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Full Length Research Paper

Differences of rhizo-bacterial diversity and the content of peimine and peiminine of *Fritillaria thunbergii* among different habits

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To explore the differences of rhizo-bacterial diversity among different habits and the relationship between the quality of *Fritillaria thunbergii* and microbial diversity, the soil bacterial diversity was analyzed by denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) and the content of peimine and peiminine in *F. thunbergii* was detected by high performance liquid chromatographic method with evaporative light scattering detection (HPLC-ELSD). For the DGGE analysis, the genetic diversity based on Shannon index of rhizo-bacterial community was the highest in Nantong (NT), followed by Chunan (CA), Panan (PA) and Ningbo (NB). Furthermore, it was significantly higher in rhizosphere soil than in non-rhizosphere soil, which indicated that some kinds of bacteria related to the growth of *F. thunbergii* were accumulated in rhizoshpere. Bacterial diversity based on principal component analysis (PCA) showed significant differences among all samples obtained from different habits and are divided into three major taxa: NB, CA and PA. It was found that the highest content of peimine (0.1364%) and peiminine (0.0518%) in NT, while the lowest peimine in NB (0.0646%) and the lowest peiminine (0.0316%) in PA. Moreover, the rhizo-bacterial diversity was relatively high related to the alkaloid concentration of *F. thunbergii.*

Key words: Traditional Chinese medicine, *Fritillaria thunbergii*, 16S r DNA, PCR-DGGE, microbial diversity, HPLC-ELSD, alkaloid.

INTRODUCTION

Traditional Chinese medicinal plants with the best quality are always distributed in relatively narrow areas, which are also called 'geo-authentic crude drugs' such as *Fritillaria thunbergii*. It is located in Zhejiang, Jiangsu, Jiangxi and Hunan Province in China. However, Zhejiang Province is recognized as the authentic origin of *F. thunbergii*. It is a perennial herb and belongs to Liliaceae. It is used in clinic for upper respiratory tract infection and bronchitis etc (Chinese Pharmacopoeia, 2005). Cai et al. (2003) found that it was almost the same in the content and composition of total alkaloid of *F. thunbergii*, but different in monomer alkaloid among three habits due to the local environmental factors. Zheng et al. (2005) reported that the output of *F. thunbergii* is larger in Jiangsu Province than in Zhejiang Province and it was probably due to the soil and climate factors. Ren et al. (2005) suggested that the soil factors had very important effects on the quality and quantity of genuine regional drug. Diversity of microbial communities is considered to be an important index of soil quality. Soil microbes have played a crucial role in the detoxification of noxious chemicals, recycling of plant nutrients and control of plant pests as well as plant growth. Alteration in the activity of microbes is proposed to be a sensitive indicator of anthropogenic effects on soil ecology (Brookes et al., 1995; Shi et al., 2002). However, till now, few researches about the relationship between the quality of genuine authentic crude drug and soil microbial have been done.

With the fast progress of molecular biological technology

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in the last decade, some powerful tools, such as PCR-DGGE and fluorescent in situ hybridization (FISH) et al., have been developed and now it is possible to look into bacterial community structure without relying on time-consuming bias-bearing cultivation methods (Liu et al., 2007; Ding et al., 2008). Till now, PCR-DGGE finger-printing has been successfully applied for various aspects (Zhao et al., 2005; Wang et al., 2008; Liu et al., 2008; Yan et al., 2008). To explore the differences of rhizo-bacterial diversity among different habits and the relationship between microorganism diversity and the quality of *F. thunbergii*, bacterial diversity was studied by PCR-DGGE and the content of peimine and peiminine was detected by HPLC-ELSD.

MATERIALS AND METHODS

Samples collection

Soil samples were collected from four regions of origin: Ningbo (NB), Chunan (CA), (Panan) PA in Zhejiang Province and Nantong (NT) in Jiangsu Province in May, 2008. Rhizosphere soil and non-rhizosphere soil were collected separately (Smalla et al., 2001). Rhizosphere soil was collected from the rhizosphere of Fritillaria, while non-rhizosphere soil near the field where it was not planted. The soil was sieved (< 0.45 mm) to remove plant materials, soil macrofauna and stones, placed in sterile polypropylene tubes and stored at -20°C for the soil community DNA extraction. Each sample was triplicate.

DNA extraction and purification

Total soil community DNA from the rhizosphere soil was extracted from 1.0 g soil using a bead beating method (UltraClean™ Soil DNA Isolation Kit, MoBio Laboratories, Inc., USA) following the manufacturer's instructions. Detect DNA concentration using spectrophotometer (3100 pro UV/VI Spectrophotometer). Remove 1 µl DNA solution and dilute to about 20 – 50 ng as a PCR reaction.

PCR–DGGE

For bacteria DGGE analysis, the V3 region of 16S rDNA was amplified with the primers F357GC clamp and R518. The GC-clamp was added to the forward primer F357 to facilitate the DGGE (Muyzer et al., 1993). PCR reaction was executed in a final volume of 50 µl. The reaction mixture contained 25 pmol of both primers, 2.5 mM of each dNTPs, 0.1 mM $MgCl_2$ solution (Sigma), 10 × PCR buffer and 2.5 U of Taq polymerase (applied Biosystems, USA). The reaction was carried out as follows: 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min and DNA extension at 72°C for 1 min. Cycling was completed by a final elongation step of 72°C for 7 min. Amplified DNA was verified by running the PCR product on a 1.0% agarose gel stained with SYBR™ GREEN (Sigma, USA).

The obtained PCR products were then subjected to DGGE analysis using a denaturing gradient of 30 to 60% denaturants (100% denaturant contained 7 M urea and 40% (v/v) formamide) in 8% polyacrylamide gel in 1 × TAE buffer (pH 8.0). 45 µl of PCR products were electrophoresed in 1 × TAE buffer at 60°C for 6.5 h at a constant voltage of 160 V (Dcode™ Universal Detection System, Bio-Rad, USA). After electrophoresis, the gels were stained with SYBR™ GREEN I for 30 min and digitized with quantity one software (Bio-Rad, USA).

Biodiversity determination at any organization level is a heuristic tool in environmental management. The Shannon biodiversity index stands out among the various available indexes that combine both components of the concept: species number and their relative abundance (Laura, 2004). Here it was used to analyze difference of bacterial diversity among soil samples from different habits and calculated from DGGE band data as:

$$H = \sum_{i=1}^{s} pi \ln pi$$

Where S represents the richness or total number of bands, p_i is the proportion of the total intensity accounted for by the *i*th band and ln is the natural logarithm. The exp (H) is equivalent to the number of the equally common species required to produce the observed uncertainty, which is the value of H given by the sample.

Alkaloid determination by HPLC-ELSD

Analyses were performed using an Agilent 1100 Series HPLC system (HP Corporation, USA) equipped with Alltech ELSD-3300 ELSD system (HP Corporation, USA). The chromatography was carried out on a Bartlett ODS column (5 µm, 250 × 4.6 mm) at a column temperature of 30°C and flowrate of 0.6 ml/min using (A) acetonitrile and (B) water containing 0.3% diethylamine as mobile phase. Parameters for the ELSD were set: S.C. (Spray Chamber) = 45℃, D.T. (Drift Tube) = 70℃, GAS = 50 psi. Specific method of sample preparation and alkaloid determination was in accordance with reference (Xue et al., 2005; Cong et al., 2008).

Statistical analysis

Digital images of the gels were obtained and analyzed using image analysis software (Quantity One 4.0.1, Bio-Rad, USA). PCA analyses of the DGGE bands were carried out based on band position and presence (presence/absence) and then the correlation matrix principal component analysis and ANOVA performed by SPSS 16.0 (SPSS for Windows, Version 16.0, USA). Differences between values at $P < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Total soil DNA extraction and PCR amplification of 16S rDNA

Extracted with the kit, polyphenols, humic acids, salts and other substances in the soil sample could be removed effectively. The total DNA and PCR products were detected by 1% agarose gel electrophoresis. As the results shown in Figures 1 and 2, the molecular weights of DNA were between 15 - 23 kb and PCR products about 250 bp.

Difference of rhizo-bacterial diversity using Shannon-Weiner index (H)

Species diversity refers to the variety of species. Aspects of species diversity can be measured in a number of ways. Most of these ways can be classified into three

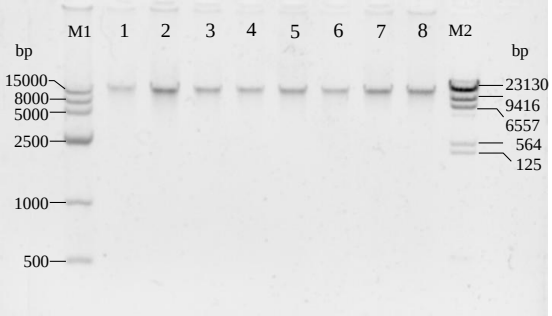

Figure 1. Soil DNA extraction from different habits of *F. thunbergii.*

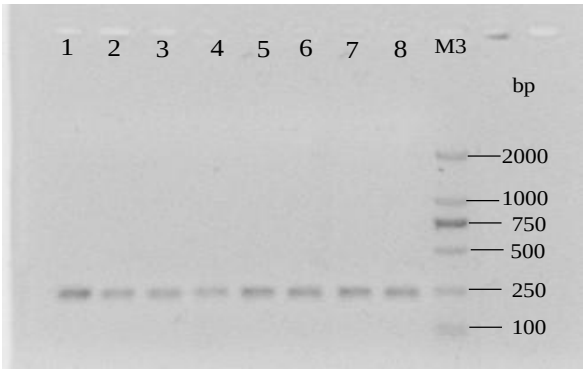

Figure 2. PCR based on V3 region of 16S rDNA. (Figures 1 and 2 are representative of soil samples from different habits. 1: Rhizoshpere soil in NB; 2: Non-rhizoshphere soil in NB; 3: Rhizoshpere soil in PA; 4: Non-rhizoshphere soil in PA; 5: Rhizoshpere soil in NT; 6: Non-rhizoshphere soil in NT; 7: Rhizoshpere soil in CA; 8: Non-rhizoshphere soil in CA.1-8 in the following charts represents for the same meaning. M1: γ-Hind Ⅲ digest DNA Marker; M2: wide range DNA Marker (500-15000); M3: DL2000 DNA Marker)

groups of measurement: species richness, species abundance and taxonomic or phylogenetic diversity (Magurran, 1988). Shannon index was calculated according to the number and intensity of bands in the DGGE plots (Table 1 and Figure 3). The composition of bacterial community in different habits was significantly different. Furthermore, the brighter bands which represented for the dominant species in the community were obviously different in amount and location. Among these samples, the uniformity of bacterial populations was very close (0.85 - 0.99), while the abundance varied, the highest on the 5th sample (collected from the rhizosphere soil in NT) for 42, the lowest on the first sample (collected from the rhizosphere soil in NB) for 24. The functional diversity based on the Shannon index of rhizosphere soil was in the following order: NT> CA > PA > NB. In addition, the genetic diversity based on Shannon index was higher in the rhizosphere soil than in non-rhizosphere soil, which indicated microbial metabolic activities in rhizosphere were particularly strong. Root exudates provided a lot of nutrients for the soil microbes and energy materials and therefore not only the types and quantities of rhizo-microbes were higher than the non-rhizosphere soil, but also its metabolic activity was higher than the non-rhizosphere microorganisms (Micallef et al., 2009) as well.

The pH value has complicated effects on the structure of soil microbial community, which can affect the availability of nutrition, microbial adsorption, production and secretion of extra cellular enzyme. Moreover, it also can affect the growth of microorganism. For example, weakly alkaline soil is suitable for bacteria and actinomycetes, while acid soil for fungi. So there is relatively large number of fungi in most acidic soils when pH value is lower than 5.0 (Hu et al., 2006). The pH value of rhizosphere soil was slightly alkaline in NT and then the highest diversity index of bacteria community was found in NT. Meanwhile, the soil samples in the other places were all acid and the lower diversity was found there. From Table 1 it also could be seen that the pH value of soil ranged from 4.29 to 7.23, which meant *F. thunbergii* could tolerate relatively wide range pH. Many researches found that pH was lower in rhizosphere soil than in non-rhizosphere soil for most plants (Zhang et al., 2004). Our findings coincided with previous researches. Changes of pH was due to CO_2 released by root respiration as well as the active absorption of ion and secretion of protons and organic acids by elongated root tip cell)(Fang et al., 2007). In these soil samples the Shannon index were all higher in non-rhizosphere soil than in rhizophere soil, but pH value just the opposite.

Difference of rhizo-bacterial diversity using PCA

PCA using both band position and presence/absence as parameters were performed to further analyze DGGE. The position and brightness of band on DGGE plots were digitized, 1 for the band appeared and 0 for no band, thus a quadratic matrix was generated for PCA by SPSS 16.0. The PCA plots showed three clear categories: NB group, PA group and CA group, indicating the altered structure in bacterial diversity among different habits. Using the presence of the bands data, the first two principal components (PC1 and 2) were sufficient to explain 28.2 and 19.4% of the variance for bacterial diversity and the different soil, respectively (Figure 4). Rhizosphere soil showed a high degree of similarity to that in non-rhizosphere soil for the same habit as indicated by PCA analysis, except NT. The rhizosphere soil in NT had relatively higher similarity with NB group, while non-rhizosphere soil in NT with CA. In a word, PCA results showed more significant differences in structures of bacterial community from different habits, but little between rhizosphere soil and non-rhizosphere soil in the same region.

Table 1. Shannon-weinner index for the different soil samples.

Soil type	Shannon index	abundance	uniformity	pH
Rhizoshpere soil in NB	$3.16^{\alpha} \pm 0.056^{\beta}a^{\gamma}$	0.99	24	4.48
Non-rhizoshphere soil in NB	2.95 ± 0.022b	0.86	31	5.45
Rhizoshpere soil in PA	3.17 ± 0.091ab	0.90	34	4.57
Non-rhizoshphere soil in PA	3.021 ± 0.128ab	0.86	33	4.97
Rhizoshpere soil in NT	3.36 ± 0.018b	0.90	42	7.45
Non-rhizoshphere soil in NT	2.81 ± 0.014a	0.85	25	7.73
Rhizoshpere soil in CA	3.31 ± 0.0001ab	0.90	39	4.29
Non-rhizoshphere soil in CA	3.13 ± 0.142ab	0.87	39	5.29

α: The arithmetic mean of 3 replicates; β: The arithmetic standard deviations; γ: significant differences (P < 0.05).

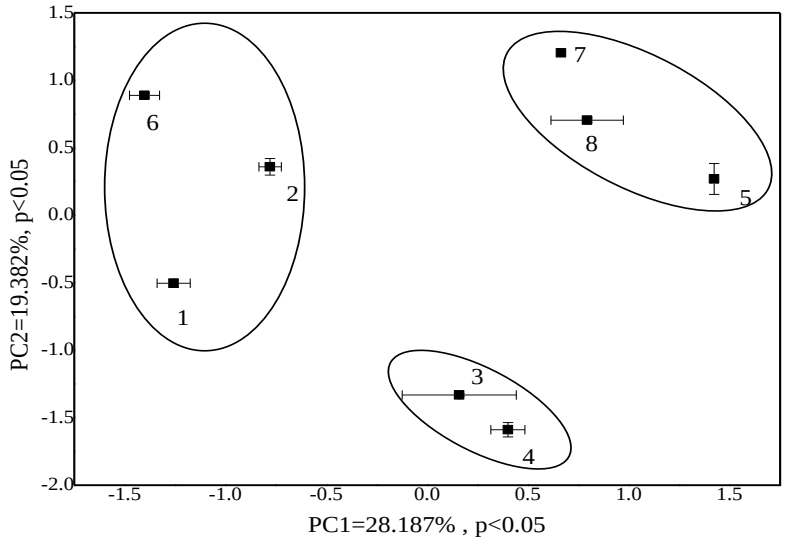

Figure 4. Score plots from PCA based on data from different soil samples of *F. thunbergii*.

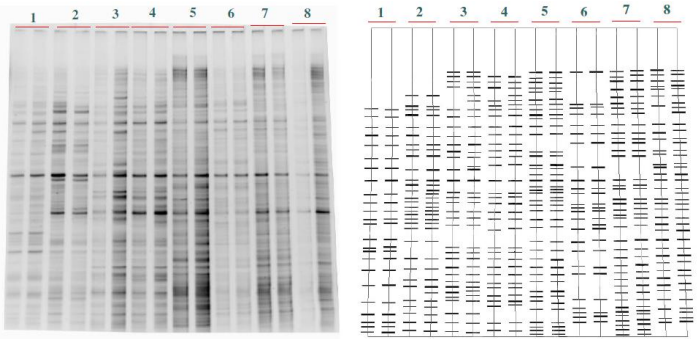

Figure 3. PCR-DGGE plot of soil microbial community from different habits.

The relationship between concentration of peimine and peiminine and rhizo-bacteria diversity

The concentration of peimine and peiminine of *F. thunbergii* was determined by HPLC-ELSD. It showed the method of HPLC-ELSD was suitable for detection of alkaloid of *F. thunbergii* because the error of each duplicate varied only from 0.0001 - 0.0005%. From Figures 5 and 6 it could be seen that the differences of the content of monomer alkaloids. The content of peimine in NT reached 0.1364%, while the lowest in NB, only 0.0646%. The level of the content of Peiminine was also the highest in NT, reaching 0.0518% and the lowest in PA (0.0316%), showing less regional differences than that of peimine. The sum of the content of peimine and peiminine in *F. thunbergii* must be above the level permitted (0.08%) by the Chinese Ministry of Health. All samples met national requirement and it was the highest in NT, followed by CA, PA and NB. As the authentic origin of *F. thunbergii*, NB showed the lowest level of the content of peimine and peiminine. The order of bacterial diversity in rhizoshpere was as follows: NT > CA > PA > NB, just as the same as the order of the content of alkaloid, showing a positive correlation. That is to say, the higher contents of peimine

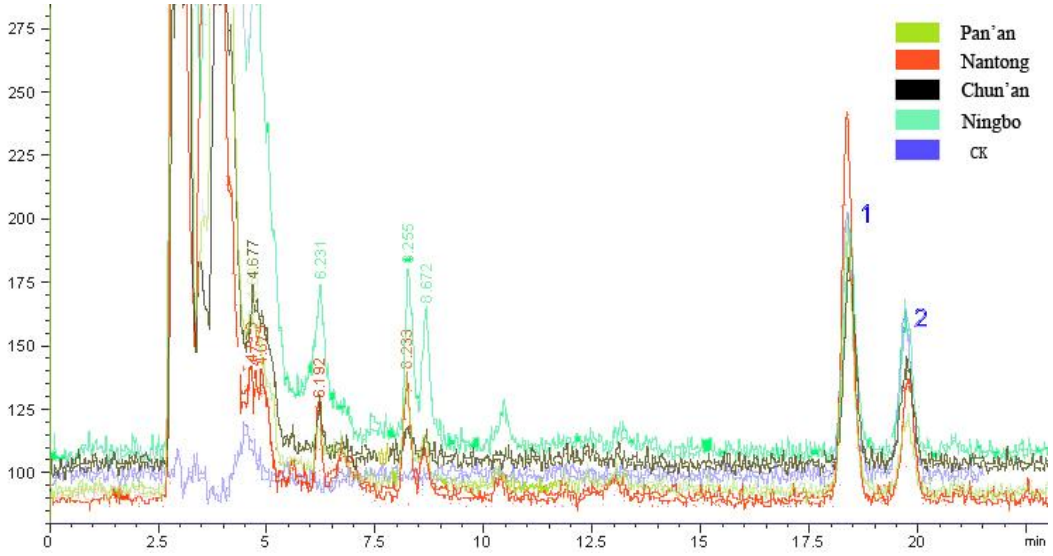

Figure 5. The content of peimine and peiminine of *F. thunbergii* .1.peimine, 2. peiminine.

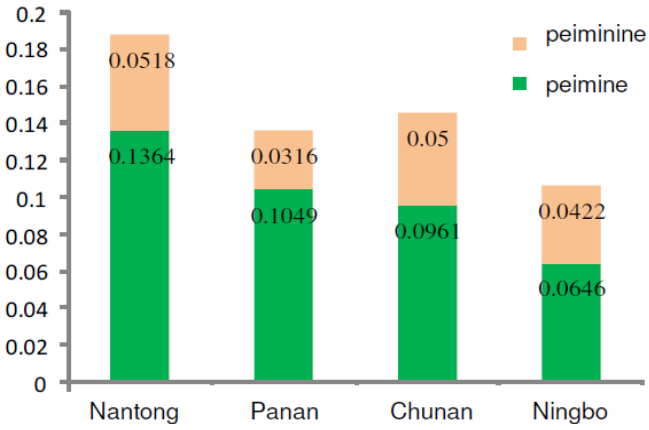

Figure 6. Chromatogram of HPLC-ELSD of peimine and peiminine from different babits of *F. thunbergi*

and peiminine, the higher bacterial diversity in rhizoshpere. However, it needed further experiment to confirm whether it was an inevitable trend for this positive correlation between alkaloid and biodiversity.

Conclusion

Plant species is considered to be one of the most important factors in shaping rhizo-bacterial communities, but specific plant-microbe interactions in the rhizosphere are still not fully understood (Micallef et al., 2009). The PCR–DGGE procedure has been proved to be sensitive and highly reproducible in analyzing microbial community compositions in various soils (Duineveld et al., 2001; Yao et al., 2000; Chen et al., 2006). In this study, we explored the differences of soil bacterial community from 4 habits of Fritillaria by PCR-DGGE. The DGGE plot was interpreted using Shannon index and PCA in which data was transformed in two ways, the former taking the relative intensity into account and the latter the presence of the bands. We found that the structure of soil bacterial communities differed in different habits and between rhizoshphere and non-rhizosphere soil in the same origin. Furthermore, rhizo-bacterial diversity was relatively highly related to the content of alkaloid concentration of *F. thunbergii*. We can improve efficacy of medicinal plant by increasing the bacterial diversity if these results make sense. The next step, we will further confirm the relationship between rhizo-bacteria and the quality of *F. thunbergii* and analyze the types of bacteria and fungi by clone and sequencing to find the specific bacteria and fungi related to efficacy.

Furthermore, NT is not authentic habits of Fritillaria but nowadays it becomes a region with the biggest yield of *F. thunbergii* (Zhen, 2005). The reason may be complicated, probably related to soil physical and chemical properties and microbial activities of soil.

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