diverse, with representatives from the *Alpha-*, *Beta-* and *Gamma-proteobacteria*, as well as Gram-positive bacteria such as *Clostridium*. Mutualism may exist between these diazotrophs and non-leguminous grasses, however, the ecological interactions do not appear to be species-specific symbioses, and colonization of grasses by endophytic diazotrophs does not lead to development of root nodules as in the legume-rhizobium symbiosis (68). Those findings imply that the nitrogen fixing process and efficiency may also be different in non-legume plants, and the environmental factors may affect colonization and nitrogen fixation in a different way.

Recent work has demonstrated that significant N fixation processes exist in some non-legumes, such as sugarcane, wheat and maize (22, 67, 130). For some specific sugar cane cultivars, the estimated fixed N can represent up to 70% percent of the total plant nitrogen (15). However, results from sugarcane have high variance and are influenced by the sugarcane genotype and environmental conditions (130). Up to now, many species of *Azospirillum* (8), *Herbaspirillum* (107), and *Burkholderia* (49), have been isolated from sugarcane. However, those diazotrophs can be isolated from only a few variety of sugarcane, and most of them are Brazilian. The performance of those diazotrophs can be affected by environmental gradients, such as soil moisture, P and K availability (15, 130).

The nitrogen fixation efficiency varies in different crops. Some studies have reported N-fixation rates in other grasses – such as rice wheat and maize – that are as high as found in sugarcane. Shrestha and Ladha examined the capabilities of N fixation in 70 genotypes of rice, and their result shows that plant nitrogen derived from air is ranged from 0-20.2% (119). Iniguez et al. used ^{15}N isotope dilution to show that up to 42% of plant N needs in wheat (cv. Trenton) were contributed by a diazotroph strain *Klebsiella pneumoniae* 342 (Kp342) (67).

Currently, little research has explored the nitrogen-fixing bacteria associated with *Miscanthus*, not to mention the environmental factors that drive the diazotroph communities. Previously reported endophytic diazotrophs associated with *Miscanthus* belong to genera *Herbaspirillum*, *Azospirillum*, and *Clostridium* (40, 70, 95). These bacteria can be isolated from sterilized root of *Miscanthus* by N free medium, or detected through

molecular methods. Their results show that those bacteria have the ability to fix nitrogen. Thus, the diazotroph community in the rhizome and rhizosphere can be considered as potential nitrogen sources for *Miscanthus*. *In planta* N fixation and plant growth promotion by diazotrophs has not yet been demonstrated for *Miscanthus*.

1.6 Ecological drivers of N fixation

From the work done with other non-legume crops, one can conclude that environmental and ecological factors play very important roles in influencing the diazotroph communities associated with those crop and their performances. Influence of niches, soil management and plant cultivars are discussed here.

The niches where diazotrophs colonize (rhizosphere soil, root, stem and leaves) have determinative effects on the richness and diversity of diazotroph communities. Rhizosphere soils generally harbor more diazotrophs than plant tissues like roots and stems (103). The diazotrophs in rhizosphere soil are also more diverse. A large portion of OTUs appears to be restricted to the rhizosphere. Across all the different niches within and near maize, only about 1% diazotroph OTUs are detected in all niches (110). Soares et al. investigated the diazotrophs associated with oat using RFLP. Diazotroph communities from root and rhizosphere showed similar RFLP bands, while a different RFLP profile was observed from leave samples (124). Roesch and associates generated *nifH* clone libraries to characterize the diazotroph communities associated with maize rhizosphere, roots and leaves. Their results showed that maize rhizosphere harbor more diverse diazotroph communities than the endophyte. The roots had a diazotroph community that was intermediate in composition between rhizosphere and leave samples (110) [ref roesch, 2008].

Soil management factors such as tillage and fertilization play an important role in governing the abundance and composition of diazotroph assemblages. For diazotrophs associated with oat, the number of diazotrophs is greater in no-till fields than in tilled fields (124). Fertilization has a long-term effect on diazotrophs, and the nitrogen is usually the overriding influential element. Sorghum cultivars receiving different levels of nitrogen fertilizer showed significantly different diazotroph community patterns (27). In addition, more diazotrophs were detected in unfertilized field then fertilized ones (103). One study

of the rice diazotroph community demonstrated that available nitrogen can severely affect the expression of the *nifH* gene, and some OTUs only appear at a high abundance after a low dosage of mineral N fertilizer at the beginning of the growth season (72).

Diazotroph communities are also influenced by plant cultivar to some extent, and the results differ across different crops. The different sorghum cultivars impact the occurrence of some but not all diazotrophs. For the community structure of *Paenibacillus*, the sorghum cultivar type was the most influential factor, while for other bacteria species, N-fertilizer level is a stronger driver (27). Expression of *nifH* in rice roots showed a different result – that plant cultivar has great influence on the diazotroph communities. Although all the rice cultivar types grew under unique conditions, the functional diversity of diazotrophs associated with different cultivars are different, even for the cultivars selected as the sister lineages (72).

1.7 Recent work

Miscanthus has high efficiency in recycling and conserving nitrogen. Investigations of the nitrogen balance of *Miscanthus* grown in Illinois have demonstrated that if harvested at appropriate time, the nitrogen removed with *Miscanthus × giganteus* above-ground biomass can be reduced from 300 Kg ha^{-1} for early harvest to 40 Kg ha^{-1} for late harvest while the average nitrogen fertilizer input is only 25 Kg ha^{-1} (58). Although *M. × giganteus* removes a minimal amount of nitrogen each year, from a long-term sustainability perspective, adding fertility maybe necessary to maintain the soil properties.

Davis and co-workers used DAYCENT model simulations to evaluate biogeochemical cycles associated with cultivation of *M. × giganteus* (35). Model results demonstrated a deficit in the nitrogen budget – that is, output N exceeds the input N. After testing all other potential nitrogen sources of nitrogen, the authors hypothesized that nitrogen fixation may contribute up to 25 g N $m^{-2}y^{-1}$ of the nitrogen requirement of *M. x giganteus*. In support of the hypothesis, seven *nifH*$^{+}$ strains of *Enterobacteriaceae* were isolated from *Miscanthus* rhizome and stem tissues, and *in vitro* nitrogenase activity was examined. Acetylene reduction assays carried out on *Miscanthus* rhizomes provided additional evidence in support of nitrogen fixation (35).

The results show the endophytic diazotrophs in root are potential nitrogen sources for *Miscanthus*. However, *in planta* N fixation and plant growth promotion by N fixers has not yet been demonstrated for *Miscanthus*.

Besides nitrogen fixation, diazotrophs possess other potential functions to enhance plant growth. One interesting study shows that salt tolerance of *M. sinensis* is enhanced after inoculating the crop with nitrogen fixers *Clostridium* sp. Kas201-1 and *Enterobacter* sp. B901-2. Endophytic *Clostridium* was detected from the host plant. The research demonstrated *Clostridium* accompanied by *Enterobacter* improved salinity tolerance of the host plant (138).

Fluorescence tagging techniques have provided evidence showing that diazotrophic Betaproteobacterium *Herbaspirillum frisingense* can form endophytic root colonization within *M. x giganteus*. This strain has demonstrated the ability to produce indole-3-acetic acid, which is an important auxin that promotes plant growth and development (113). Although no significant plant growth stimulation was observed, *H. frisingense* shows the potential to benefit plant growth (113).

In this study, we investigated the presence and community composition of diazotrophs associated with *Miscanthus* using both classical culture and molecular methods. We compared diazotrophs in native and agricultural *Miscanthus*, and the environmental factors that drive the diazotroph community composition were also explored.

Objectives

In order to explore the association of diazotrophs with *Miscanthus*, we will address the following objectives:

1. Isolate the N-fixing bacteria associated with native *Miscanthus* rhizomes.
    a. Isolate the nitrogen-fixing bacteria by enriching on nitrogen deficient medium.
    b. Confirm N-fixing capability of the isolates by detection of *nifH* gene and with acetylene reduction assay.
    c. Identify cultured isolates based on DNA sequence of the 16S rRNA gene and *nifH* gene.
2. Identify the diazotroph populations associated with *Miscanthus* grown in different environmental conditions using molecular methods.
    a. Compare the diazotroph species obtained from culture-dependent methods with those detected using culture-independent methods.
    b. Identify and compare diazotroph species associated with *Miscanthus* rhizome and rhizosphere soil.
    c. Determine if environmental factors, such as edaphic factors and plant species influence diazotroph community composition based on phylogenetic information.

Materials and methods

Study sites

Native *Miscanthus* rhizomes and rhizosphere soil samples were collected from 19 sites in Taiwan in July 2008. Sites were selected to represent gradients in pH, soil moisture, fertility level, elevation, and plant species. (Table 1, Figure 1).

To compare the diazotroph communities from native and agricultural *Miscanthus*, *Miscanthus x giganteus* rhizomes and rhizosphere soil samples were collected summer 2009 from 8 sites in IL, USA (Table 1, Figure 1).

Rhizome processing was carried out using the methods of Chelius and Triplett (22) with modification. Rhizosphere soil was washed and collected from rhizomes using 40 ml sterile deionized water. The rhizosphere soil was then frozen at -80 °C in sterile containers. The soil was lyophilized prior to DNA extraction. For collection of endophytic microbes, rhizomes were first surface-sterilized within 1L containers containing 100 mL 95% ethanol and shaken for 30 s. The ethanol was then replaced with 5.25% sodium hypochlorite and shaken for 15 min at 150 rpm. Sterilized rhizomes were rinsed three times with 300 ml sterile deionized water. Rhizomes were then macerated in a sterilized blender cup containing 50 ml of PBS and 0.1% Tween 80. Pulverized rhizomes were washed gently to release endophytic bacteria following the methods of Brulc et al. with modification (17). Ground rhizomes were shaken gently (100 rpm) on ice for 1 hour, and plant material was removed by filtration through a sterile 3-inch No. 25 US Standard Test Sieve (Newark). Endophytic bacteria contained in the filtrate were concentrated by centrifugation prior to DNA extraction.

DNA extraction and purification

Total genomic DNA was extracted from freeze-dried rhizosphere soil samples collected from all rhizomes using the FastDNA SPIN Kit for Soil (MP Biomedicals). Genomic DNA was further purified using a cetyl trimethyl ammonium bromide (CTAB) extraction procedure to remove contaminating humic acids (114). Genomic DNA was extracted from concentrated endophytic bacterial samples using the FastDNA Spin Kit (MP Biomedicals)

following the manufacturers protocol. DNA concentration for all samples was adjusted to a standard concentration of 10 ng/µl prior to DNA analyses.

Isolation and characterization of nitrogen fixing bacteria

Extracts containing endophytic microbes were used to inoculate 10 ml of semi-solid N deficient AcD medium (19). Pellicle-forming cultures were transferred three times to fresh medium in order to enrich the nitrogen-fixing bacteria. Individual colonies were then isolated and purified on AcD agar plates. Individual colonies with distinct morphological appearance were picked and stored at -80 °C in 20% glycerol.

Polymerase chain reaction (PCR) targeting the *nifH* gene was carried out for all isolated bacterial strains (102) using primer PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3'). Potential diazotrophs were identified by successful *nifH* amplification in triplicate PCR reactions.

To obtain phylogenetic information for the *nifH*-positive colonies, the 16S rDNA gene was amplified by PCR from genomic DNA of each bacterial isolate. PCR primers for amplification of the 16S rRNA gene included 8F (5'-AGAGTTTGATCMTGGCTCAG-3'; bacteria-specific 16S rRNA gene) and 1492R (5'GGYTACCTTGTTACGACTT-3'; universal 16S rRNA gene) (75). . Each reaction contained PCR buffer consisting of 50mM Tris buffer [pH 8.3], 0.25 mg of bovine serum albumin per mL, and 3 mM $MgCl_2$ (Idaho Tech, Salt Lake City, Utah, USA), 0.25 mM of dNTPs, 0.4 µM of each primer, 0.05 U/µl of Taq polymerase (Promega, Madison, WI, USA) and 5 µL of bacterial cells suspended in sterile deionized water solution, in a final volume of 25 µL. PCR cycles included an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 35 s, 55 °C for 45 s, and 72 °C for 2 min, with a final extension carried out at 72 °C for 2 min. Reactions were carried out in an Eppendorf MasterCycler Gradient (Eppendorf AG, Hamburg, Germany). The DNA was sent to UIUC Biotechnology Center for sequencing. DNA sequences were edited with Geneious Pro 4.7.6.

Acetylene Reduction Assay

Bacterial isolates possessing the *nifH* gene were inoculated into 10 ml semi-solid AcD medium, and then allowed to grow at room temperature for three days. Three

replicate cultures were prepared for each isolate. Diazotroph strain IL-12, previously isolated from *Miscanthus,* was included as positive control. *Escherichia coli* was included as negative control. Strain IL-12 incubated in a nitrogen-rich medium was also included as a negative control. At the beginning of the acetylene reduction assay, each tube was flushed with air, sealed with a rubber septum, and 10% of the headspace was replaced with acetylene. After 24-28 h incubation at room temperature, 500 ul of headspace was collected from each tube and injected into a gas chromatograph with a GS-Alumina column. The ethylene content of each gas sample was detected and quantified by the flame ionization detector.

Construction of nifH clone library

A *nifH* clone library was constructed from endorhizosphere samples collected from 4 out of a total of 19 sites in Taiwan (The Gate, Mazao Bridge, Datunshan Peak and Alishan Area) as well as 4 sites in IL (Brownstown, Dixon Spring, DeKalb and Havana) selected to span environmental gradients present in the native and agricultural ecosystems. The primers used for *nifH* PCR were PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3') (102). PCR reaction buffer contained 1X GoTaq buffer, 0.5 mg of bovine serum albumin per mL, and 2 mM $MgCl_2$ (Idaho Tech, Salt Lake City, Utah, USA), 0.20 mM of dNTPs, 0.5 µM of each primer, 0.05 U/µl of Taq polymerase (Promega, Madison, WI, USA) and 2 µL of DNA template in a final volume of 25 µL. A touchdown PCR program was performed as follows: an initial step at 94 °C for 5 min, followed by 35 cycles each of denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at variable temperatures for 45 s, and extension at 72 °C for 45 s. In the first cycle, the annealing temperature was 64 °C. The 2nd and 3rd cycles had an annealing temperature of 62°C. The 4th to 6th cycles had an annealing temperature of 60 °C, and the 7th to 11th cycles used an annealing temperature of 58 °C. The last 24 cycles used an annealing temperature of 56 °C. The reactions were carried out in an Eppendorf MasterCycler Gradient (Eppendorf AG, Hamburg, Germany). PCR products from replicate samples (samples collected at the same site) were pooled together and concentrated using the MinElute 96 UF PCR purification Plate (QIAGEN, Valencia, CA, USA). PCR products were

then extracted and purified with QlAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). In order to increase the efficiency of ligation into the pGEM-T Easy vector (Promega, Madison, WI, USA), the PCR products were A-tailed using dATP and *Taq* polymerase at 72 °C for 15 minutes. Ligation and transformation of *nifH* PCR products into the pGEM-T Easy vector was carried out following the manufacturer's protocol (Promega, Madison, WI). White colonies were picked and screened for inserts using *nifH* PCR.

DNA templates for sequencing were generated by PCR amplification of plasmid inserts directly from cells using M13 forward and reverse primers, 5'-GTTTTCCCAGTCACGAC-3' (M13F) and 5'-CAGGAAACAGCTATGAC-3' (M13R) (98). The DNA amplicons were submitted for sequence analysis at the Keck Center for Comparative and Functional Genomics (UIUC). DNA sequences were edited with Geneious Pro 4.7.6 (Biomatters, Auckland, NZ). The sequences were aligned and trimmed using the PolF and PolR primers, producing an edited sequence length of approximately 355bp. Endophyte *nifH* sequences were compared to previously described sequences in GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) (2). The software package mothur (v. 1.10.0) was used to calculate rarefaction curves for each sample site (115). A UniFrac tree was generated with online UniFrac analysis tool (83). Soil chemical data were averaged, log transformed and normalized for each site. A Euclidean distance matrix was built to represent the similarity of soil factors among sampling sites. Spearman's rank correlation was used to evaluate the relationship between the soil chemical factors (Euclidean distance) and the diazotroph communities (UniFrac distance).

Pyrosequencing

Libraries for 454 sequencing of the *nifH* gene and 16S rDNA were constructed from samples from 8 representative locations from Taiwan and 4 locations from IL. Samples from Cingtiangang, Neughuanhu Ecological Conservation Zone, Datunshan Nature Center, Jhongpu, Mazao Bridge, Datunshan Peak, Pingyang and Fengshan were chosen to represent different environmental gradients (pH, plant species, geographic distribution) across Taiwan. Samples from Brownstown, Dixon Spring, Urbana, and Havana were chosen in

order to compare the geographic influence on endophytic and rhizosphere whole bacteria and nitrogen fixer assemblages.

In order to add the A and B adapters required for 454 pyrosequencing (81), special primers were synthesized for PCR of 16S rRNA genes and *nifH* genes. Barcodes were incorporated into the primer sequences in order to identify DNA sequences generated from each sample. The basic construction of the forward primers is 5'-Adapter A – Barcode – Forward Primer for targeted gene-3'. The reverse primer is constructed as 5'- Adapter B – Barcode – Reverse Primer for targeted gene – 3'. The sequences of adapters are: adapter A 5'–CGTATCGCCTCCCTCGCGCCATCAG–3' and adapter B 5'–CTATGCGCCTTGCCAGCCCGC TCAG–3'.

Equal amounts of PCR product amplified from twelve replicate samples collected at each site were pooled together and concentrated using the MinElute 96 UF PCR purification Plate (QIAGEN, Valencia, CA, USA). PCR was then carried out on the pooled DNA. Primers 519F (5' – CAGCMGCCGCGGTAATWC – 3') and 926R (5' – CCGTCAATTCMTTTRAGTT – 3') were chosen for PCR from the 16S rRNA gene (88). The PCR amplification to generate pyrosequencing template contains 1X HF PCR Buffer (New England Biolabs), 0.5 mg/ml bovine serum albumin, 200 uM of each dNTP, 0.4 uM of each primer, 0.05 U/ul Phusion High Fidelity polymerase (New England Biolabs) and 2 μL of DNA template at 10 ng/ul in a final volume of 25 μL. The PCR program starts with 2 min initial denaturation at 98 °C, followed by 25 cycles of 98 °C for 10s, 54 °C for 30s, 72 °C for 20 s and a final extension of 72 °C for 5 min.

For *nifH* PCR, the PolF and PolR primers described above (with barcodes and A and B adapters added for pyrosequencing) were used as forward and reverse. PCR used a step-down thermocycle program similar to that used for the *nifH* clone library above, with the denaturation temperature adjusted to 98 °C to meet the requirement of Phusion High Fidelity polymerase. The PCR amplification contained 1X HF PCR Buffer (New England Biolabs), 0.5 mg/ml bovine serum albumin, 200 uM of each dNTP, 0.4 uM of each primer, 0.05 U/ul Phusion High Fidelity polymerase (New England Biolabs) and 2 μL of DNA template in a final volume of 25 μL.

After amplification of the target gene, I purified the PCR product with QIAquick PCR Purification Kit (QIAGEN) according to the manual, quantified the purified PCR products

using a Qubit fluorometer (Invitrogen), and pooled equal amounts of PCR products from different samples. Pooled PCR products were purified using the QlAquick Gel Extraction Kit (QIAGEN). The purified samples were submitted to the Keck Center for Comparative and Functional Genomics (UIUC) for sequence analysis using the Roche 454 Genome Sequencer FLX-Titanium platform.

Data analyses of pyrosequencing

Perl scripts edited by Dr. Yuejian Mao were used to segregate data according to the barcodes (http://as-mackielab1.animal.uiuc.edu/list.html). The sequence data were then imported into Ribosomal Database Project (RDP) for analysis. The 16S rRNA gene sequences were aligned and clustered using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/). Similar processing was carried out for the *nifH* sequences by the RDP FunGene pipeline for functional gene analysis (http://fungene.cme.msu.edu/). Shannon and Chao1 diversity indices were calculated for both genes from each sample. The *nifH* database was downloaded from FunGene.

To determine the bacterial taxa present in each sample, each 454 sequencing read was compared against a reference database provided by RDP at 95% threshold using the BLAST algorithm (3, 29). The *nifH* protein sequences were compared to the FunGene *nifH* database using the BLAST tool supported by the Institute for Genomic Biology, University of Illinois.

Representative sequences were selected based on the 97% similarity at the nucleotide (16S rRNA gene) or amino acid (*nifH* gene) level. Neighbor-joining phylogenetic trees with 100 bootstraps were constructed using Geneious 5.4. The trees were imported into online FastUniFrac for community analysis (http://bmf2.colorado.edu/fastUniFrac/). Community structures, represented by the sequence reads, were compared to each other using UniFrac (54, 84). Jackknife Environmental Clusters were used to calculate the phylogenetic distance between pairs of samples. Jackknife analysis was performed to determine the robustness of every node on the cluster with 1000 permutations. . Distance matrices generated by UniFrac were imported into R for analysis of similarity (ANOSIM) (26) using the vegan package in R.

Results

1. Isolation and acetylene reduction assay

1.1 Isolation of diazotrophic endophytes from native Miscanthus

186 endophytic bacteria were isolated on nitrogen deficient AcD medium from native *Miscanthus* rhizome samples collected throughout Taiwan (Table 1). The presence of the *nifH* gene was detected by PCR in 32 of these isolates. Details about the isolated diazotrophs have been listed in Table 3. No diazotrophs were recovered from following 5 sites: Siaoyoukeng Hot Springs, Cingtiangang, the base of Cising Mountain, Mazao Bridge Hot Springs and Datunshan peak. One to five *nifH*$^{+}$ isolates were recovered per site for the remaining 14 locations. The 32 *nifH*$^{+}$ isolates were further characterized using acetylene reduction assay (ARA) and phylogenetic analysis based on the 16S rRNA gene.

1.2 Nitrogen fixing ability

All *nifH* positive isolates were tested for their ability to reduce acetylene to ethylene in semi-solid AcD N-deficient medium, an indicator of nitrogenase activity (Figure 3). Nitrogenase activity was detected in all isolates except four: N4-3, I1-1, Q3-1, and N3-0. The detected nitrogenase activity of those diazotrophs is higher than the negative controls but lower than the positive control IL-12, which is a previously isolated diazotroph.

1.3 Sequence and phylogenetic analysis of isolates

The 16S rRNA gene of each diazotroph isolate was amplified by PCR, and partial 16S rRNA gene sequencing was used to identify the diazotrophs (Table 3). Among the thirty-two diazotrophic isolates, 30 strains are classified as *Gammaproteobacteria*, while only 2 strains are classified as *Betaproteobacteria*. At genus level, the greatest number of isolates was identified as *Klebsiella* (10 strains, 31%), followed by *Raoultella* (7 strains, 22%), *Enterobacter* (6 strains, 19%), *Serratia* (5 strains, 16%), *Citrobacter* (2 strains, 6%), *Herbaspirillum* (1 strains, 3%), and *Burkholderia* (1 strain, 3%). No genus was common among all the sites.

A neighbor-joining tree was generated for the diazotrophs isolated from *Miscanthus* rhizomes in Taiwan, as well as some diazotrophs isolated previously from *Miscanthus* in

Illinois (Figure 2). Related sequences from *Proteobacteria* and some other common soil diazotrophs were included as reference.

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2. nifH clone library

2.1 Influence of biogeographic factors to the nifH gene pool

Following screening for sequence quality, a total of 135 clones from native *Miscanthus* samples (Taiwan) and 129 clones from agricultural *Miscanthus* samples (Illinois) were retained for analysis. Similar to the culture-based analyses, a number of diazotrophs were identified as *Proteobacteria*. However, a greater diversity of diazotrophs was detected in the clone library. Based on the phylogenetic assignments of the *nifH* sequences, the *nifH* clones from native *Miscanthus* samples can be grouped into 7 clusters: *Alpha-*, *Beta-*, *Gamma-* and *Delta-proteobacteria, Nitrospirae, Firmicutes*, and unclassified bacteria. Clones derived from agricultural *Miscanthus* samples can be grouped into similar clusters, except that no OTUs from Phylum *Nitrospirae* were detected. Although the *nifH* clones from both native and cultivated sites form similar taxonomic clusters, the relative abundance of sequences in each cluster is different. *Beta-proteobacteria* (51.85%) is the most abundant class in the clones from agricultural *Miscanthus*, while for native *Miscanthus* clones, *Alpha-* (23.26%), *Beta-* (16.28%), *Gamma-proteobacteria* (32.56%) are of similar abundance.

2.2 Phylogenetic information for the nifH clone sequences

A phylogenetic tree containing the clones and isolates from native and agricultural *Miscanthus* samples was constructed using the unweighted pair group method with arithmetic mean (UPGMA) (Figure 4A). Cloned *nifH* sequences from both native and agricultural samples encompass broader diversity than the cultivated samples. Focusing just on the cloned sequences, *nifH* sequences from native *Miscanthus* displayed a more diverse pattern than *nifH* clones from agricultural *Miscanthus* on the tree. A group of *nifH* clones from native *Miscanthus* are identified to be *nifH* gene at low alignment score (<200), and the most closely related culturable sequences for this group is *Paenibacillus* from

Firmicutes. Although all 4 Taiwan sites contributed some clone sequences to this unique cluster, the majority of them are from Datunshan Peak.

Jackknife clustering was used to better visualize the phylogenetic relationship among the endophytic diazotroph community from different sampling sites. UniFrac analysis shows that the composition of the endophytic diazotroph community is distinct among native (Taiwan) and agricultural (IL) *Miscanthus*. Diazotroph communities associated with agricultural *Miscanthus* tend to cluster more closely than those from native *Miscanthus* samples (Figure 4B). Clones from Brownstown samples are similar with those from DeKalb (P=0.700) and Dixon Springs (P = 0.320). All the other comparisons indicated a significant (P= 0.010) or highly significant result (P<0.001). Spearman rank correlation (ρ=0.479) indicates a good agreement between patterns of similarity among the diazotrophs assemblages and soil chemical factors, suggesting that environmental conditions play a role in shaping diazotroph communities.

3. Total bacterial community composition

Samples from representative plots of native *Miscanthus* were selected for pyrosequencing based on community patterns previously generated with automated ribosomal intergenic spacer analysis (ARISA) (Figure 9A) (45). Both rhizosphere and endorizosphere samples from each of the eight locations were included in the pyrosequencing analysis to investigate how niche and environmental factors can affect bacterial community composition. After removing low quality sequences and sequences derived from plant DNA, a total of 8,134 reads were obtained.

Species richness, and evenness analyses (Table 4) were used to compare the bacterial communities from these samples. A threshold of 97% sequence similarity was used to estimate microbial community richness. For the endorhizosphere samples, the Chao1 index for rhizome samples ranges from 88.75 to 1162.72 operational taxonomic units (OTUs); the Shannon index (H') ranges from 3.5 to 5.6; the evenness ranges from 0.87 to 0.97. Among all the three indices, samples from Pingyang Village and Fengshan have the lowest values for microbial diversity, which may due to a large number of reads derived from plant DNA. Higher values of the three indices were observed for rhizosphere samples:

Chao1 (1413 - 3768 OTUs), H' (5.5, 6.5), and Evenness (0.94 - 0.99) Both the Chao1 index and Shannon diversity index indicate that the endorhizosphere samples have significantly lower microbial diversity and richness than the rhizosphere samples ($P_{chao1}<0.001$, $P_{H'}<0.001$).

3.1 Phylogenetic analysis

Most bacterial taxa detected in the samples can be classified into *Bacteroidetes, Proteobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes, Firmicutes, Actinobacteria* phyla, or unclassified bacteria. Additionally, we classified *Proteobacteria* to the class level (Figure 5). It was observed that the bacteria from rhizomes and rhizosphere have different community composition. At the phylum level, the taxa detected in rhizosphere samples are similar to those detected within the rhizomes. However, the relative abundance of each taxon differed among niches (Figure 5). Bacteria from phylum *Proteobacteria* are the most abundant for all the endorhizosphere samples. *Proteobacteria* are also abundant in rhizosphere samples. However, large proportion of the reads recovered from rhizosphere samples were classified as unclassified bacteria.

At the genus level, the most abundant and widely detected genera from endorhizosphere and rhizosphere samples were listed separately (Table 5). *Burkholderia* was the most abundant and widely detected genus in both endorhizosphere samples and rhizosphere samples. Other abundant genera in native rhizome samples were: *Acidocella*, *Fulvimonas*, *Oxalicibacter* and *Methyloversatilis*. The other genera that were abundant in rhizosphere samples include *Fulvimonas*, *Oxalicibacterium*, *Singulisphaera*, *Raoultella* and *Pseudomonas*.

3.2 Comparison of total bacterial communities

Bacterial community composition based on representative 16S rRNA gene sequences (97% similarity) was compared using UniFrac. The PCoA plot clearly shows that the bacterial communities from endorhizosphere and rhizosphere form separate clusters (Figure 6) (ANOSIM R=0.9626, p<0.001). The jackknife cluster also indicated that bacterial communities colonizing the rhizome are distinct from those colonizing the rhizosphere

with only one exception that rhizosphere sample from Jhongpu (Site P) is more similar to the rhizomes from same location and distinct from the other rhizosphere samples.

4. Diazotroph community composition

4.1 Richness and evenness of the diazotroph communities

A total of 11,118 *nifH* sequences were recovered after removing low quality sequences. However, very few sequences were recovered from rhizome samples from Datunshan Peak, Datunshan Natural Center, and Pingyang Village, while ~5000 sequences were recovered from the rhizosphere samples from Fengshan.

Amino acid sequences translated from the DNA sequences were used for diazotroph community analysis. The Chao1 diversity estimator and the Shannon diversity Index (H') were used to evaluate species richness and evenness for the diazotroph communities of the 16 samples (Table 4). Endorhizosphere samples (TME) have a significantly lower number of OTUs at the 97% threshold than rhizosphere samples (TMR) ($p = 0.030$). Correspondingly, the Chao1 index of TME samples is significantly lower than TMR samples ($P = 0.034$). Diazotroph communities from all the locations have a medium to high H' value ranging from 2.5 to 6.2. The highest H' value was observed in rhizosphere samples from Fengshan.

4.2 Phylogenetic analysis of diazotrophs

All the unique protein sequences obtained were compared with the FunGene database of *nifH* protein sequences using the BLAST algorithm (3, 11). The BLAST analysis results confirmed that all the reads matched the nitrogenase protein sequence fragment. *Proteobacteria* was the most abundant phylum in both rhizome and soil samples. A few *nifH* sequences from other phyla - *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Nitrospirae*, *Spirochaetes*, and *Verrucomucrobia* - were also detected in this study. Sequences from those other phyla made up to less than 5% of the total unique protein bacteria.

Sequences affiliated with all four classes of *Proteobacteria* were detected in both rhizomes and rhizosphere soil. For soil samples, *Alpha-proteobacteria* made up the largest

fraction of about 61.41%. On average, *Beta-*, *Gamma-*, and *Delta-proteobacteria* comprised 16.37%, 6.96%, and 10.73% of total unique protein sequences, respectively.

The taxonomic analysis for endophytic microbial communities revealed a more even distribution of OTUs from different classes of *Proteobacteria*. *Alphaproteobacteria* are still dominant (35.93% of the sequences). *Beta-*, *Gamma-*, and *Delta-proteobacteria* comprised 25.93%, 15.33%, and 8.77% of total unique protein sequences, respectively.

At genus level, we classified the *nifH* protein sequences to 57 genera for rhizome samples, and 73 genera for rhizosphere samples (Table 6). Similar to the rhizome samples, *Burkholderia*, *Bradyrhizobium* and *Xanthobacter* are among the most abundant diazotroph genera in rhizosphere samples, representing 28.1, 17.0, and 10.4% of the sequences. *Azorhizobium*, *Anearomyxobacter* are also abundant in the rhizosphere soil samples.

In addition to those top abundant genera, a large number of other genera showed capability to colonize the rhizomes and rhizosphere of *Miscanthus*. They were detected in low numbers in our sequences (< 5%). Those genera include *Desulfovibrio*, *Azospirillum*, *Geobacter*, and *Sphingomonas*.

4.3 Comparison of the diazotroph communities

Several unweighted UniFrac approaches were used to compare the diazotroph communities, and all the approaches generated similar results. Analysis of similarity (ANOSIM) was conducted based on the distance matrix of each samples generated by UniFrac. There was less distinction among diazotroph communities from rhizosphere and *Miscanthus* rhizomes (R=0.2612, p=0.016) than what was observed for the overall bacterial communities. The unweighted PCoA plot (Figure 8A) shows similar information except that the three samples with low coverage tend to be distinct from all the other communities. The Jackknife cluster (Figure 8B) was used to determine the robustness of every node of the cluster with 100 permutations. The Jackknife fraction for the node separating TMRQ and TMRR with all the other samples was 100%, while all the other nodes have a Jackknife fraction less than 50% (not significant).

Discussion

1. Diazotroph isolates

Miscanthus is one of the most promising candidate crops for biofuel production. More efficient and sustainable bioenergy production calls for the investigation of possible strategies that reduce the artificial energy and fertilizer input. Previous research indicated that nitrogen sources other than fertilizer and direct decomposition might contribute the growth of *Miscanthus*, and inferred that biological nitrogen fixation processes might be carried out by diazotrophs to contribute to the nitrogen requirements of *Miscanthus* (25, 35). Biological fixed nitrogen is a potential bio-fertilizer that can benefit grasses and improve yield (13). Biological nitrogen fixation (BNF) processes have been detected in a number of non-legume crops, including related gramineous crops such as sugarcane, maize, and rice, supporting our hypothesis that *Miscanthus* may also benefit from BNF (15, 22, 67, 68, 96). Diazotrophs enhance the plant growth and yield for hosts. For specific sugarcane species, diazotrophs that naturally associate with the plant can contribute up to 70% of the total nitrogen requirement (14). Yanni et al discovered that diazotrophs can be used as inoculants to replace a portion of the nitrogen fertilizer that required by rice (137). Their study demonstrated that an inoculated *Rhizobium* increased the rice biomass, nitrogen content and grain yield. Riggs et al demonstrated that diazotrophic bacterial strains were able to consistently increase the yield of field-grown maize in the presence of low levels of N fertilizer (39). One of the strains they used - *Klebsiella pneumoniae* 342 (Kp 342) – was later demonstrated be able to contribute up to 42% of plant N for wheat (67). Up to this point, a few diazotroph strains from genera *Herbaspirillum*, *Azospirillum,* and *Clostridium* have been isolated from *Miscanthus*, and results from those studies showed that those bacteria have the ability to fix nitrogen *in vitro* (40, 71, 95).), but *in planta* nitrogen fixation has not yet been demonstrated in *Miscanthus*. The environmental factors that influence colonization and activity of the *Miscanthus*-associated diazotroph community are largely unknown. Based on those previous studies, it is clear that BNF in *Miscanthus* is worth investigating from both environmental and economic perspectives. If the ecological drivers of diazotroph community structure and function can be elucidated, they could be manipulated to enhance the sustainability of this prospective biofuel crop. In this study,

both classical pure culture and molecular methods were used to determine the occurrence and distribution of diazotrophs associated with *Miscanthus* rhizome samples as a first step toward understanding the ecology of this beneficial plant-microbe interaction.

Environmental factors such as pH can limit the colonization of endophytic diazotrophs. In this study, thirty-two endophytic diazotrophs were isolated from the *Miscanthus* rhizome tissue, confirming the presence of diazotrophs associated with *Miscanthus*. Bacteria endophytes were isolated from rhizomes of native *Miscanthus at* all sample collection sites, but diazotrophic endophytes were detected from only 14 out of 19 sites. Those 5 sites where no diazotrophic endophytes were isolated are mainly from extreme environments like mountain peaks and hot springs. One common character of the 5 sites is that the soils all have low pH value ranging from 2.9 to 4.2. Highly acidic soil (pH <4.0) is usually low in phosphorus, calcium and high in aluminum and manganese, which are toxic to diazotrophs (16). Although few reports have investigated the effect of pH on diazotrophs associated with non-legume crops, acidic conditions can limit both survival and persistence of nodule-forming diazotrophs in soil (16, 50, 55). This suggests that diazotrophs associated with *Miscanthus* may be less abundant or be inhibited in those extreme environments due to acidity. In addition, in this study, the AcD medium we used to isolate diazotrophs has a pH value that is close to neutral. Use of this single isolation medium may constrain our ability to culture nitrogen-fixers that are adapted to acidic conditions.

The nitrogenase activity of the *nifH*$^{+}$ isolates was detected by the acetylene reduction assay. Twenty-eight strains demonstrated the ability to reduce acetylene to ethylene, inferring those strains have active nitrogenase enzymes and possess the potential to contribute to the nitrogen needs of *Miscanthus*. However, due to the nature of this assay, where bacteria were inoculated by loop into semi-solid agar, bacterial biomass likely varied in this assay. The results we obtained are qualitative but not quantitative. Still, some valuable observations can be made on the results of this assay. First, variation in nitrogenase activity was seen among those isolates after considering statistical variations. Moreover, the nitrogenase activities of isolates seem to be unrelated to physiology. For instance, both strain N3-1 and O1-0 are *Enterobacter*, O1-0 shows greater acetylene reduction activity than N3-1. The variance observed may be due to the fact that we only

used one carbon source for the ARA. Santos et al. demonstrated that the nitrogen-fixing ability of diazotrophs might change depending on the selective carbon source and microaerophilic conditions (42). This variance may also mean that abundance and identity of diazotrophs cannot fully predict their nitrogen fixing ability (72, 87, 142).

The phylogenetic tree of the isolates implies that despite the geographic isolation, different environmental conditions, and differences in *Miscanthus* species, the diazotrophs associated with rhizome tissue collected from native *Miscanthus* in Taiwan are similar to the diazotrophs associated with agricultural *Miscanthus* rhizomes. There may be a core set of diazotroph taxa that are capable of associating with *Miscanthus.* In this study, the closest relatives for our isolates include *Gammaproteobacteria*, represented by *Enterobacter* and *Betaproteobacteria* such as *Burkholderia* and *Herbaspirillum*. Previously, *Azospirillum*, *Clostridia*, *Herbaspirillum* have been isolated from *Miscanthus* (40, 70, 94). *Azospirillum*, *Enterobacter* and *Herbaspirillum* as diazotrophs were also documented to be isolated from other non-legume crops such as rice (69, 74, 139).

Giving the fact that most endophytic bacteria are uncultivated (128), culture-based methods limited our ability to further explore the different diazotrophs that may contribute to BNF in *Miscanthus*. Ueda et al. detected 23 *nifH* clones from rice roots, but none of those clones are identical to any previously described cultured nitrogen-fixing strains (129). Hurek and associates conducted a careful greenhouse experiment to investigate the nitrogen fixing ability of *Azoarcus*. Their results showed that *Azoarcus* contributed fixed nitrogen to the host plant in an unculturable state, implying unculturable diazotrophs may play important role in contributing fixed nitrogen to the plant (66).

Additionally, the taxa detected through culturing are sensitive to the culture methods and media used. Reinhold and associated found that the carbon source used in the N-free medium could influence the growth of diazotroph isolates (106). For example, they found that *Azospirillum lipoferum* grew on medium using glucose as carbon source, but not on the medium with sucrose. The culture methods we used limited the taxa we could recover. For example, clostridia are obligate anaerobic gram-positive bacteria that are ubiquitous in soil (21, 30). Previous work reported diazotrophs identified as *Clostridium* were isolated from *Miscanthus sinensis* (95). The culture methods employed in the current study would not have recovered *Clostridium* because we did not use anaerobic culture

conditions. Those previous studies imply that the diazotroph community present in nature is much more diverse and complicated than what culture methods can detect.

2. Clone library

The molecular techniques allowed us to exam the diversity of diazotrophs more thoroughly than conventional culture-based methods. The results of the *nifH* clone library provided a more complete picture of the diazotroph communities that are associated with *Miscanthus* in both native and agricultural sites. The recovered *nifH* sequences were affiliated with several major groups of bacteria. Both clones from native and agricultural samples were dominated by *Proteobacteria*, as has been reported before as the dominant endophytic diazotroph group in many other non-legume crops (23, 56, 103, 110, 124). In this study, the main classes recovered were *Alpha-*, *Beta-* and *Gammaproteobacteria*. Despite geographic distance and variation in plant species, it is interesting to note that similar diazotroph taxa are colonizing *Miscanthus* in each location. It is not clear if *Miscanthus* is actively recruiting these taxa, or if only certain taxa are suited for plant entry and colonization of the endorhizosphere. Previous work showed endophytes seem to be naturally selected, and strong correlation between rhizosphere colonization and endophyte colonization were observed (39, 112). Plant genotype, plant age and the tissue sampled may also influence the endophyte communities (112).

From this preliminary sequence analysis, it appears that the diazotroph community composition of native (Taiwan) samples is more diverse than observed in agricultural (Illinois) *Miscanthus* samples. In our study, the UniFrac tree showed that the *nifH* sequences from agricultural samples are more similar with each other than native samples (Figure 4). Previous work has shown that edaphic factors such as pH, nitrogen content, as well as agricultural practices, such as tillage and fertilization play a major role in determining the diversity and structure of the soil bacteria community (27, 72, 103, 124). However, many factors besides agricultural practice differ among the native and agricultural plots of *Miscanthus*, including geographic isolation, plant species and heterogeneity of the soil conditions (Table 1). All of these may potentially affect the observed patterns in microbial community composition (118). The Spearman rank

correlation result supports the hypothesis that soil chemical factors (Table 1, Figure 4D) influence the diazotroph community composition (Figure 4C).

For native *Miscanthus* samples, UniFrac clustering showed greater dissimilarity in diazotroph clones among sites compared to the agricultural samples, and Datunshan Peak (H) has the most different diazotroph clones from the other sites. Unlike the other sites where *Proteobacteria* are dominant, the clones recovered from Datunshan Peak are mostly unidentified nitrogenase-encoding sequences that most closely related to *Paenibacillus* from *Firmicutes.* In general, the soil chemistry of Datunshan Peak (site H in Table 1) was different from all the other native sites (Figure 4D), and this might explain why different diazotroph species were recovered from Datunshan Peak (Figures 4B and 4C).

3. 16s Pyrosequencing of 16S rRNA genes

We employed a pyrosequencing approach that allowed a large number of 16S rRNA gene fragments to be sequenced (89). This technique greatly enhanced our ability to access the phylogenetic information of the species and communities associated with *Miscanthus* compared to culture and cloning methods. Although the pyrosequencing data have a relatively shorter length than conventional clone library, the community comparison methods such as UniFrac are very robust to the length variations (81). This allows us to detect the similarities and differences between samples with sufficient resolution. The results illustrate that there is a great deal of similarity among endorhizosphere and rhizosphere samples collected from different locations. The main finding in this pyrosequencing study is that the plant niche (rhizosphere versus endorhizosphere) is the most determinant factor influencing the composition of the overall bacterial community.

Previous studies indicate that many factors influence the bacterial populations, including host genotype (72), geographic separation (109) and niches (110). Our analysis showed that the bacterial communities of rhizome samples are significantly different from those of rhizosphere sample. None of the other factors, such as plant species, geographic distance has such significant impact to the bacterial communities.

The results of the 454 sequencing of 16S rRNA genes agree with previous results generated by automated ribosomal intergenic spacer analysis (ARISA). ARISA is a rapid and sensitive method that developed for investigating microbial diversity and community

composition (45). The ARISA results indicate that despite of the different soil fertility conditions, soil pH and geographic distribution, the strongest influential factor is the plant niche. The consistent results obtained from different methods confirmed our conclusion about the determinant role of plant niche to the bacterial community composition.

Besides the major differences between rhizosphere and endorhizosphere, some other patterns were observed from the Jackknife sample clusters. In the cluster of endorhizosphere, samples collected from north Taiwan (sites C, D, H, G, J) tend to be more similar with other than those collected from south Taiwan (sites R and Q). A similar pattern was observed for the rhizosphere clade. Additionally, samples collected from Pingyang Village and Fengshan have the farthest distance with other samples. Two possible reasons may explain the observed pattern. Edaphic factors may contribute to the variation in bacterial community composition in this study. For instance, pH value is known to have impact on bacteria community composition and alter the interaction between fungal and bacterial communities (6, 105, 111). In our case, the 5 locations in northern Taiwan have a significantly lower pH than the 3 locations in south Taiwan ($p<0.001$). Comparable differences of north and south Taiwan soil were observed for other soil factors, such as total N ($p<0.001$) and calcium ($p<0.001$). Such differences may contribute to the bacterial community pattern we observed. Additionally, the plant samples collected from north Taiwan are *Miscanthus sinensis*, and samples from south Taiwan are *Miscanthus floridulus*. Thus, plant species and cultivar may also contribute to the "North-South" differences in microbial community composition.

Sequences closely related to *Burkholderia* species are the most abundant in samples from both niches. *Burkholderia* is a genus of *Betaproteobacteria*. *Burkholderia* species are documented to be versatile species that occupy diverse ecological niches (28). The majority of *Burkholderia* is known as soil bacteria, and has non-pathogenic interactions with plants, but some species are known as plant and human pathogen, such as *Burkholderia cepacia* (37). Genus *Burkholderia* was first described in 1992 by Yabuuchi (136). This genus went through many changes and comprises more than 30 species now. Some *Burkholderia* species have been investigated for their potential in agricultural and environmental applications such as nitrogen fixation, plant growth promotion, and biocontrol (28, 42, 48). High abundance of different *Burkholderia* species has been reported associated with many

non-legumes such as maize, rice, coffee and wheat (7, 33, 42). Additionally, *Burkholderia cepacia*, a common plant-associated species, is also known as a human and plant pathogen (7). *Burkholderia cepacia* can cause soft rot in onion, and outbreak among cystic fibrosis patients (62). Those findings highlighted the importance of exploring the diversity of *Burkholderia* in plant rhizosphere and endorhizosphere in order to maximum the benefit from this species and to avoid the harm it may bring.

Pseudomonas is the second most abundant bacterial genus found in both endorhizosphere and rhizosphere soil. *Pseudomonas* is a genus within the *Gammaproteobacteria*, and like *Burkholderia*, it is widespread in water, soil, and plant seeds (36). Pseudomonas species are known as plant pathogens and biocontrol agents (59, 60). They have the capability to colonize rhizosphere soil (85, 86) and roots of some non-legume crops (59, 91). *Acidisoma* and *Acidocella* were among the abundant *Alphaproteobacteria* detected in the sequence library. These are acetic acid bacteria, a group of bacteria that are common in low pH environments. These two genera are common among the sequences recovered from low pH plots (pH < 4), but barely present in the medium pH plots in this study, supporting the conclusion that soil factors such as pH have influence on the bacterial community composition.

In summary, the 454 sequencing results of 16S rRNA genes revealed that the strongest driver of bacterial community composition associated with native *Miscanthus* was driven by the plant niche (rhizome vs. rhizosphere). Other factors, such as geographic distribution and soil pH also showed some impact on bacterial community composition.

4. nifH pyrosequencing

Diazotrophs that associate with non-legumes have the potential to greatly benefit the plant. However, our knowledge of diazotroph communities that are associated with rhizomes and rhizosphere of *Miscanthus* is poor. Previous studies on nitrogen-fixing bacteria have focused on the culturable diazotrophs or *nifH* clone libraries (40, 71, 95). Here we investigated *Miscanthus*-associated diazotroph communities from a range of different environmental conditions using a pyrosequencing approach. This approach provides a greater depth of comparison of the taxonomic diversity of diazotrophs inside and outside of *Miscanthus* rhizomes. By investigating the diazotrophs colonizing different

niches, we hope to enhance our understanding about the potential for non-legume plants to select endophytic diazotrophs, and to address the environmental factors that influence the diazotroph communities.

The detected diazotroph OTUs associated with native *Miscanthus* plants and their rhizosphere were dominated by *Proteobacteria*, especially *Alphaproteobacteria*. This result contrasts with our culture-based results, but agrees with previous studies investigating diazotrophs in root and soil. Chelius and Triplett investigated the diazotrophs associated with maize root, and they found that among the 74 phylotypes they isolated, the majority were classified as *Alpha-proteobacteria* (23). Roesch, who constructed *nifH* clone libraries for diazotrophs associated with maize roots and rhizosphere soil further confirmed the dominance of *Proteobacteria* (110). Coelho and associates also found that *Proteobacteria*, especially *Alpha-proteobacteria,* were the dominant taxa in the sorghum rhizosphere (27). Similar results were found by Chowdhury who investigated the *nifH* gene diversity in the root and rhizosphere soil of a grass - *Lasiurus sindicus* (24).

The genus *Burkholderia* was found to be most abundant genus of diazotroph in all of our samples. *Burkholderia vietnamiensis* was the first nitrogen-fixing species described from this genus. It was isolated from rhizosphere of rice cultivated in Vietnam in 1995 (48). Later, nitrogen-fixing abilities were detected in other *Burkholderia* species, including *B.cepacia, B. tuberum, B. phymatum, B. tropica, B. unamea* (20, 92, 108, 131). *Burkholderia* diazotroph species are capable of colonizing both legume and non-legume plants in plant endophyte and rhizosphere (20, 28, 92, 99, 108). Additionally, *Burkholderia* species were demonstrated to have a wide geographic distribution and the capability to fix nitrogen for diverse non-legume hosts including maize, sugarcane, rice and coffee plant (20, 48, 99). Estrada-de los Santos and associates recovered 25 diazotrophic *Burkholderia* strains from maize and coffee plants in a wide geographic range in Mexico (42). In this study, a large amount of *Burkholderia* species were detected in both rhizomes and rhizosphere soil, however, *nifH* sequences classified as *Burkholderia* were more dominant in the rhizome compare with the rhizosphere samples.

Although in relatively lower abundance, *nifH* sequences classified as *Azospirillum* were detected in almost all the samples. *Azospirillum* are a genus of gram-negative free-living bacteria that belong to the *Alphaproteobacteria*. It is considered to be a promising

genus that participates in important plant-microbe interactions (9, 10). *Azospirillum* has been demonstrated to significantly increase the nitrogen content of hundreds of cereal and non-cereal crops, such as maize and wheat (10). Besides nitrogen fixation, *Azospirillum* also participates in ammonification and nitrification. This capacity to contribute to several transformations within the N cycle enables these diazotrophs to adapt to the complex rhizosphere environment (10). *Azospirillum* is also known to produce hormones to promote plant growth. Those hormones include indole acetic acid (IAA) and gibberellins (126).

In this study, some genera of diazotrophs were exclusively detected in soil samples (including *Amorphomonas*, *Beijerinckiaceae*, *Cyanothece*, *Frankia*, *Erwinia*), while 4 genera were detected only in rhizome samples (*Rhodoblastus*, *Alkaliphilus*, *Azospira*, *Chloroherpeton*). Most of such genera were found in low abundance. A few taxa, like *Anaeromyxobacter*, were abundant in soil samples, but rarely found in rhizome samples. These results lead to the conclusion that the rhizome and rhizosphere soil niches recruit different group of diazotrophs.

Species richness and evenness are closely related to depth of sequencing. Rarefaction curves can be used to evaluate how thoroughly the microbial assemblages were sampled by the pyrosequencing approach (10). The curves should represent the taxonomic diversity within a sample if none of the DNA manipulation was biased (65). In addition, the taxonomic diversity also depends on the similarity threshold that was employed. In this study, we defined an OTU as sequences with 97% similarity in amino acid sequence. The rarefaction curves leveled off towards an asymptote, indicating the number of sequences we recovered was sufficient to represent the total diazotrophs within each community except for the three samples that yielded low number of reads. For future work, additional sampling effort is necessary to capture the rest taxonomic units for those three samples.

Diversity measures were applied to the *nifH* sequences detected in this study. Rarefaction analysis suggested that the rhizosphere samples contain significantly more OTUs than the endorhizosphere samples. Non-paramentic Chao1 estimation also indicates that the bacterial community richness of the rhizosphere soil is greater than that within the rhizomes. This result is in agreement with a previous study conducted by Roesch et al. that

investigated the diazotrophs within the soil, root, and stem of field-grown maize by constructing *nifH* clone libraries (127). The author detected that the rhizosphere soil yielded the most diverse diazotroph community and a higher Chao1 index than microbial communities within maize tissues. The rhizosphere is a biologically active zone, where many microbial processes are occurring, including nutrient cycling and decomposition of organic matter. Plant substrate transferred from plant root to soil forms a nutrient-rich microenvironment (110). In our study, rhizosphere soil harbors more diverse diazotroph communities than the *Miscanthus* rhizome, and this may be explained by that exudation produced by the host plant provides higher carbon content in rhizosphere than in bulk soil. Molecular analyses have revealed that plant defense responses can suppress bacterial colonization of the rhizome tissues (123) Krause et al reported the complete genome sequence of an *Azoarcus* sp. (strain BH72), a nitrogen-fixing bacterium that colonizes host plants with remarkably high efficiency (112). They compared the coding sequences with plant pathogens and found that strain BH72 may have novel plant recognition and colonization factors that facilitate its endophytic lifestyle (73). Only bacteria that can evade or overcome plant defense mechanisms are able to enter the root tissue. The lower diversity of endophytes (both total bacteria and diazotrophs) seems to indicate that endophytic taxa are selected from the diverse rhizosphere populations, and that some bacteria are more competitive in colonizing the plant tissue. Some plant growth promoting endophytes can suppress the invasion of plant pathogens. Those plant-microbe interaction and microbe-microbe interactions can be the reasons of low diversity detected inside of the plant.

In our study, difference in composition was detected between the nitrogen-fixing communities colonizing the rhizosphere and endorhizosphere. But the difference is not very strong based on the r value. This result is similar with work carried out by Rosche et al (73, 93). Their results showed that similar diazotroph communities were observed in maize roots and rhizosphere. Conversely, our result differs from another previous work conducted by Vollu and associates (110). They studied the diazotrophs associated with *Chrysopogon zizanioides* (L.) using DGGE, and the DGGE bands shows different pattern for rhizosphere and endophyte simples. Some endophytes are seed-borne, but most of them are recruited from the pool of soil and rhizosphere bacteria (135). The root is the primary

site where diazotrophs enters the plant tissue. This may contribute to the observation that assemblages of nitrogen-fixing bacteria in *Miscanthus* rhizomes are not distinctly different from those in the rhizosphere. In addition, this is similar to what we observed from our previous work using *nifH* terminal restriction fragment length polymorphism (T-RFLP), which indicated plant niches have influence to the diazotroph communities to some extent, but not strong (ANOSIM R = 0.199, p=0.001).

In this study, diazotroph community composition from rhizosphere soil and endorhizosphere didn't show as great of a difference as the total bacterial communities did. One possible reason for the different patterns observed between diazotroph communities and the total bacterial community is that only a small portion of the *Miscanthus*-associated bacteria, representing a fraction of the diversity of the microbiome, have the potential to fix nitrogen. Thus, the ecological factors that affect the total bacterial communities and produce the patterns of community composition observed in this study are not necessarily the same factors that influence the diazotroph communities associated with *Miscanthus*. Methodology may offer another explanation for the different patterns observed in the total bacterial and diazotroph communities. In order to compare the total bacterial communities, a phylogenetic tree was built based on 16S rRNA sequences, while translated protein sequences were used to construct the *nifH* phylogenetic tree. The 454 sequencing platform was reported to have a relatively high insertion and deletion error rate of about 3.3% per individual read (47, 112). Using protein sequences that corrected the frameshifts to align reduced the risk of inaccurate alignment due to 454 sequencing error. However, in this study, the divergence of taxa is small – the protein sequences have lower capacity to distinguish taxa than rRNA gene sequences. At the protein level, phylogenetic structure has less resolution than that at the DNA level (89). Thus, compared to DNA sequences, the less diverse protein sequences may contribute to the non-significant niche effect we observed for the diazotrophs.

To conclude, the *nifH* pyrosequencing results didn't show strong rhizosphere versus endorhizosphere clusters as the 16S rRNA gene sequences did, but the results still show diazotroph communities respond to geographic distribution. Rhizosphere soil harbor more diverse nitrogen fixers than endorhizosphere. Although the composition of diazotrophs

from these two niches is similar at the phylum level, many genera are restricted to rhizosphere.

Conclusion

In this study, we confirmed the presence of diazotrophs associated with *Miscanthus*, and demonstrated that those diazotrophs have great potential to fix nitrogen for the plant. We identified and compared representative diazotroph communities from variety sites. We found that plant niche has the strongest influence on the total bacterial commuting but exerts very little influence on the diazotroph community. Additionally, it was observed that other environmental factors such as geographic distribution and edaphic factors have influences on the diazotroph communities. To evaluate the capacity for the *Miscanthus*-associated diazotrophs contribute to plant nitrogen needs, and to examine how individual environmental factors affect the diazotroph community, well-design greenhouse and field experiments are needed. Understanding how microbial diversity and composition correlate with environmental variables will enable agronomic practices that effectively utilize beneficial microbes for sustainable crop production.

Tables and Figures

Site Code	Site Name	Site Location	Plant Species	Latitude	Longitude	Elevation
A	Siaoyoukeng Hot Springs	North Taiwan	*M. sinensis*	25.17558	121.54726	121.54726
B	The Gate	North Taiwan	*M. sinensis*	25.16615	121.57548	121.57548
C	Cingtiangang	North Taiwan	*M. sinensis*	25.16531	121.57629	121.57629
D	Menghuanhu Ecological Conservation Zone	North Taiwan	*M. sinensis*	25.16566	121.55976	121.55976
E	Mt. Cising (base)	North Taiwan	*M. sinensis*	25.17559	121.5582	121.5582
F	Mazao Bridge Hot Springs	North Taiwan	*M. sinensis*	25.17747	121.56193	121.56193
G	Mazao Bridge	North Taiwan	*M. sinensis*	25.17793	121.56252	121.56252
H	Datunshan peak	North Taiwan	*M. sinensis*	25.17943	121.52219	121.52219
I	Datunshan middle	North Taiwan	*M. sinensis*	25.18251	121.52402	121.52402
J	Datunshan Nature Center	North Taiwan	*M. sinensis*	25.18743	121.52163	121.52163
K	Houlong/Miaoli (West Coast)	Central Taiwan	*M. floridulus*	24.60799	120.75047	120.75047
L	Yushan NP	Central Taiwan	*M. sinensis*	23.48448	120.8964	120.8964
M	Lulin Mountain (peak)	Central Taiwan	*M. floridulus*	23.46774	120.88077	120.88077
N	Alishan Area	Central Taiwan	*M. floridulus*	23.49433	120.7938	120.7938
O	Between Alishan and Chiayi	Central Taiwan	*M. floridulus*	23.41466	120.64457	120.64457
P	Near Jhongpu	Central Taiwan	*M. floridulus*	23.4473	120.58086	120.58086
Q	Pingyang Village	South Taiwan	*M. floridulus*	23.07891	120.37367	120.37367
R	Fengshan	South Taiwan	*M. floridulus*	22.91804	120.63941	120.63941
S	Pingtung	South Taiwan	*M. floridulus*	22.70931	120.5798	120.5798
DXS	Dixon Springs	Illinois	*M. ×giganteus*	37.45375	88.72316	144
BST	Brownstown	Illinois	*M. ×giganteus*	38.95077	88.95972	178
DKB	DeKalb	Illinois	*M. ×giganteus*	41.84418	88.85248	263
HAV	Havana	Illinois	*M. ×giganteus*	40.2951	89.9438	150

Table 1: Site name, site code, locations, and plant species of the sampling sites.

Site Code	Moisture	P (ppm)	K (ppm)	Ca (ppm)	Fe (ppm)	Total C (%)	Total N (%)	C/N ratio	pH
A	30.4 (25.1, 35.2)	19 (5.5, 27)	49.2 (26, 109.3)	84.1 (44.7, 171.5)	397.9 (183.9, 562.5)	0.6 (0.2, 1.7)	0.1 (0, 0.1)	7 (4.3, 12.3)	3 (2.9, 3.2)
B	37.6 (36.7, 38.5)	2.4 (1, 4)	76.5 (57.2, 98.2)	62.7 (29.6, 89.2)	212.7 (106.8, 339.4)	3.7 (2.3, 4.9)	0.3 (0.2, 0.4)	13.3 (12.8, 13.5)	4.5 (4.3, 4.6)
C	38.8 (29.9, 45.9)	3.5 (1, 7.5)	91.8 (65.2, 106.6)	47.4 (29.8, 76.4)	379.7 (230.9, 533.7)	5.6 (2.3, 7.9)	0.4 (0.2, 0.5)	13.5 (12.2, 15.4)	4.1 (3.8, 4.2)
D	60.4 (53.3, 64.7)	7.8 (4.5, 13)	163.6 (126.1, 197.8)	104.6 (91.4, 117.9)	380 (238.6, 499.8)	13 (10.6, 15.3)	0.8 (0.7, 1)	15.4 (15.2, 15.7)	3.7 (3.5, 4)
E	44.3 (28.1, 53.5)	56.3 (7.5, 110.5)	166.8 (43.6, 299.2)	75.1 (57.8, 108.6)	596.7 (345.1, 927.5)	17.8 (3.1, 39.9)	0.8 (0.2, 1.7)	20.1 (16.2, 23.9)	3.6 (3.3, 4.1)
F	49.6 (33.7, 65.6)	38.6 (6.5, 85.5)	40.3 (7.8, 88.6)	763.4 (189.7, 1512)	555.4 (254.7, 893.5)	1.4 (0.6, 3.1)	0.1 (0.1, 0.2)	10.8 (8.2, 16.4)	3.1 (2.9, 3.6)
G	46.4 (28.1, 65.6)	47.7 (6.5, 110.5)	112.5 (7.8, 299.2)	422.7 (57.8, 1512)	644.7 (254.7, 1476.5)	9.8 (0.6, 39.9)	0.5 (0.1, 1.7)	15.3 (8.2, 23.9)	3.3 (2.9, 4.1)
H	63.7 (53.6, 72)	36.6 (19.5, 65.5)	286.5 (245.8, 323.8)	200.5 (155.2, 268.7)	137.4 (117.3, 181.5)	19.8 (16.8, 25.4)	1.3 (1.1, 1.7)	14.6 (14.3, 15.3)	4.1 (4.1, 4.2)
I	30.6 (19, 40.2)	41.5 (4.5, 138.3)	131.6 (117.6, 164.3)	1298.8 (205.8, 3696)	96.4 (80.9, 121.4)	6.3 (4.8, 8.2)	0.5 (0.4, 0.7)	14.1 (12.6, 17.2)	5.3 (4.3, 7.5)
J	28.8 (26.9, 30.4)	4.9 (3, 6.5)	171.1 (156, 185)	1059.6 (433.6, 2120.5)	77.4 (61.3, 97.2)	3.5 (2.9, 4.3)	0.3 (0.3, 0.4)	11.5 (9.7, 12.1)	5.6 (5.2, 6.2)
K	24.3 (8, 67)	8 (7, 9)	79.3 (57.9, 103.9)	914.9 (800, 1001)	116.6 (100.3, 130.5)	0.6 (0.4, 1)	0.1 (0.1, 0.1)	5.6 (4.4, 7.5)	6.1 (5.7, 6.4)
L	16.3 (9.5, 25)	5.4 (3, 9)	56.7 (25, 134.4)	1657 (1308.5, 2178.5)	183.6 (130.8, 256.9)	2.7 (1, 6.9)	0.2 (0.1, 0.6)	9.5 (7.9, 12.5)	6.9 (5.1, 7.9)
M	68.9 (64.2, 75.5)	71.8 (19.5, 220)	549.1 (218.6, 1465.5)	868 (543.5, 1793)	272.9 (155, 334.6)	20.5 (14.3, 34)	1.2 (1, 1.4)	16.4 (13.6, 24.3)	4.1 (4.1, 4.1)
N	26.9 (21.3, 33)	17.4 (10.3, 26)	133.6 (115.1, 153.7)	905.3 (451.2, 1671.5)	135.4 (92.6, 171.6)	2.4 (1.6, 3.6)	0.2 (0.2, 0.3)	12.6 (10.2, 14.2)	5.2 (4.6, 6.1)
O	19.2 (18.1, 20.2)	7.8 (6.5, 9)	102.5 (91.9, 119.5)	972.1 (651.5, 1190)	190.4 (165.1, 210.3)	1.6 (1.1, 1.9)	0.2 (0.1, 0.2)	8.4 (8, 9)	5.3 (4.9, 5.6)
P	18.9 (15.5, 24.5)	76.4 (45, 108.5)	137.5 (52.8, 292)	1051.9 (960.5, 1265.5)	235.4 (213.8, 260.6)	1.2 (0.7, 1.9)	0.1 (0.1, 0.2)	8.5 (7.2, 10.7)	5.8 (5.7, 5.9)
Q	26.1 (22, 33.2)	44.4 (23.5, 56.5)	159.1 (94, 297.6)	4011.6 (3016.5, 5495)	273.8 (212, 391.3)	3.4 (2.6, 4.2)	0.3 (0.2, 0.3)	12.8 (10.4, 19.6)	7.7 (7.6, 7.9)
R	23.2 (18, 26.6)	107.9 (55, 133.5)	74.1 (39.2, 122)	699.5 (566, 821.5)	652 (479.1, 767)	1.5 (0.9, 2)	0.2 (0.2, 0.3)	6.8 (5.4, 8)	5.2 (5.1, 5.4)
S	14.5 (10.8, 21)	31.1 (18.5, 53)	174.6 (45.7, 475.4)	5461.9 (2124.5, 14075)	333.8 (281.3, 380.7)	1.6 (0.8, 2.5)	0.2 (0.1, 0.2)	9 (5.9, 12.3)	7.9 (7.8, 7.9)
DXS	23.2 (22.2, 25.0)	21.5 (17.5, 25.5)	57.3 (51, 64)	2134.4 (1899, 2331)	240.4 (215.7, 255.4)	1.6 (1.5, 1.8)	0.1 (0.1, 0.2)	11 (10.2, 12)	7.2 (6.8, 7.4)
BST	17.1 (16.6, 17.8)	14.1 (9.5, 21)	94.1 (56.5, 129)	666.2 (350.8, 1154.5)	297.7 (272.6, 312.8)	1.6 (1.4, 1.8)	0.2 (0.2, 0.2)	9.5 (9.1, 9.7)	5.3 (4.9, 6.1)
DKB	21.2 (19.6, 23.5)	25.3 (18.5, 38.5)	154.8 (102, 254)	4855.7 (3641.5, 6125)	147.8 (91.8, 197.4)	3.3 (2.7, 3.9)	0.3 (0.3, 0.4)	10.6 (9.6, 11.5)	6.2 (5.8, 7.3)
HAV	7.2 (5.3, 9.1)	48.8 (6, 80.5)	120.9 (62.5, 182.5)	1151.3 (541.5, 1895.5)	174.2 (125.6, 202.5)	0.8 (0.5, 1.1)	0.1 (0.1, 0.1)	11.5 (7.8, 13)	7.2 (7, 7.5)

Table 2: The edaphic values of the sampling sites. The soil chemical values listed are mean (minimum, maximum).

Site ID	Isolate ID	Phylogenetic assignments (genus) based on 16S rRNA genes
B	TME_B11	*Raoultella*
D	TME_D41	*Herbaspirillum*
G	TME_G1-1	*Raoultella*
	TME_G3-0	*Raoultella*
	TME_G4-0	*Raoultella*
I	TME_I1-1	*Serratia*
	TME_I3-1	*Klebsiella*
	TME_I3-2	*Klebsiella*
J	TME_J3-0	*Raoultella*
K	TME_K4-1	*Enterobacter*
L	TME_L3-0	*Serratia*
	TME_L4-1	*Serratia*
M	TME_M3-0	*Enterobacter*
N	TME_N1-0	*Serratia*
	TME_N2-0	*Serratia*
	TME_N3-0	*Enterobacter*
O	TME_O1-0	*Enterobacter*
	TME_O2-0	*Burkholderia*
P	TME_P1-0	*Raoultella*
	TME_P2-2	*Raoultella*
	TME_P3-0	*Klebsiella*
Q	TME_Q1-0	*Klebsiella*
	TME_Q2-4	*Klebsiella*
	TME_Q3-1	*Citrobacter*
	TME_Q3-2	*Enterobacter*
	TME_Q4-1	*Citrobacter*
R	TME_R1-1	*Klebsiella*
	TME_R4-1	*Klebsiella*
S	TME_S1-0	*Enterobacter*
	TME_S1-1	*Klebsiella*
	TME_S2-0	*Klebsiella*
	TME_S4-0	*Klebsiella*

Table 3: Phylogenetic assignments at the genus level for bacterial diazotroph strains isolated from native *Miscanthus* rhizomes.

Sample ID	16S rDNA			*nifH*		
	Chao1 Index	Shannon Index	Evenness	Chao1 Index	Shannon Index	Evenness
TMEC	1253.16	5.54	0.97	194.00	4.13	0.93
TMED	485.14	4.46	0.94	109.00	3.42	0.90
TMEG	546.71	3.69	0.95	183.80	3.89	0.91
TMEH	770.17	4.69	0.96	11.50	2.11	0.96
TMEJ	1024.18	5.05	0.97	81.00	2.51	0.93
TMEP	1055.91	5.25	0.90	159.00	4.26	0.92
TMEQ	173.08	1.70	0.88	55.00	2.66	0.96
TMER	93.33	1.75	0.90	155.69	4.19	0.93
TMRC	3768.26	6.54	0.99	242.78	4.70	0.93
TMRD	3747.59	6.47	0.98	110.05	3.91	0.91
TMRG	3044.06	6.21	0.98	275.37	4.67	0.95
TMRH	1842.49	5.86	0.97	174.04	4.44	0.93
TMRJ	2419.88	6.05	0.98	154.39	4.43	0.92
TMRP	1774.21	5.48	0.94	319.00	4.60	0.92
TMRQ	3101.00	6.07	0.99	528.80	5.46	0.95
TMRR	1413.57	5.71	0.97	1070.82	6.24	0.95

Table 4: Estimated Chao1, Shannon diversity, and evenness indices for total bacterial and diazotroph communities colonizing the rhizome and rhizosphere of native *Miscanthus*, based on pyrosequencing of the 16S rRNA gene and *nifH* gene. Sample IDs starting with TME represent native *Miscanthus* endorhizosphere samples, while sample IDs beginning with TMR represent rhizosphere samples collected from native *Miscanthus*. The final letter of each sample ID corresponds to site codes in Tables 1 and 2.

Genus	Phylum/Class	Proportion in endorhizosphere samples (%)	Proportion in rhizosphere samples (%)
Acidocella	*Alphaproteobacteria*	2.9	
Burkholderia	*Betaproteobacteria*	8.4	4.3
Oxalicibacterium	*Betaproteobacteria*	2.1	1.4
Methyloversatilis	*Betaproteobacteria*	1.9	
Dickeya	*Gammaproteobacteria*	1.4	
Fulvimonas	*Gammaproteobacteria*	2.7	1.6
Pantoea	*Gammaproteobacteria*	1.2	
Pseudomonas	*Gammaproteobacteria*		1.3
Rahnella	*Gammaproteobacteria*	1.3	
Raoultella	*Gammaproteobacteria*		1.3
Samsonia	*Gammaproteobacteria*	1.6	
Desulfonatronovibrio	*Deltaproteobacteria*	1.3	
Singulisphaera	*Planctomycetes*		1.4
Micrococcineae	*Actinobacteria*		1.2
Ktedonobacter	*Chloroflexi*		1.2

Table 5: Abundant genera among total bacterial communities in endorhizosphere and rhizosphere of *Miscanthus*. Class is indicated for genera within the *Proteobacteria*, phylum is indicated for other genera.

Genus	Class	Proportion in endorhizosphere samples (%)	Proportion in rhizosphere samples (%)
Azorhizobium	*Alphaproteobacteria*		5.8
Bradyrhizobium	*Alphaproteobacteria*	5.8	17
Sinorhizobium	*Alphaproteobacteria*	5.7	
Xanthobacter	*Alphaproteobacteria*	5.4	10.4
Burkholderia	*Betaproteobacteria*	20.7	28.7
Herbaspirillum	*Betaproteobacteria*	5	
Klebsiella	*Gammaproteobacteria*	5.7	
Anaeromyxobacter	*Deltaproteobacteria*		5.7

Table 6: Dominant genera (> 5% of *nifH* sequences) among diazotroph communities colonizing *Miscanthus* endorhizosphere and rhizosphere.

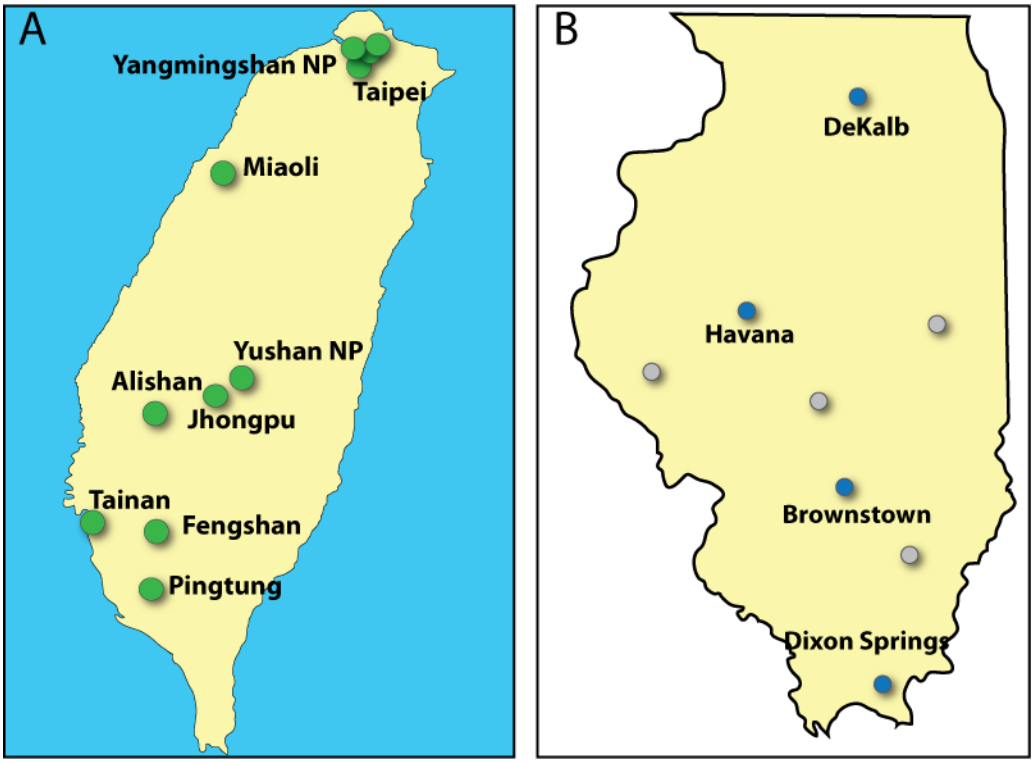

Figure 1. Map of sample collection sites for native *Miscanthus* in Taiwan (A) and agricultural *Miscanthus* in Illinois (B). Each point represents a sampling site. The agricultural *Miscanthus* samples included in this study were collected from the Illinois locations represented in blue. Detailed information about each location is contained in Table 1.

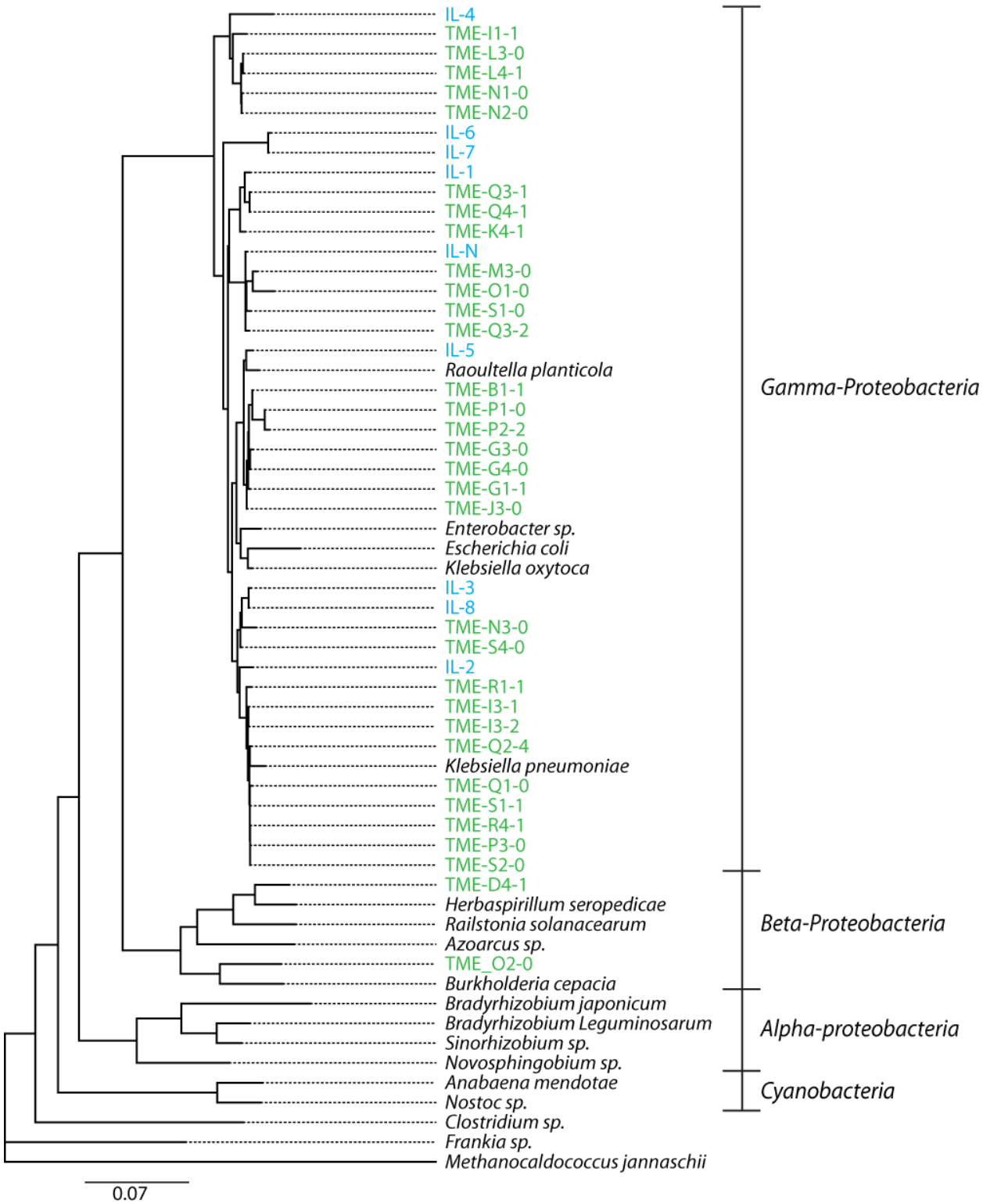

Figure 2: Phylogenetic tree of the diazotroph isolates recovered from native and agricultural *Miscanthus* rhizome tissues. The tree is built based on the 16S rDNA, generated by the weighted neighbor-joining tree-building algorithm (5). Diazotroph strains from native and agricultural *Miscanthus* rhizomes are similar, and the majority of the isolated strains are from

Gammaproteobacteria. Only two strains isolated from native *Miscanthus* rhizomes were assigned to *Betaproteobacteria*. Additional details about each isolate are contained in Table 2.

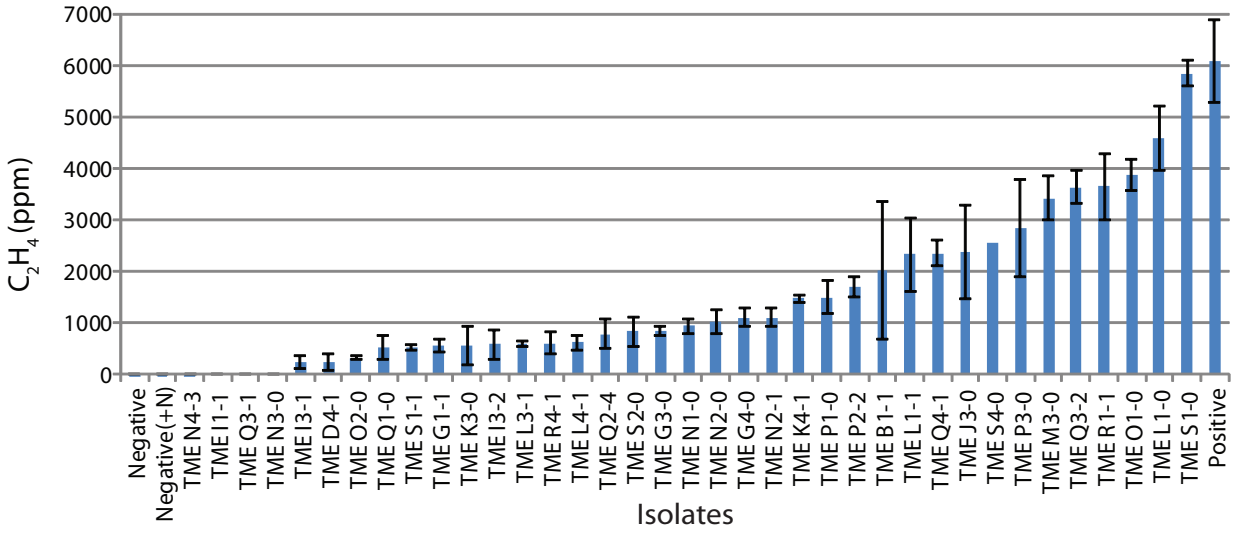

Figure 3: All diazotrophs isolated from native *Miscanthus* rhizomes were tested for their nitrogenase activity using the acetylene reduction assay. Values represent the average ethylene detected in triplicate assays. Error bars represent standard error. Nitrogenase activity was detected in the majority of the isolates, and that the level of acetylene reduction activity in this assay varies among the isolates. The positive control was a previously isolated *Gammaproteobacteria* diazotroph, strain IL-12. Negative controls included *E. coli* (a non-nitrogen-fixing bacterium), and the positive control strain incubated with available N to inhibit expression of nitrogenase. Additional details about each isolate are contained in Table 2.

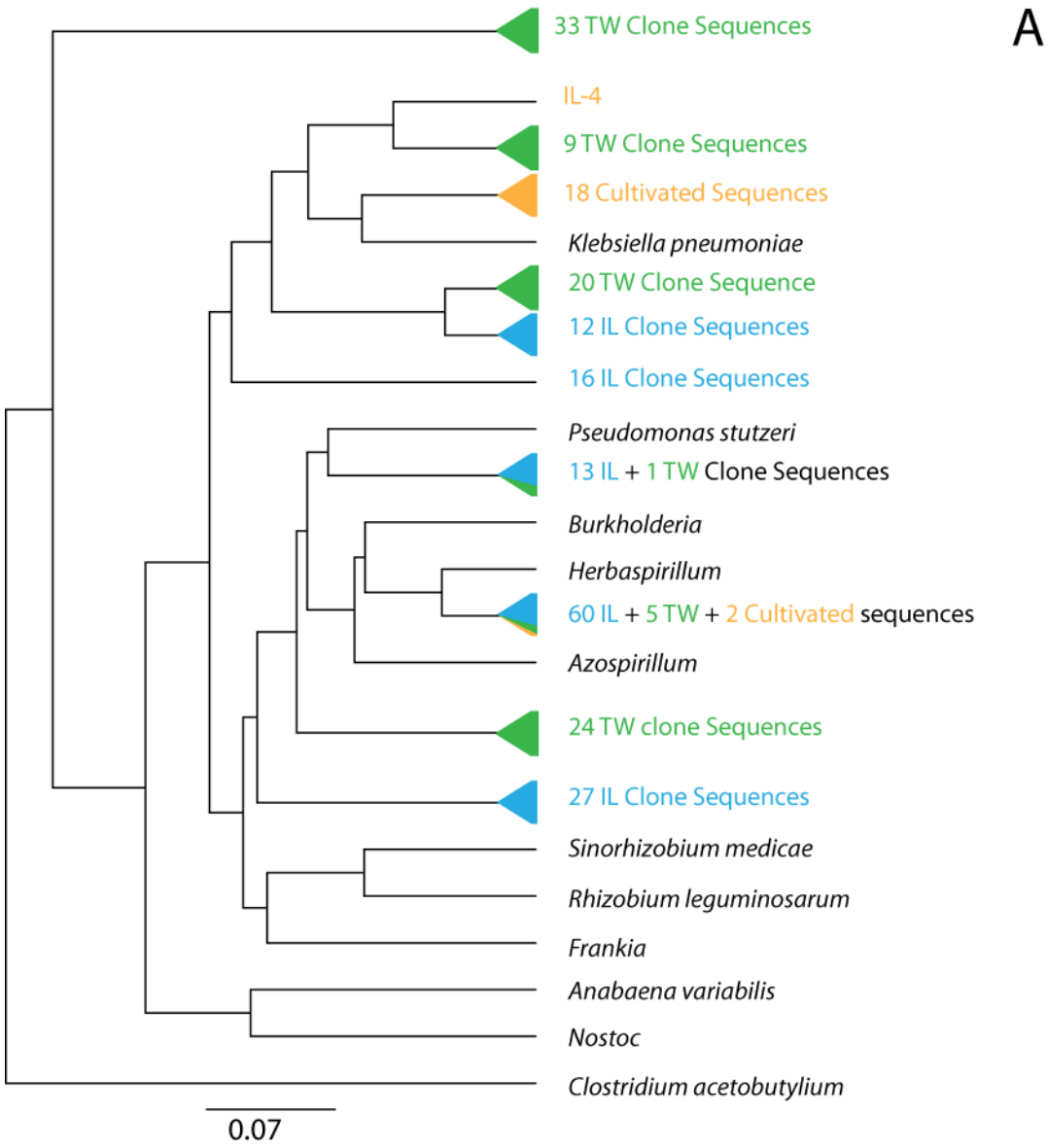

Figure 4 (cont. on next page)

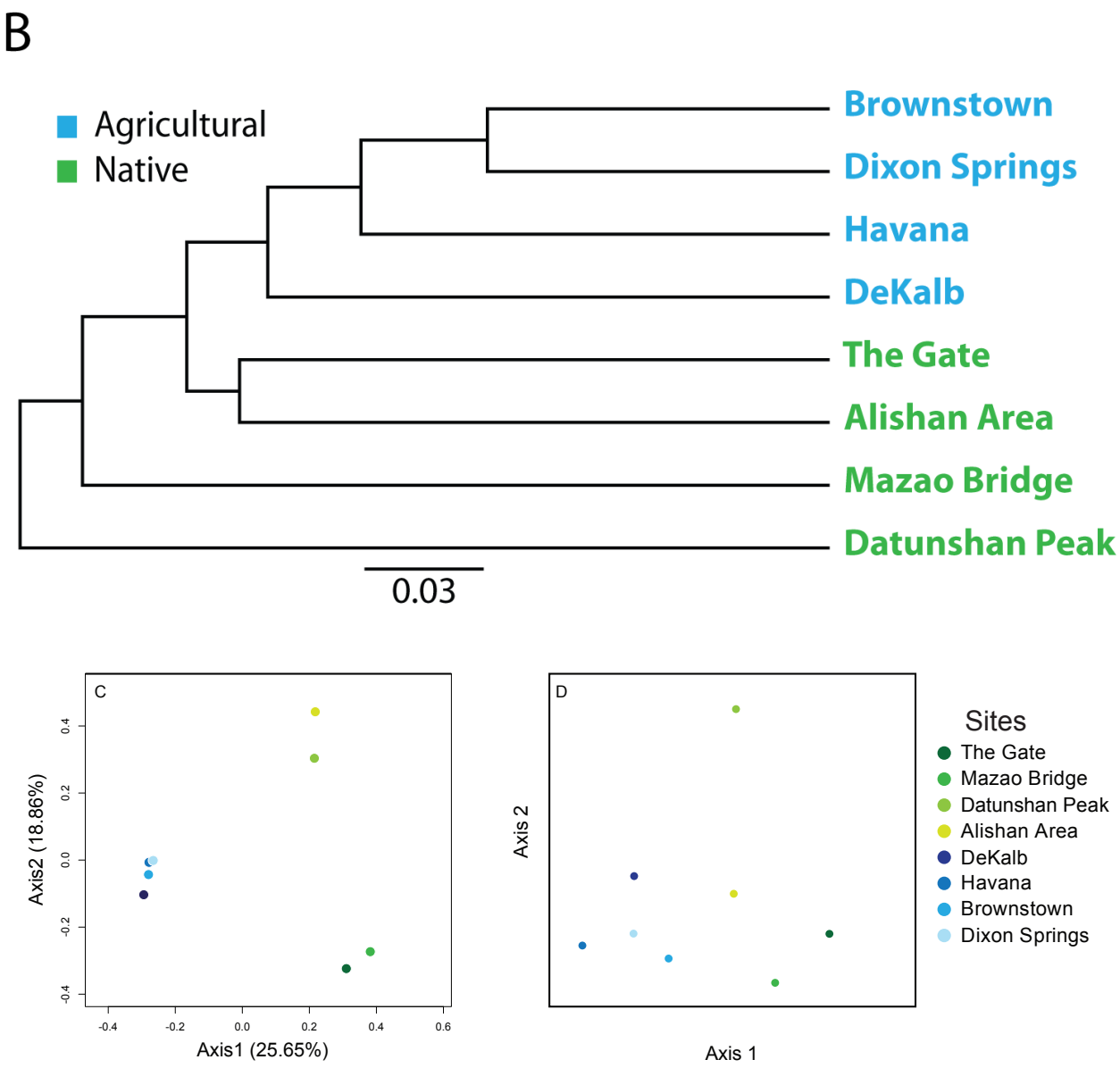

Figure 4: (A) Phylogenetic tree of the diazotrophic clones and isolates recovered from native and agricultural *Miscanthus* rhizome tissues. The tree is built based on the *nifH* gene, generated by the weighted neighbor-joining tree-building algorithm (18). Additional details about each isolate are contained in Table 2. Scale bar represents change per nucleotide.

(B) UniFrac tree representing phylogenetic relationships among endophytic diazotroph communities from representative native and agricultural *Miscanthus* rhizomes in different sample sites based on *nifH* gene sequences obtained from *nifH* clone libraries. Distinct diazotroph community composition was observed in native and agricultural *Miscanthus*. Diazotroph assemblages associated with agricultural *Miscanthus* were more similar across these four locations than the diazotrophs associated with native *Miscanthus*. Scale bar represents UniFrac distance.

(C) Principal coordinate analysis (PCoA) representing the relationship among endophytic diazotroph communities from representative native and agricultural *Miscanthus* rhizomes in

different sample sites based on *nifH* gene sequence obtained from *nifH* clone libraries. The PCoA analysis and plot were based on the UniFrac metric. Distinct diazotroph community composition was observed in native and agricultural *Miscanthus* (ANOSIM R = 0.885, p = 0.029). The key indicated the samples presented here.

(D) Non-metric multidimensional scaling ordination based on soil chemical factors (Table 1) was carried out for sampling sites that were used to construct the clone library. The key indicated the samples presented here. The green points represent samples collected from sites in Taiwan, while the blue points represent samples collected from sites in IL. The Spearman rank correlation was used to compare the diazotroph assemblages and the soil chemical factors; Spearman's ρ=0.479, indicating a good correlation between the patterns of similarity among the diazotroph assemblages and the soil chemical factors.

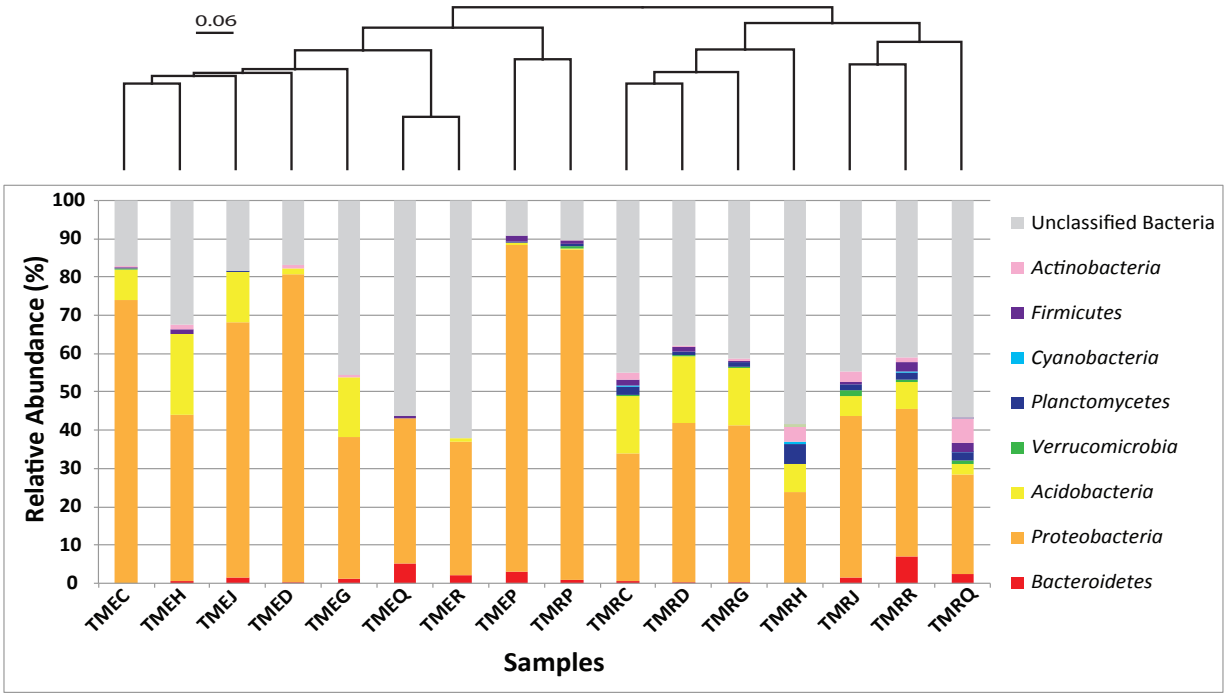

Figure 5: The proportion of pyrosequencing reads assigned to different phyla are shown to compare sequences from *Miscanthus* rhizomes and rhizosphere soil collected from different sample locations. Sample labels correspond to Table 4. The phyla presented are indicated in the key. The samples are ordered according to a Jackknife Environmental Cluster analysis performed using UniFrac (shown in Figure 6B).

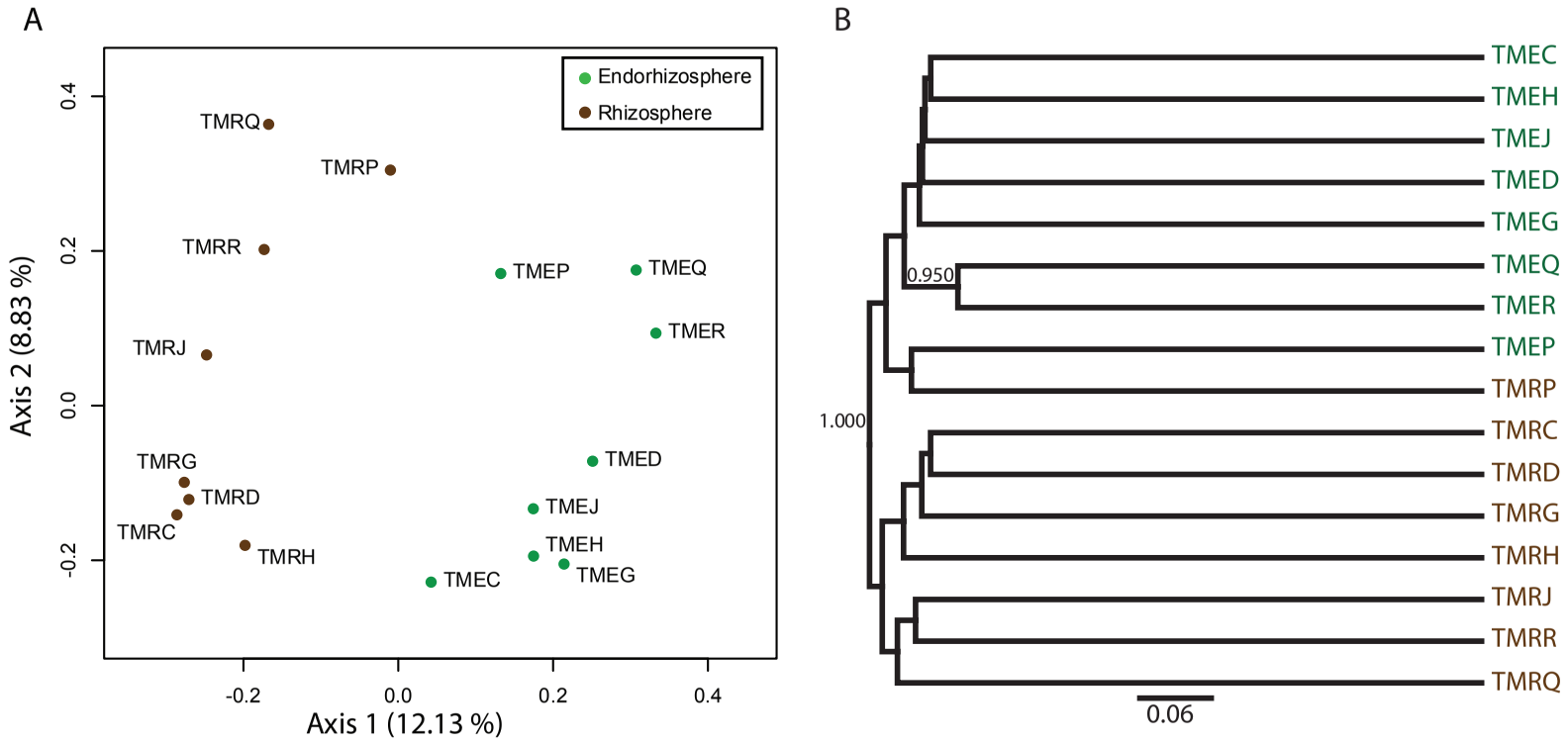

Figure 6: Patterns of similarity among bacterial communities colonizing *Miscanthus* rhizomes and rhizosphere based on UniFrac analysis of pyrosequencing reads from 16S rRNA genes. (A) Principal coordinates analysis (PCoA) shows distinct total bacterial communities were formed in native *Miscanthus* rhizomes and rhizosphere ($p = 0.001$). Each point represents one composite sample of the microbial community from each site indicated; sample codes are described in Table 4. Green points represents sample from *Miscanthus* rhizome, and brown points represent samples from rhizosphere. Percent of variance explained is indicated for each axis. (B) Jackknife clusters were generated to show the phylogenetic relationships among different samples. Jackknife fractions larger than 0.500 (indicating a significant node) are shown on the cluster plot. Scale bar indicates UniFrac distance.

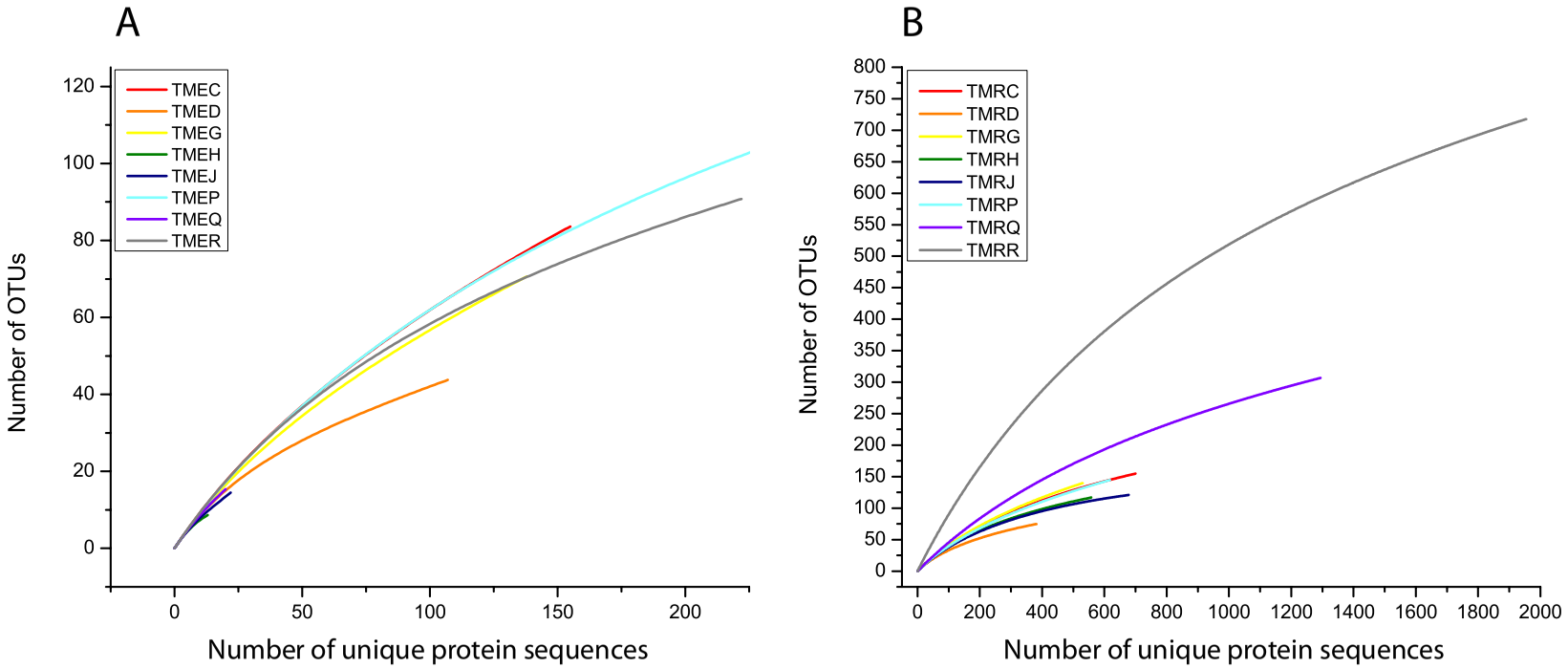

Figure 7: Rarefaction curve of diazotroph population from *Miscanthus* endorhizosphere (A) and rhizosphere (B) based upon translated protein sequences for *nifH* gene. The samples presented are indicated in the key, sample codes are described in table 1. The number of different kinds of diazotrophs (classified as Operational Taxonomic Units – OTUs – based on 97% similarity of amino acid sequences) in each sample are plotted versus the number of unique sequences sampled.

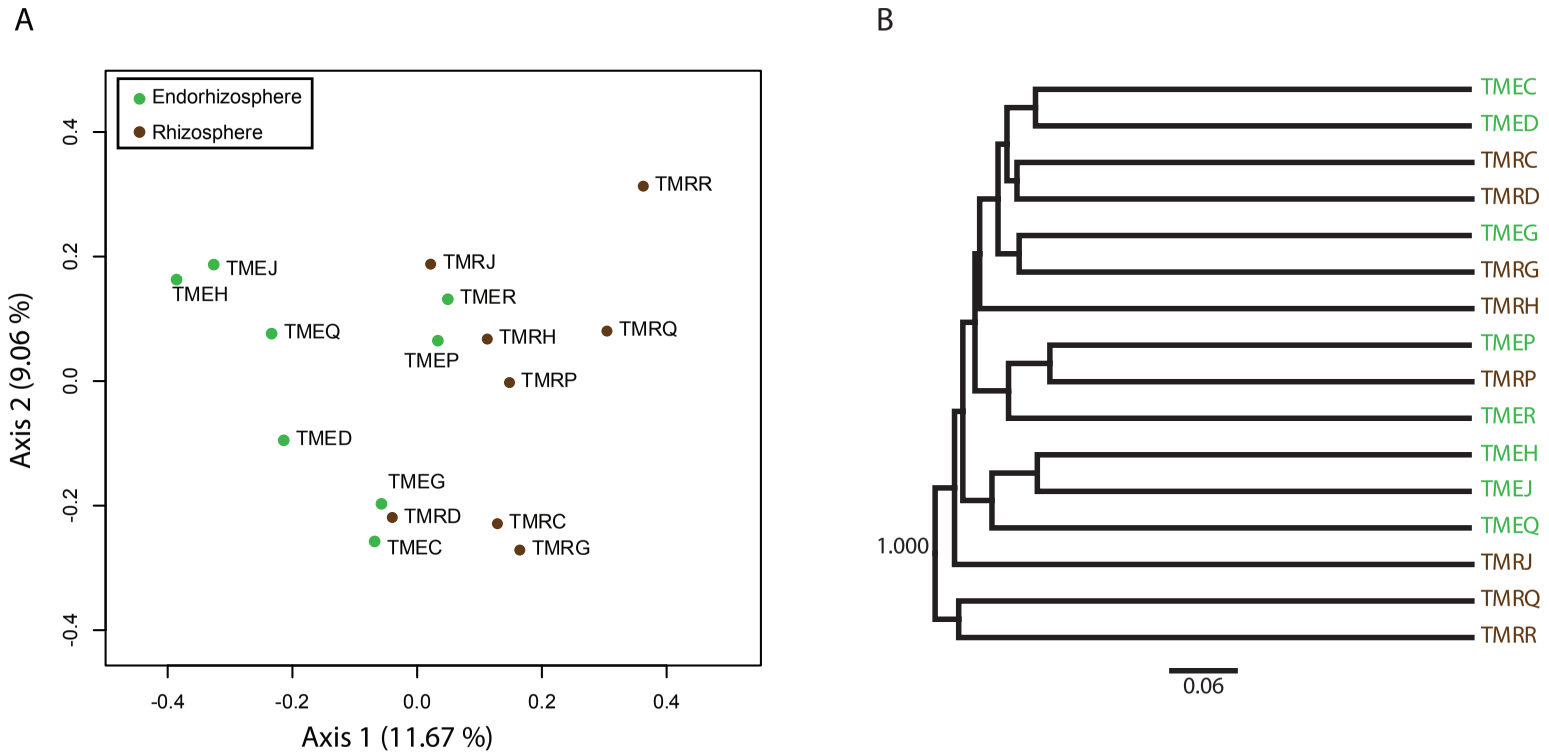

Figure 8: Patterns of similarity among diazotroph communities colonizing *Miscanthus* rhizomes and rhizosphere (UniFrac). (A) Principal Coordinates analysis (PCoA) shows diazotroph communities from native *Miscanthus* rhizomes were not completely distinct from those in rhizosphere samples (p = 0.016). Each point represents one composite sample of the diazotroph community from each location. Green points represent samples from *Miscanthus* rhizomes, and brown points represent samples from rhizosphere. (B) Jackknife clusters were generated to show the phylogenetic relationships among different samples. Jackknife fractions larger than 0.500 (indicating a significant node) are shown on the cluster plot. Scale bar indicates UniFrac distance.

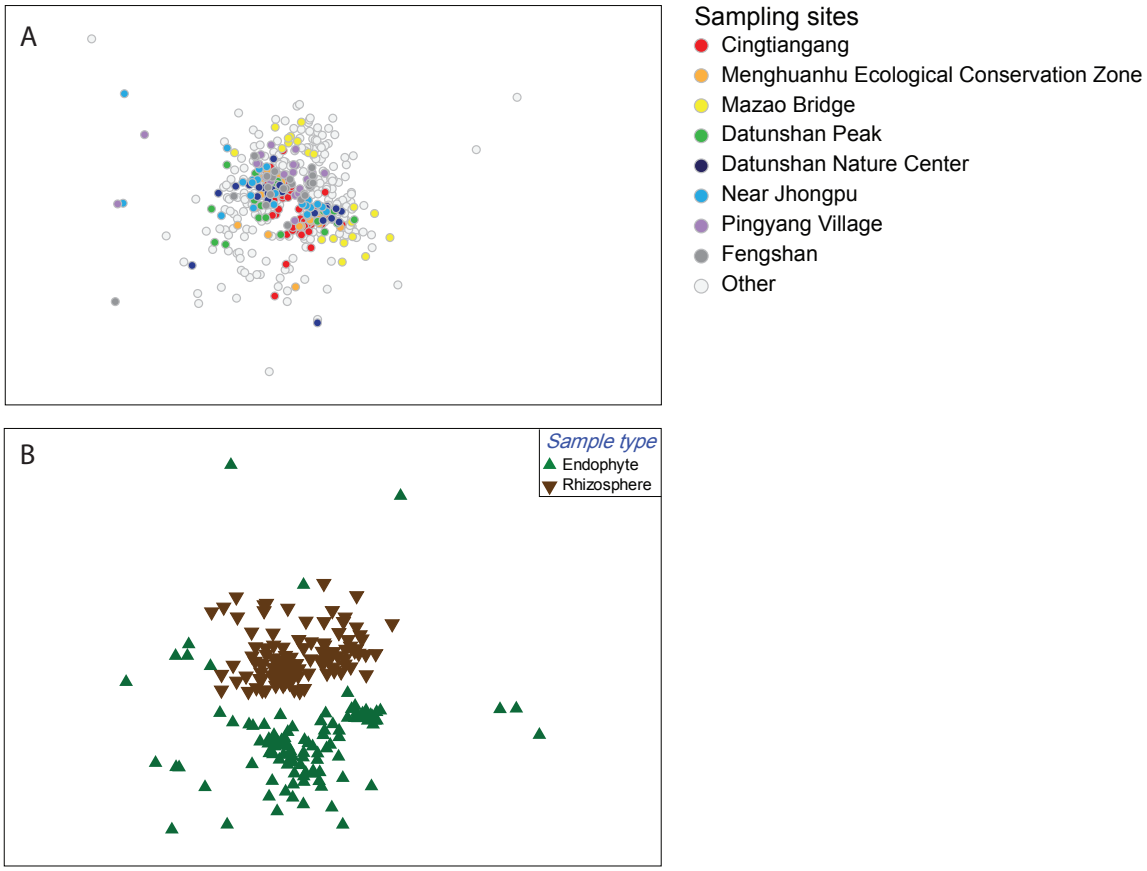

Figure 9: ARISA community fingerprints based on the bacterial rRNA operon were generated for all the samples collected from native *Miscanthus* samples (A. Kent, unpublished data). (A) Patterns of bacterial communities in samples selected for pyrosequencing compared with all the other samples. The samples selected in pyrosequencing are indicated in the key. "Other" represents samples that were not chosen for pyrosequencing (see Table 1 for sample information). (B) For the eight selected sampling sites, bacterial communities from the rhizosphere are significantly different than those from endorhizosphere (ANOSIM R= 0.199, p=0.01).

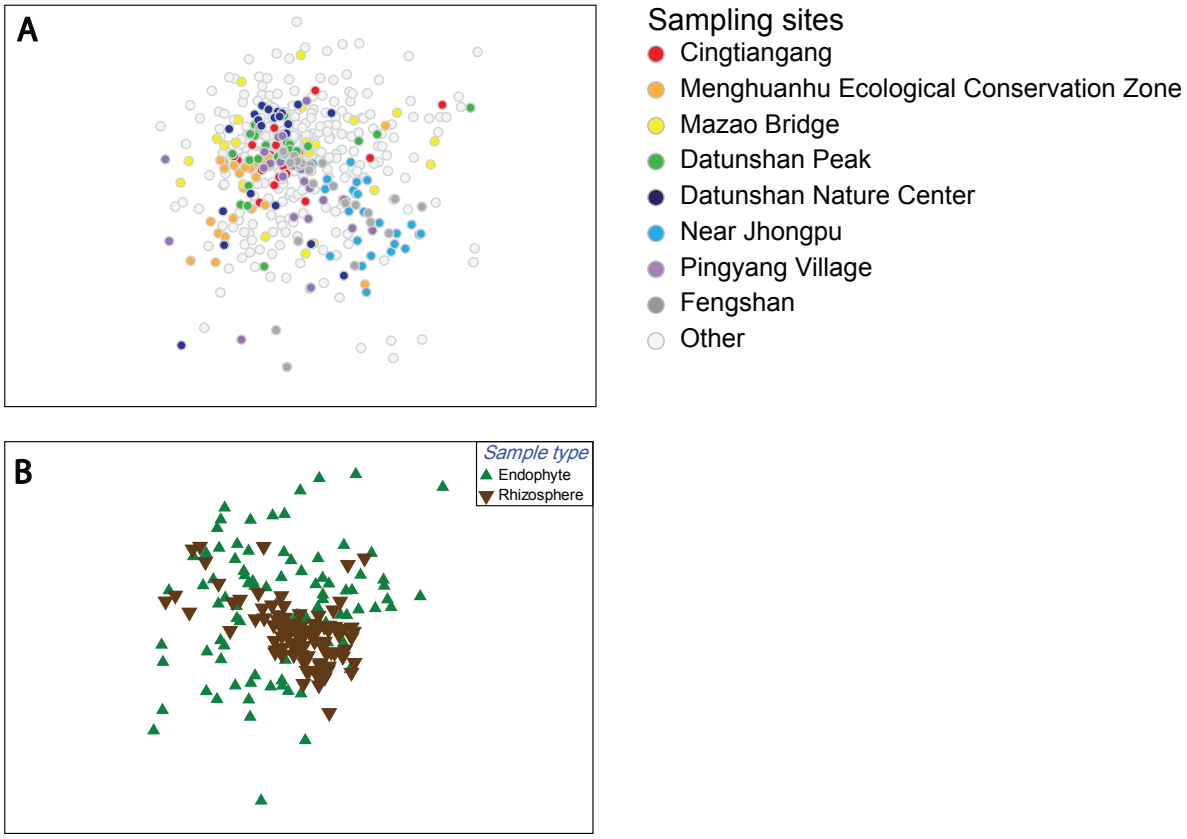

Figure 10: T-RFLP community fingerprints were generated from *nifH* genes in all native *Miscanthus* samples (D. Keymer, unpublished data). (A) Patterns of diazotroph communities in samples selected for pyrosequencing compared with all the other samples. The samples presented are indicated in the key. "Other" represents samples that were not included in the pyrosequencing analysis. (B) For the eight selected sampling sites, the diazotroph communities from the rhizosphere are somewhat distinct from those in the endorhizosphere (ANOSIM R= 0.199, p=0.01).

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