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Microbial Community on Healthy and Diseased Leaves of an Invasive Plant *Eupatorium adenophorum* in Southwest China

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Invasive plants have caused great economic losses and environmental problems worldwide. *Eupatorium adenophorum* is one of the most invasive weeds in China. To better understand its invasive mechanisms, in the present paper, the microbial communities of healthy and diseased leaves of *E. adenophorum* were obtained using both culture-independent and -dependent methods and their diversities were compared. The bacteria obtained from culture-independent method belong to Proteobacteria (95.8%), Actinobacteria (2.1%), and Firmicutes (2.1%) and fungi belong to Ascomycota (65.2%) and Basidiomycota (34.8%). Very few overlapped microbial species were found by culture-dependent and -independent methods. Healthy leaves display higher bacterial diversity than diseased leaves. Phylogenetic structures are very different between healthy and diseased phyllosphere microbial communities. Bacteria close to *Acinetobacter* and *Pseudomonas* were dominant on healthy leaves, whereas those close to *Shigella* were dominant on diseased leaves. 52.9% of fungal clones from healthy leaves were Ustilaginomycetes, close to *Rhodotorula phylloplana* and uncultured basidomycete; by contrast, 60% of clones from diseased leaves were Lecanoromycetes, close to *Umbilicaria muehlenbergii*. No bacteria but four fungal strains phylogenetically close to *Myrothecium* sp. and *Alternaria alternate* were pathogenic to seedlings and detached leaves of the invasive plant. Therefore, this plant may be resistant to pathogens from bacteria but not fungi in its introduced range.

***Keywords*:** *E. adenophorum*, invasive plant, phyllosphere, microbes, pathogenicity

Invasive plants have caused great economic losses and environmental problems worldwide (Pimentel *et al*., 2000; Duncan *et al*., 2004). In China, a few of invasive species cost 57.4 billion Yuan (RMB) annually (Ma *et al*., 2003). Crofton weed *Eupatorium adenophorum* (also called *Ageratina adenophora*) is one of the most invasive weeds in China. This plant native to Central America was first recorded in Yunnan province in 1940s. After outbreak in 1960, it has spread north- and east-ward about 20 km annually (Wang and Wang, 2006) to Guizhou, Sichuan, and Guangxi provinces in Southwest China and distributed in more than 30 countries and regions (Qiang, 1998; Lu *et al*., 2005; Ding *et al*., 2008).

The invasive mechanisms and control of *E. adenophorum* have been always attractive to biologists and ecologists. Previous studies have focused on its biological characteristics. High level of propagation rate and widely biogeographic adaptation are considered as major causes for its invasiveness (Qiang, 1998; Lu *et al*., 2005). In addition, its allelopathic effect on native plant species has been suggested as another cause (Yu *et al*., 2004; Li *et al*., 2007).

Currently the most popular explanation for the rapid establishment and proliferation of exotic species is the enemy release hypothesis (ERH). ERH suggests the abundance of some exotic plant species is related to the scarcity of natural enemies (including leaf pathogens and herbivores) in the introduced range compared with their native range (Keane and Crawley, 2002; Wolfe, 2002). Moreover, it has been demonstrated that the successful invasion of a plant is determined not only by release from its native fungal and viral enemies but also by less accumulation of its microbial pathogens in naturalized range (Mitchell and Power, 2003). In deed, many previous researches have demonstrated the possibility of *E. adenophorum* accumulating less natural enemies in naturalized range. For example, *E. adenophorum* contains cadinene, which antifeeds herbivore *Philasomia ricini* (Bordoloi *et al*., 1985) and *Pieris rapae* (Zhou *et al*., 2003). Moreover, the petroleum ether-extracts from *E. adenophorum* leaves have strong inhibitory effects on hypha growth of *Phytophthora infestan*, a potato fungal pathogen, and application potentials as biocontrol agent for plant fungal disease (Zhang *et al*., 2006). Our field investigations of 17 populations across Yunnan province have demonstrated that fewer diseases occur at top five pairs of leaves, among which, 8%-18% of the diseased leaves have only one leaf spot with a diameter <1 cm (Zhang *et al*., unpublished data). Based on these data, we postulate fewer microbes inhabit on its phyllosphere due to the release from their native enemy or the accumulation of fewer enemies in its introduced range. Therefore, studies on the phyllosphere microbial communities may provide new clues to understand *E. adenophorum* invasiveness. However, only a few of studies on the phyllosphere microbes and their potential pathogenicity to *E. adenophorum* have been carried out (Wan *et al*., 2001).

To further explore the phyllosphere microbial communities and their differences between healthy and diseased leaves, in

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the present report, we investigated the microbial species compositions of healthy and diseased leaves from *E. adenophorum*. Considering most phyllosphere microbes are unable to grow on culture medium (Yang *et al*., 2001), we characterized the phylogenetic compositions of phyllosphere microbes using integrated culture-independent and -dependent methods and further evaluated their pathogenicity to *E. adenophorum*.

Materials and Methods

Collecting leaves

The sampling site was located at 25°05′401″ N and 102°46′227″ E at an altitude of 1965 m at Jindian forest park, Kunming, China. This site is dominated by *E. adenophorum* with a population coverage of >90%. The top 5 pairs of healthy and diseased leaves were collected from the plant shoots. Diseased leaves were identified by the appearance of leaf spot. All collected leaves were immediately put into sterile plastic bags and stored at 4°C. Samples were used within two days.

Collecting and culturing phyllosphere microbial cells

To obtain phyllosphere microbial cells, about 50 g of healthy or diseased leaves were softly washed twice in 500 ml sterile water to remove dust, then submerged into washing buffer (0.1 M potassium phosphate buffer, pH 7.0) and sonicated for 7 min in a water bath with ultrasound to dislodge microbes (Yang *et al*., 2001). Part of the microbial suspension was smeared on potato dextrose agar (PDA) plate (containing 20 μg/ml penicillium and 40 μg/ml streptomycin to inhibit the growth of bacteria) to grow culturable fungi or on tryptic soy agar (TSA) plate to grow culturable bacteria, respectively. The remaining suspension was centrifuged to collect microbes.

Establishment of rRNA gene libraries from culture-independent sources

The above collected microbes were directly used to extract genome DNAs as described previously (Zhou *et al*., 1996). To construct genomic libraries, bacterial 16S rRNA gene was amplified from total microbial genomic DNA using the conserved primer pairs F984 and R1401 (Nübel *et al*., 1996; Filion *et al*., 2004) and fungal 18S rRNA genes were amplified with a nested PCR using primers NS1-NS6 (White *et al*., 1990), SSU-1196 and SSU-1536 (Borneman and Hartin, 2000). The PCR products were inserted into the pMD18-T vector (TaKaRa, China) and transformed into competent *Escherichia coli* HB101 cells (Boyer and Roulland-Dussoix, 1969). Positive clones were identified by blue-white plaque screening and stored in 20% glycerol at -70°C.

Establishment of rRNA gene libraries from cultured microbes

Bacterial genome DNA was extract from bacteria obtained from TSA plates using a bacterial genome DNA extraction kit according to the manufacturer's protocol (Watson, China) and fungal DNA was extracted from fungi on PDA plate using CTAB method (Zolan and Pukkila, 1986). Bacterial 16S RNA genes were amplified using primer pairs F984 and R1401 and fungal 18S rRNA genes were amplified with SSU-1196 and SSU-1536 (Borneman and Hartin, 2000). Further cloning of PCR products were performed exactly as described above.

Restriction fragment length polymorphism (RFLP) analysis

Target rDNA fragment was directly amplified from cell suspension resuscitated from the positive clone stock at -70°C by PCR using primers M13-M4 and M13-RV. Two microliter amplicons of each clone were digested with 1.0 U *Rsa*I, *BsuR*I, and *Hinf*I (Bio Basic, Canada), respectively, in 5 μl reaction mixtures (Filion *et al*., 2004), separated by 3% agarose gels electrophoresis, visualized with ethidium bromide and photographed using GIS system (Tanon, China). Clones with unique digestive fragment pattern were considered as an operational taxonomic unit (OTU). One representative clone from each OTU was commercially sequenced by BGI-SEQUENCING (Shenzhen, China).

Phylogenetic analysis

The sequences obtained were further edited by deleting primer sequences using Editseq and Seqman softwares in DNAstar package. Chimeras were picked up by CHIMERA_CHECK program (RDP Database, http://rdp8.cme.msu.edu/html/analyses.html). Bacterial species were defined by RDP II SEQUENCE MATCH based on Pei's method (Pei *et al*., 2004). The species boundary was determined by a RDP II similarity score of 0.873, which is nearly equal to 97% sequence homology of 16S rDNA (Pei *et al*., 2004). Fungal phylogenetic position was characterized by the closest relatives, which were found by sequence blasting on NCBI database. Phylogenetic trees were constructed using PHYLIP package (Felsenstein, 1989) with neighbor-joining method. Bootstrap analysis was carried out with 1,000 replicates. Bacterial sequences that are not classifiable by the current 16S database at RDP II and representative fungal sequence from each OTU were deposited in GenBank as accession numbers from GQ253325 to GQ253366. Species rarefaction curves and Chao1 species estimates were calculated using DOTUR program (Schloss and Handelsman, 2005). Differences of species compositions between libraries were estimated using the ∫-LIBSHUFF program (Schloss *et al*., 2004). Sampling coverage was calculated according to the formula reported previously (Good, 1953).

Pathogenicity testing

About 1×10^4 spores/ml fungus suspension and 1×10^8 cells/ml bacterium suspension were used in microbial pathogenicity testing. For the pathogenicity on leaves, the third pair of healthy leaves from the top of the shoot of *E. adenophorum* were detached and sterilized by soaking in 70% ethanol for 45 sec. After washed 3 times with sterile water, and air-dried for 30 min on the sterile filter paper, each leaf was wounded on the surface using sterile toothpick at three spots with ten points per spot. Each spot was inoculated 100 μl microbial cell suspension in testing group or 100 μl sterile water in control group. The leaves were kept at 28°C and the developed disease symptoms were observed every day for 7 days. To test pathogenicity on seedlings, 20 surface-sterilized *E. adenophorum* seeds were put in a can with 10 cm in diameter and 15 cm in height containing MS medium (Sigma), and cultured at 25°C in an incubator under 12 h/12 h light-dark cycle to germinate. After 15 days' cultivation, ten 2-leaf stage seedlings of *E. adenophorum* obtained in each can were sprayed with microbial suspension and the other ten seedlings were treated with sterile water. All experiments were performed in triplicate.

Results

Phyllosphere microbial diversity

As listed in Table 1, total four libraries were established by culture-independent methods: healthy leaf bacterial library (HB), diseased leaf bacterial library (DB), healthy leaf fungal library (HF), and diseased leaf fungal library (DF). Excluding low quality sequences and 19 possible chimeras, total 95 bacterial and 69 fungal sequences were obtained, among

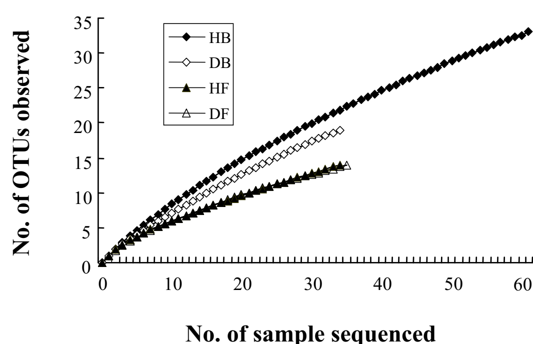

Fig. 1. Rarefaction curves from DOTUR analysis using the furthest neighbor assignment algorithm. HB and DB represent bacterial clone libraries constructed from healthy and diseased leaves, respectively. HF and DF represent fungal clone libraries constructed from healthy and diseased leaves, respectively.

which, 61 from HB, 34 from DB, 34 from HF, and 35 from DF. Based on the fragment patterns of 16S rRNA gene digested by *Rsa*I, *BsuR*I, and *Hinf*I, bacterial sequences were divided into 45 OTUs: 33 OTUs were identified from HB and 19 OTUs were from DB; based on the fragment patterns of 18S rRNA gene digested by *Rsa*I, *BsuR*I, and *Hinf*I, fungal sequences were divided into 26 OTUs: 14 from each library. The sample coverage for all libraries were >50%.

If all clones from each OTU were considered as the repeats of its representative sequence, total 164 deduced sequences were obtained from four libraries. The OTU richness of each library was estimated by rarefaction. Healthy leaves displayed higher bacterial and fungal richness than diseased leaves (Fig. 1). Chao1 Species Estimate indicated that 66 bacterial and 36 fungal species inhabited on healthy leaves, whereas only 37 bacterial and 26 fungal species did on diseased leaves. ∫-LIBSHUFF analysis showed that phylogenetic structures were significantly different between healthy and diseased phyllosphere microbial communities ($P<0.0001$ for bacterial libraries and $P<0.0003$ for fungal libraries, data not shown).

Bacterial phylogenetic diversity

Of the total 95 bacterial clones, 76 had a similarity score larger than 0.873 in strain type and 4 had a similarity score larger than 0.873 in 16S rRNA gene derived from a fully defined cultured nontype strain. These 80 clones (84.0%) were classified as culture-defined bacterial species. Of the remaining 15 clones (16.0%), only 4 (representing 3 OTUs) had a similarity score larger than 0.873 in 16S rDNA clones generated from culture-independent source. The other 11 sequences (representing 11 OTUs) with similarity score less than 0.873 were heterogeneous to 16S rRNA gene sequences generated from both culture-dependent and -independent sources and classified as unknowns.

The taxonomic assignments were further confirmed by phylogenetic analysis (Fig. 2). These bacterial OTUs belonged to 3 divisions, including Proteobacteria (95.8%), Actinobacteria (2.1%), and Firmicutes (2.1%). Proteobacteria were further divided into 4 subdivisions, including Gammaproteobacteria (74.7%), Betaproteobacteria (18.7%), Alphaproteobacteria (5.5%), and Deltaproteobacteria (1.1%).

Species compositions between healthy and diseased leaves were different. β-Proteobacteria accounted for 21.3% in HB, but only account for 11.8% in DB. At low taxonomic level, clones close to *Acinetobacter johnosii* were not found from DB, whereas 11 (18%) clones were found in HB. Clones close to *Pseudomonas* accounted for 18% of total clones in HB. By contrast, clones close to *Shigella* were dominant and accounted for 35.3% of total clones in DB (Fig. 2). All clones in Firmicutes were from HB (Fig. 2), and those close to Alphaproteobacteria and Deltaproteobacteria were from DB.

Fungal phylogenetic diversity

Typically, approximately 400-bp rRNA gene sequence of one representative clone from each OTU was used to evaluate the fungal phylogenetic diversity. Sequences with the highest similarity to each representative OTU were downloaded from NCBI database and used to construct phylogenetic tree (Fig. 3). All clones were divided into Ascomycota (65.2%) and Basidiomycota (34.8%). Clones from Basidiomycota were divided into to classes Hymenomycetes and Ustilaginomycetes and clones from Ascomycota were divided into 6 classes including Sordariomycetes, Eurotimycetes, Lecanoromycetes, Dothideomycetes, Saccharomycetes, and Taphrinomycetes. 52.9% of clones from HF were from Ustilaginomycetes and close to yeast *Rhodotorula phylloplana* and uncultured *Basidomycete*. By contrast, 60% of clones from DF were from Lecanoromycetes and close to *Umbilicaria muehlenbergii*.

Culturable microbes and their pathogenicity

Total 22 bacterial and 19 fungal isolates from healthy and diseased leaves were obtained and identified based on rRNA gene sequences (Figs. 2 and 3). All bacterial isolates had similarity scores larger than 0.873 to 16S rRNA gene from fully defined cultured type strain in RDP database. Different compositions were observed in species obtained by culture-dependent, and -independent methods. Betaproteobacteria, Alphaproteobacteria, and Deltaproteobacteria were obtained only by culture-independent method, whereas Flavobacteria, phylogenetically close to *Myroides odoratimimus*, was obtained only by culture-dependent method. Culturable Actinobacteria and Firmicutes were close to *Arthrobacter* and *Bacillus*, respectively, but the uncultured clones of these two phyla were close to *Nocardioides* and *Exiguobacterium*, respectively. Some cultured isolates distributed in gammaproteobacteria were close to *Serratia*, *Providencia*, *Proteus*, etc., whereas, most uncultured clones were close to *Shigella*, *Pseudomonas*, *Pantoe*, and *Erwinia*. Only 1 strain was phylogenetically close to *Cedecea davisae*, and its DNA was similar to the uncultured clones 2ZW106 and 2ZW86.

Culturable dominant fungi from either healthy (6 strains) or diseased leaves (6 strains) were close to *Eupenicillium javanicum*. Other strains from healthy leaves were mainly close to *Taphrina deformans* and *Myrothecium* sp., and those from diseased leaves were close to *Paraphaeosphaeria michotii*, *Phoma medicaginis*, and *Alternaria alternate*. Again, very few of species overlapped between microbes revealed by culture-dependent and -independent methods.

Surprisingly, bacteria from neither healthy nor diseased leaves were pathogenic to *E. adenophorum*. However, fungi J3 and J1 from healthy leaves and B13 and B18 from diseased

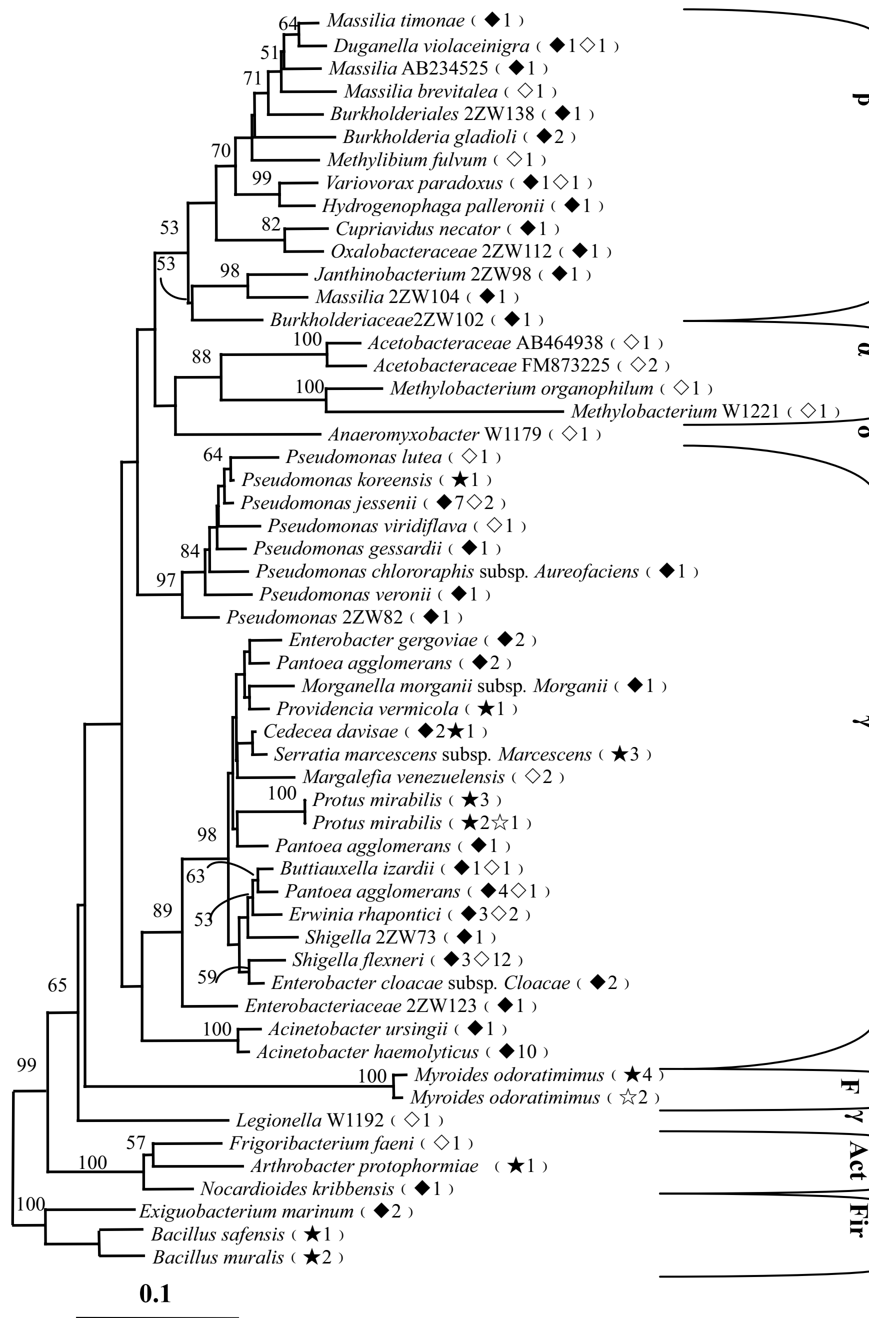

Fig. 2. Phylogenetic relationship of representative 16S rRNA gene sequences.
α, Alphaproteobacteria; β, Betaproteobacteria; γ, Gammaproteobacteria; δ, Deltaproteobacteria; Act, Actinobacteria; F, Flavobacteria; Fir, Firmicutes. Bootstrap values (1,000 replications) equal to or greater than 50% are indicated at branch nodes. Species names of OTUs classified as belonging to culture-defined bacterial species are located at the termination of each branch. Three OTUs (represented by 4 clones) classified as 16S rRNA gene clones are named as the putative genus or higher taxon followed by the GenBank accession no. of the best-matched rRNA gene clone. The OTUs classified as unknowns are represented by the putative genus or higher taxon that is most closely resembled, followed by the clone number used in this study. Solid and open diamonds represent clones obtained by culture-independent methods from healthy and diseased leaves, respectively. Solid and open stars represent strains isolated from healthy and diseased leaves, respectively. The number followed these symbols represent the clone/strain number. The scale bar represents 10% estimated sequence divergence.

leaves were pathogenic to both *E. adenophorum* detached leaves (Fig. 4) and its seedlings (data not shown). To further confirm the fungal pathogenicity, Koch's postulates were performed and these four fungal strains were re-isolated on diseased leaf spot. As shown in Fig. 3, the morphologies and 18S rRNAs' RFLP fragment patterns were identical to their

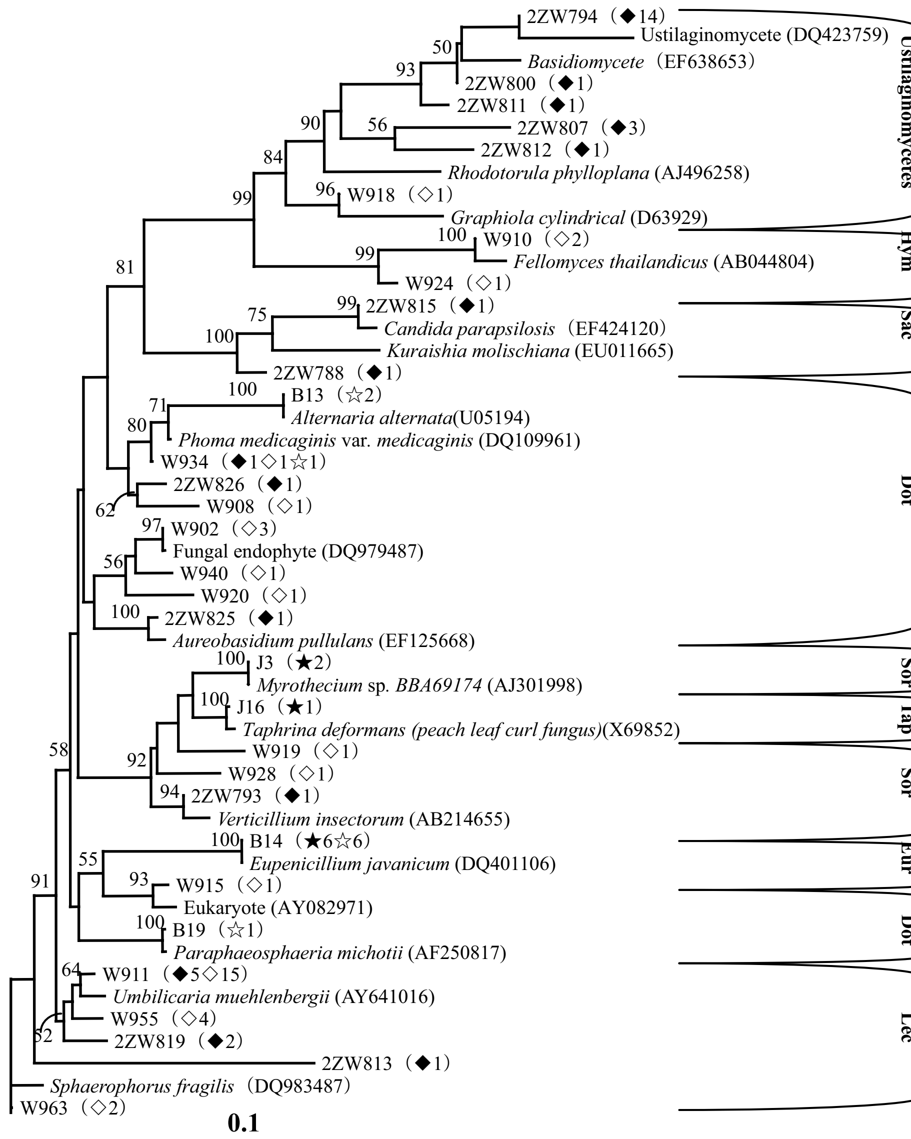

Fig. 3. Phylogenetic relationship of representative 18S rRNA gene sequences affiliated with fungal relatives from GenBank. Each OTU followed by its occurrence time in parentheses is located at the termination of each branch. Lec, Lecanoromycetes; Hym, Hymenomycetes; Sac, Saccharomycetes; Sor, Sordariomycetes; Tap, Taphrinomycetes; Eur, Eurotiomycetes; Dot, Dothideomycetes. See other notes in Fig. 2.

inoculated original strains and similar phylogenetical closeness to *Myrothecium* sp. (J1 and J3) and *Alternaria alternate* (B13 and B18) were observed.

Discussion

Large microbial populations in phyllosphere can affect plant growth by both positive and negative interactions (Lindow and Brandl, 2003). In this study, we investigated the phyllosphere microbes obtained by both culture-independent and -dependent methods in an invasive plant, *E. adenophorum*. Culture-independent method revealed that 66 and 37 bacterial species inhabited on healthy and diseased leaves, respectively (Table 1). Currently, comparison of such diversity in other plant species is difficult because analysis of phyllosphere microbial populations using rRNA-based method is still rare. Study on phyllosphere bacterial communities of a tropical Brazilian forest has revealed that at 97% similarity of bacterial sequence boundary, average 54-265 species are identified from phyllospheres of different plant species (Lambais *et al.*, 2006). In comparison, the number of phyllosphere bacterial species from *E. adenophorum* in this study is slightly small. Considering that the rarefaction curves are far from saturation (Fig. 1), this number may be underestimated by the relatively small sample size.

Proteobacteria, the dominant microbe in phyllosphere of several plant species, including *Thlapsi*, *Trichilia*, *Campomanesia*, *Zea*, *Capsicum*, *Solanum*, and *Crocus*, reportedly account for 45%-86% of total microbes on phyllosphere (Weidner *et al.*, 2000; Whipps *et al.*, 2008). In this study, 95.8% of clones

Table 1. Coverage of RFLP OTUs in rRNA gene clone libraries

Parameter	Bacteria			Fungi		
	HB	DB	Total	HF	DF	Total
Total No. of patterns	33(61)[a]	19(34)	45(95)	14(34)	14(35)	26(69)
No. of singletons[b]	23	14	34	10	9	18
% of sample coverage[c]	62.3	58.8	64.2	70.6	74.3	73.9
Chao1 species estimated[d]	66(44, 128)	37(24, 84)		36(19, 111)	26(17, 68)	

HB, healthy leaf bacterial library; DB, diseased leaf bacterial library; HF, healthy leaf fungal library; DF, diseased leaf fungal library
[a] the values in parentheses are the numbers of clones.
[b] OTUs represented by single clone in a clone library.
[c] Good coverage (Good, 1953). Coverage calculated by Cx=1-(Nx/n), where Nx is the number of singletons and n is the total number of sequences.
[d] The values in parentheses are the interval of 95% of confidence

obtained by culture-independent method belong to proteobacteria (Fig. 2), higher than those described above. At low taxonomic level, the most populated clones are the commonly seen phyllosphere microbes *Pseudomonas*, *Acinetobacter*, *Pantoea*, and *Erwinia* (Fig. 2) (Thompson *et al*., 1993; Yang *et al*., 2001; Alamri, 2008; Namasivayam and Sahayaraj, 2009). Noticeably, *Pseudomonas* alone accounts for 15.8% of culture-independent clones (Fig. 2). Its dominance may rely on its good capability of surfactant release which results in increased wettability of leaf surfaces and consequently leads to the improved growth of *Pseudomonas* (Bunster *et al*., 1989).

Up to date, knowledge about phyllosphere fungal diversity is mainly obtained through culture-dependent methods, by which the commonly observed species are *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Acremonium*, *Mucor*, *Phoma*, *Monodictyis*, and *Aspergillus* (Fenn *et al*., 1989; Ziedan, 2006; Alamri, 2008; Whipps *et al*., 2008). Consistently, most cultural fungi in our study are phylogenetically close to *Alternaria*, *Phoma*, and *Eupenicillium* (Fig. 3). However, many culture-independent fungal clones are close to those not frequently seen on leaves. For example, 20 clones are phylogenetically close to uncultured ustilaginomycete. In addition, some clones are close to yeast commonly seen on leaves, such as *Aureobasidium* and *Rhodotorula* (Levetin and Dorsey, 2006; Whipps *et al*., 2008). Therefore, culture-independent method is able to reveal wider phylogenetic diversity than culture-dependent method.

Rarefaction curve is widely used to compare the richness of observed species among different sites, treatments or habitats that are unequally sampled (Hughes *et al.*, 2001). Therefore, it is used in the present study to compare the OTU richness due to significant difference in the size of tested bacterial libraries between healthy and diseased leaves (Table 1). Rarefaction clearly indicates that healthy leaves have higher bacterial diversity than diseased leaves (Fig. 1). Similar results have been seen in bacterial and actinomycete populations of groundnut leaves (Namasivayam and Sahayaraj, 2009). Dominant bacterial species may consume ecological niches, thus block the colonization of fungi, especially potential pathogens. Although no antagonism was directly tested in the current study, the bacterial species composition may provide some clues to this point. More pseudomonas were found on the healthy leaves than the diseased leaves (18% vs 11.8%) (Fig. 2). Similarly, *P. fluorescens* has been reported in all but the diseased leaves of groundnut (Namasivayam and Sahayaraj, 2009). Many members of *Pseudomonas*, such as *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, and *P. putida*, are potential biocontrol agents for plant diseases (McSpadden Gardener, 2007). Meanwhile, all members from genus *Bacillus*, the potential suppressor of phytopathogens (McSpadden Gardener, 2004), inhabit on healthy leaves (Fig. 2). Therefore, further studies are needed to explore the antifungal effects of phyllosphere bacteria of invasive plants.

The exotic plants are thought to gain a competitive advantage over natives that may suffer disproportionately from natural enemy attacks (Keane and Crawley, 2002; Wolfe, 2002). The present study shows that bacteria from both the healthy and diseased leaves are not pathogenic to *E. adenophorum*, in consistence with our previous observation showing that none of the 74 bacterial strains isolated from leaves of 15 *adenophorum* populations are pathogenic (Zhang *et al.*, unpublished data). According to ERH theory (Keane and Crawley, 2002; Wolfe, 2002), it is plausible that none or few of bacterial pathogens contribute to the invasiveness of *E. adenophorum*. Contrarily, *E. adenophorum* may be attacked by pathogenic fungi, such as *Myrothecium* spp. and *Alternaria alternate* (Fig. 4). *Alternaria alternate* has been previously reported to produce toxin to *E. adenophorum* (Wan *et al*., 2001), but whether *Myrothecium* is toxic to *E. adenophorum* has not been explored. Further studies are needed to investigate their host-specificity and potential applications as biocontrol agents.

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Fig. 4. Symptom of fungal strains J1, J3, B13, and B18 on detached leaves of *E. adenophorum.*

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