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Low Frequency of Plants Associated with Symbiotic Nitrogen-Fixers Exhibits High Frequency of Free-Living Nitrogen Fixing Bacteria: A Study in Karst Shrub Ecosystems of Southwest China

Yueming Liang [1], Xunyang He [2,3], Xiangbi Chen [2,3], Yirong Su [2,3,*], Fujing Pan [4] and Lening Hu [1,2,5]

[1] Key Laboratory of Karst Dynamics, Ministry of Natural and Resources & Guangxi Zhuangzu Autonomy Region, Institute of Karst Geology, Chinese Academy of Geological Science, Guilin 541004, China; yueming0919@163.com (Y.L.); hulening@126.com (L.H.)
[2] Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China; hbhpjhn@isa.ac.cn (X.H.); xbchen@isa.ac.cn (X.C.)
[3] Huanjiang Observation and Research Station for Karst Eco-Systems, Chinese Academy of Sciences, Hechi 547000, China
[4] Guangxi Key Laboratory of Theory and Technology for Environmental Pollution Control, Guilin University of Technology, Guilin 541000, China; panfujing@glut.edu.cn
[5] College of Environment and Resource, Guangxi Normal University, Guilin 541000, China
* Correspondence: yrsu@isa.ac.cn; Tel.: +86-731-8461-5222

Abstract: Plants associated with symbiotic nitrogen-fixers and soil free-living nitrogen-fixing bacteria are good indicators for detecting the source of nitrogen in natural ecosystems. However, the community composition and diversity of plants associated with symbiotic nitrogen-fixers and soil free-living nitrogen-fixing bacteria in karst shrub ecosystems remain poorly known. The community composition and diversity of soil free-living nitrogen-fixing bacteria and plants, as well as the soil physical–chemical properties were investigated in 21 shrub plots (including different topographies and plant types). The frequency of plants associated with symbiotic nitrogen-fixers was found to be low in the 21 shrub plots. The soil free-living nitrogen-fixing bacterial community structure varied among the 21 shrub soils. Based on a variance partitioning analysis, topography, plant type, and soil pH explained 48.5% of the observed variation in bacterial community structure. Plant type had a predominant effect on community structure, and topography (aspect and ascent) and soil pH had minor effects. A negative correlation between the abundance of the soil free-living nitrogen-fixing bacterial community and the richness index for plants associated with symbiotic nitrogen-fixers was observed. The result of the low frequency of plants associated with symbiotic nitrogen-fixers highlights the importance of sources of fixed nitrogen by soil free-living nitrogen-fixing bacteria in the nitrogen limitation shrub ecosystem of the karst regions.

Keywords: nitrogen limitation shrub ecosystem; nitrogen-fixing plant; vegetation restoration

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1. Introduction

Nitrogen is an important limiting factor for plant growth [1,2]. It is particularly limited during the early stages of the natural recovery of degraded soils. Similarly, in the karst shrub ecosystem, nitrogen is considered a key limiting factor for shrub growth [3,4]. The primary source of soil nitrogen is biological nitrogen fixation by nitrogen-fixing bacteria [5], which plays a critical role in nitrogen cycling.

Nitrogen-fixing bacteria are highly diverse and are divided into two groups: free-living nitrogen-fixing bacteria (e.g., *Azotobacter*, *Azospirillum*, and *Pseudomonas*) and symbiotic nitrogen-fixing bacteria (e.g., *Rhizobia* and *Frankia*; [6]). Approximately 80% of the total biological nitrogen fixation is fixed by rhizobia symbiotic association with leguminous

plants [5]. The fixation of atmospheric nitrogen by free-living nitrogen-fixing bacteria is significant in a few types of soil [7,8] and is the most important nitrogen source for natural ecosystems [9,10]. Therefore, in nitrogen-limited natural ecosystems lacking legumes, soil free-living nitrogen-fixing bacteria may be particularly important nitrogen sources for plant functioning.

Karst shrub growth is more severely restricted by low nitrogen content compared with forest and primary forest [4]. Simultaneously, less nitrogen fixation by rhizobia symbiotic association with leguminous plants makes the condition worse [11]. Therefore, soil free-living nitrogen-fixing bacteria play important roles in karst shrub growth and may be useful for shrub restoration. Shrubs are widely distributed in the karst region of southwest China and are uniquely adapted for survival in conditions of drought, rocky establishments, and excessive calcium [12,13]. Accordingly, they have important applications for karst vegetation restoration [11,14].

Nitrogen-fixing bacteria are influenced by many factors, such as plant type [15,16], soil nutrients [17–19], and soil pH [20]. However, these parameters have usually been treated as independent environmental factors that affect communities of soil free-living nitrogen-fixing bacteria [10,21,22]. Few studies have explored the effects of composite factors on soil free-living nitrogen-fixing bacterial communities in a given ecosystem [23]. In karst shrub ecosystems, soil properties and plant type show higher levels of spatial heterogeneity compared with forest and primary forest ecosystems [24]. This indicates that factors influencing soil free-living nitrogen-fixing bacteria are more complex. However, the specific factors driving changes in the community structure of free-living nitrogen-fixing bacteria in shrub ecosystems are poorly known; symbiotic nitrogen-fixing bacteria are well-documented [11]. Our previous study reported that vegetation types impacted this bacterial community, along with the vegetation restoration [15]. Therefore, we hypothesized that plant type had the greatest effect on this bacterial community structure in the karst shrub ecosystem and that a lower frequency of plant association with symbiotic nitrogen-fixers would result in a higher frequency of free-living N-fixing bacteria. Soil free-living nitrogen-fixing bacterial communities were analyzed by quantitative PCR and T-RFLP in 21 shrub soils collected from the karst shrub ecosystems in the present study. Our objectives were to (1) characterize the community structure of soil free-living nitrogen-fixing bacteria and plants associated with symbiotic nitrogen-fixers in karst shrub ecosystems; (2) identify the critical parameters affecting the community structure of soil free-living nitrogen-fixing bacteria.

2. Materials and Methods

2.1. Study Area

This study site was laid at Huanjiang County in the Guangxi Zhuang Autonomous Region, southwestern China (107°51′ to 108°43′ E, 24°44′ to 25°33′ N). This region is a subtropical mountainous monsoon climate. The mean annual rainfall and the mean annual air temperature are 1389 mm and 18.5 °C, respectively. The wet season starts in April and lasts until August, accounting for 70% of the annual precipitation [25]. Soil average depth in depressions and on hillslopes is 50–80 cm and 10–30 cm, respectively.

2.2. Survey of Plant and Collecting of Soil Sample

In June 2012, 21 shrub plots were established in Huanjiang County. The plot establishment considered environmental factors (i.e., slope position, aspect, and ascent (As); Table S1). Each plot (10 m × 10 m) was divided into four subplots (5 m × 5 m) for plant surveys. For the shrub vegetation survey, the subplot was divided into the shrubby layer and the herbaceous layer. All the individual trees with DBH > 1 cm were identified. Simultaneously, plant height, cover, and density were measured. Each potential plant associated with symbiotic nitrogen-fixers was surveyed for nodules during the plant surveys. Briefly, all small roots and 1/4 of the large roots of individuals were surveyed. The surface soil around roots was gently removed, and then we observed plants for nodules.

The plant diversity is calculated according to Ma et al. described in reference [26]. The Shannon–Wiener diversity (H') and evenness indices (E) were applied to calculate the plant diversity index. The equations were as follow:

$$H' = -\sum_{i=1}^{S} Pi \log_2 Pi,\ E = H'/H_{\max} = H'/\ln S$$

In these equations, S indicates total plant species in each community and is referred to as the plant species richness. P represents the importance value (IV), and thus Pi is the importance value (IV) of the ith plant species. The equation of importance value (IV) was as follow: IV = (relative height + relative abundance + relative coverage)/3. A coverage percentage of trees is the proportion of the elliptical area of the tree crown within all the covered areas to the area of a quadrat.

Each plot was divided into four subplots (5 m × 5 m), and five soil cores (diameter, 5 cm) in each subplot were collected. A total of 20 soil cores (depth, 0–15 cm) were collected from each plot and thoroughly mixed to form one composite soil sample. Twenty-one soils were sampled in the shrub ecosystems. Soil samples were removed from stones, animals, roots, and plant material through 2-mm sieves. The sieved soil sample was divided into two portions. One portion was kept at −70 °C for molecular analysis. The other portion was air-dried for soil physicochemical properties analysis.

2.3. Extraction and Purification of Soil DNA

Soil microbial DNA was extracted from 0.5 g of freeze-dried soil using the sodium dodecyl sulfate-guanidine isothiocyanate-polyethylene glycol (SDS-GITC-PEG) method according to Liang et al. described in reference [15]. The 50 µL of sterilized water was used to dissolve soil DNA. The concentration of DNA was measured using a spectrophotometer (NanoDrop; PeqLab, Erlangen, Germany). The extracted DNA was stored at −20 °C for further use.

2.4. Amplification of Polymerase Chain Reaction (PCR) and Analyses of Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The *nifH* gene, encoding a subunit of the nitrogenase enzyme, has been widely applied to study the distribution and diversity of soil free-living nitrogen-fixing bacteria [27]. The primer pair PolF (5′TGCGAYCCSAARGCBGACTC3′) and PolR (5′ATSGCCATCATYTCRCCGGA3′) was used to amplify *nifH* [10] to determine this bacterial community structure by T-RFLP. The 5′ end of the forward primer was labeled with 6-carboxy-fluorescein (FAM; Invitrogen, Shanghai, China). The 50 µL of PCR reaction mixtures contained: 25 µL 2× PCR Premix (0.5 mM deoxyribonucleoside triphosphate (dNTPs); 0.1 U of Prime STAR HS DNA polymerase), 1 µL genomic DNA (20 ng), 1 µL each primer (10 pM), and 19 µL H_2O. The PCR conditions were as follows [10]: 95 °C for 2 min; followed by 35 cycles of 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s; and a final extension of 10 min for 72 °C. The PCR products were purified with QIAquick PCR Purification Kit (Tiangen Biotech Ltd., Beijing, China) and quantified with NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). Approximately 200 ng of PCR products were digested with *Hae*III enzymes and then sent to the Sunny Company (Shanghai, China) for T-RFLP analysis with an automated sequencer (model 373A; Applied Biosystems, Weiterstadt, Germany).

T-RFLP data were analyzed using GeneScan Analysis Software (version 2.1; Applied Biosystems). Peak areas of terminal restriction fragments (T-RFs < 2 bp) were summed and defined as a T-RF. A T-RF relative abundance (RA) was calculated as follow: RA = (ni/N) × 100, where ni represents a T-RF peak area, and N represents total T-RFs peak areas in each sample. During the statistical analyses, the relative abundance of a peak area <1% was considered a minor peak, which was deleted as background noise [28]. The database T-RFLP was established based on 40 *nifH* sequences from karst regions (accession numbers KF859859 to KF859898), which was applied to identify taxa of free-living nitrogen-fixing bacteria according to the Aldrich-Wolfe et al. described in reference [29].

2.5. Abundance of nifH Genes

The abundance of the *nifH* gene was measured by quantitative PCR (qPCR; ABI 7900, ABI, Foster City, CA, USA) with the PolF/PolR primers. The PCR reaction mixture with 10 µL included: 5 µL 1× Synergy SYBR Premix ExTaq (Takara Bio, Shiga, Japan), 0.2 µL each primer (10 pM; Invitrogen, China), 1 µL DNA template (5 ng μL^{-1}), 0.2 µL Rox (Takara Bio, Shiga, Japan), and 3.4 µL sterilized water. The PCR conditions were as follows: 95 °C for 20 s; 5 cycles each of 95 °C for 15 s, 64 °C for 20 s, 72 °C for 15 s; 35 cycles each of 95 °C for 15 s, 60 °C for 25 s, and 72 °C for 15 s. This PCR condition was slightly modified according to Poly et al. [10]. A standard curve (ranging from 10^2 to 10^8 μL^{-1}) was generated using a plasmid from *Bradyrhizobium* sp. ISA1601 (KF859886) containing the *nifH* gene. The reactions in a single 384-well plate concluded soil DNA samples, positive control samples (plasmid samples for standard curve), and negative control samples without template DNA. Each sample concluded four technical replicates. The data were automatically processed with SDS 2.3 software containing the real-time PCR system. The amplification efficiency and R^2 value for the standard curve was 99% and 0.99, respectively.

2.6. Determination of Soil Physicochemical Parameters

Soil pH was measured using a pH meter (Delta 320; Mettler-Toledo Instruments Ltd., Shanghai, China) with a soil/deionized water ratio of 1:2.5 (*w*/*v*). Olsen-P was extracted with sodium bicarbonate (0.5 M) and determined using the Mo-Sb colorimetric method [30]. Soil organic carbon was measured by oxidation with $K_2Cr_2O_7$-H_2SO_4 and then titration with $FeSO_4$. The total nitrogen was determined using a flow injection analyzer (FIAstar 5000, FOSS Company, Stockholm, Sweden) according to the Kjeldahl method [31]. Nitrate N (NO_3^--N) and ammonium N (NH_4^+-N) were extracted with 2 M KCl and measured with a flow injection analyzer.

2.7. Analyses of Statistics

The SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data analyses. The level of significance was defined as $p < 0.05$ with the least significant difference (LSD) test. Data were prior to $\log_{(x+1)}$-transformed without being normally distributed by SPSS 19.0. The Pearson correlation analysis was used to evaluate relationships between environmental factors and *nifH* gene abundance. Ordination techniques were used in a correspondence analysis (CA) to assess bacterial community structure (CANOCO 5.0; Microcomputer Power, Inc., Ithaca, NY, USA). Forward selections, using redundancy analysis (RDA; CANOCO 5.0, Microcomputer Power, Inc., Ithaca, NY, USA), were applied to choose the significant environment parameters effect on bacterial community structure. Following the forward selection, the contribution of individual and interaction significant parameters to the bacterial community structure variation was determined by a variance partitioning analysis with CANOCO 5.0. A path analysis (using the plspm package in R) was applied to explore all significant parameters directly and indirectly influencing bacterial structure.

3. Results

3.1. Composition Community of Soil Free-Living Nitrogen-Fixing Bacteria from Different Plot Soils in Karst Shrub Ecosystems

A total of 27 T-RFs for soil free-living nitrogen-fixing bacteria were obtained (Figure 1). The dominant T-RFs (≥3%) included 180, 157, 75, and 66 bp, accounting for 75% of the bacterial T-RFs in different plot soils. The dominant T-RFs of 180, 157, and 75 bp were closely related to *Bradyrhizobium japonicum*, *Bradyrhizobium* sp. ISA1601, and *Bradyrhizobium* sp. CCBAU 101065, respectively.

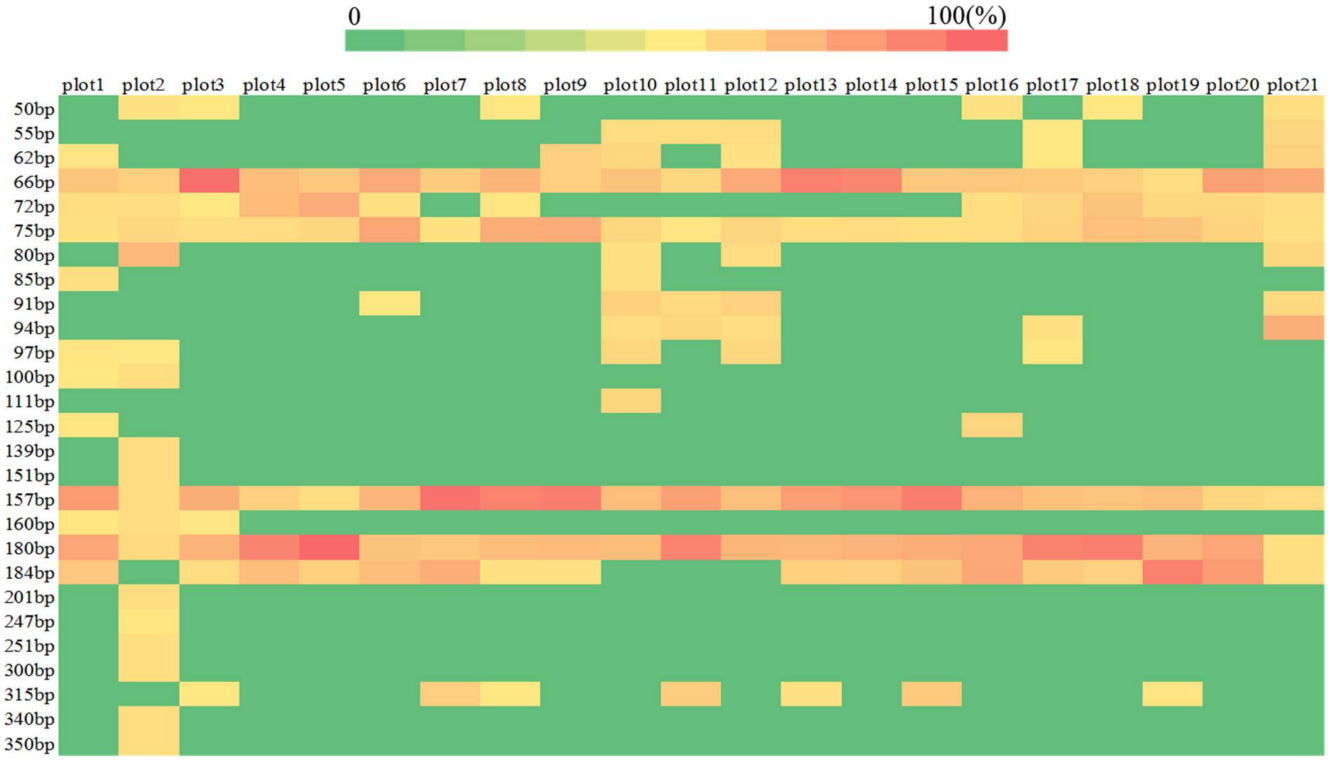

Figure 1. The average relative abundance of soil free-living nitrogen-fixing bacteria based on terminal restriction fragments (T-RFs, *Hae*III enzymes digestion) in different plot soils. The numbers of 1, 2, 3, . . . , 21 represented the plot1, plot 2, plot 3, . . . , plot 21, respectively.

3.2. Factors Influencing Soil Free-Living Nitrogen-Fixing Bacterial Community Structure in Karst Shrub Ecosystems

The low frequency of plants associated with symbiotic nitrogen-fixers was observed in 21 shrub plots (Table 1). Based on the correspondence analysis, the structure of the soil free-living nitrogen-fixing bacterial community varied among the 21 shrub soils (Figure 2). The Shannon diversity index ($F = 3.7$, $p = 0.002$) and richness index ($F = 2.9$, $p = 0.007$) for all plants; Shannon diversity index for plants associated with symbiotic nitrogen-fixers ($F = 2.0$, $p = 0.049$), aspect ($F = 2.6$, $p = 0.019$), ascent ($F = 2.3$, $p = 0.039$), and pH ($F = 2.1$, $p = 0.047$) contributed remarkably to soil free-living nitrogen-fixing bacterial community structure (Figure 3).

Variance partitioning showed that up to 43.9% of the variation in soil free-living nitrogen-fixing bacterial community structure was explained by pH, the richness index for all plants, Shannon diversity index for all plants and plants associated with symbiotic nitrogen-fixers, aspect, and ascent ($p < 0.05$; Figure 4). Plant type had a predominant effect on soil free-living nitrogen-fixing bacterial community structure, and topography (aspect and ascent), and soil pH had minor effects. Additionally, the direct contribution of the main factors to soil free-living nitrogen-fixing bacterial community structure was larger than their interaction effects.

A path analysis indicated that plant type positively influenced soil nitrogen-fixing bacterial community structure, while soil parameters negatively affected bacterial structure (Figure 5). Topography indirectly affected nitrogen-fixing bacterial community structure via plants and soil (Figure 5).

Table 1. Dominant plants and plants associated with symbiotic nitrogen-fixers from all sample plots.

Plot	Dominant Species	Plants Associated with Symbiotic Nitrogen-Fixers	Richness of all Plant	Shannon Diversity ofall Plant	Richness of N_2-Fixing Plant	Shannon Diversity of N_2-Fixing Plant
Plot1	*Croton lachnocarpus, Mahonia fortunei, Ficus tinctoria*	*Bauhinia hypochrysa, Dalbergia hancei*	42	4.69	2	0.6
Plot2	*Loropetalum chinense, Cyclobalanopsis glauca, Croton lachnocarpus*	*Bauhinia acuminata, Bauhinia hypochrysa, Pterolobium punctatum, Albizia odoratissima, Derris fordii*	56	4.91	5	0.67
Plot3	*Sinosideroxylon pedunculatum, Derris fordii, Chukrasia tabularis*	*Bauhinia hypochrysa, Pterolobium punctatum, Albizia odoratissima, Derris fordii*	50	4.95	4	0.5
Plot4	*Litsea coreana, Pyrus calleryana, Syzygium championii*	-	38	4.11	0	0
Plot5	*Viburnum triplinerve, Loropetalum chinense, Pyrus calleryana*	*Campylotropis delavayi, Sophora tonkinensis*	31	4.51	2	0.11
Plot6	*Pyracantha fortuneana, Rosa laevigata, Mallotus repandus*	*Bauhinia championii, Indigofera atropurpurea*	53	5.00	2	0.15
Plot7	*Pterolobium punctatum, Alchornea trewioides, Xylosma controversum*	*Bauhinia hypochrysa, Pterolobium punctatum*	68	5.52	2	0.56
Plot8	*Bauhinia hypochrysa, Millettia pachycarpa, Ficus tinctoria*	*Millettia pachycarpa, Bauhinia hypochrysa, Pterolobium punctatum*	61	5.20	3	0.73
Plot9	*Chaydaia rubrinervis, Sinoadina racemosa, Xylosma controversum*	*Bauhinia hypochrysa, Pterolobium punctatum*	61	5.45	2	0.42
Plot10	*Litsea coreana, Pittosporum tonkinense, Syzygium championii*	*Millettia pachycarpa, Bauhinia championii, Albizia odoratissima*	48	4.40	3	0.12
Plot11	*Loropetalum chinense, Pittosporum tonkinense, Syzygium championii*	*Indigofera atropurpurea*	34	4.43	1	0.13
Plot12	*Loropetalum chinense, Litsea coreana, Syzygium championii*	*Campylotropis delavayi, Albizia odoratissima*	40	4.78	2	0.14
Plot13	*Syzygium championii, Pyracantha fortuneana, Viburnum triplinerve*	*Millettia eurybotrya, Campylotropis delavayi*	49	5.21	2	0.09
Plot14	*Pittosporum tonkinense, Syzygium championii, Litsea coreana*	*Bauhinia championii, Indigofera atropurpurea, Campylotropis delavayi*	50	4.99	3	0.18
Plot15	*Litsea coreana, Pittosporum tonkinense, Itea chinensis*	*Bauhinia championii*	35	4.47	1	0.22
Plot16	*Loropetalum chinense, Pyracantha fortuneana, Pyrus calleryana*	*Albizia odoratissima, Gelsemium elegans*	41	4.74	2	0.21
Plot17	*Loropetalum chinense, Litsea coreana, Pyrus calleryana*	*Dendrolobium triangulare,*	40	4.36	0	0
Plot18	*Loropetalum chinense, Viburnum fordiae, Viburnum triplinerve*	-	47	4.57	0	0
Plot19	*Alangium chinense, Pyracantha fortuneana, Alchornea trewioides*	*Dendrolobium triangulare, Gelsemium elegans, Bauhinia championii*	61	5.09	3	0.28
Plot20	*Loropetalum chinense, Pyrus calleryana, Pyracantha fortuneana*	*Gelsemium elegans, Campylotropis delavayi*	36	4.46	2	0.25
Plot21	*Loropetalum chinense, Pyracantha fortuneana, Pistacia weinmannifolia*	*Pterolobium punctatum*	34	4.34	1	0.06

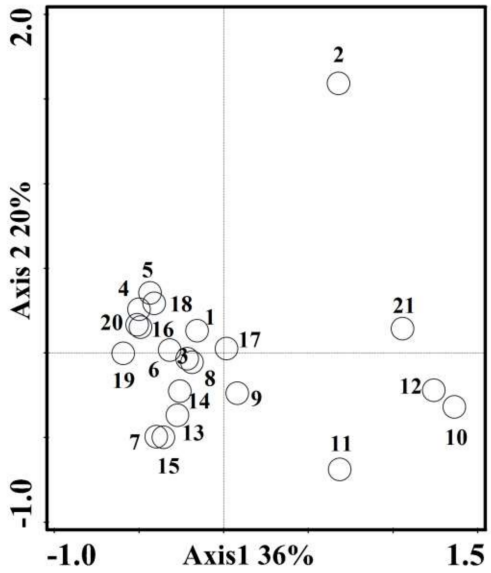

Figure 2. CA analysis of soil free-living nitrogen-fixing bacterial community structures in the shrub ecosystems based on T-RFLP profiles using *Hae*III enzymes. The numbers of 1, 2, 3, . . . , 21 represent the plot1, plot 2, plot 3, . . . , plot 21, respectively.

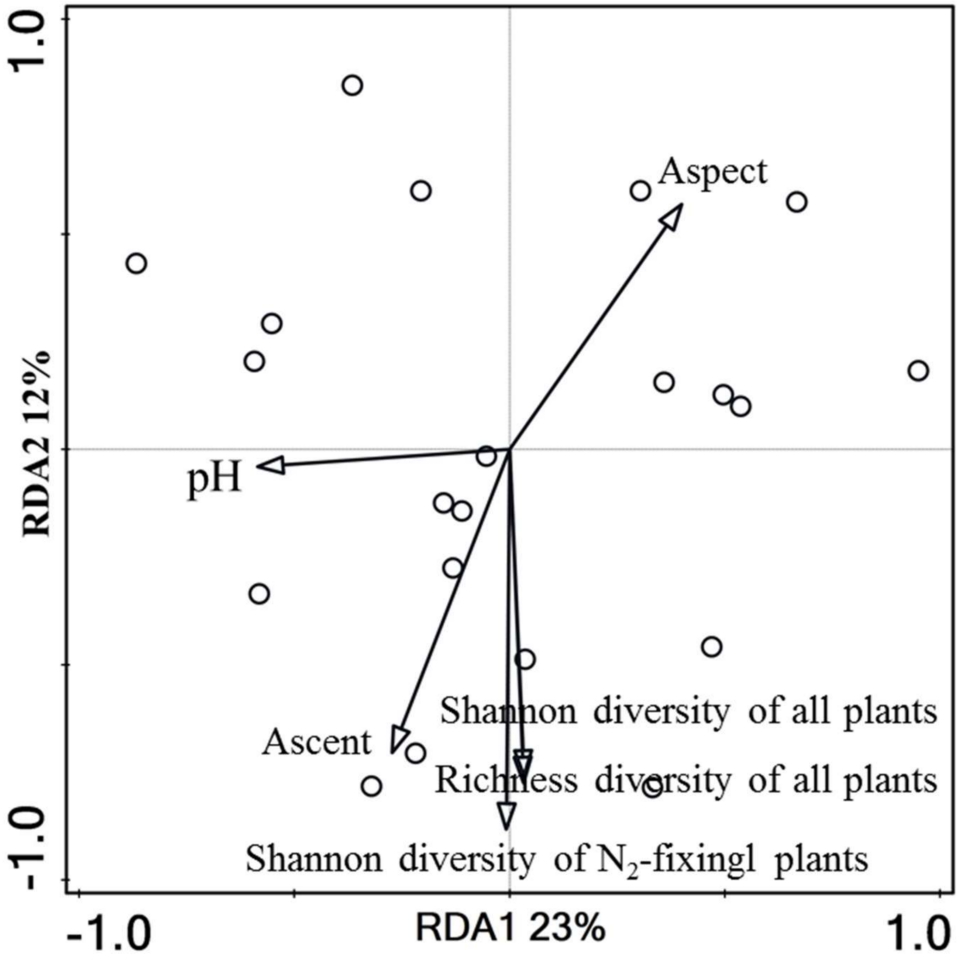

Figure 3. RAD analysis of environment factors influencing structures of soil free-living nitrogen-fixing bacteria in the shrub ecosystems. The bacterial distributions were shown in the figure based on T-RFLP profiles using *Hae*III enzymes.

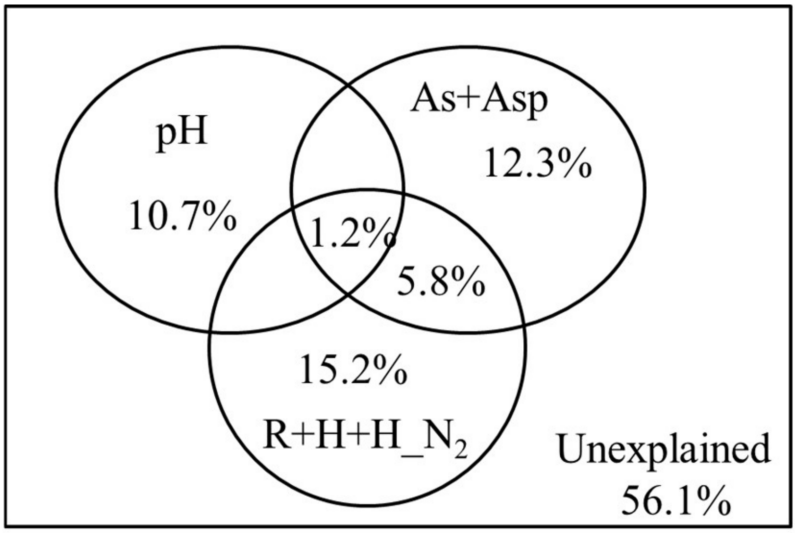

Figure 4. Topographical factor, plant and soil physicochemical properties of each significant parameter to the proportion of variation explained (%) for the community structure of soil free-living nitrogen-fixing bacteria by pH, all of plant richness index (R), all of plant Shannon diversity index (H), Shannon diversity index of plants associated with symbiotic Nitrogen-fixers ($H_{\_N2}$), aspect (Asp) and ascent (As).

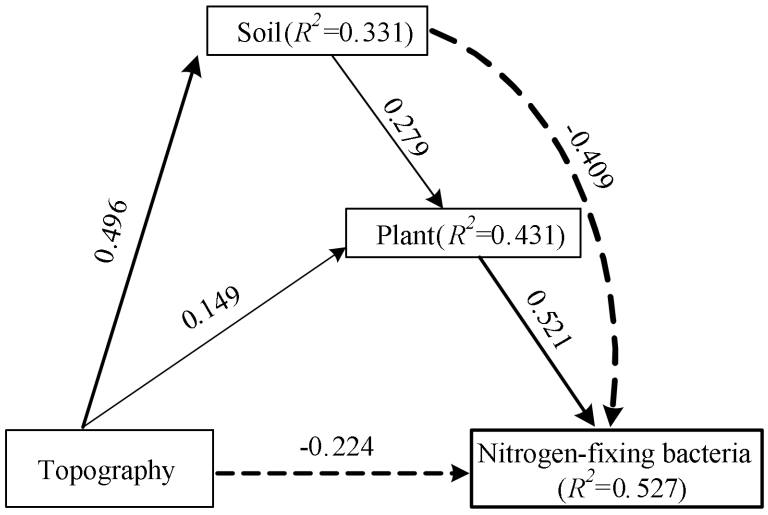

Figure 5. Path model analysis of plant, soil physicochemical parameters, and topography directly and indirectly affect the community structure of soil free-living nitrogen-fixing bacteria in the karst shrub ecosystems. Value in the arrow is a path coefficient and indicates the independent variable directly influencing the dependent variable.

3.3. Factors Influencing the Community Abundance of Free-Living Nitrogen-Fixing Bacteria in the Karst Shrub Ecosystems

The free-living nitrogen-fixing bacterial abundance ranged from 4.69×10^5 to 5.01×10^7 (Table 2). Significant negative relationships were observed between the abundance of nitrogen-fixing bacterial community and soil Olsen-P ($F = -0.561$, $p < 0.05$), pH ($F = -0.719$, $p < 0.01$), and the richness index for plants associated with symbiotic nitrogen-fixers ($F = -0.443$, $p < 0.05$; Table 3). This bacterial abundance was positively correlated with C/N ($F = 0.475$, $p < 0.05$), NH_4^+-N ($F = 0.574$, $p < 0.01$).

Table 2. Soil physicochemical properties and *nifH* gene abundance in all sample plots.

Plot	Olsen-P ($mg \cdot kg^{-1}$)	pH	Total Nitrogen ($g \cdot kg^{-1}$)	Soil Organic Carbon ($g \cdot kg^{-1}$)	C/N	NH_4^+-N ($mg \cdot kg^{-1}$)	NO_3-N ($mg \cdot kg^{-1}$)	*nifH* Gene Abundance
Plot1	7.16	7.6	12.53	120.7	9.63	34.23	4.55	3.80×10^6
Plot2	5.43	7.32	4.71	57.1	12.11	17.25	5.63	4.78×10^6
Plot3	5.59	7.38	8.77	87.42	9.97	29.53	4.87	4.07×10^6
Plot4	3.48	7.14	5.35	55.89	10.45	18.56	5.57	1.61×10^6
Plot5	5.34	7.44	4.12	65.16	15.81	23.24	4.98	5.29×10^5
Plot6	5.3	7.81	4.32	63.09	14.59	22.68	4.69	6.65×10^5
Plot7	4.78	7.92	4.46	58.73	13.17	24.84	6.45	4.04×10^6
Plot8	5.43	7.32	6.76	78.21	11.56	27.47	4.32	3.89×10^6
Plot9	4.02	7.88	3.99	57.54	14.42	19.54	5.86	1.51×10^6
Plot10	4.56	7.59	5.78	66.78	11.55	23.46	5.02	5.34×10^6
Plot11	6.44	7.8	5.56	67.94	12.23	23.52	5.45	2.27×10^6
Plot12	5.46	7.82	6.26	64.75	10.34	24.48	5.12	5.27×10^6
Plot13	6.57	7.98	4.93	71.45	14.5	24.89	4.86	1.32×10^6
Plot14	6.75	7.87	6.25	72.86	11.66	25.35	4.68	2.98×10^6
Plot15	6.24	7.83	6.35	75.53	11.89	26.46	4.56	2.42×10^6
Plot16	5.22	7.77	4.35	65.67	15.09	25.68	5.36	1.19×10^7
Plot17	3.74	7.27	3.76	56.5	15.01	18.56	5368	2.87×10^7
Plot18	2.79	6.52	2.54	45.37	17.84	46.36	5.87	5.01×10^7
Plot19	4.73	7.78	4.1	57.35	14	24.87	5.96	5.36×10^5
Plot20	3.71	7.95	3.16	50.09	15.86	16.78	6.54	4.98×10^6
Plot21	4.16	8.03	4.51	69.21	15.36	25.45	5.67	4.69×10^5

Table 3. Pearson correlations among diversity of all plants and plants associated with symbiotic N-fixers, soil physicochemical properties, free-living nitrogen-fixing bacterial abundance.

	***nifH* Abundance**	**Shannon Diversity Index of Plants Associated with Symbiotic N-Fixers**	**Richness Index of Plants Associated with Symbiotic N-Fixers**	**Shannon Diversity Index of all Plants**	**Richness Index of all Plants**	**C/N**	**SOC**	**TN**	**pH**	**AP**	**NO_3^--N**
NH_4^+-N	0.574 **	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NO_3^--N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-
AP	−0.516 *	ns	ns	ns	ns	−0.534 *	0.727 **	0.662 **	ns	-	
pH	−0.719 *	ns	ns	ns	ns	ns	ns	ns	-		
TN	ns	0.468 *	ns	ns	ns	0.796 **	0.686 **	-			
SOC	ns	ns	ns	ns	ns	−0.61 **	-				
C/N	0.475 *	ns	ns	ns	ns	-					
Richness index of all plants	ns	0.602 *	0.502 *	0.857 **	-						
Shannon diversity index of all plants	ns	0.601 *	0.509 *	-							
Richness index of plants associated with symbiotic N-fixers	−0.443 *	0.676 *	-								
Shannon diversity index of plants associated with symbiotic N-fixers	ns	-									
nifH abundance	-										

Note: ns means not significant; significance levels are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$.

4. Discussion

The genus *Bradyrhizobium* is dominant in karst shrub regions, although the T-RFLP technique is limited in identifying the taxon. This result is at odds with a previous study on the same karst region based on a high-throughput sequencing technique [32]. *Bradyrhizobium* is highly adaptable [33], thus it is widely distributed and dominates in the karst and non-karst regions [11,34]. Additionally, *Bradyrhizobium* could import nitrogen through symbiotic and non-symbiotic relationships with plants [11,34]. Therefore, understanding the role of *Bradyrhizobium* can provide a basis for alleviating nitrogen limitations in karst shrub ecosystems.

In the present study, soil free-living nitrogen-fixing bacterial community composition was related to the Shannon diversity and richness indices of the total plant community. The results were consistent with many previous studies [15,25,35]. Two possible reasons can explain this. Firstly, nitrogen-fixing bacteria can promote plant growth and maintain the productivity and diversity of plants by directly impacting nitrogen availability [36]. Secondly, higher plant diversity is accompanied by greater root exudates and leaf litter input into soils [37], which would increase diazotrophic diversity. Therefore, the feedback between plants and soil free-living nitrogen-fixing bacteria drives change for these bacteria and plant diversity in the karst shrub ecosystem. Besides plant diversity, plant functional groups (such as plants associated with symbiotic nitrogen-fixers and plants not associated with symbiotic nitrogen-fixers) could also influence soil free-living nitrogen-fixing bacterial communities. Growing plants can consume carbohydrates, which are mainly derived from photosynthesis. The fate of photosynthesized C is influenced by N input [38,39]. The contribution of photosynthesized C to SOC pools is closely related to plant species [40]. In natural ecosystems, the primary source of nitrogen is biological nitrogen fixation by legume–rhizobium symbiosis [5]. Inefficient N use due to too few N_2-fixing plant species in a given ecosystem is related to weakening photosynthetic capability by decreasing chlorophyll and Rubisco activity [41,42], both of which are involved in photosynthesis.

This would reduce carbon translocation from plants to belowground communities and rhizodeposition, resulting in limited plant growth. In the present study, a negative correlation between the richness of plants associated with symbiotic nitrogen-fixers and free-living nitrogen-fixing bacterial abundance was observed in the karst shrub ecosystem. Few plants associated with symbiotic nitrogen-fixers existed in the karst shrub ecosystem, and thus provided less nitrogen and carbon input for plant growth. In this condition, soil free-living nitrogen-fixing bacterial abundance increased and enhanced nitrogen/carbon input. The plant growth would be promoted by nitrogen and carbon transfer from root-to-root contact or mycorrhizal networks. Therefore, the importance of soil free-living nitrogen-fixing bacteria as a pathway of nitrogen and carbon translation from plants to soil was observed in the karst shrub ecosystem, suggesting these bacteria play key roles in the vegetation restoration of the karst region.

Soil physicochemical properties also influence soil free-living nitrogen-fixing bacterial communities [17,35,43]. Among the measured soil physicochemical parameters, pH was the most significant factor influencing the composition and abundance of soil free-living nitrogen-fixing bacteria in the present study, consistent with previous reports [34,43]. Soil pH influences microbes by affecting the pH homeostasis of microbial cells or regulating soil nutrient availability. Therefore, soil pH might exert stress indirectly on free-living nitrogen-fixing bacteria. Soil pH ranged from 6.5 to 8.0 in the present study, and other physicochemical conditions varied, thus shaping soil free-living nitrogen-fixing bacterial community structures. The result was in agreement with a previous study [43], in which soil pH ranged from 5.08 to 5.53 and was much narrower than the range of soil pH values in our study. Additionally, soil C/N was also a key factor affecting soil free-living nitrogen-fixing bacterial abundance. Soils with low C/N have high rates of nitrogen mineralization [44] and provide more nutrients for soil free-living nitrogen-fixing bacterial use.

Topography plays a key role in the distribution of microbial communities. This may be explained by the effects of topography on water distribution, leaching infiltration,

and runoff potential [45,46], as well as its effects on the erosion and redistribution of fine soil particles and thus the partial redistribution of plants. Therefore, topography indirectly influences microbial communities mainly by affecting soil physicochemical properties [47,48] and plant communities [49–51]. This is partially consistent with our previous research reporting that the composition and abundance of soil free-living nitrogen-fixing bacteria are strongly influenced by differences in soil pH and plant diversity among topographies. Karst is characterized by a distinctive topography, the action of acidic water on carbonate bedrock, steep geological features, leading to a distinctive composition of soil free-living nitrogen-fixing bacteria. High-throughput sequencing will be used to identify the taxa of soil free-living nitrogen-fixing bacteria in the future.

Additionally, the unexplained variation in soil free-living nitrogen-fixing bacterial community structure may be explained by factors that were not considered in this study. For example, rock exposure is greater in the karst region than in other regions. The exposed rocks can form different rain-funneling structures. These rain-funneling structures can produce heterogeneous soil nutrients from rocks to open soil [52] and thereby affect the soil free-living nitrogen-fixing bacterial community structure.

Previous studies have shown that soil free-living nitrogen-fixing bacterial community structure can be shaped by multiple environmental conditions, including soil parameters [20], geography [19], and plant type [15]. The positive effect of plant type on soil nitrogen-fixing bacterial community structure is found in the present study, while soil parameters negatively influence these bacteria. This result is not in agreement with a previous study on the karst region by Xiao et al. [32]. They have reported that the effect of plant species on soil dominant nitrogen-fixing bacterial taxa is little compared with soil property. The possible reason for this is that the study by Xiao et al. [32] is based on an artificial experiment, and only two leguminous plants were introduced. It is interesting that soil free-living nitrogen-fixing bacterial abundance is negatively corrected with the richness index for plants associated with symbiotic nitrogen-fixers. According to our present study result, a lower frequency of plants associated with symbiotic nitrogen-fixers was observed. This indicated that nitrogen fixation by soil free-living nitrogen-fixing bacteria was the most important nitrogen source for karst shrub ecosystems. Thus, these bacteria play an important role in relieving the nitrogen-limited karst shrub ecosystem. Except for plants, soil parameter, i.e., soil molybdenum content, has a greater effect on soil nitrogen-fixing bacterial communities [53]. This could explain why nearly half of the observed variation in the free-living nitrogen-fixing bacteria community structure has not been explained by the environmental variables measured in this study. Additionally, the effect of a single parameter (i.e., plant species) on soil free-living nitrogen-fixing bacterial community structures was greater than their interaction effects, similar to a previous study of microbial community structure [54]. This may mean the dominant genus *Bradyrhizobium* in our study could form a symbiotic association with leguminous plants [11].

5. Conclusions

This study was a large-scale investigation of soil free-living nitrogen-fixing bacterial communities in the karst shrub ecosystems of southwestern China. The genus *Bradyrhizobium* is dominant in the karst shrub ecosystems. The topography, plant type, and soil pH were key factors shaping the soil free-living nitrogen-fixing bacterial community structure. This is the first step toward the quantitative evaluation of the relative contributions of environmental parameters to the observed distribution of these bacteria. The environmental factors examined in this study accounted for approximately half of the observed variations of soil free-living nitrogen-fixing bacterial community structure. Plant type and topography were particularly important factors for the soil free-living nitrogen-fixing bacterial distribution in the karst shrub ecosystems of southwestern China. The important sources of fixed nitrogen by soil free-living nitrogen-fixing bacteria as a pathway of restoration vegetation in the karst regions is clearly demonstrated by the negative correlation

between soil free-living nitrogen-fixing bacterial abundance and the richness index for plants associated with symbiotic nitrogen-fixers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13020163/s1, Table S1: Plot basic information.

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