Microbial diversity of various agricultural soil sites using denaturing gradient gel electrophoresis (DGGE) community profiling
(Kepelbagaian mikrob bagi pelbagai kawasan tanah pertanian menggunakan DGGE profil komuniti)

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Abstract
PCR-DGGE was applied in this project to compare the diversity of the bacterial populations in two different plots, Tuscany and Basilicata. Tuscany consists of forest and agricultural site while Basilicata consists of biological agricultural and conventional agricultural site. The soil sample from each plot was extracted for the DNA using the combination protocols of conventional method. The DNA was then amplified for the target 16S rRNA genes using the universal primer of P1 (forward) and P2 (reverse) for 35 cycles using the hot start technique. The PCR product was then run on 10% (w/v) of polyacrylamide gel on the denaturing gradient gel electrophoresis (DGGE) to observe the diversity profile with the gradient ranging from 40% to 75%. From the dendogram image, the PCR-DGGE bands in Tuscany showed that there were different soil microbial communities dominant for each site although there was no major difference in banding pattern between the biological agricultural and conventional plots or disturbed and undisturbed forest. In Basilicata, the soil from this site can be categorized as a heterogeneous soil. The correlation analysis indicated that when the aridity index increased and water content decreased, the bacterial number increased although no impact on microbial diversity was observed.

Introduction
Microorganisms are found in large number in soil. The availability of nutrients is limiting the growth of some microorganisms in soil. Soil microorganisms form a robust community capable of surviving and functioning under extremes of temperature, water availability, pH, energy resources, nutrient and salt concentration (Conklin 2002). The culturable microorganisms were only 0.1–1.0% recovered from the culture dependent method that used most of the laboratory media.

Diversity is composed of two elements: i) richness and ii) evenness. From these two elements, the highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Torsvik and Ovreas 2002). Most of the microbial diversity studies conducted in complex ecosystem, such as soil have been biased essentially by

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the unculturability of many microorganisms and the lack of sensitivity of traditional microbiological methods (Hugenholtz et al. 1998). However, the methods of molecular analysis of microbial communities rely not only on the extraction of DNAs but also on factors related to PCR, such as the choice of primers, the concentration of amplified DNA, errors in the PCR or even the method chosen for analysis (Miller et al. 1999).

The 16S rRNA gene is a phylogenetic marker that is frequently used to describe the microbial community in natural environments without a need for cultivation (Felske et al. 1999). Methods that rely on the 16S rRNA gene to characterize the microbial community structure include denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and amplified ribosomal DNA restriction analysis (ARDRA). These methods are frequently used to study bacterial communities in soil ecosystems (Henckel et al. 1999; Kowalchuk et al. 1999).

DGGE of PCR amplified ribosomal DNA was introduced by Muyzer et al. (1993) as one of the successful fingerprinting techniques for microbial ecology. By using the DGGE, 50% of sequence variants can be detected in DNA fragments up to 500 bp. DGGE is based on the electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In DGGE gels, DNA fragments of the same length but with different base pair sequences can be separated (Muyzer et al. 1993). The aim of this study was to investigate microbial diversity in soil with the community profiling for 16S rRNA conserved gene.

Materials and methods

Site sampling

The Tuscany plot was divided into two sites, i.e. the forest and agricultural. The forest site consists of disturbed forest which has a boar feeding place near the sampling plot and undisturbed forest. The agricultural site consists of biological agriculture (the field was applied with green manure) and conventional agriculture (the field was applied with mineral fertilizer such as ammonium nitrate and pesticide).

The Basilicata plot also consists of biological agriculture and conventional agriculture. In biological agriculture, the soil was cropped with *Triticum durum* since year 2000. In the conventional agriculture, the soil was cropped with *T. durum* for 4 years. Fertilizer used was a mineral complex with 25% N, 15% P, added as ammonium nitrate and ammonium phosphate (Garcia and Hernández 2004).

All soil samples were collected and stored at −20 °C.

Soil DNA extraction

The soil DNA extraction was based on established methods by Griffiths et al. (2000) and Calvo-Bado et al. (2003). In the Griffiths method, 0.5 g of sample was weighed in the blue cap ribolyzer tube (BIO 101 System). Then, 0.5 ml of 5% CTAB buffer and 0.5 ml of phenol:chloroform:isoamyl was added. The tube was ribolyzed for 30 sec at speed 5.5. The tube was then put on ice and centrifuged at full speed (13,000 x rpm) for 5 min. The top layer was extracted and transferred into a new eppendorf tube. An equal volume of chloroform:isoamyl alcohol to the volume of the liquid from the top layer was pipetted and mixed before centrifuging at full speed (13,000 x rpm) for 5 min. The top layer was extracted and 2 volumes of PEG solution were added.

The solution mixture was left for 2 h at room temperature and then centrifuged at full speed for 10 min. Supernatant from the tube was poured off and pellet washed with 70% ethanol. After another spin, pellet was air dried for 20 min and resuspended in 50 μl of TE buffer and stored at 4 °C. In the combination protocol, 0.5 g of soil sample was weighed into ribolyzer tube and 1 ml of 0.1 M NaH₂PO₄ buffer and 100 μl of
lysozyme were added. Tubes were incubated in a water bath for 1 h at 37 °C.

The samples were then ribolyzed for 30 sec at speed 5.5. Then, 10 μl of 10% SDS was added immediately and the samples were left on ice for 10 min. A volume of 1 ml of phenol:chloroform:isoamyl alcohol was added to the tube, mixed and centrifuged for 15 min (Calvo-Bado et al. 2003). The top layer was extracted and DNA precipitated by adding 2 volumes of PEG solution as in the Griffiths protocol. All samples were run on 1% agarose gel at 120 V.

**Polymerase chain reaction (PCR)**

PCR was performed using the Hybaid Thermal Gradient PCR Express machine. Universal primer pair P1 and P2 was used to amplify part of the 16S rRNA gene of P1 (forward) 5'-CCTACGGGAGGCAGCAG-3' and P2 (reverse) 5'-ATTACCGCGGCTGCTGG-3' (Muyzer 1993). The reagent for PCR mixture contained (in total volume of 100 μl): 2 μl dNTPs, 10 μl 10 x Taq PCR Buffer + KCl-MgCl₂, 6 μl of 25 mM MgCl₂, 10 μl BSA, 5 μl DMSO, 1 μl of primer P1 (50 pM), 1 μl primer P2 (50 pM), 1 μl DNA, 8 μl Taq polymerase 500 u and 53 μl of sterilized distilled water.

Hot start technique was used to ensure proper denaturation and amplification of all DNA: 94 °C for 5 min followed by hold temperature 80 °C during which the diluted Taq polymerase was added. Then the samples were amplified as follows: 35 cycles of 94 °C for 1 min, 64 °C for 1 min and 72 °C for 2 min with a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide and photographed using the UV transilluminator.

**Denaturing gradient gel electrophoresis (DGGE)**

PCR samples were loaded on 10% (w/v) polyacrylamide gel in 1 x TAE buffer using denaturing gradient ranging from 40% to 75%. A volume of 100 ml of

100% denaturant solution consisted of 42 g of urea, 40 ml of formamide, 25 ml of 40% (v/v) acrylamide/Bis (37:5:1) and 2 ml of 50 x TAE buffer while a volume of 100 ml of 0% denaturant contained 25 ml of 40% (v/v) acrylamide/Bis (37:5:1), 2 ml of 50 x TAE buffer and mark up with distilled water until it reached 100 ml.

The electrophoresis was run at 60 V for 18 h at 60 °C. After the electrophoresis, the gel was stained with SYBR Green 1 nucleic acid stain and an image was taken under the UV transilluminator (GeneGenius BioImaging System, UK). All gel was screened and analysed by using Gene Tool and Gene Directory (Syngene, UK) software that contribute dendograms based on similarities in banding patterns.

**Results and discussion**

**DGGE analysis**

All DGGE gel was analysed using Gene Tool Software (Syngene, UK). This software detected more banding patterns that were not visualized clearly when gel picture was taken under UV transilluminator.

**Tuscany plot**

The dendogram of DGGE image of Tuscany (Figure 1) clearly showed that sample from agricultural site and forest was distinguished by the separated branch (except track 6 was out of the forest group) which had a closely similar banding pattern. Based on this, there are different soil microbial communities dominant for each site although there is no major difference in banding pattern between the agricultural and conventional plot or disturbed and undisturbed forest. Soil for the analysis was taken from a different point in both treatments. But with the banding pattern shown by DGGE, conventional agricultural site and disturbed forest were homogenous. Therefore, the effect of the pesticide application, fertilizer and animal population gave an impact on the diversity of the microorganisms in soil in both the conventional agriculture and disturbed forest treatment. Soil from biological agriculture
Microbial diversity using community profiling

Tuscany site

Basilicata site

and undisturbed forest were heterogeneous which means both treatments did not affect the microbial diversity in soil.

**Basilicata plot** The DGGE banding pattern in both biological and conventional agriculture produced patterns closely identical to each site. From the DGGE image (*Figure 1*), the branch separating biological and conventional agricultural can be distinguished clearly. But there were replicates (from biological agriculture – track 2 and from conventional agriculture – tracks 7 and 13) from both treatments which separated from their group. So, the soil in this site can be categorized as a heterogeneous soil. These results indicate that the dominant microbial genera were not much affected by the application of either fertilizer or pesticide application in this soil.
Correlation analysis
The correlation analysis was done to compare bacterial number with a few important factors that influenced microbial diversity in soil; organic matter content, water content, aridity index and plant cover. All data was provided by INDEX team members and not shown in this paper.

No correlation appeared between bacterial numbers and organic matter content (Figure 2). The $R^2$ value was less than 1% which showed that there is a little or no correlation between bacterial number and organic matter. The bacterial numbers increased with the increasing value of the aridity index (Figure 2). No correlation appeared between bacterial numbers and plant cover (Figure 3). It was a weak relation with the $R^2$ values being less than 1%. A weak correlation also was presented with bacterial numbers and water content (Figure 3).

Conventional and biological agricultural practices appeared to have an impact on microbial diversity. Tuscany and Basilicata sites both were agricultural but different results were indicated by the DGGE image profiles (Figure 1). Soil from conventional agriculture in Tuscany showed similar dendrogram patterns for different treatments but those in Basilicata showed that banding patterns were highly heterogeneous.

The banding pattern represents the different microbes DNA which migrates through the gel and stop when denatured within the gradient range. There is different limitation for each microbe to denature and this is an indication of species richness and evenness which were the two elements in the diversity.

The banding pattern in Basilicata showed that diversity within treatments was quite heterogeneous and therefore no conclusions could be drawn. However, in the Tuscany treatments, replicates were quite reproducible. The banding pattern profile from DGGE image indicated by the dendrogram showed that most replicates of
each agriculture type clustered separately distinguishing between the biological and conventional agricultural treatment. Two replicates from conventional agriculture and one replicate from biological agriculture did not cluster with their groups.

The influence of pesticide on the soil microbial diversity has been reported by Sims (1990). Microbial activity is a term used to indicate the vast range of activities carried out by microorganisms in soil, whereas biological activity reflects not only microbial activities but also the activities of other organisms in the soil, including plant roots (Nannipieri et al. 1990). When the soil activity is influenced by any mechanism involved in the soil, the microbial diversity can also be affected (Gelsomino et al. 1999).

Four main factors were correlated with data generated in this project; correlation between bacterial number with i) organic matter, ii) aridity index, iii) plant cover and iv) water content. Aridity is determined by the simultaneous scarcity of rain and high evaporation that removes moisture from the soil. Aridity may be characterized by an aridity index, which compares the incoming moisture flow (rainfall) with the potential outflow (evaporation) (Sciortino 2001).

There was a relationship between bacterial number and the aridity index, with $R^2$ value of 0.247. When the aridity index increased and water content decreased, the bacterial number was also increased. It can therefore be hypothesized that an increase in organic matter content decreases diversity independently of water content, but has no effect on bacterial numbers; while water content (also a factor in aridity index) is negatively correlated with bacterial number.

**Conclusion**

The use of microbial fingerprinting analysis could illustrate an important finding that the different treatment applied to the soil could give some impact to the microbial diversity. This impact should be correlated with some physicochemical studies such as water content and organic matter to give more accurate indicator of communities profiling.

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**Abstrak**

PCR-DGGE telah diaplikasikan dalam projek ini untuk membandingkan kepelbagaian populasi bakteria di dua plot yang berlainan, Tuscany dan Basilicata. Di Tuscany, terdapat tapak hutan dan tapak pertanian manakala di Basilicata terdapat tapak pertanian biologi dan pertanian konvensional. Sampel tanah dari setiap plot diekstrak untuk mendapatkan DNA tanah menggunakan protokol gabungan kaedah lazim. DNA tanah kemudian diperluas untuk sasaran gen 16S rRNA menggunakan pencetus universal (primer) P1 (ke hadapan) dan P2 (ke belakang) untuk 35 kitaran menggunakan teknik permulaan yang panas. Produk PCR kemudian diletakkan di atas 10% (berat/isi padu) gel poliakrilamida untuk digunakan pada DGGE bagi memerhatikan kepelbagaian profil dengan julat gel antara 40% hingga 75%. Daripada imej dendogram, jalur PCR-DGGE di Tuscany menunjukkan terdapat komuniti mikrob tanah yang berbeza bagi setiap kawasan walaupun tidak terdapat perbezaan besar antara jalur kawasan pertanian biologi dengan konvensional atau hutan terganggu dengan tidak terganggu. Di Basilicata, tanah dari kawasan ini boleh dikategorikan sebagai tanah yang heterogen. Analisis korelasi menunjukkan bahawa apabila indeks kekeringan meningkat dan kandungan air berkurangan, jumlah bakteria meningkat walaupun tiada kesan terhadap kepelbagaian mikrob.

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