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ORIGINAL ARTICLE

Diversity and characterization of *Azotobacter* isolates obtained from rice rhizosphere soils in Taiwan

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Abstract *Azotobacter* species, free-living nitrogen-fixing bacteria, have been used as biofertilizers to improve the productivity of non-leguminous crops, including rice, due to their various plant growth-promoting traits. The purposes of this study were to characterize *Azotobacter* species isolated from rice rhizospheres in Taiwan and to determine the relationship between the species diversity of *Azotobacter* and soil properties. A total of 98 *Azotobacter* isolates were isolated from 27 paddy fields, and 16S rRNA gene sequences were used to identify *Azotobacter* species. The characteristics of these *Azotobacter* strains were analyzed including carbon source utilization and plant growth-promoting traits such as nitrogen fixation activity, indole acetic acid production, phosphate-solubilizing ability, and siderophore secretion. Of the 98 strains isolated in this study, 12 were selected to evaluate their effects on rice growth. Four species of *Azotobacter* were identified within these 98 strains, including *A. beijerinckii*, *A. chroococcum*, *A. tropicalis*, and *A. vinelandii*. Of these four species, *A. chroococcum* was predominant (51.0%) but *A. beijerinckii* had the highest level of nucleotide diversity. Strains within individual *Azotobacter* species showed diverse profiles in carbon source utilization. In addition, the species diversity of *Azotobacter* was significantly related to soil pH, Mn, and Zn. Members of the same *Azotobacter* species showed diverse plant growth-promoting traits, suggesting that the 98 strains isolated in this study may not equally effective in promoting rice growth. Of the 12 strains evaluated, *A. beijerinckii* CHB 461, *A. chroococcum* CHB 846, and *A. chroococcum* CHB 869 may be used to develop biofertilizers for rice cultivation because they significantly promoted rice growth. This study contributes to the selection of suitable *Azotobacter* strains for developing biofertilizer formulations and soil management strategies of *Azotobacter* for paddy fields.

Keywords Nitrogen-fixing bacteria · Rice · Diazotropic bacteria · Nitrogenase · Diversity

Introduction

Rice is one of the main food crops in the world, being consumed by about 50% of world and 85% of Asian populations (Sahoo et al. 2014). With reducing rice production areas due to urbanization and other cultural challenges, improvement in productivity is vital to ensure food security. Moreover, it has been suggested that rice yields need to be increased by 40% or even more in the next few decades to meet the requirements of a rapidly increasing world population (Khush 2005). One of technological methods used to increase rice yields is to apply more fertilizers to high-yield rice varieties. Of these chemical fertilizers, nitrogen fertilizers considerably affect rice yields because rice growth highly depends on nitrogen fertilizers that are closely related to the photosynthetic capacity of leaves, development of tillers, differentiation of spikelets, and properties of grains (Choudhury et al. 1997; Duan et al. 2007; Singh et al. 2011; Deng et al. 2014). However, nitrogen deficiency is often a limiting factor for rice growth, due partly to low nitrogen use efficiency caused by ammonia volatilization, denitrification, and leaching losses that cause environmental problems (Ponnamperuma 1972; Choudhury and Kennedy 2004). In addition, application of excessive nitrogen fertilizers to increase yield may result in reducing grain yield and quality,

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low N use efficiency, and environmental pollution (Deng et al. 2014). Moreover, the price of chemical nitrogen fertilizers has been increasing. Therefore, it is pertinent to find alternative nitrogen sources to avoid these problems.

Biological nitrogen fixation (BNF) can substitute for some chemical nitrogen fertilizer use in rice cultivation (Choudhury and Kennedy 2004), thus reducing the afore-mentioned environmental problems to some extent. Nitrogen-fixing bacteria can transform atmospheric nitrogen into fixed nitrogen, which will be further used by plants. Rice is a monocot, non-leguminous crop, suggesting that associative N_2-fixing microbes play a key role in in situ nitrogen fortification (Zaki et al. 2009; Sahoo et al. 2014). Of non-symbiotic free-living nitrogen-fixing bacteria, *Azotobacter* has been proved to fix nitrogen for rice plants and to promote rice growth through release of some beneficial compounds (Piao et al. 2005; Das and Saha 2007; Sahoo et al. 2014). The genus *Azotobacter* belongs to the γ-subclass of the Proteobacteria and includes *A. armeniacus*, *A. beijerinckii*, *A. chroococcum*, *A. nigricans*, *A. paspali*, *A. salinestri*, *A. tropicalis*, and *A. vinelandii* (Jiménez et al. 2011; Özen and Ussery 2012). *Azotobacter* can fix at least 10 mg N/g carbohydrate and provide 19–47% of total nitrogen requirement in rice (Choudhury and Kennedy 2004; Tejera et al. 2005), reducing the use of chemical nitrogen fertilizers. In addition to nitrogen fixation, *Azotobacter* can improve plant growth via increasing availability of nutrients, such as phosphorus, and producing growth hormones such as indole acetic acid (IAA), gibberlins, and cytokinins, siderophores and antifungal compounds (Sahoo et al. 2014). However, environmental conditions influence the performance of BNF, suggesting that indigenous nitrogen-fixing bacteria may better adapt local niches than inoculant microorganisms, and thus native strains would be more appropriate for use as biofertilizers for regional crops (Martinez-Toledo et al. 1985; Kannan and Ponmurugan 2010). In Taiwan, neither the diversity of *Azotobacter* species in paddy soils nor their biofertilizer potential has been thoroughly investigated.

The purposes of this study were to identify *Azotobacter* species isolated from rice rhizospheres in Taiwan and to characterize plant growth-promoting properties of *Azotobacter* spp. The results of this study may be of importance in allowing better utilization of *Azotobacter* biofertilizers.

Materials and methods

Soil sampling and sample preparation

Twenty-seven paddy fields of main rice-growing areas in Taiwan were selected between the years 2013 and 2014. Five rice plants were uprooted, with rhizosphere soils, from each field. After removing visible root debris, these field moisture rhizosphere soils were stored in sterile bottles at 4°C. Parts of these soil samples from each field were air-dried and sieved (2 mm) for analyzing soil properties as described below.

Isolation of *Azotobacter* species

Azotobacter species were isolated using the soil grains-sowing technique as described previously (Aquilanti et al. 2004; Jiménez et al. 2011). Briefly, 30 g fresh soil was saturated with sterile water, and 3 g mannitol was added to the soil paste. The soil paste was then transferred and pressed into about 3 mm-diameter grains in a Petri dish using a sterile spatula. A total of 16 soil grains was placed on each Ashby-Sucrose agar plate (Jiménez et al. 2011), and each soil sample consisted of three replicates. The plates were incubated at 28°C for 7 days, after which sticky and glistening colonies around the grains were streaked and incubated on Ashby-Sucrose agar three times for purification. These purified isolates were stored in 15% glycerol at −80 °C for long-term storage and further characterization. In addition, *A. armeniacus* DSM 2284, *A. beijerinckii* DSM 378, *A. nigricans* DSM 375, *A. salinestris* DSM 11553, *A. vinelandii* DSM 2289, and *Pseudomonas fluorescens* ATCC 13525 were obtained from Bioresource Collection and Research Center, Hsinchu, Taiwan, for 16S rRNA gene sequencing and phylogenetic analysis as described below.

Genomic DNA extraction and 16S rRNA gene amplification and sequencing

DNA was extracted using the hexadecyltrimethylammonium bromide method, as previously described (Ausubel et al. 1998). The quantity and quality of DNA were determined using a NanoDrop ND-2000c UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA was stored in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0) at −20 °C. The 16S rRNA gene of *Azotobacter* spp. was amplified using the primers fD1 and rD1 and PCR conditions as previously described (Weisburg et al. 1991). Amplifications were performed in an Analytik Jena AG FlexCycler^2 thermocycler (Analytik Jena, Göttingen, Germany). Amplicons were sequenced using BigDye Terminator Cycle Sequencing Chemistry and ABI 3730 XL DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences were edited using CodonCode Aligner version 5.0.2 (CodonCode Corporation, Centerville, MA). Accession numbers of the sequences obtained in this study for 16S rRNA gene are KX709968–KX710065.

Phylogenetic analyses

The alignment of 16S rRNA gene sequences was performed using Clustal X version 2.0.6 (Larkin et al. 2007) and adjusted by eye using BioEdit version 7.2.5 (Hall 1999). Gaps were considered missing data. Prior to phylogenetic analyses, sequence diversity, including numbers of haplotypes, numbers of polymorphic sites, and nuclear diversity were determined among all isolates of *Azotobacter* using DnaSP version 5 (Librado and Rozas 2009). Parsimony analyses were conducted using MEGA6 (Tamura et al. 2013). The most parsimonious tree was obtained using the close-neighbor-interchange algorithm with search level 1 in which initial trees were obtained with the random addition of sequences (1000 replicates). Clade stability was evaluated using 1000 bootstrap replicates. For maximum likelihood (ML) and Bayesian analysis, jMODELTEST version 2.1.7 was used to determine appropriate models of nucleotide substitution (Posada 2008). The Tamura-Nei (TrN) model with γ-distributed rate variation across sites and a proportion of invariable sites was chosen based on the Akaike Information Criterion (Huelsenbeck and Rannala 1997). ML was analyzed using GARLI version 2.0.1 (Zwickl 2006), and a bootstrap analysis was performed using 1000 replicates. Bayesian analysis was carried out using MrBayes version 3.2.6 (Ronquist and Huelsenbeck 2003). The Markov chain Monte Carlo (MCMC) analysis was run with four chains for 10,000,000 generations, sampling every 100 generations and starting with a random tree. The first 25,000 trees with low likelihoods were discarded as the burn-in. The remaining 75,000 trees were imported to PAUP* 4.0b10 (Swofford 2001) to generate a 90% majority-rule consensus tree. Phylogenetic trees derived from the three methods were rooted with the outgroup, *P. fluorescens* ATCC 13525.

Soil property analysis

The pH and electrical conductivity (EC) of rhizosphere soils were determined in 1:1 (w/v) soil:H_2O extracts (Smith and Doran 1996). Total organic carbon was determined by the Walkey-Black method (Walkley and Black 1934). Available iron, manganese, copper, and zinc were extracted by 0.1 *N* HCl (Tening and Omueti 2011) and analyzed with an inductively coupled plasma-mass spectrometer (ICP/MS; Perkin Elmer, Waltham, MA).

Characteristics of *Azotobacter* strains

Test of utilization of carbon sources

In the carbon source utilization test, 10 mL phenol red broth was dispensed into sterile test tubes. Then, 0.5% (w/v) each of galactose, glucose, maltose, mannitol, or rhamnose, was separately added and inoculated in the tubes with a loopful of 24-h-old culture. The tubes were incubated at 28°C, 200 rpm in a shaking incubator for 7 days. The change of color to yellow indicated the ability of *Azotobacter* spp. to utilize the carbohydrates (Jiménez et al. 2011).

Nitrogenase activity

Nitrogenase activity of *Azotobacter* strains isolated from different rice fields was determined by acetylene reduction assay (ARA) (Hardy et al. 1973). The bacteria were grown in flasks with septa and nitrogen-free Ashby-Sucrose medium at 28°C for 3 days, and uninoculated medium served as a control. After incubation, 10% of air from the flasks was replaced by acetylene using a syringe and incubated for 24 h. A volume of 0.5 mL gas was withdrawn from the flasks, and ethylene formation was determined using a gas chromatograph (Hewleit Packard 5890 series II; Hewlett-Packard, Wilmington, DE) fitted with flame ionization detector and a Porapak N column.

Determination of plant growth-promoting traits

Enzymatic degradation of cellulose and lignin was determined using medium supplied with carboxymethyl cellulose and azure B as a sole carbon source, respectively (Kausar et al. 2011). Production of IAA was evaluated as previously described (Ahmad et al. 2005). *Azotobacter* strains were cultured in the tryptic soy broth supplied with 200 μg L-tryptophan/mL for 3 days. Cultures were centrifuged at 5500 *g* for 10 min, and 1 mL supernatant was mixed with 0.1 mL orthophosphoric acid and 4 mL Solawaski's reagent (Ehmann 1977) and incubated at room temperature for 30 min in the dark. The level of IAA produced was determined at 530 nm using a spectrophotometer, and estimated using a standard curve of pure IAA. Sterilized Pikovskaya's medium (Sundara Rao and Sinha 1963) was used to determine the phosphate-solubilizing ability of *Azotobacter* spp. The solubilization halo around bacterial colonies on the plates served as an indicator for phosphate solubilization. The production of siderophores by *Azotobacter* was determined using chrome azurol S (CAS) agar plates (Schwyn and Neilands 1987). The presence of orange, purple, or yellow halos indicated the ability of the strains to secrete siderophores (Alexander and Zuberer 1991).

Effect of selected Azotobacter strains on rice growth

Twelve strains of *Azotobacter* species isolated in this study were selected to evaluate their effects on rice growth, including three strains of *A. beijerinckii*, four strains of *A. chroococcum*, three strains of *A. tropicalis*, and two strains of *A. vinelandii*. The plant growth-promoting traits of these 12 strains were shown on Table 1. Seeds of *Oryza sativa* L. var.

Table 1 *Azotobacter* strains used for evaluating their inoculation effect on rice growth. Their plant growth-promoting traits are shown. Acetylene reduction assay was used to determine N_2 fixation activity expressed as nmol ethylene/h. Indole-3-acetic acid (IAA) production was expressed as μg/ml

Isolate	Closest species	N_2 fixation activity	IAA production	Phosphate solubilization	Siderophore production	Cellulose degradation	Lignin degradation
CHB 195	*A. chroococcum*	19.3	6.01	−	+	−	−
CHB 461	*A. beijerinckii*	0.26	4.43	−	−	+	−
CHB 475	*A. vinelandii*	3.39	1.62	+	+	−	−
CHB 598	*A. tropicalis*	76.6	3.45	−	+	+	−
CHB 626	*A. vinelandii*	23.4	2.62	−	+	−	−
CHB 652	*A. tropicalis*	119	2.81	−	+	+	−
CHB 838	*A. tropicalis*	14.7	4.55	−	+	−	−
CHB 846	*A. chroococcum*	61.9	2.17	−	+	+	+
CHB 851	*A. chroococcum*	136	3.01	−	+	+	+
CHB 869	*A. chroococcum*	14.2	8.94	−	−	+	−
CHB 900	*A. beijerinckii*	120	1.19	−	+	−	+
CHB 939	*A. beijerinckii*	185	3.38	+	−	−	−

TNGS22 were immersed in 55°C hot water for 30 min to kill plant pathogens and then soaked in 1% (v/v) sodium hypochlorite (NaOCl) for 1 min for surface sterilization. Finally, the seeds were washed three times with sterile water. The sterilized seeds were soaked in bacterial suspensions containing respective *Azotobacter* strains at a concentration of 10^8 CFU/ml for 24 h. The inoculated seeds were sowed on Petri dish plates with filter papers saturated with sterile water and incubated in the dark. Non-inoculated seeds served as a control. One week after sowing, seedlings were transplanted to 200 ml-polyethylene cups each filled with 150 g paddy soil [textural class, sandy clay loam; pH (1:1 *w/v*) in water, 5.01; EC (1:1 w/v), 0.78 dS/m; organic C, 18.3 g/kg; total N, 1.50 g/kg; available N, 64.6 mg/kg; available P, 34.0 mg/kg; exchangeable K, 101 mg/kg; exchangeable Ca, 930 mg/kg; exchangeable Mg, 170 mg/kg]. One rice seedling was planted in a cup, and there were five replicates per treatment arranged in a complete randomized design in a greenhouse with an average daily temperature of 30°C. The rice plant in each cup were fertigated with 56 mL Yoshda's nutrient solution (Yoshida et al. 1976) every week after transplanting. Deionized water was used to irrigate rice plants as needed. Four weeks after transplanting, the plants were harvested, washed in deionized water, and dried in paper bags for 72 h at 80°C. Dry weight and plant height were determined for each replication.

Statistical analyses

Species diversity of *Azotobacter* in paddy fields was determined using the Shannon-Weiner Index (Spellerberg and Fedor 2003). Regression analysis was used to evaluate the relationship between the Shannon-Weiner Index of *Azotobacter* species in paddy fields and soil properties using SIGMAPLOT version 12.0 (Systat Software, Chicago, IL). Generalized linear models employed in PROC GLIMMIX of SAS (version 9.4; SAS Institute, Cary, NC) were used to analyze the effect of treatments on response variables. The least squared means (LSMEANS) statement of the GLIMMIX procedure in SAS was used to compare treatment means at the 5% level of significance according to Fisher's least significant difference (Fisher's LSD).

Results

Isolation and identification of *Azotobacter* species

Ninety-eight isolates were isolated from rice rhizosphere soils and identified based on the near full length of bacterial 16S RNA sequences (Table 2). Of these isolates, 50 isolates were identified as *A. chroococcum*, which was the dominant species in this study; 30 isolates could be assigned to *A. beijerinckii*, and 16 isolates were identified as *A. tropicalis*. However, only two isolates of *A. vinelandii* were obtained from this study. Overall, four species of *Azotobacter* were found in the rice rhizosphere soils of Taiwan. Twenty-one haplotypes were found for these 98 isolates based on sequence diversity estimates of 16S rRNA, with the highest number of haplotypes found in *A. beijerinckii*. In addition, *A. beijerinckii* showed considerable polymorphic variation and the highest level of nucleotide diversity (Table 2).

Results of phylogenetic analyses from maximum parsimony, ML, and Bayesian inference were congruent for these 21 haplotypes of *Azotobacter* species, suggesting that there were five phylogetic groups (Fig. 1). Clades I and II consisted of isolates of *A. beijerinckii*, whereas clades III, IV, and V were

Table 2 Sequence diversity estimates of 16S rRNA for isolates of *Azotobacter* species collected from rice rhizosphere soils in Taiwan. Nucleotide diversity was calculated based on the average number of nucleotide differences per site between two sequences (Nei 1987)

Species	Number of isolates	Number of haplotypes	Number of polymorphic sites	Nucleotide diversity
A. beijerinckii	30	8	36	9.26×10^{-3}
A. chroococcum	50	7	17	2.50×10^{-3}
A. tropicalis	16	4	9	0.85×10^{-3}
A. vinelandii	2	2	1	0.75×10^{-3}
Combined	98	21	84	1.59×10^{-2}

classified as *A. chroococcum*, *A. tropicalis*, and *A. vinelandii*, respectively.

Relationship between soil properties and species diversity of *Azotobacter*

Rhizosphere pH of the paddy soils used for isolating *Azotobacter* spp. ranged from 4.82 to 7.63, with an average of 6.06. EC values ranged from 0.32 dS/m to 1.13 dS/m, with an average of 0.72 dS/m. The organic matter content of the rhizosphere soil had a range of 1.77–6.67%, with an average of 3.44%. Some paddy fields showed micronutrient deficiencies due to low levels of micronutrients extracted by 0.1 *N* HCl. Available Fe ranged from 0.47 mg/kg to 1132 mg/kg with an average of 670 mg/kg, available Mn ranged from 4.15 mg/kg to 209 mg/kg with an average of 60.9 mg/kg, available Cu ranged from 0.13 mg/kg to 19.5 mg/kg with an average of 6.55 mg/kg, and available Zn ranged from

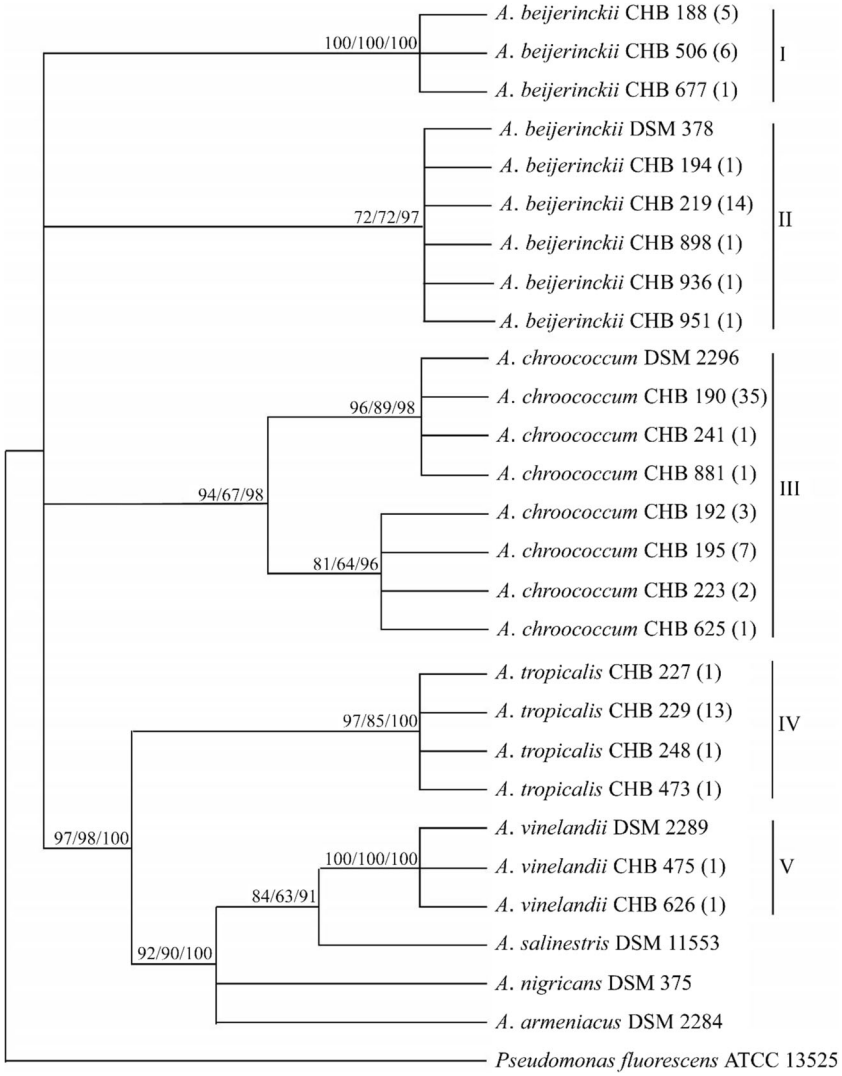

Fig. 1 Phylogenetic analysis of isolates of *Azotobacter* species collected from rice rhizosphere soils in Taiwan based on 16S rRNA sequences. Numbers on nodes represent bootstrap support values for maximum parsimony (*front*), maximum likelihood (*middle*), and Bayesian posterior probabilities presented as percentage (*back*). Numbers in *parentheses* represent the total isolates of each haplotype

0.02 mg/kg to 26.3 mg/kg with an average of 5.86 mg/kg. Regression analysis suggested that a quadratic model best described the relationship between species diversity of *Azotobacter* and rhizosphere soil pH (Fig. 2). However, linear models were observed to describe their significantly positive relationships between species diversity of *Azotobacter* and rhizosphere soil Mn (Fig. 3) and Zn (Fig. 4).

Characteristics of *Azotobacter* strains

Carbon source utilization

Strains within individual *Azotobacter* species obtained from this study showed diverse profiles in carbon source utilization. Up to 76.5% of these 98 strains of *Azotobacter* species could utilize mannitol, but only 42.9% of these strains could utilize rhamnose (Table 3). Moreover, *A. beijerinckii*, *A. chroococcum*, and *A. tropicalis* showed their preferential utilization of mannitol over the other four carbon sources. Although the two strains of *A. vinelandii* obtained in this study could not use galactose and rhamnose as a single carbon source, they all could utilize glucose. Of these 98 strains collected, 6 strains of *A. beijerinckii*, 11 strains of *A. chroococcum*, and 6 strains of *A. tropicalis* showed their ability to utilize all five carbon sources (data not shown).

Evaluation of nitrogenase activity of Azotobacter spp.

These 98 strains differed in the levels of the nitrogenase activity, ranging from no detection to 185 nmol ethylene/h with an average of 23.8 nmol ethylene/h (Table 4). Using these results, the 98 strains tested were arbitrarily classified as low- (0–50 nmol ethylene h^{-1}), medium- (51–100 nmol ethylene h^{-1}), and high- (>101 nmol ethylene h^{-1}) nitrogen fixers. Although up to 79.6% of the *Azotobacter* strains were categorized as low nitrogen fixers, five strains were categorized as high nitrogen fixers including *A. beijerinckii* CHB 939, *A. chroococcum* CHB 192, *A. chroococcum* CHB 851, *A. chroococcum* CHB 900, and *A. tropicalis* CHB 652.

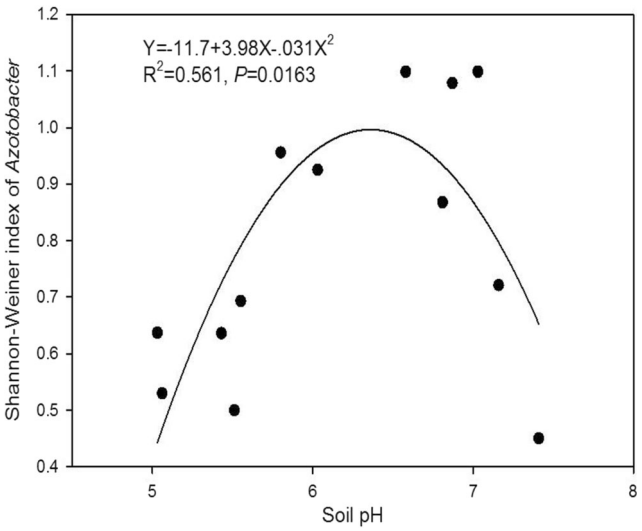

Fig. 2 Relationship between rhizosphere soil pH of paddy fields and Shannon-Weiner index of *Azotobacter* species

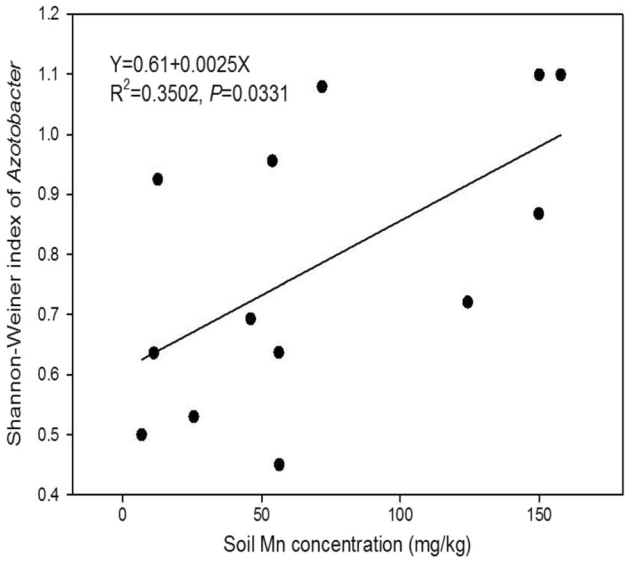

Fig. 3 Relationship between rhizosphere soil Mn of paddy fields and Shannon-Weiner index of *Azotobacter* species

Determination of plant growth-promoting traits

Except CHB 190 and CHB 224, the other 96 isolates produced various levels of IAA (0.14–8.94 μg/mL) with an average of 2.83 μg/mL (Table 4). *Azotobacter chroococcum* strain CHB 941 displayed the highest level of IAA at

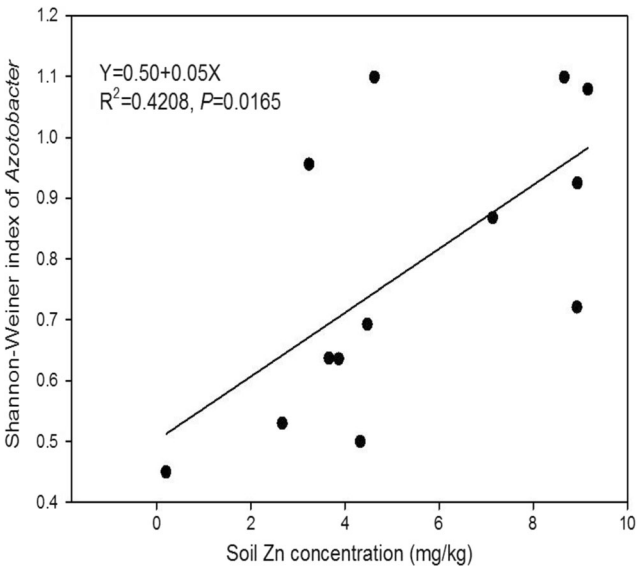

Fig. 4 Relationship between rhizosphere soil Zn of paddy fields and Shannon-Weiner index of *Azotobacter* species

Table 3 Carbon source utilzation by *Azotobacter* species isolated from rice rhizosphere soils in Taiwan expressed as a percentage of the total of each species. *Azotobacter* species were grown in the phenol red broth amended with 0.5% (w/v) each of galactose, glucose, maltose, mannitol, and rhamnose

		Carbon source				
Species	Number of isolates	Galactose	Glucose	Maltose	Mannitol	Rhamnose
A. beijerinckii	30	56.7	60.0	70.0	80.0	36.7
A. chroococcum	50	60.0	68.0	62.0	74.0	42.0
A. tropicalis	16	50.0	50.0	68.8	81.3	62.5
A. vinelandii	2	0	100	50.0	50.0	0
Combined	98	56.1	63.3	65.3	76.5	42.9

8.94 μg/mL. Of these 98 strains, 32.7% *Azotobacter* strains displayed the ability to utilize cellulose. A higher proportion of *A. beijerinckii* strains could utilize cellulose (40.0%). However, only 15 strains could use lignin as a sole carbon source. Interestingly, 8 of these 15 strains also could utilize cellulose (data not shown). Up to 57.1% of the 98 strains exhibited the ability to secrete siderophores. Moreover, 86.0% of *A. chroococcum* strains and the two *A. vinelandii* strains showed the ability to secrete siderophores. Of these 98 strains, 24 *Azotobacter* strains could dissolve tricalcium phosphate, as evidenced by a solubilization halo around bacterial colonies as grown on Pikovskaya's medium plates (Table 4).

Effect of selected *Azotobacter* strains on rice growth

Azotobacter inoculation significantly affected plant height and the dry weight of rice plants (Table 5). Rice plants inoculated with *A. chroococcum* CHB 195 and *A. beijerinckii* CHB 900 showed a significantly higher plant height than the non-inoculated control, with an increase of 5.56–8.39%. However, these two strains did not significantly increase the dry weight of rice plants compared to the control. In contrast, *A. beijerinckii* CHB 461, *A. chroococcum* CHB 846, and *A. chroococcum* CHB 869 significantly increased the dry weight of rice plants by 25.0–36.1% in comparison with the control.

Discussion

To our knowledge, this is the first study to investigate the species diversity and characterization of *Azotobacter* spp. isolated from rice rhizosphere soils in Taiwan. In this study, 98 *Azotobacter* strains were isolated and could be identified as *A. beijerinckii*, *A. chroococcum*, *A. tropicalis*, or *A. vinelandii* based on the near full length of bacterial 16S RNA sequences. Of these four species, *A. chroococcum* was predominant in the rice rhizosphere soils. Although considerable variation in carbon utilization existed within each *Azotobacter* species, most strains could utilize mannitol as a sole carbon source. Interestingly, rhizosphere soil pH, Mn, and Zn significantly affected the species diversity of *Azotobacter* spp. In addition, this study has evaluated plant growth-promoting traits of these *Azotobacter* strains, providing some potential strains to develop biofertilizers for rice cultivation. Of the 12 strains tested in this study, *A. beijerinckii* CHB 461, *A. chroococcum* CHB 846, and *A. chroococcum* CHB 869 significantly increased dry weight of rice plants. The results of this study may contribute to the development of *Azotobacter* biofertilizers.

The species diversity of *Azotobacter* strains may be associated with sampling locations. For northern and central Taiwan, *A. chroococcum* was dominant, but *A. beijerinckii* was more commonly isolated from rice rhizosphere soils of southern and eastern Taiwan. However, only two *A. vinelandii* strains were obtained from paddy soils of southern Taiwan. In

Table 4 Plant growth-promoting traits of *Azotobacter* strains isolated in this study. Acetylene reduction assay was used to determine N_2 fixation activity expressed as nmol ethylene h^{-1}. Traits of cellulose degradation, lignin degradation, siderophore production, and phosphate solubiolization are expressed as a percentage of the total for each species

Species	Number of isolates	N_2 fixation activity	IAA production (μg/ml)	Cellulose degradation	Lignin degradation	Siderophore production	Phosphate solubilization
A. beijerinckii	30	22.5±39.2	2.22±1.18	40.0	3.33	6.66	40.0
A. chroococcum	50	22.8±30.2	3.05±1.62	26.0	22.0	86.0	22.0
A. tropicalis	16	31.6±33.3	3.35±1.19	22.9	18.8	56.3	0
A. vinelandii	2	13.4±14.1	2.12±0.71	0	0	100	50.0
Combined	98	23.8±33.0	2.83±1.47	32.7	15.3	57.1	24.5

Table 5 Effect of *Azotobacter* inoculation on rice growth. Values with different letters in the same column are significantly different, as denoted by the LSMEANS statement of the GLIMMIX procedure in SAS v9.4 at the 5% level of significance according to Fisher's least significant difference (Fisher's LSD)

Strain	Plant height (cm)	Dry weight (g)
CHB 195	15.5 a	0.37 bc
CHB 461	14.6 b-d	0.45 ab
CHB 475	14.6 b-d	0.41 a-c
CHB 598	14.5 b-d	0.44 a-c
CHB 626	14.3 cd	0.44 a-c
CHB 652	14.8 bc	0.46 a-c
CHB 838	14.1 d	0.37 c
CHB 846	14.6 b-d	0.49 a
CHB 851	14.5 b-d	0.39 bc
CHB 869	14.9 a-c	0.49 a
CHB 900	15.1 ab	0.43 a-c
CHB 939	14.3 cd	0.40 bc
Non-inoculated control	14.3 cd	0.36 c

contrast to our finding, *A. vinelandii* is predominantly obtained from rice fields of India, and shows a higher level of nitrogenase activity (Sahoo et al. 2014). Although *A. vinelandii* is commonly isolated from alkaline soils (Becking 2006), the two *A. vinelandii* strains were isolated from rhizosphere soils with pH 6.49 and pH 6.91 in this study. The *A. vinelandii* strains obtained from slightly acidic soils may result from the opposite relationship between rhizosphere and the bulk soil pH due to greater net excretion of H^+ by plant roots that accompanies greater uptake of cations than anions (Rengel 2015). In addition, *A. vinelandii* is halophilic to some degree (Becking 2006). However, the EC values of all the rhizosphere soils used for isolation of *Azotobacter* were less than 1.2 dS/m, which is unfavorably selective for the occurrence of *A. vinelandii*. Variations observed in *Azotobacter* species are probably attributed to their adaption and performance in specific niches such as climate conditions, environments, microhabitats, and soil characteristics (Bhatia et al. 2008; Rubio et al. 2013).

Moreover, long-term application of different fertilizers significantly influence the population of free-living diazotrops (Islam et al. 2010). It has been suggested that soil organic matter influences the abundance and diversity of diazotrophs (Limmer and Drake 1998; Poly et al. 2001; Green et al. 2006). However, our results did not observe a significant relationship between the species diversity of *Azotobacter* and soil organic matter, probably due to a higher organic matter content for rhizosphere soils used in this study (an average of 3.44%). In contrast, the species diversity of *Azotobacter* was significantly associated with soil pH, Mn, and Zn. Most *Azotobacter* species are sensitive to acidic soils such that their growth is favored by a neutral or alkaline medium (Becking 2006; Jiménez et al. 2011). Although Mn and Zn are required for the growth of soil microorganisms (Sylvia et al. 2005), their availability is related to soil pH (Rengel 2015). Since soil properties influence the species distribution and characteristics of *Azotobacter*, the functionality of biological nitrogen fixation may be location-specific (Kannan and Ponmurugan 2010). Resident *Azotobacter* strains would be well suited for specific rice-growing regions.

A question that may be asked is whether selective media influenced the population structure of *Azotobacter* revealed in this study. Our study utilized mannitol as the carbon source to enrich *Azotobacter* species, and sucrose as the carbon source in Ashby-Sucrose agar (Jiménez et al. 2011) for isolation of the selective microbes. Although a total of 98 *Azotobacter* strains was isolated from rice rhizosphere soils in this study, up to 79.6% of the *Azotobacter* isolates were categorized as low nitrogen fixers (0–50 nmol ethylene h^{-1}). It has been suggested that the carbon source is important for the growth of diazotrophic bacteria (Islam et al. 2010). Therefore, we cannot rule out the possibility that N-free media with different carbon sources select varying *Azotobacter* species. It is appropriate to isolate diazotrophic bacteria using rhizosphere soils (Martinez-Toledo et al. 1985; Muthukumarasamy et al. 2007) as performed in this study, but it is not well known whether a correlation exists between nitrogenase activity of *Azotobacter* isolates and the selective media used for isolating the diazotroph. Only five strains were categorized as high nitrogen fixers (>101 nmol ethylene h^{-1}) obtained in this study, with dominance of *A. chroococcum*. Therefore, it is necessary to further investigate how to isolate *Azotobacter* species with high nitrogenase activity. Knowledge of the isolation of *Azotobacter* species in paddy soils may contribute to an understanding of their diversity and utilization in rice cultivation as inoculants.

Evaluation of plant growth-promoting traits in bacterial strains is an important task in selecting appropriate strains for developing biofertilizer formulations (Rubio et al. 2013). The plant growth-promoting traits tested were considerably variable for the members of each *Azotobacter* species, suggesting that the 98 strains isolated in this study may not be equally effective in promoting rice growth. It has been shown that *Azotobacter* species with a higher level of nitrogenase activity also exhibit better plant growth promoting traits such as IAA production, phosphorus solubilization, and siderophore secretion (Sahoo et al. 2014). However, the five high nitrogen fixers isolated in this study did not produce higher levels of IAA, and did not all show the ability to both secrete siderophore and solubilize tricalcium phosphate. Consequently, polyvalent plant growth-promoting functions of *Azotobacter* spp. may be uncommon. Our findings are in agreement with previous studies suggesting that the functions of plant growth-promoting rhizobacteria including

Azotobacter spp. vary both qualitatively and quantitatively (Choudhury and Kennedy 2004; Jiménez et al. 2011; Saharan and Nehra 2011; Sahoo et al. 2014). Since the plant growth-promoting traits of *Azotobacter* spp. are location-specific, application of these strains to paddy fields may need to consider the indigenous niches where they were originally isolated. Our study reveals the population structure and plant growth-promoting traits of *Azotobacter* spp. in main rice-growing areas of Taiwan, resulting in better development of *Azotobacter* biofertilizers for rice plants.

Of plant growth-promoting traits, the ability to utilize cellulose along with N_2 fixation activity and IAA production may play an important role in promoting rice growth. Our results showed that *A. beijerinckii* CHB 461, *A. chroococcum* CHB 846, and *A. chroococcum* CHB 869 could significantly increase the dry weight of rice plants. In addition to N_2 fixation activity and IAA production, all three strains can utilize cellulose. Cellulose and lignin are two main structural components in the plant biomass. The ability to utilize complex carbohydrates such as cellulose and lignin contributes to rhizosphere competence of rhizobacteria including *Azotobacter* (Sylvia et al. 2005). To colonize the root surface or rhizosphere, rhizobacteria must be rhizosphere competent. Therefore, the three strains able to promote rice growth may be attributed to their ability to degrade cellulose, consequently increasing their rhizosphere competence and exerting their plant growth-promoting traits on rice roots.

Conclusions

This study reveals the distribution and diversity of *Azotobacter* species and their physiological characteristics, allowing us to develop them as biofertilizers for various rice-growing areas in Taiwan. Four *Azotobacter* species including *A. beijerinckii*, *A. chroococcum*, *A. tropicalis*, and *A. vinelandii* were identified among 98 strains isolated from rice rhizosphere soils of Taiwan. Of these four species identified, *A. chroococcum* is dominant. The *Azotobacter* strains isolated in this study vary considerably in plant growth-promoting traits that would constitute a biofertilizer, revealing potential candidates for improving rice growth. Of the 12 strains evaluated, *A. beijerinckii* CHB 461, *A. chroococcum* CHB 846, and *A. chroococcum* CHB 869 may be used to develop biofertilizers for rice cultivation because they can promote rice growth. This study contributes to the selection of suitable *Azotobacter* strains for developing biofertilizer formulations and soil management strategies of *Azotobacter* for paddy fields in Taiwan. In addition to using indigenous *Azotobacter* strains, it is necessary to further evaluate the effects of combination of different *Azotobacter* strains with various plant growth-promoting traits on rice growth.

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