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Full Length Research Paper

Diversity and stress-tolerance of symbiotic nitrogen-fixing bacteria from legumes in the dry-hot valleys of southwest China

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The Dry-Hot valleys in Southwest China are hostile environments for living organisms. The dominant plants in the valleys are drought-resistant legumes. Here, we examined the diversity and physiology of the nitrogen-fixing bacteria associated with these legumes. Thirty-three strains were isolated from the nodules of six legume species located in five valleys. Analyses of their 16S rRNA gene sequences indicated that each strain had a unique 16S rRNA gene sequence and none of the strains had a sequence identical to those reported in the GenBank. The 16S rRNA gene sequence showed little clustering based on host tree species or geographic locations. PCR fingerprinting confirmed the genetic uniqueness of these strains. Physiological tests showed that these strains were all capable of growing at 35℃ or above and at hypertonic environm ents. All strains formed root nodules on *Acacia richii*, the most common legume in these valleys. The bacterial strains obtained here could help future reforestation efforts in these valleys and other environments with similar conditions.

Key words: *Rhizobium* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., 16S rRNA, high temperature resistance, drought resistance.

INTRODUCTION

The term dry-hot valley (DHV) is a direct translation of the Chinese name “Gan Le He Gu”, coined for a specific type of ecological terrain found in southwestern China. High mountains enclose these valleys, blocking moisture clouds and rain from entering into these areas and creating extremely dry conditions for extended periods of time through the year (Zhang, 1992; Ma and McConchie, 2001). The land upheaval from the collision between the Indian Plate and the Eurasian Plate and the large amount of water flowing down from the Qinghai-Tibetan Plateau have created many rivers in southwestern China, with four international rivers running through Yunnan province. DHVs are found along all four large rivers in Yunnan: Hong River (upstream of the Red River), Nu River (upstream of Salween), Lanchang River (upstream of Mekong), and Jinsha River (upstream of Yangtze). In addition, many of the tributaries of these and other river systems in the Hengduan mountain region of the Qinghai-Tibet plateau can also be extremely dry for extended periods of time.

Among these rivers in Southwest China, Jinsha River in the upper Yangtze is the most northern. Here, the mean temperature of the coldest month in the DHVs is over 12℃, while that of the warmest is between 24 to 28℃, both significantly higher than the averages of surrounding areas (Ma and McConchie, 2001). The mean temperatures in other DHVs in Yunnan are comparable to or higher than those along Jinsha River. Each year, about

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350 days have temperatures greater than 10℃ in these areas. The high temperatures drive up evaporation and transpiration rates and create extremely dry condition in the valley (Zhang, 1997a). Another reason for the dry condition is low rainfall. For example, in the Jinsha River DHVs around Panzhihua Municipality and Yuanmou County, the annual evaporation rate is about 3 847.8 mm while the mean annual rainfall is only about 634 mm (ranging from 287.4 to 906.7 mm) (Zhang, 1992; Ma and McConchie, 2001). Based on meteorological data in Yuanmou County, between 1949 and 1996, there were 91 rain days per year with 90% of the precipitation occurring between June and October. During the six-month dry season (November to April), evaporation rates exceed rainfall by at least an order of magnitude and the relative air humidity in these areas is close to zero. Under such harsh climatic conditions, plants are under significant stress with many species unable to survive, particularly young tree seedlings.

Plants that succeeded in growing in the DHVs are mostly legumes. The Leguminosae is one of the largest plant families with over 19,000 species. One of the unique features of legumes is their ability to form symbiotic relationships with nitrogen-fixing bacteria (NFB), enabling the legumes to grow in nitrogen-poor soils. It has been suggested that symbiotic NFB is among the main drivers of speciation and the high species richness in legumes (Martinez-Romero, 2009). Indeed, it has been suggested that the symbiotic relationship between legumes and NFB could facilitate the adaptation of both partners to harsh conditions (Marino et al., 2007; McDowell et al., 2008; Zahran, 1999), such as those found in DHVs.

The objective of this study is to examine the diversity of nitrogen-fixing bacteria associated with legumes in the DHVs and to assess whether these bacteria have unique physiological features such as growth at relatively high temperatures and being resistant to osmotic stresses. We isolated a total of 33 strains from six legume species in five DHVs in Southwest China. Our analyses identified abundant novel diversity of symbiotic nitrogen-fixing bacteria. In addition, these strains were very tolerant to high temperature and were adaptive at growing in hypertonic environments.

MATERIALS AND METHODS

Isolation of nitrogen-fixing bacteria from legume roots

The nodules on the roots of legumes from the DHVs in Southwest China were collected. In the field, the nodules were kept in sterile tubes filled with silica gels. Immediately after returning to the lab, the nodules were cleaned by first rinsing with tape water and then sterile water. They were then sterilized by immersing in 70% ethanol for 1 min, in a sodium hypochlorite solution containing 1% chlorine for 1 min, and then in 1% mercuric chloride for 6 min. Each sterilized nodule was then put into 1 ml sterilized distilled water in a sterile Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse the bacteroids in the nodule. About 20 ml sterilized potato-dextrose-agar (PDA) medium was cooled to 40 to 45℃ and poured into each Petri dish and mixed with the contents of the crushed nodule through gentle shaking. The mixture was then incubated at 25℃ in the dark for 3 to 5 days. One single colony from each nodule was streaked onto a new plate to obtain pure culture for further analyses described subsequently.

In total, 33 legumes belonging to six species were sampled in five DHVs. Two DHVs were located along Jinsha River, the upper section of the Yangtze River: Yuanmou (YM, in Yunnan Province, about 250 km northwest of Kunming, the capital of Yunnan) and Panzhihua (PZ, in Sichuan Province, about 380 km northwest of Kunming). One DHV was located along Pudu River in Luchuan (LC, about 80 km north of Kunming). Pudu River is a tributary of Jinsha River. One DHV was along the Hong River in Yuanjiang (YJ) County, about 220 km southwest of Kunming. The fifth DHV was from Dongchuan (DC) District in Yunnan, about 150 km northeast of Kunming, along Xiaojiang River. In addition, two strains from legumes in Xishuanbana, an area with abundant moisture and rain in southern Yunnan were obtained for comparison. Pure NFB isolates were permanently stored in a -70℃ freezer.

Genome DNA extraction

To obtain genomic DNA for sequence analysis and genotyping, each isolate from the -70℃ stock was first streaked onto TY (tryptone/yeast extract) agar plate and incubated at 30℃ for 3 to 5 days. For each strain, a single colony was picked to inoculate into liquid Yeast Extract-Mannitol-Agar (YMA, per liter: 10 g D-Mannitol, 0.25 g K_2HPO_4, 0.8 g yeast extract, 0.2 g $MgSO4 \cdot 7H_2O$, and 0.1 g NaCl, and 15 g Agar, pH 6.8 to 7.0). Cells were incubated at 30℃ with constant agitation at 120 rpm and harvested by centrifugation when the population density reached an OD420 reading between 0.8 and 1.0. Genomic DNA was extracted using a method previously described for *S. meliloti* (Guo et al., 2009). The quantity and quality of DNA were assessed using the UltraSpec 2000 pro spectrophotometer (Fisher Scientific).

Primers, PCR, and DNA sequencing

To examine the phylogenetic diversity of the obtained NFB strains, we amplified a portion of the 16S small ribosomal RNA gene using the following primers: 5'-CAA GAT CCA GAG TTT GAT CCT GGC TCA GAA CGA ACG CT-3' (forward) and 5'-CAA GAT CCT ACG GCT ACC TTG TTA CGA CTT CAC CCC-3' (reverse). A typical PCR reaction contained 6 µl of diluted genomic DNA template (~20 ng), 0.5 U Taq DNA polymerase, 1 mM each primer and 200 mM of each of the four deoxyribonucleotide triphosphates, in a total volume of 30 µl. The following PCR conditions were used: 3 min at 94℃, followed by 30 cycles of 45 s at 94℃, 45 s at 56℃, 75 s at 72℃, and finally 6 min at 72℃. After confirmation by agarose gel electrophoresis, PCR products were cleaned using the DiaMed PCR cleanup kit according to the manufacturer's manual (Burlington, Ontario, Canada). The purified PCR products were then sequenced at the MoBix Laboratory, McMaster University, Canada, using an Applied BioSystems Prism 3100 automated sequencer with dRhodamine-labeled terminators (PE Applied BioSystems), following the manufacturer's instructions.

Analyses of the 16S rRNA sequences

The putative identities of these strains were obtained through BLAST searches against all existing sequences in the GenBank. To determine the relationships among the strains and between them and other species within the broad NFB symbionts, we retrieved GenBank sequences of symbiotic NFB strains in legumes

representing all species in *Rhizobium, Mesorhizobium, Sinorhizobium*, and *Bradyrhizobium*, the genera closely related to the strains isolated in this study based on 16S rRNA sequences (see results). Whenever possible, 2 to 3 representative strains with sequences similar to the analyzed portion for our strains were retrieved from each species. In addition, sequences from the following nitrogen-fixing bacteria were also retrieved for referencing and comprehensive comparison: *Methylobacterium nodulans, Azorhizobium caulinodans, Azorhizobium doebereinerae, Shinella kummerowwiae, Devosia neptuniae, Cupriavidus taiwanensis, Herbaspirillum lusitanum*, two species in *Ochrobactrum (O. cytisi* and *O. lupini*), four species in genus *Phyllobacterium* (*P. leguminum, P. ifriqiyense, P. trifolii, and P. bourgognense*), and seven species in *Burkholderia (B. cepacia, B. mimosarum, B. nodosa, B. tuberum, B. sabiae, B. caribensis,* and *B. phymatum*). In total, 128 sequences of the 16S rRNA gene were downloaded from GenBank and compared to the 35 sequences obtained in our study. The combined 163 sequences of the 16S rRNA gene were aligned by the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

The aligned sequences were imported into the phylogenetic software program PAUP4.0b10 (Swofford 2002) and analyzed using the Neighbor-Joining algorithm, the maximum-likelihood method, and the maximum parsimony method. In our analyses, gaps were treated as missing data and the Kimura-2-Parameter distance measure was used for tree construction.

Establishing a symbiotic relationship between NFB isolates and *Acacia richii*

To determine whether the isolated NFB strains could establish symbiotic relationships and form nodules on the roots of legumes, we inoculated seedlings of the most common legume in our sites, *Acacia richii*. Seedlings were prepared and inoculated with each of the 35 NFB strains, following the protocols of Trujillo et al. (2005). The plants were grown under natural light in a greenhouse and checked for nodule formation and potential nitrogen-fixing ability after 4 to 6 weeks. Pure NFB strains from the nodules of these inoculated legume plants were then re-isolated and their DNA extracted following a previously described protocol (Guo et al. 2009). Their genotypes were compared to those originally used for inoculation through PCR fingerprinting described subsequently.

PCR fingerprinting

The genotypes of the 35 strains obtained here were determined by PCR fingerprinting using two common primer pairs previously shown to be effective at discriminating natural strains of bacteria (de Bruijn 1992; Nick and Lindstrom 1994). The primer sequences for the REP primer pair are 5' – IIIICGICGICACIGGC – 3' (REP1R-1) and 5' – ICGICTTATCIGGCCTAC – 3' (REP2R) and those for the ERIC primer pair are 5' – ATGTAGCTCCTGGGGATTCA – 3' (ERIC1R-1) and 5' – AAGTAAGTGACTGGGGTGAGCG – 3' (ERIC2-1). These primers were synthesized by InVitrogen Biotechnology Corporation Ltd in Shanghai. The PCR reactions were carried out as described by (Bruijn, 1992; Nick and Lindstrom, 1994). After PCR, the amplification products from each reaction were separated on 1.5% agarose gels at 60 V for 3 h, stained with ethidium bromide, and photographed through a UV illuminator. PCR fingerprinting profiles were scored and strain genotypes were compared following protocols described earlier (Bruijn, 1992; Nick and Lindstrom, 1994; Wu et al., 2010).

High temperature growth

Most NFB strains and species reported so far have maximum growth temperatures around 35°C, with an optimum between 25 to 30°C. To examine their growth at high temperature, strains from DHVs obtained in this study were incubated at three different temperatures, 28, 35 and 40°C, respectively. Briefly, actively growing liquid cultures of these strains were spot-plated (5 µl of 10^3 dilution of OD420 = 1.0) on YMA agar plates. The plates were then incubated at the three selected temperatures and the appearance of actively growing colony 10 days after incubation was recorded as capable of growing at that temperature. Three repeats were done for each strain at each temperature.

Tolerance to osmotic stress

Following the protocol established in an earlier study (Creus et al., 1997), the tolerance of NFB strains to osmotic stress was determined by growing these strains at five concentrations of Polyethylene glycol (PEG) 6000 (final concentration, weight/volume): 0, 5, 10, 20 and 30% in the Yeast Extract - Mannitol broth (YMB). The corresponding water potential at 35°C for these five treatments were 0, -0.34, -1.13, -4.04 and -8.71 bar, respectively. Experiments were performed in 10 ml tubes containing 5 ml YMB with corresponding concentrations of PEG6000. For each tube, 10 µl of actively growing bacterial cells (10^3 dilution of OD420 = 1.0 culture) were inoculated. Three replicates were performed for each treatment for each strain. The cultures were placed in a shaker-incubator at 35°C with 120 rpm. The optical densities of these cultures were measured 5 days after incubation.

RESULTS

Strain isolation

We successfully obtained pure strains from 33 sampled legume plants from five DHVs in Southwest China. The strains, their host legume species, their geographic origins, and the local evaporation/rainfall ratios are listed in Table 1. Fourteen of the 33 strains from DHVs were from Panzhihua (PZ) Municipality in Sichuan Province; thirteen from Yuanmou (YM) County; four from Yuanjiang (YJ) County, and one each from Luchuan (LC) County and Dongchuan (DC) District. In addition, two strains were isolated from Xishuanbanna (XS), a warm and wet region in southern Yunnan.

The 33 strains from the DHVs were isolated from six legume species: *Acacia dealbata* (1 strain), *Acacia richii* (14 strains), *Albizia mollis* (6 strains), *Leucaena glauce* (4 strains), *Leucaena guauca* (4 strains), and *Tephrosia candida* (4 strains). The two strains from Xishuanbanna were from two different legume species, *Indigofera forlunei* (strain XS-If-95-58) and *Robinia pseudoacacia* (strain XS-Rp-87-11).

Phylogenetic analyses of the 16S rRNA gene sequence

We successfully obtained the 16S rRNA gene sequences from all 35 strains analyzed here. The GenBank accession numbers for these sequences are JF974144-JF974178. Our phylogenetic analyses of these strains

Table 1. Symbiotic nitrogen-fixing bacteria obtained and analyzed in this study. Relevant information about legume hosts and collection sites are also presented.

Strain name	Host plant	Location (county/ municipality)	Altitude (meters above sea level)	Evaporation/rainfall ratio
YM-Lgl-8-17	*Leucaena glauce*	Yuanmou	1350	> 6
YM-Tc-8-14	*Tephrosia candida*	Yuanmou	1350	>6
YM-Tc-8-13	*Tephrosia candida*	Yuanmou	1350	> 6
YM-Lgl-8-12	*Leucaena glauce*	Yuanmou	1350	> 6
YM-Am-7-1	*Albizia mollis*.	Yuanmou	1200	> 6
YM-Ar-6-2	*Acacia richii*	Yuanmou	1200	> 6
YM-Am-6-1	*Albizia mollis*.	Yuanmou	1200	> 6
YM-Tc-5-6	*Tephrosia candida*	Yuanmou	1350	> 6
YM-Lgl-5-3	*Leucaena glauce*	Yuanmou	1350	> 6
YM-Ar-4-4	*Acacia richii*	Yuanmou	1300	> 6
YM-Am-4-3	*Albizia mollis*.	Yuanmou	1300	> 6
YM-Ar-4-2	*Acacia richii*	Yuanmou	1300	> 6
YM-Lgu-4-1	*Leucaena guauca*	Yuanmou	1300	> 6
YJ-Ar-5-3	*Acacia richii*	Yuanjiang	700	> 4
YJ-Lgu-5-2	*Leucaena guauca*	Yuanjiang	700	> 4
YJ-Ar-5-1	*Acacia richii*	Yuanjiang	700	> 4
YJ-Ar-4-1	*Acacia richii*	Yuanjiang	600	> 4
LC-Ar-3-4	*Acacia richii*	Luchuan	600	> 4
PZ-Lgu-6-5	*Leucaena guauca*	Panzhihua	1400	> 5
PZ-Ar-6-11	*Acacia richii*	Panzhihua	1500	> 5
PZ-Ar-5-6-8	*Acacia richii*	Panzhihua	1400	> 5
PZ-Ar-5-6	*Acacia richii*	Panzhihua	1400	> 5
PZ-Tc-5-4	*Tephrosia candida*	Panzhihua	1300	> 5
PZ-Lgl-5-3	*Leucaena glauce*	Panzhihua	1300	> 5
PZ-Ar-5-1-2	*Acacia richii*	Panzhihua	1300	> 5
PZ-Ar-5-1	*Acacia richii*	Panzhihua	1300	> 5
PZ-Am-4-5	*Albizia mollis*.	Panzhihua	1200	> 5
PZ-Am-3-8	*Albizia mollis*.	Panzhihua	1200	> 5
PZ-Ar-3-7	*Acacia richii*	Panzhihua	1200	> 5
PZ-Lgu-3-5	*Leucaena guauca*	Panzhihua	1200	> 5
PZ-Am-3-2	*Albizia mollis*	Panzhihua	1200	> 5
PZ-Ar-3-1	*Acacia richii*	Panzhihua	1200	> 5
DC-Ad-5-3	*Acacia dealbata*	Dongchuan	1300	> 4
XS-If-95-58	*Indigofera forlunei*	Xishuanbanna	<300	~1
XS-Rp-87-11	*Robinia psendoacacia*	Xishuanbanna	<300	~1

[1]Strain names include a two-letter code for geographic locations (PZ, Panzhihua; YM, Yuanmou; YJ, Yuanjiang; LC, Luchuan, DC, Dongchuan, XS, Xishuanbanna), two to three letter code for host legume species (shown in column 2), and two to three numbers as an Rhizobium strain isolation code.

with the 128 retrieved from the GenBank identified that the 35 strains were distributed in three genera *Bradyrhizobium, Mesorhizobium* and *Rhizobium.* However, the large number of sequences analyzed prevented the generation of a readable figure including all 163 strains. Instead, only the 35 sequences generated in this study along with a subset of the retrieved reference sequences closely related to our sequences were included in Figure 1. Interestingly, none of our sequences were identical to each other or to any of the deposited sequences in the GenBank. The most closely related species to our sequences were *Bradyrhizobium liaoningense* (9 of our strains), *Bradyrhizobium jicamae/B. elkanii* (3 strains), *Mesorhizobium plurifarium/M. amorphae* (8 strains), and *Rhizobium leguminosarium* (14 strains).

Each of the three 16S rRNA sequence clusters with multiple strains contained samples from multiple geographic locations and host legume species (Figure 1). For example, the 9 strains in the cluster closely related to

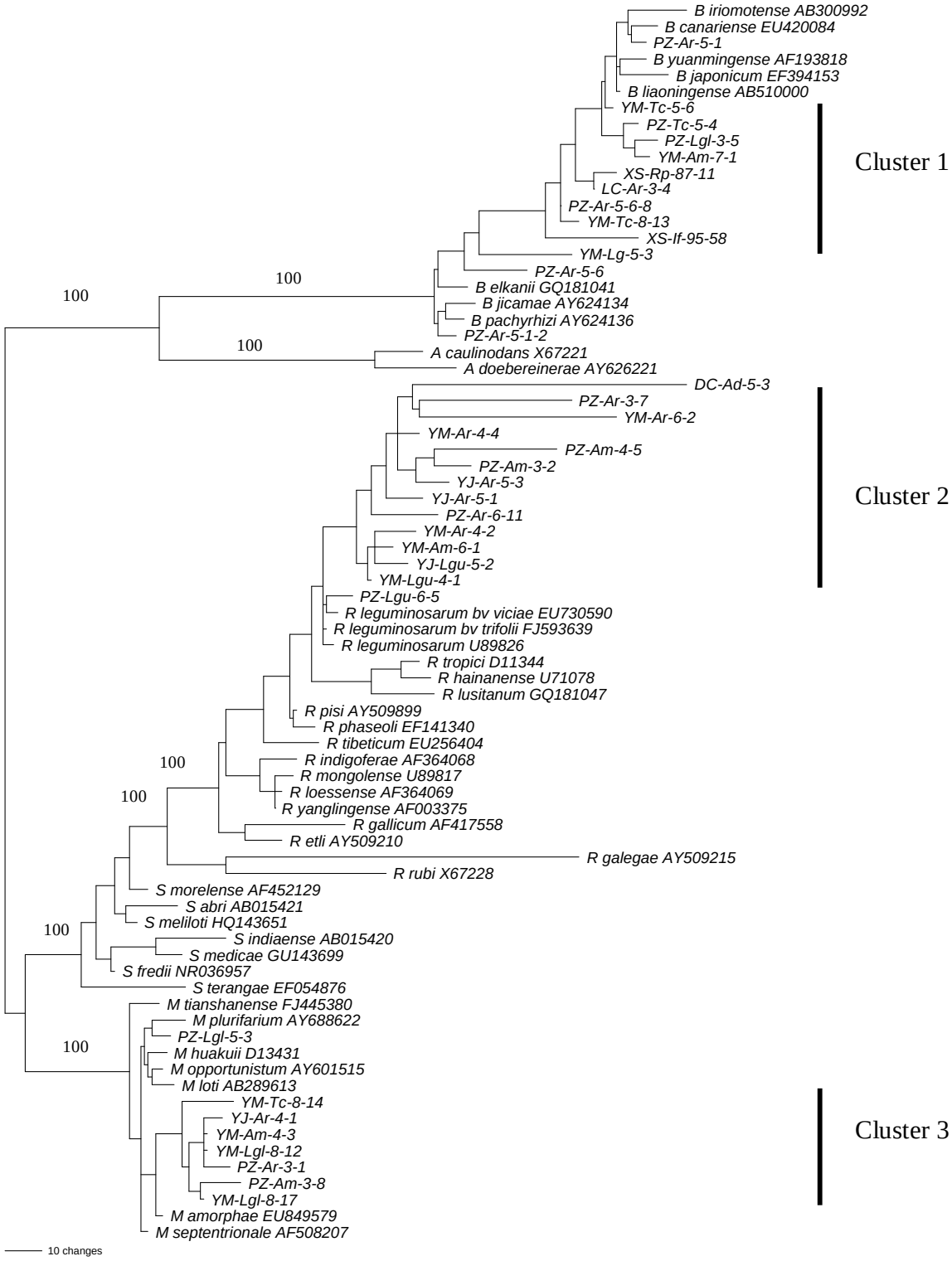

Figure 1. Phylogenetic relationships based on the 16S rRNA gene sequences among our 35 strains analyzed here and with the 41 representative strains of nitrogen-fixing bacteria closely related to ours retrieved from the GenBank. Names for the 35 strains obtained in this study follow those in Table 1. Abbreviations for genera for those retrieved from GenBank: A, *Azorhizobium*; R, *Rhizobium*; B, *Bradyrhizobium*; S, *Sinorhizobium*; and M, *Mesorhizobium*. The genera names were followed by species names and individual GenBank accession numbers. Number above branches indicates 100% bootstrap support. The maximum parsimony tree length is 1406, with a consistency index of 0.594 and retention index of 0.867.

B. liaoningense were from six host legume species and four of the five DHVs (two strains not from the DHVs but from Xishuanbanna were also clustered in this group, Cluster 1 in Figure 1). Similarly, the three strains closely related to *B. jicamae/B. elkanii* were from two DHV locations along the upper stream of Yangtze River, with each from a different legume species. The 14 strains in the cluster related to *R. leguminosarium* were from four legume species at four DHV locations (Cluster 2 in Figure 1). Finally, the eight strains in the cluster closely related to *M. plurifarium/M. amorphae* were from four tree species at three DHV locations (Cluster 3 in Figure 1). As shown in Figure 1, many of our sequences within each of the clusters showed pairwise divergence similar to or greater than those between many known pairs of sister species. Such a result suggests that there are potentially many new species of symbiotic nitrogen fixing bacteria in the DHVs in southwest China.

PCR fingerprinting using the REP and ERIC primers

Our PCR fingerprinting results identified that each of the 35 strains had a different banding pattern when amplified by each of the two primer pairs. Indeed, few bands were shared among the strains. Figure 2 showed a representative gel generated using the ERIC primer pair. No obvious correlation was found between the PCR fingerprinting banding pattern and host geographic location or host tree species. Due to the very limited band sharing, further analyses of the fingerprinting patterns were not conducted. Our PCR fingerprinting results confirmed the phylogenetic diversity as revealed by 16S rRNA sequencing and suggest tremendous genetic diversity of symbiotic nitrogen fixing bacteria in the Dry-Hot Valleys of southwest China.

Tolerance to high temperature

At the 28℃ environment, all 35 strains obtained here grew well. In addition, all 35 strains grew at the 35℃ environment, with most showing comparable growth to that at the 28℃ environment. The strains showing significantly reduced growth at 35℃ were PZ-Ar-3-7 , PZ-Ar-5-1-2, PZ-Ar-5-6, YM-As-6-1, and YM-Tc-8-14. At the 40℃ environment, all strains still showed residual growth with several showing comparable growth to those at the 28℃ and/or the 35℃ environments. Specifically, strain PZ-Ar-6-11 showed comparable growths across all three temperatures while strains PZ-Ar-5-6, YM-As-6-1, and PZ-Ar-3-7 showed comparable growths between the 35 and 40℃ environments (though slower than those at 28℃). As expected, two strains from Xishuanbanna in southern Yunnan (with a hot and humid climate) showed similar growth abilities to that of strain PZ-Ar-6-11 at all three temperature environments. These results indicate variable but significant tolerance to high temperature for strains from DHVs in Southwest China.

Tolerance to osmotic stress

The results of osmotic stress test showed variable but significant growth at all four concentrations of PEG6000 that we tested for the strains. Overall, the 33 NFB strains from the five DHVs grew to much higher population densities than the two strains from the non-DHV environment, across all four PEG6000 concentrations ($P < 0.05$ for all four tests). The population densities of a few representative strains across the five treatments are presented in Table 2. Unlike the two strains XS-If-95-58 and XS-Rp-87-11 that showed decreasing population density with increasing concentrations of PEG6000, a total of 26 strains showed significantly higher population density at PEG6000 concentrations of 5 and/or 10% than those without any PEG6000 (Table 2). Five of the 26 strains (PZ-Ar-6-11, PZ-Ar-3-7, PZ-Lgl-5-3, YM-Lgl-8-12, and YM-As-6-13) are shown in Table 2. These results suggest that a significant proportion of the NFB strains from DHVs have developed a preference for environments with high osmolarity and low water potential. However, at PEG6000 concentrations of 20 and 30%, none of the strains isolated here grew to greater population densities than those at lower PEG6000 concentrations (Table 2).

Nodulation and confirmation of strain genotypes by PCR fingerprinting

At 4 to 6 weeks after inoculation, all 35 strains formed nodules on the roots of *Acacia richii*. In addition, the centers of the nodules were pinkish, consistent with the presence of leghemoglobin and nitrogen fixation in these nodules. The NFB strains were re-isolated from the roots and their genotypes compared with those of the originally inoculated parental strains by PCR fingerprinting using the REP and ERIC primers, following protocols described earlier. Our analyses confirmed that the re-isolated strains were genetically identical to those originally inoculated (data not shown).

DISCUSSION

The legumes are very diverse in morphology, habitat and ecology, ranging from arctic annuals to tropical trees (Faria et al., 1989). These legumes are commonly nodulated by nitrogen fixing bacteria, forming one of the most important symbiotic relationships on Earth (Eardly and Xu, 2010). This symbiosis not only fixes atmospheric nitrogen for the two interacting partners but also supplies organic and inorganic nitrogenous compounds for other

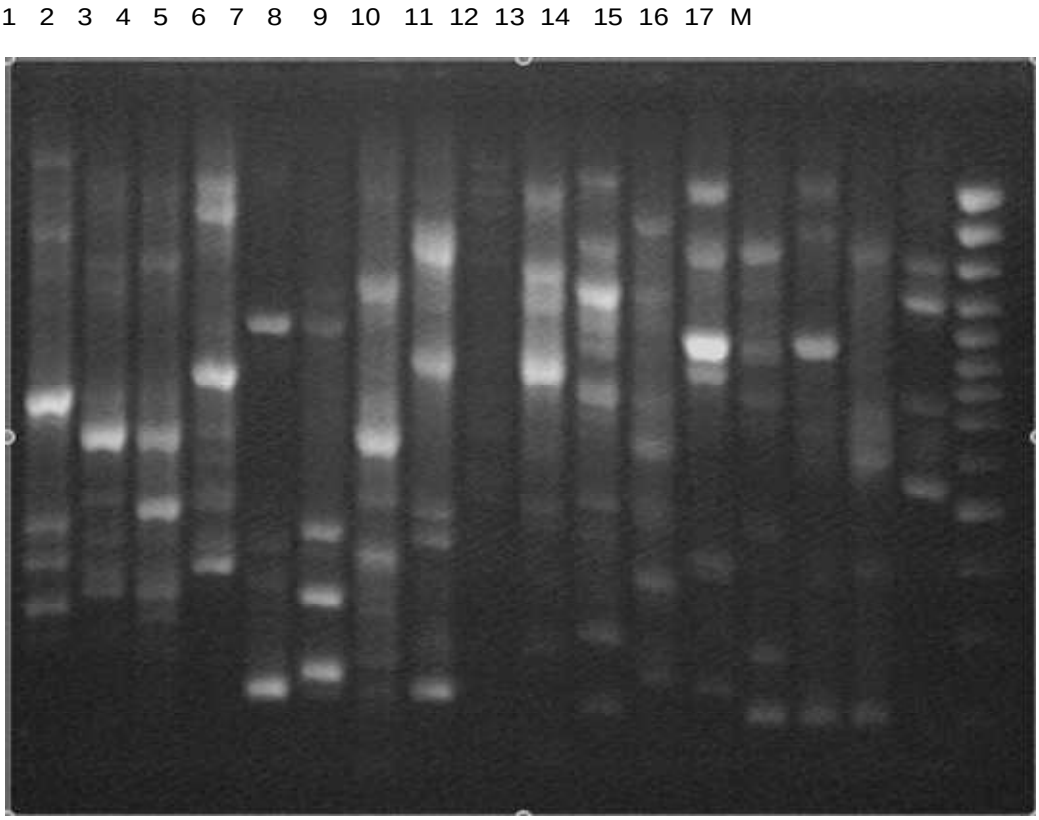

Figure 2. The PCR results produced using the ERIC primer pair. Lanes 1 to 17 correspond to strains YM-Lgl-8-17, PZ-Lgl-5-3, YM-Lgl-8-12, PZ-Ar-5-1, PZ-Ar-5-6-8, PZ-Ar-5-1-2, PZ-Ar-5-6, PZ-Ar-6-11, PZ-Ar-5-7, PZ-Tc-5-4, YM-Tc-8-14, YM-Tc-8-13, DC-Ad-5-3, YM-Tc-5-6, YJ-Ar-3-4, YM-Lgl-5-3, XS-Rp-87-11 respectively. M is the DNA size ladder, from top to bottom: 3kb, 2kb, 1.5kb, 1031bp, 900bp, 800bp. 700bp, 600bp, 500bp, 400bp, and 300bp. Note that no two strains had identical banding pattern.

organisms in their respective environments, for almost all ecological niches on Earth. Indeed, the symbiosis plays one of the most important roles in elemental and nutrient cycling in natural ecosystems (vanRhijin and Vanderleyden, 1995).

The broad phylogenetic distribution of the nitrogen-fixing bacteria isolated here suggested that different phylogenetic groups of NFB are capable of evolving the specialized physiological adaptations to high temperature and dry environments. Such a result is consistent with those found in other studies where a diversity of NFB could be found in individual environments (Zhang et al., 1991; Moreira et al., 1998; Liu et al., 2007). However, unlike those in the earlier studies where many of their strains had identical or near-identical 16S rRNA sequences to each other or to those of known species, none of our 35 strains had 16S rRNA gene sequences identical to each other or to those deposited in the GenBank. For example, Moreira et al. (1998) found most of their strains from natural Brazil forest legumes were identical or near identical to each other and to those of known NFB species in agricultural fields. Our survey thus indicated an extremely diverse collection of potential novel NFB species in the DHVs in Southwest China. However, more extensive sampling and the assessment of other genetic, physiological and biochemical features need to be conducted before the potential number of new NFB species in these DHVs could be accurately determined.

Aside from the broad phylogenetic distribution of the strains from the DHVs in southwest China, the lack of geography–based distribution seen from their 16S rRNA gene sequences within each of the phylogenetic groups also suggests historic and/or current gene flow among these DHV sites. Significant gene flows have been documented for many groups of microorganisms, including nitrogen-fixing bacteria (Xu, 2010). For example, Stepkowski et al. (2005) reported that European genotypes of *Bradyrhizobium* spp. could be isolated from the agricultural soils of both Australia and South Africa, likely resulted from anthropogenic transfers of crops across geographic regions. However, most studies indicated some level of geographic structuring among natural populations of symbiotic NFB from

Table 2. Mean optical density values of selected NFB strains from DHVs grown in Yeast extract-Mannitol-Broth (YMB) at various concentrations of PEG6000. The strains were incubated at 35℃ for 5 days.

Strain	0% PEG	5% PEG	10% PEG	20% PEG	30% PEG
Bradyrhizobium spp.					
PZ-Ar-5-1	2.135	1.323	1.456	0.273*	0.246*
PZ-Ar-5-6-8	1.903	1.309*	1.310*	0.294**	0.165**
PZ-Tc-5-4	1.237	1.136	0.664*	0.275*	0.126*
XS-If-95-58	1.412	0.915*	0.303**	0.085**	0.033**
XS-Rp-87-11	1.367	0.982*	0.404**	0.244**	0.133**
Rhizobium spp.					
PZ-Ar-6-11	1.335	1.512*	1.259	0.484**	0.273**
PZ-Ar-3-7	1.379	1.548*	1.379	0.845*	0.464**
YM-As-6-1	1.008	1.489*	1.373*	0.308**	0.179**
Mesorhizobium spp.					
PZ-Lgl-5-3	0.723	1.058*	0.647	0.256*	0.142*
YM-Lgl-8-12	1.082	1.348*	1.102	0.323*	0.093*
YM-Lgl-8-17	1.681	1.302*	0.462**	0.298**	0.083**
YM-Tc-8-14	1.514	1.415	0.335**	0.103**	0.080**

* and ** indicate statistically significant difference in growth between treatment and 0% PEG control at 0.01<P<0.05 and P<0.01, respectively.

agricultural crops, with the highest diversities found in centers of crop diversity and crop origins (Eardly and Xu, 2010). At present, little is known about population structuring of symbiotic NFB from non-crop legumes.

Gene flows among sites create conditions for genetic exchange and recombination among natural strains of bacteria. Indeed, signatures of genetic exchange and recombination have been identified for populations of all nitrogen-fixing bacteria examined so far (Eardly and Xu, 2010). Such genetic exchange might be responsible for the apparent lack of host legume species-based phylogenetic pattern observed in the current study. In the majority of symbiotic nitrogen-fixing bacteria, the genetic determinants for host specificity and nitrogen fixation in the microbial genomes are located on symbiotic plasmids (MacLean et al., 2007). These plasmids (or parts of them) have shown capability of transferring among strains of symbiotic NFB (Sun et al., 2006), and often among those from different genera or families (Sullivan and Ronson, 1998). Once a strain has acquired such a plasmid(s), its host range could change/expand (Young and Johnston, 1989). In contrast, the 16S rRNA gene is located on the chromosome and its possibility for horizontal gene transfer is very limited in bacteria. As a result, the phylogeny based on 16S rRNA gene sequences can be very different from those based on genes involved in host-bacteria interactions or genes from host legume species (Moreira et al., 1998; Guo et al., 2009; Eardly and Xu, 2010).

The patterns of dispersal and genetic interactions among NFB strains could be influenced by many factors. Legumes emerged about 60 million year ago and their root nodulation with NFB about 58 millions year ago (Sprent, 2007). During this tertiary period, many river valleys in Southwest China were intensively incised when the Qinghai-Tibet plateau was rapidly rising from the collision between the Indian Plate and the Eurasia Plate (Zhang, 1992). As a result, the terrain and climate in southwest China can be extremely striking: even within individual DHVs, vegetation and climate can change rapidly along with elevation. The deepest valley in the region ranges in elevation from about 1000 m to over 5000 m above sea level, with these two elevation points within 1 km of each other if measured horizontally. At any given time, the bottom of the valley could be very hot and dry (that is, the hot-dry valley) while at the top very cold and permanently covered in snow. The co-occurrence of geographic/geological changes and the emergence NFB-legume symbiosis could have impacted the evolution and co-evolution of both the legumes and the NFB in this region. Specifically, we hypothesize that the extremely high levels of genetic diversity found among our NFB strains have likely been impacted by these extreme geographic, geological, and/or climate factors in these valleys. Previous studies have identified that environmental factors could influence the abundance, distribution, and patterns of genetic variation of symbiotic nitrogen foxing bacteria (for a recent review, see Eardly and Xu, 2010). For example, Woomer et al. (1988) found that factors such as the existence of suitable host plants, annual rainfall, the abundance and coverage of legumes in the area, the flowering time of plants, temperature, pH,

and biologically available phosphorus concentration all influenced rhizobia abundance in the soil. However, the detailed influences of each of these factors on the patterns of genetic variation in NFB in the DHVs await further investigation. More NFB samples from more geographic areas as well as detailed measures of vegetation, climate and soil conditions from each of the sites will need to be recorded. In addition, DNA sequences from more genes will be needed to fully quantify the patterns of genetic variation and to partition such variation to individual ecological factors.

Our stress test results showed that several strains were extremely tolerant to both high temperature and high osmotic stress. Among these, two strains stood out with both from the nodules of *Acacia richii* in Panzhihua Municipality: PZ-Ar-6-11 and PZ-Ar-3-7. Both strains showed comparable growth at 28, 35 and 40℃. Furthermore, in the 30% PEG6000 environment, they maintained 30 to 40% of growth abilities of those in the medium without any PEG6000. NFB strains with comparable stress-tolerance abilities from other host plants in other geographic areas have been reported previously (Hartel and Alexander, 1984; de Lajudie et al., 1994; Kuykendall, 2005). At present, the molecular mechanisms for stress tolerance for the NFB strains isolated here are unknown. However, the naturally stress-resistant NFB strains recovered here (especially strains PZ-Ar-6-11 and PZ-Ar-3-7) could be of significant applied values in reforestation efforts in these DHVs and in other environments with similar ecological and climate conditions.

Indeed, reforesting the DHVs in Southwest China as well as other areas along the Yangtze River has been a high priority of all levels of governments. Because of long periods of logging and deforestation, there had been serious environmental degradation along the Yangtze River (Cha and Li, 1998; Zhang, 1997b). Back in 1989, the Chinese government started the "*Project for the construction of a shelter-forest network for the middle and upper reaches of the Yangtze River*" and the *"Project for water and soil conservation for the middle and upper reaches of the Yangtze River*" (Wu and Ma, 1996). Several billion RMB were invested in re-vegetation and water and soil conservation. Following the catastrophic flooding along the Yangtze River in 1998, the Chinese government placed even more emphasis on environmental recovery in the middle and upper reaches of the Yangtze River (Ma, 1999). For example, there have been two long-term ongoing major initiatives from the central and provincial governments: *"The project of natural forest protection"* and *"The project for the ecological recovery of the middle and upper reaches of the Yangtze River"* (data from Yunnan Provincial Department of Forestry 1999). After significant work, vegetation on most of the hills along the upper and middle Yangtze River catchments has improved dramatically, soil erosion has decreased significantly, and flooding has been less frequent and less severe (http://www.china.org.cn/english/MATERIAL/170393.htm). However, the reforestation efforts using plants succeeded in other areas have largely been ineffective for the DHVs (Wu and Ma, 1996). The harsh climate and soil conditions in DHVs mean that recovery techniques need to be specifically targeted for these areas to make it successful. Planting legumes that have naturally succeeded in these areas along with inoculating them with stress-resistant BNF strains could help improve reforestation success rate in these areas. Our preliminary results here suggest that such a strategy should work.

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