Full Paper

Effect of *Ageratina adenophora* invasion on the composition and diversity of soil microbiome

(Received June 26, 2016; Accepted August 10, 2016; J-STAGE Advance publication date: February 24, 2017)

Yunhong Kong,¹ James Kong,² Dingkang Wang,¹ Heping Huang,³ Kaiyou Geng,³ Yonxia Wang,⁴ and Yun Xia^{3,*}

¹ Key Laboratory of Special Biological Resource Development and Utilization of Universities of Yunnan Province, Kunming University, Kunming, China

² Department of Computer Science, Waterloo University, Waterloo, Canada

³ Agricultural School, Kunming University, Kunming, China

⁴ Microbiology Institute of Yunnan University, Kunming, China

In the present study, high throughput 16S rRNA gene sequencing was used to investigate soil invaded by the aggressive weed *Ageratina adenophora* to determine its effect on the species composition, distribution, and biodiversity of the bacterial communities. Soil samples from 12 micro-sites containing a monoculture of *A. adenophora* plants, mixtures of *A. adenophora* and different native plant species, and native species alone were studied. We found that the invasion of this weed resulted in a selection of bacteria belonging to phyla *Acidobacteria* and *Verrucomicrobia* and the lack of bacteria belonging to phyla *Actinobacteria* and *Planctomycetes*, but did not affect significantly the percentage abundances of members of other phyla. A similar bacterial population selection was also observed at genus or subgroup levels. The NO₃⁻-N level was an important factor affecting soil bacterial communities and contributed to the dominance of *A. adenophora*. However, the numbers of total bacterial species, and the diversity and structure of soil bacterial microbiome did not ($P > 0.05$) change significantly following invasion by this weed.

Key Words: *Ageratina adenophora*; high throughput sequencing; undersurface soil microbiome; weed invasion

Introduction

Ageratina adenophora (Sprengel) (Synonym: *Eupatorium adenophorum* Sprengel), a notorious weed originat-

ing in Mexico and Costa Rica, has now invaded more than 30 tropical and subtropical countries and regions (Xie et al., 2001). It has spread throughout much of Southwestern China, including Yunnan, Guizhou, Sichuan, Guangxi, Xizang provinces and Chongqing (Xie et al., 2001), and is continuing to spread eastwards and northwards at an estimated 20 km per annum (Wang and Wang, 2006). *A. adenophora* replaces native crops and trees by outcompeting them for the available fertilizer, water, sunlight and space, and very rapidly becomes the single predominant plant species (McGeoch et al., 2010). Its dominance leads to the destruction of local nutritional cycles, hydrological conditions, and the energy budget, causing severe economic losses to the agriculture, forestry and livestock industries. It also negatively impacts on the ecology of the native habitat. This weed is responsible for an annual loss of 150 million US dollars from livestock production decline and 400 million US dollars from the service function of grassland ecosystems in China (Xu et al., 2006).

Existence of a positive interaction between *A. adenophora* and its associated rhizosphere microbiome is speculated to play a key role in its successful establishment and dominance in its invasion of native habitats (Yu et al., 2014). Once an area is invaded, it seems likely that *A. adenophora* alters the structure of its rhizosphere microbiome such that its growth is favored and native plant species are displaced (Wan et al., 2010). Bacteriostatic effects of its root exudates and associated plant litter have been confirmed (Liu et al., 2010) and many antibacterial chemicals have been identified there (Zhang et al., 2013). Previous studies suggest that its colonization of soil increases levels of soil bacteria and fungi, and the structure of the soil microbiome is altered (Li et al., 2011; Yu et al.,

*Corresponding author: Yun Xia, Agricultural School, Kunming University, Kunming, China.

Tel: 86 871 65098537 E-mail: xiayun22@hotmail.com

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

2014). Consequently, the levels of available P, K, NO_3^- , NH_4^+ and organic C in the affected region are substantially higher than those in non-invaded soils. However, most of these data come from using culture-dependent microbiological techniques. It is well known that less than 0.1% of soil (Hill et al., 2000) and 10% of rhizosphere bacteria (Hirsch et al., 2010) can be recovered as pure cultures. The limitation of these culture-dependent techniques is such that meaningful information about the diversity and population composition of the soil microbiome located in close proximity to *A. adenophora* is still lacking. Furthermore, it remains unclear to what extent invasion of *A. adenophora* may affect the native soil microbiome. We believe this information is an essential first step in understanding interactions between *A. adenophora* and its immediate soil microbiome, before proposing new measures to control this weed.

In recent years, as a result of the accelerated advances in sequencing technology, metagenomic analysis has been increasingly used in the study of the interaction of soil-plant-microorganisms. Soil metagenomics of the 16S rRNA gene and/or the ITS1 region, using next generation sequencing technologies (Niedringhaus et al., 2011), has revealed an unsurpassed diversity of soil microbiota (Nesme et al., 2016). Soil high-throughput sequencing, combined with soil physicochemical analysis, has been successfully used to investigate the effects of soil pH (Zhalnina et al., 2014), plant type (Prober et al., 2014) and crop rotation (de Quadros et al., 2012), on soil microbial diversity and composition. In this study, we have investigated the composition and diversity of the microbiome in soil invaded by *A. adenophora* using 16S rRNA gene high throughput sequencing to assess the possible influence of the invasion of *A. adenophora* on the composition and diversity of the soil microbiome. Also, the key factors controlling the soil bacterial microbiota invaded by *A. adenophora* have been examined using redundancy analysis.

Materials and Methods

Site description and sampling. The study site was situated in a foothill of Maomao Qing, Xishan, Kunming (24°55'29" N, 102°36'36" E), where the invasion of *A. adenophora* began in 1987. It had an obvious gradient of *A. adenophora* invasion intensity with vegetation consisting of *A. adenophora* as monoculture, a mixture of *A. adenophora* (40–50% coverage) and native species *Elsholtzia ciliata*, *Artemisia indica* and *Setaria plicata*, and native species only. The foothill has an area of ca. 400 × 400 m² with a mean altitude of 2170 m (from 2150 to 2190 m) without tree coverage. The study site has a mountain red soil and an average annual temperature of 15.2°C (from 14.3–16.1°C), and average annual precipitation of 975 mm (from 937–1058 mm). To investigate the effects of *A. adenophora*'s invasion on the soil microbiome, four micro-site samples were collected in May 2015 from each zone, with *A. adenophora* as a monoculture, a mixture of *A. adenophora* and native species *Elsholtzia ciliata*, *Artemisia indica* and *Setaria plicata*, and native species only. The sample micro-sites

were spaced by at least 2 m intervals and located at the same altitude. In total, twelve soil samples were collected. Before soil collection, all surface vegetation and litter layers were removed with a shovel, which was thoroughly cleaned with 70% ethanol after each sampling. At each micro-site, soil samples were collected at a depth of 0–10 cm and 10–20 cm. The collected soil samples were wrapped in autoclaved foil, placed into sterile Whirl-Pak sample bags (Nasco, Fort Atkinson, WI, USA), and transported to the laboratory within 1 h. About a 500 g subsample of each soil sample was homogenized (medium treatment for 1 min) in a Waring heavy-duty blender (Lab-Biogen, Kunming, China) and passed through a 2-mm sieve. A 250 g subsample collected at a 0–10 cm depth was mixed with the same amount of subsample collected at a 10–20 cm depth at the same micro-site (by the same blender, medium treatment for 1 min) and the mixture was used for physicochemical analysis.

Physicochemical analysis of soil samples. Soil organic matter composition was determined using the $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 oxidation method (Nelson and Sommers, 1996). The total phosphorus nitrogen was measured using the Kjeldahl method. Soil inorganic N was extracted with 2 mol KCl, and concentrations of NO_3^- -N and NH_4^+ -N in the KCl extracts were determined with hydrazine sulfate colorimetry and indophenol blue colorimetry, respectively (Mulvaney, 1996). The total phosphorus and available phosphorus were determined using the colorimetric Moblue-method (Kuo, 1996). The pH value (1:2.5 solution of soil to water) was measured using a pH meter (Mettler-Toledo International Inc., China).

DNA extraction, PCR amplification and illumina sequencing. Soil DNA was extracted using a PowerSoil DNA Isolation Kit (Anbiosci, Shenzhen, China) according to the manufacturer's protocol. The same amount of DNA extracted from a 0–10 cm and 10–20 cm depth from the same micro-site was mixed, and the mixture was used for downstream analyses. The crude DNA was purified on 0.8% (wt/vol) low melting point agarose gel in a TAE buffer to obtain genomic DNA. The DNA bands were excised, extracted with QIAquick Gel Extraction Kits (Qiagen Inc., Mississauga, ON, Canada), and then used as the template for PCR amplification. The V4 hypervariable region of bacterial 16S rRNA gene (Biddle et al., 2008) was amplified using 2 × KAPA HiFi HotStart ReadyMix (Shanghai Dobio, Shanghai, China) with the primer set: 341F (CCTACGGGNGGCWGCAG) (Muyzer et al., 1993) and 785R (GACTACHVGGGTATCTAATCC) (Lee et al., 1993) with the following PCR conditions: initial denaturation at 94°C for 3 min followed by 5 cycles of denaturing at 94°C for 10 s, annealing at 55°C for 15 s and extension at 72°C for 30 s before a final extension at 72°C for 5 min. The 16S rRNA gene amplicons obtained were attached after cleanup with AMPure XP beads to dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina China, Shanghai) with the following PCR (also prepared with 2 × KAPA HiFi HotStart ReadyMix) condition: initial denaturation at 95°C for 30 s followed by 8 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s,

Table 1. physiochemical characterization of soil invaded by *Ageratina adenophora* at different degrees.

Site description	pH	Organic C (mg Kg ⁻¹)	NH ₄ ⁺ -N (mg Kg ⁻¹)	NO ₃ ⁻ -N (mg Kg ⁻¹)	Total N (%)	P (mg Kg ⁻¹)	K (mg Kg ⁻¹)
Monoculture ^a 1	5.04	78.1	15.92	5.42	0.28	7.7	278
Monoculture 2	5.28	73.6	14.85	6.10	0.24	7.8	295
Monoculture 3	5.49	81.5	20.33	4.28	0.20	7.4	250
Monoculture 4	5.42	67.0	17.34	5.99	0.28	6.5	258
Mixed-species ^b 1	5.83	50.7	14.09	5.31	0.20	6.7	299
Mixed-species 2	6.02	52.7	12.57	5.11	0.25	6.1	241
Mixed-species 3	6.33	55.0	24.78	4.34	0.21	5.6	237
Mixed-species 4	5.62	62.2	23.04	3.41	0.29	7.5	214
Native-species ^c 1	6.18	47.6	17.96	3.12	0.27	5.4	251
Native-species 2	5.28	50.5	20.71	2.27	0.36	5.6	246
Native-species 3	5.99	51.4	18.06	3.65	0.41	6.8	243
Native-species 4	6.73	57.7	18.24	3.83	0.22	5.5	279

^{a,b,c} represent the soil micro-sites with three aboveground vegetation types: ^a *Ageratina adenophora* as monoculture, ^b a mixture of *Ageratina adenophora* and native plant species, and ^c only native species.

Table 2. Biodiversity index and similarity of bacterial communities in soils invaded by *Ageratina adenophora* at different degrees.

Sample description	Number of raw sequences	Number of HQ* sequences	Number of OTUs	Cho1 index	Shannon index	Coverage
Monoculture ^a 1	26,860	26,598	3,670	5,814	6.573	0.92
Monoculture 2	30,728	30,449	3,749	6,045	5.932	0.93
Monoculture 3	30,019	29,794	4,208	6,970	6.491	0.92
Monoculture 4	23,196	22,978	2,992	5,144	5.613	0.92
Mixed-species ^b 1	31,840	31,473	3,157	4,991	5.530	0.94
Mixed-species 2	25,944	25,787	3,663	6,393	6.372	0.91
Mixed-species 3	36,396	36,164	4,843	7,606	6.478	0.92
Mixed-species 4	35,036	34,868	4,545	6,737	6.894	0.93
Native-species ^c 1	33,067	32,939	4,263	5,928	6.944	0.93
Native-species 2	24,313	24,169	3,771	6,105	6.557	0.91
Native-species 3	24,966	24,808	3,870	6,109	7.073	0.91
Native-species 4	26,744	26,589	3,379	5,189	6.640	0.93

*HQ represents high quality.

^{a,b,c} represent the soil micro-sites with three aboveground vegetation types: ^a *Ageratina adenophora* as monoculture, ^b a mixture of *Ageratina adenophora* and native plant species, and ^c only native species.

before a final extension at 72°C for 5 min. The PCR was done in triplicate for each sample and PCR products were again cleaned up with AMPure XP beads, quantified using an Agilent Technologies 2100 Bioanalyzer, normalized and pooled. Then, the 16S rRNA gene amplicons obtained were diluted to 4 nM with 10 mM Tris (pH 8.5) before being denatured with NaOH and sequenced (with 5% PhiX as an internal control) on a MiSeq Illumina Platform (PE250) at Genergy Biotechnology (Shanghai, China).

Phylogenetic analyses. The V4 amplicons of 16S rRNA genes were pair-end assembled and checked using the software Flash (Magoc and Salzberg, 2011) to ensure that their sequences perfectly matched in the index sequences, had no more than one 1 mismatch error present in the forward primer sequences, and the trimmed sequences were longer than 200 bp. Trimmed high-quality sequences were uploaded into QIIME (Caporaso et al., 2010) to perform OTU clustering through the ‘pick_otus_through_otu_table.py’ pipeline using the 97% identity threshold (3% dissimilarity levels), and to carry out alpha diversity analyses. The OTUs grouped were annotated by using RDP classifier (Cole et al., 2014). Any OTU represented by ≤3 sequences was removed. The 16S rRNA amplicon sequences

have been deposited in the NCBI Sequence Read Archive under the following accession number SAMN04251321: Agtkyh2015 (TaxId: 410658). Biodiversity indices, including the Cho1 index, Sharron index, coverage ratios, and Venn figures, were calculated with Mothur (Schloss et al., 2009) by following the procedures provided, again applying a 97% identity threshold. Phylogenetic trees showing distance and OTU abundances of the different taxa were constructed with MEGAN4 (Huson et al., 2011) following the procedures recommended by the provider. A heatmap for showing the clustering of sequences and OTU abundances for individual genera was calculated and constructed with the software Cluster 3.0 (de Hoon et al., 2004).

Redundancy analysis. Redundancy analysis (RDA) was carried out to investigate the relationships between environmental variables and bacterial community. To test whether RDA analysis was appropriate for the dataset, the data were first tested for normality. Detrended correspondence analysis (DCA) was used first to determine the character of variability in the assemblages using the following criterion: if the length of the first gradient was greater than 3 standard deviations (SD) we assumed a unimodal variation, and a length less than 3 SD indicates a linear

variation. The length of the first gradient calculated for the bacterial communities was 0.7 SD, indicating a linear variation and justifying redundancy analysis. Statistical calculations were performed using CANOCO for Windows 4.5 software. The significance of the relationship was assessed using Monte Carlo permutation tests provided in the software. The significance of the first ordination and canonical axes was assessed in permutation tests with 999 unrestricted Monte Carlo permutations ($P < 0.05$).

Statistical analysis. Statistical analyses were carried out using a Student *t*-test, and, when necessary, normalization of data to be compared was carried out in SPSS (version 16.0). The significance was declared at $P < 0.05$.

Results

Soil physiochemical properties

The invasion of *A. adenophora* affected the physiochemical characters (Table 1) of the colonized soil. The pH values of micro-sites with a monoculture of *A. adenophora* (abbreviated as monoculture micro-sites hereafter) were lower ($P = 0.01$) than those occupied by native species (abbreviated as native-species micro-sites hereafter). The former contained more organic C than both the latter ($P = 0.001$) and the micro-sites occupied by mixtures of *A. adenophora* and native species (abbreviated as mixture micro-sites hereafter) ($P = 0.002$). Soil NO_3^- -N and available phosphorus levels at the monoculture micro-sites were higher than those at the native-species micro-sites (P values of 0.01 and 0.01, respectively). No significant differences ($P > 0.05$) in soil NH_4^+ -N, available K and total N were found between these three sites.

Effect of invasion of *A. adenophora* on biodiversity and composition of soil bacterial microbiome

16S rRNA amplicon sequences (Table 2) retrieved from the 12 micro-sites colonised with the three vegetation types (four replicas for each) were used to investigate any effect the invasion of *A. adenophora* might have on the composition of each local soil microbiome. High quality sequence reads of 22,978 to 36,164 were obtained from individual samples taken from these 12 micro-sites. Phylogenetic analyses showed they belonged to between 2992 and 4843 OTUs. Chao1 analyses (Table 2) estimated that these site communities individually contained 4991 to 7606 OTUs with a coverage range of 0.91 to 0.94.

In total, 10,758 OTUs were identified. Their distributions in different genera of bacterial communities of the 12 micro-sites are shown in Table S1. The bacterial communities at the monoculture micro-sites contained more species (8715 OTUs) than those at both the mixed-species micro-sites (7408 OTUs) and the native-species micro-sites (7314 OTUs) (Fig. 1). Vegetation type did not affect significantly ($P > 0.05$) the numbers of OTUs detected at the four micro-sites (Table 2). The bacterial communities at the monoculture micro-sites shared 6202 OTUs with those at the mixed-species micro-sites, which is higher than those (5799 OTUs) shared with OTUs at the native-species micro-sites. The bacterial communities at the mixed-species micro-sites shared 4456 OTUs with those at the native-species micro-sites. Communities from

Fig. 1. Venn diagram produced using 16S rRNA gene sequences obtained in this study.

The Venn diagram is produced with soft Mothur to show the numbers of OTUs of bacterial communities in soil with *A. adenophora* as a monoculture (abbreviated as Monoculture), a mixture of *A. adenophora* and native plant species (abbreviated as Mixed-species) and only native plant species (abbreviated as Native-species), respectively, and the numbers of OTUs shared among the bacterial communities with different vegetation types.

soils colonised with the three different vegetation types shared 3778 OTUs. No significant differences in the Shannon indices of these microbial communities were determined.

Bacterial OTUs belonging to members of 18 phyla were identified from these 12 micro-sites (Fig. 2). In descending average percentage abundances, they represent members of the phyla *Acidobacteria* (22.4%), *Proteobacteria* (14.6%), *Verrucomicrobia* (9.3%), *Actinobacteria* (6.2%), *Bacteroidetes* (6.1%), *Planctomycetes* (3.7%), *Gemmatimonadetes* (0.8%), *Chloroflexi* (0.43%), *Firmicutes* (0.40%), OD1 (0.21%), *Chlamydiae* (0.20%), TM7 (<0.01%), *Nitrospira* (<0.01%), WS3 (<0.01%), OP10 (<0.01%), BRC1 (<0.01%), *Cyanobacteria* (<0.01%) and *Spirochaetes* (<0.01%). Unclassifiable bacteria contributed an average of 33.6% to the total bacterial OTUs. The orders and families identified for each of the main phyla, their relative abundances, and their phylogenetic relationship are shown in Fig. S1.

Invasion of *A. adenophora* affected the relative abundances of members of these phyla. Thus, bacterial communities at the monoculture micro-site soils contained higher abundances of members of *Acidobacteria* [34.7(±6.2)% cf. 22.0(±4.6)%, $P = 0.008$] and *Verrucomicrobia* [26.9(±10.0)% cf. 11.4(±6.4)%, $P = 0.01$], but fewer members of *Actinobacteria* [3.2(±0.6)% cf. 6.7(±0.4)%, $P = 0.002$], *Planctomycetes* [1.3(±0.3)% cf. 3.1(±0.7)%, $P = 0.01$] and *Bacteroidetes* [3.0(±2.7)% cf. 11.68(±7.7)%, $P = 0.08$], than those in soils at the native-species micro-sites. No significant differences in the percentage abundances of members of the phyla *Proteobacteria* [11.8(±2.2)% cf. 15.8(±3.7)%, $P = 0.11$] were found between the microbial communities at the different vegetation sites.

Fig. 2. Phylum composition of bacterial communities at 12 soil micro-sites invaded by *A. adenophora* at different densities.

Monoculture, Mixed-species and Native-species represent surface vegetation with *A. adenophora* as a monoculture, a mixture of *A. adenophora* and native plant species, and only native plant species, respectively.

Impacts of this invasion on the soil microbiome are also apparent at the genus and group levels. Of the OTUs identified for each microbiome at the 12 micro-sites (Table 2), 29–81% could be classified reliably at the genus level. These belong to 159 genera, and their distribution and relative abundances in the different bacterial communities are shown in Fig. S2. Of these, 34 genera are assessed to be relatively abundant, with each accounting for at least an average of 0.25% of the total OTUs detected per micro-site, and they are listed in Table S1.

OTU community distributions varied in soil samples taken from the different vegetation types. Thus, percentage abundance values for *Bradyrhizobium* at the *A. adenophora* sites were higher than those in soils at the native-species [7.8(±1.3)% cf. 2.8(±0.7)%, $P = 0.03$] and mixed-species sites [7.8(±1.3)% cf. 5.3(±0.7)%, $P = 0.01$]. Similarly, percentage abundance values of *Singulisphaera* [2.0(±0.6)% cf. 0.6(±0.3)%, $P = 0.01$], *Phenylobacterium* [2.1(±1.9)% cf. 0.7(±0.2)%, $P = 0.01$], *Prosthecomicrobium* [0.6(±0.3)% cf. 0.2(±0.1)%, $P = 0.05$] and *Ktedonobacter* [2.9(±4.0)% cf. 0.2(±0.1)%, $P = 0.05$], at the *A. adenophora* sites also exceeded those at the native-species sites. In contrast, the *A. adenophora* soil communities contained fewer members of *Nocardioides* [0.8(±0.2)% cf. 7.3(±2.1)%, $P = 0.001$], *Terrimonas* [1.0(±0.5)% cf. 4.8(±3.5)%, $P = 0.05$], *Flavobacterium* [0.1(±0.01)% cf. 4.1(±3.9)%, $P = 0.05$], *Solirubrobacter* [0.3(±0.2)% cf. 3.1(±1.1)%, $P = 0.001$], *Pirellula* [0.4(±0.3)% cf. 2.6(±0.8)%, $P = 0.001$], *Sterohidobacter* [0.3(±0.2)% cf. 1.1(±0.1)%, $P = 0.02$], *Adhaeribacter* [0.1(±0.01)% cf. 1.7(±0.9)%, $P = 0.001$], *Pseudonocardia*

[0.3(±0.1)% cf. 1.3(±1.0)%, $P = 0.01$], *Nitrospira* [0.2(±0.2)% cf. 0.8(±0.3)%, $P = 0.02$], *Lysobacter* [0.1(±0.03)% cf. 0.7(±0.2)%, $P = 0.001$], *Marmoricola* [0.1(±0.09)% cf. 0.6(±0.4)%, $P = 0.03$], and *Arthrobacter* [0.2(±0.1)% cf. 0.5(±0.2)%, $P = 0.001$], than those at the native-species micro-sites. However, the percentage values of members of the genera *Opitutus*, *Gemmatimonas*, *Kribbella*, *Conexibacter*, *Mycobacterium*, *Mucilaginibacter*, *Gemmata*, *Planctomyces*, *Variovorax*, *Zavarzinella*, *Sphingosinicella*, *Chitinophaga* and *Solitalea* were not significantly different in soil samples taken at the 3 different vegetation sites.

Acidobacteria and *Verrucomicrobia* members were highly abundant in all examined soil samples. In total, 2,404 acidobacterial OTUs were identified belonging to subgroups GP1–GP7, GP10, GP11, GP13, GP15–GP18, GP20, GP22 and GP25. Of these, subgroups GP1–GP6 were relatively abundant, with each accounting for an average of at least 0.26% of the total OTUs per micro-site. The communities at the *A. adenophora* micro-sites contained more members of GP1 [7.7(±0.8)% cf. 1.9(±0.5)%, $P = 0.001$], GP2 [4.4(±1.2)% cf. 0.6(±0.4)%, $P < 0.001$], GP3 [1.5(±0.2)% cf. 0.6(±0.2)%, $P < 0.001$] and GP6 [7.7(±0.8)% cf. 1.9(±0.5)%, $P < 0.001$], but fewer members of GP4 [2.0(±0.2)% cf. 2.6(±0.4)%, $P = 0.04$] and GP5 [0.5(±0.1)% cf. 0.7(±0.1)%, $P = 0.04$], than those at the native-species micro-sites. A total of 882 verrucomicrobial OTUs were retrieved. Most belonged to unclassified *Spartobacteria*_genera_incentae_sedis (359 OTUs) and subdivision 3_incentae_sedis members (343 OTUs). Communities at the *A. adenophora* micro-sites

Fig. 3. Redundancy analysis figure produced with CANOCO 4.5 to reveal the relationship between the composition of bacterial community in soils invaded by *A. adenophora* at different densities and environmental variables.

The environmental variables examined and the bacterial phyla are shown as red arrows and blue arrows, respectively. The vector orientations represent the correlation between bacterial community and environmental variables, and the vector lengths represent the relative importance of individual factors examined. Thus, a positive correlation between two factors is expressed as relatively long vectors pointing approximately in the same direction, whereas a negative correlation is indicated by arrows pointing in the opposite direction. The longer the vector of an environmental variable, the stronger is its relationship with the bacterial community. The larger the angle among the arrows, the smaller the correlation.

contained more *Spartobacteria_genera_incertae_sedis* species than those at the native-species micro-sites [$2.3(\pm 0.4)\%$ cf. $1.4(\pm 0.2)\%$, $P = 0.03$], but about the same abundances of Subdivision 3_incertae_sedis species [$1.7(\pm 0.4)\%$ cf. $1.7(\pm 0.2)\%$, $P = 0.6$].

Clustering analyses showed that no clear patterns (Fig. S2) could be recognized for the communities at the four micro-sites with the same vegetation type, or among the 12 communities in soil colonised by the different vegetation types. Thus, differences in vegetation type did not appear to influence significantly the structures of their soil microbiomes.

Relationship between environmental factors and composition of bacterial communities

RDA was applied to reveal the relationship between the bacterial community and environmental factors. The environmental factors in the first two RDA axes, respectively,

explained 85.3% and 7.1% of the total variance on bacterial community (Fig. 3). Soil NO_3^- -N ($P = 0.0010$, $F = 5.41$, 999 Monte Carlo permutations) significantly correlated with the abundance of bacterial communities, indicating that it was the important determinant to bacterial community. Soil pH had a tendency ($P = 0.0910$, $F = 1.83$, 999 Monte Carlo permutations) to contribute to the composition of bacterial communities, while soil available P and K, TN, TOC, NH_4^+ -N and temperature did not show any significant community-environment relationship with P values of 0.1680, 0.7100, 0.2910, 0.2330, and 0.4550, respectively, showing a lesser importance in affecting the composition of bacterial communities.

Discussion

In this study, we have used high-throughput 16S rRNA amplicon sequencing to investigate the composition and

diversity of soil bacterial communities invaded by the aggressive weed *A. adenophora* at different population densities. Our study has focused on the potential influences of such an invasion on the localised soil microbiome by taking bulk soil samples under different vegetation types (see Section “Materials and Methods” for more detail) but not strictly rhizosphere samples.

We were able to show that members of the *Acidobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Bacteroidetes* and *Planctomycetes* were dominant in all the soil samples examined. This was not unexpected as these bacteria are considered to be core components of the soil microbiome (Janssen, 2006). However, the data presented here clearly show that this invasion appeared to result in the favourable selection of bacteria belonging to the phyla *Acidobacteria* and *Verrucomicrobia*, and the exclusion of bacteria belonging to the phyla *Actinobacteria* and *Planctomycetes*. Rhizosphere selection of *Acidobacteria* and *Verrucomicrobia* by *A. adenophora* has been reported (de Rocha et al., 2013). *Acidobacteria* have been attributed an important role in the soil C cycle due to their ability to degrade cellulose and lignin (Ward et al., 2009) and *Verrucomicrobia* may play an important role in methanol transformation (Fierer, 2015). Their selection in bulk soil below *A. adenophora*, found in the current study, indicates that invasion of this weed significantly modified soil microbiota close to its rhizosphere, which could be mainly due to plant litters produced by the fast growth of *A. adenophora*.

We also noticed that *A. adenophora*'s invasion selected bacterial species from α -Proteobacterial genera *Bradyrhizobium*, *Phenyllobacterium* and *Prosthecomicrobium*, *Planctomycetes* genus *Singulisphaera*, and *Chloraflex* genus *Ktedonobacter*. However, except in the case of the genus *Bradyrhizobium* in which *B. japonicum* (100% match in 16S rRNA sequence), a nitrogen-fixing bacterium when living either free in the soil or endosymbiotically in infected host cells of the central nodule tissue (Preisig et al., 1993; Saito et al., 1998), accounted for 1.2% of the total bacteria (data not shown) at the monoculture sites, thus implying a role in nitrogen fixation, it is impossible to explain the enrichment of members from other bacterial genera by *A. adenophora*. Xu et al. (2012) reported that soils heavily invaded by *A. adenophora* had greater cultivable non-symbiotic nitrogen-fixing bacteria number and species diversity than non-invaded ones. Our result is generally in line with theirs.

The NO_3^- -N level of soil is an important factor contributing to the dominance of *A. adenophora*. RDA analyses performed in our study showed that NO_3^- -N levels played an important role in the composition of bacterial communities, and our physiochemical analyses also showed that the soil NO_3^- -N levels detected at the monoculture sites are significantly higher than those detected at the native-species sites. These analyses reinforce the fact that soil NO_3^- -N levels are of vital importance in determining *A. adenophora*'s dominance. This provides further conclusive insights which support the observations of other researchers. Perkins and Nowak (2012) discovered that ni-

trogen availability played a key role in exotic plant invasion. Moreover, Bajpai and Inderjit (2013) reported that a higher availability of soil microbial-generated nitrogenous compounds positively impacts *A. adenophora*'s biomass accumulation.

Moreover, for the first time, our results show that although invasion by this weed changed the composition of bacterial populations and increased total species biodiversity (1.2 times of that measured in soil without *A. adenophora*), it did not significantly change the structure of the underlying soil bacterial microbiome. Thus, diversity indices of bacterial microbiome at the weed monoculture sites did not differ significantly from those at the native species sites. Furthermore, clustering results failed to reveal any pattern among the bacterial microbiomes in soils colonised with different vegetation types.

In conclusion, the high-throughput sequencing conducted in this study has provided sufficient molecular evidence to support that the enrichment of select soil functional bacterial groups play a vital role in the successful establishment and eventual dominance of *A. adenophora*. Furthermore, higher levels of NO_3^- -N were also linked to the dominance of *A. adenophora*. However, the invasion of *A. adenophora* did not constitute a significant change in the structure of the underlying soil's bacterial microbiome.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31360026 and 31260073) and the Key Disciplines (Ecology) Project of Yunnan Education Department, China.

Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

References

- Bajpai, D. and Inderjit (2013) Impact of nitrogen availability and soil communities on biomass accumulation of an invasive species. *AoB Plants*, 1–27.
- Biddle, J. F., Fitz-Gibbon, S., Schuster, S. C., Brenchley, J. E., and House, C. H. (2008) Metagenomic signatures of the Peru margin seafloor biosphere show a genetically distinct environment. *Proc. Natl. Acad. Sci. USA*, **105**, 10583–10588.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D. et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, **7**, 335–336.
- Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. et al. (2014) Ribosomal Database Project: Data and tools for high throughput rRNA analysis. *Nucleic Acids Res.*, **42**, D633–D642.
- da Rocha, U. N., Plugge, C. M., George, I., van Elsland, J. D., and van Overbeek, L. S. (2013) The rhizosphere selects for particular groups of *Acidobacteria* and *Verrucomicrobia*. *PloS ONE*, **8**, e82443.
- de Hoon, M. J. L., Imoto, S., Nolan, J., and Miyano, S. (2004) Open source clustering software. *Bioinformatics*, **20**, 1453–1454.
- de Quadros, P. D., Zhalnina, K., Davis-Richardson, A., Fagen, J. R., Drew, J. et al. (2012) The effect of tillage system and crop rotation on soil microbial diversity and composition in a subtropical acrisol. *Diversity*, **4**, 375–395.
- Fierer, N. (2015) *Verrucomicrobia* and their role in soil methanol consumption. *B21J-02 Microbial Controls of Biogeochemical Cycling I, Presented at 2015 Fall Meeting*, AGU, San Francisco, CA, 14–18 Dec.

- Hill, G. T., Mitkowski, N. A., Aldrich-Wolfe, L., Emele, L. R., Jurkonie, D. D. et al. (2000) Methods for assessing the composition and diversity of soil microbial communities. *Appl. Soil Ecol.*, **15**, 25–36.
- Hirsch, P. R., Mauchline, T. H., and Clark, I. M. (2010) Culture-independent molecular techniques for soil microbial ecology. *Soil Biol. Biochem.*, **42**, 878–887.
- Huson, D. H., Mitra, S., Weber, N., Ruscheweyh, H., and Schuster, S. C. (2011) Integrative analysis of environmental sequences using MEGAN4. *Genome Res.*, **21**, 1552–1560.
- Janssen, P. H. (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.*, **72**, 1719–1728.
- Kuo, S. (1996) Phosphorus. In *Methods of Soil Analysis. Part 3, Chemical Methods*, ed. by Sparks, D. L., Page, A. L., Helmke, P. A., Loeppert, R. H., Soltanpour, P. N. et al., Madison, WI, pp. 869–919.
- Lee, S. H., Malone, C., and Kemp, P. F. (1993) Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. *Mar. Ecol. Prog. Ser.*, **101**, 193–201.
- Li, H. N., Liu, W. X., and Wan, F. H. (2011) Effect of *Ageratina adenophora* (Sprengel) and *Flaveria bidentis* (Linn.) invasion on soil microbial community and *Oryza sativa* L. growth. *Chinese J. Eco-Agri.*, **19**, 1365–1371.
- Liu, W. X., Niu, H. B., Wan, F. H., and Liu, B. (2010) Effects of leachates of the invasive plant, *Ageratina adenophora* (Sprengel) on soil microbial community. *Acta Ecol. Sinica*, **30**, 196–200.
- Magoc, T. and Salzberg, S. L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, **27**, 2957–2963.
- McGeoch, M. A., Butchart, S. H. M., Spear, D., Marais, E., Kleynhans, E. J. et al. (2010) Global indicators of biological invasion: species numbers, biodiversity impact and policy responses. *Divers. Distrib.*, **16**, 95–108.
- Mulvaney, R. L. (1996) Nitrogen-inorganic forms. In *Methods of Soil Analysis. Part 3, Chemical Methods*, ed. by Sparks, D. L., Page, A. L., Helmke, P. A., Loeppert, R. H., Soltanpour, P. N. et al., Madison, WI, pp. 1123–1184.
- Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**, 695–700.
- Nelson, D. W. and Sommers, L. E. (1996) Total carbon, organic carbon, and organic matter. In *Methods of Soil Analysis. Part 3, Chemical Methods*, ed. by Sparks, D. L., Page, A. L., Helmke, P. A., Loeppert, R. H., Soltanpour, P. N. et al., Madison, WI, pp. 961–1010.
- Nesme, J., Achouak, W., Agathos, S. N., Bailey, M., Baldrian, P. et al. (2016) Back to the future of soil metagenomics. *Front Microbiol.*, **7**, 1–5.
- Niedringhaus, T. P., Milanova, D., Kerby, M. B., Snyder, M. P., and Barron, A. E. (2011) Landscape of next-generation sequencing technologies. *Anal. Chem.*, **83**, 4327–4341.
- Perkins, L. B. and Nowak, R. S. (2012) Soil conditioning and plant-soil feedbacks affect competitive relationships between native and invasive grasses. *Plant Ecol.*, **213**, 1337–1344.
- Preisig, O., Anthamatten, D., and Hennecke, H. (1993) Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proc. Natl. Acad. Sci. USA*, **90**, 3309–3313.
- Prober, S. M., Leff, J. W., Bates, S. T., Borer, E. T., Firn, J. et al. (2014) Plant diversity predicts beta but not alpha diversity of soil microbes across grasslands worldwide. *Ecol. Lett.*, **18**, 85–95.
- Saito, A., Mitsui, H., Hattori, R., Minamisawa, K., and Hattori, T. (1998) Slow-growing and oligotrophic soil bacteria phylogenetically close to *Bradyrhizobium japonicum*. *FEMS Microbiol. Ecol.*, **25**, 277–286.
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M. et al. (2009) Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, **75**, 7537–7541.
- Wan, F. H., Liu, W. X., Guo, J. Y., Qiang, S., Li, B. P. et al. (2010) Invasive mechanism and control strategy of *Ageratina adenophora* (Sprengel). *Sci. China*, **53**, 1291–1298.
- Wang, R. and Wang, Y. Z. (2006) Invasion dynamics and potential spread of the invasive alien plant species *Ageratina adenophora* (Asteraceae) in China. *Divers. Distrib.*, **12**, 397–408.
- Ward, N. L., Challacombe, J. F., Janssen, P. H., Henrissat, B., Coutinho, P. M. et al. (2009) Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. *Appl. Environ. Microbiol.*, **75**, 2046–2056.
- Xie, Y., Li, Z. Y., Gregg, W. P., and Li, D. M. (2001) Invasive species in China—an overview. *Biodivers. Conserv.*, **10**, 1317–1341.
- Xu, C. W., Yang, M. Z., Chen, Y. J., Chen, L. M., Zhang, D. Z. et al. (2012) Changes in non-symbiotic nitrogen-fixing bacteria inhabiting rhizosphere soils of an invasive plant *Ageratina adenophora*. *Appl. Soil Ecol.*, **54**, 32–38.
- Xu, H. G., Ding, H., Li, M. Y., Qiang, S., Guo, J. Y. et al. (2006) The distribution and economic losses of alien species invasion to China. *Biol. Invasions*, **8**, 1495–1500.
- Yu, W. Q., Wan, F. H., He, X. H., Liu, W. Z., Liu, W. X. et al. (2014) Soil microbes enhance competition ability of the exotic *Ageratina adenophora* (Sprengel) against native plant species. *J. Biosafety*, **23**, 156–164.
- Zhalnina, K., Dias, R., de Quadros, P. D., Davis-Richardson, A., Camargo, F. A. O. et al. (2014) Soil pH determines microbial diversity and composition in the park grass experiment. *Microbial Ecol.*, **69**, 395–406.
- Zhang, M., Liu, W. X., Zheng, M. F., Xu, Q. L., Wan, F. H. et al. (2013) Bioactive quinic acid derivatives from *Ageratina adenophora*. *Molecules*, **18**, 14096–14104.