Short Communication

A Direct Method to Isolate DNA from Phyllosphere Microbial Communities without Disrupting Leaf Tissues

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We developed a method for direct DNA isolation from phyllosphere microbial communities, designated Direct-DIP. This method comprises DNA extraction from non-shredded leaves with benzyl chloride, and DNA purification by gel filtration. Scanning electron microscopy showed that epiphytic microorganisms were completely removed from the leaf surface after benzyl chloride treatment, while microstructures of the leaf were not damaged. Clear DGGE profiles were obtained regardless of the plant species. Shannon diversity indices of DGGE profiles by Direct-DIP were higher than those by a conventional method. Our findings suggest that Direct-DIP is a rapid, simple, and cost-effective method of extracting DNA from phyllosphere microbial communities.

Key words: phyllosphere, epiphytic bacteria, DNA isolation, DGGE

The phyllosphere, the above-ground surfaces of plants represented by leaves, is the habitat of various microorganisms including bacteria, fungi and yeasts. Epiphytic bacteria are the main inhabitants of the phyllosphere, and are estimated to have a density of 10^6 to 10^7 CFU cm⁻² on a typical leaf^{1,4}. Traditional isolation studies have shown that such bacteria include plant pathogens, ice nucleation-active bacteria, decomposers, phytohormone producers, nitrogen fixers, and antagonists of plant pathogens^{3,4,6,8,17,26}. However, recent studies have revealed that many microorganisms in this environment are non-culturable. Thus, traditional culture-based methods are insufficient to analyze *in situ* microbial communities^{24,25}. Instead, culture-independent methods, particularly molecular approaches involving DNA extracted from environmental samples, are now increasingly used to analyze microbial communities^{1,2,5,22}.

In recent years, molecular biological techniques have been used to study environmental microbial community structures. Such techniques include PCR-denaturing gradient gel electrophoresis (DGGE) and the PCR-single strand conformation polymorphism (SSCP) method^{11,19,28}. For these techniques, PCR inhibitor-free DNA should be prepared by simple, rapid, and cost-effective methods. To date, two different methods have mainly been used to isolate DNA from the phyllosphere microbial community^{10–12,21,28}. With one method^{12,28}, leaves suspended in buffer solution are sonicated to strip epiphytic microbes from leaves. After microbes are collected by centrifugation, they are disrupted by bead-beating and total DNA is isolated using a commercial DNA extraction kit (FastDNA kit, Q-BIOgene, Irvine, CA, USA). With the other method¹⁰, leaves suspended in buffer are frozen, thawed, and treated with lysozyme, proteinase K, and sodium dodecyl sulfate (SDS) to disrupt microbial cells.

After purification with chloroform, total DNA is recovered by isopropanol precipitation. The former method requires an expensive kit and mechanical disruption, while the latter is laborious and time-consuming.

Zhu *et al.*²⁹ reported a simple and rapid procedure to extract genomic DNA from plants and microbial (fungi and bacteria) pure cultures. With their method, benzyl chloride is used to destroy cell walls of plants and microorganisms, via its reaction with polysaccharides' hydroxyl groups. In addition, benzyl chloride can also extract proteins and other cell debris from the aqueous phase. Therefore, genomic DNA is released without any mechanical shearing and phenol-chloroform treatment.

In this study, we developed an efficient DNA extraction method: direct genomic DNA isolation from the phyllosphere, or Direct-DIP, for targeting DNA from phyllosphere microbial communities. The applicability of Direct-DIP was assessed by PCR-DGGE analysis using a wide variety of plant species, and its efficacy was compared with a conventional method.

Leaves from eight plant species were used in this study. Fresh leaves of Japanese cedar (*Cryptomeria japonica*) were collected in 2005 or 2006 from four sites in Japan; Nagano, Gunma, Chiba, and Hokkaido. Samples of mature leaves were collected from three individual trees at each site. Fresh leaves of Japanese cypress (*Chamaecyparis obtusa*), Chinese fir (*Cunninghamia lanceolata*), Himalayan cedar (*Cedrus deodara*), Japanese red pine (*Pinus densiflora*), oak (*Quercus myrsinaefolia*), and ginkgo (*Ginkgo biloba*) were collected from Chiba in 2006. In these cases, samples were collected from two individual trees. In addition, fresh leaves of cucumber (*Cucumis sativus*) grown in a greenhouse were collected from Chiba in 2007. Leaf samples were stored in polyethylene bags at 4°C and DNA was isolated within 24 h.

For DNA extraction from the phyllosphere microbial community, a 5 g non-shredded fresh leaf sample was placed in a

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50 mL sterilized polypropylene tube, and 5 mL of extraction buffer (100 mM Tris-HCl, pH 9.0, 40 mM EDTA), 1 mL of 10% SDS, and 3 mL of benzyl chloride (Wako Pure Chemical Industries, Osaka, Japan) were added²⁹. The tube was incubated for 15 min at 50°C by mixing repeatedly with 1-min intervals so that the two phases were thoroughly mixed. The leaves were removed from the tube, and 3 mL of 3 M sodium acetate (pH 5.0) was added. After incubation on ice for 10 min, the suspension was centrifuged (6,000×g, 15 min, 4°C). The aqueous phase was transferred to a new centrifuge tube, and DNA was precipitated by adding an equal volume of isopropanol followed by centrifugation (9,000×g, 15 min) at 4°C. After air-drying, the pellet was resuspended in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

To confirm the removal of epiphytic microorganisms from the leaf surface by the extraction step, leaves of Japanese cedar were observed using a scanning electron microscope (SEM). After the incubation with benzyl chloride, leaves were cut to approximately 10 mm in length and fixed in 3% glutaraldehyde for 12 h at 4°C. The fixed samples were dehydrated through a graded ethanol series (50, 70, 80, 90, and 100%; 15 min each), followed by 100% t-butanol. After dehydration, the samples were lyophilized and sputter-coated with gold (JFC-300 and JFC-1100, JEOL, Tokyo, Japan). Leaf surfaces were observed with an SEM (JSM-5400LV, JEOL). Fresh Japanese cedar leaves without the benzyl chloride treatment were similarly observed as a control. Filamentous fungi or yeasts (Fig. 1A and B, white arrows) and bacterial biofilm-like structures (Fig. 1B, black arrows) were observed on the surface of the control leaves, whereas no such microbe-like structures were found on the leaves treated with benzyl chloride (Fig. 1C and D). In addition, plant leaf structures such as stomata were apparently not damaged by the treatment.

Zhu *et al.*²⁹ reported that benzyl chloride extracted microbial DNA and also plant DNA, because plant cell walls are destroyed as a result of its reactivity with hydroxyl groups of polysaccharides, including cellulose and hemicellulose.

Fig. 1. Leaf surface after treatment with benzyl chloride. A and B: SEM micrograph of fresh Japanese cedar leaf surface before DNA extraction. White and black arrows indicate filamentous fungi and bacterial biofilm-like structures, respectively. C and D: SEM micrograph of leaf surface after the extraction process. White arrows indicate stomata of the host plant.

However, the SEM observations indicated that plant leaf structures such as stomata remained intact after benzyl chloride treatment (Fig. 1). As compared with the technique of Zhu *et al.*²⁹, we assumed that smaller amounts of plant DNA would be extracted by Direct-DIP because it involves no physical disruption of leaf tissues, whereas Zhu *et al.*²⁹ cut leaves into pieces approximately 1 cm².

PCRs using the crude DNA as a template gave poor results, so DNA was further purified by gel filtration. A 1 mL volume of Sepharose CL-4B (GE Healthcare UK, Buckinghamshire, England) was packed into a sterilized plastic syringe (Terumo, Tokyo, Japan), plugged with 0.1 g of siliconized glass wool at the bottom⁹. The syringe was transferred to a plastic tube (15 mL volume), centrifuged (1,000×g, 3 min) and repacked until the gel matrix reached approximately 5 cm in the column. TE buffer (50 µL) was added to the gel matrix, and excess buffer was removed by centrifugation (1,000×g, 3 min). Crude DNA (50 µL) was then loaded onto the column, and the eluent was collected by centrifugation (1,100×g, 3 min). Using this procedure, we were able to obtain approximately 250 ng of purified DNA from 5 g of Japanese cedar leaves.

To evaluate the effectiveness of the purification step, the 16S rRNA gene was amplified either from the crude DNA or from the purified DNA by PCR. PCR was conducted using the following reaction mixture: 200 µM dNTP; 0.5 U of Ampli Taq Gold (Applied Biosystems, Foster City, CA) in 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.5 mM MgCl₂; 0.5 µM primers designed for amplification of the V3 region of the 16S rRNA gene; and 2 µL of template DNA. The specific PCR primers were PRBA338f (5'-ACTCCTACGGGAGGC-AGCAG-3')¹³ and PRUN518r (5'-ATTACCGCGGCTGCTGG-3')¹⁸. For DGGE, a 40-bp GC-clamp (5'-CGCCCCGCCGCGCGCGCGGGCGGGGCGGGGGCACGGGGGG-3') was linked at the 5' end of primer PRBA338f¹⁸. After 10 min of initial preheating at 94°C, 38 cycles at 94°C for 15 s, 55°C for 45 s, and 72°C for 30 s were carried out, with a final extension at 72°C for 7 min (iCycler, Bio-Rad Laboratories, Hercules, CA). PCR products of approximately 200 bp were visually confirmed by electrophoresis on 4.0% (w/v) agarose gels, while no amplification was observed when the crude DNA was used as a template (Fig. 2).

One of the major concerns in DNA extraction from environmental samples is the co-extraction of endogenous impurities, e.g., humic substances that frequently inhibit PCR

Fig. 2. Effect of genomic DNA purification on 16S rRNA gene amplification. Lanes 1–5 show PCR products of DNA extracted from Japanese cedar, Japanese cypress, Japanese red pine, oak, and ginkgo, respectively. C: crude DNA used as a template, P: purified DNA used as a template, M: DNA size markers. The expected length of PCR product is approximately 240 bp. Inset shows purified DNA (left) and crude DNA (right) solutions extracted from Japanese cypress.

amplification^{23,27}). In our study, PCR amplification of 16S rRNA genes was strongly inhibited when the crude DNA was used as a template. Because the crude DNA was brownish in color (Fig. 2, inset), polyphenol-like PCR inhibitors may have been co-extracted from epicuticular wax on the leaf surface. However, such endogenous impurities were eliminated by the gel filtration, and the resulting DNA was sufficiently pure for amplification of 16S rRNA genes.

PCR products from the purified DNA were further separated by DGGE using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories). The products were separated on an 8% polyacrylamide gel containing a linear gradient of 30–60% denaturant (100% denaturant defined as 7 M urea and 40% formamide). The electrophoretic conditions were as follows: 170 V, 6 h, 60°C; running buffer, 40 mM Tris, 20 mM acetate, 1 mM EDTA (pH 7.4). The gel was stained with ethidium bromide after electrophoresis. Separated DNA bands on DGGE profiles were cut from the gel and eluted in 30 µL of TE buffer overnight at 4°C. The eluted DNA was re-amplified by PCR using primers PRBA338f (without a GC-clamp) and PRUN518r, under the conditions described above. Sequences of the re-amplified DNA fragments were determined using a DNA sequencing kit (Big Dye Terminator V3.1, Applied Biosystems, Foster City, CA) and a DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). The sequences obtained in this study have been deposited in the DNA Data Bank of Japan (Accession numbers AB325969 to AB325983). Similarity searches for the determined sequences were performed using the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information web server.

We obtained purified DNA samples from leaf surfaces of 12 individual Japanese cedar trees collected from four geologically different sites. When these DNA samples were subjected to PCR-DGGE of 16S rRNA genes, clear DGGE profiles were obtained for all leaf samples (Fig. 3). Variations in banding patterns were observed among individual trees and sampling sites. Nucleotide sequences of dominant bands in the DGGE profiles were determined and compared with gene databases (Table 1). Of 15 sequences determined, 11 showed highest similarity to the 16S rRNA gene sequences of uncultured bacterial clones obtained from environmental samples.

Fig. 3. PCR-DGGE profiles of epiphytic bacteria from phyllospheres of Japanese cedar collected from four different sites. Lanes 1–3, 4–6, 7–9, and 10–12 show DGGE profiles of samples collected from Nagano, Gunma, Chiba, and Hokkaido, respectively. Three lanes for each site represent DGGE profiles from three individual trees.

The sequence of band B was 100% homologous to that of the chloroplast 16S rRNA gene of *Cryptomeria fortunei*, a species that is phylogenetically close to Japanese cedar (*Cryptomeria japonica*). The sequences of bands C, D and E showed highest similarities to that of the 16S rRNA gene of the uncultured bacterium clone BPH3074, which was found in phyllosphere microbial communities in tree canopies of the Atlantic forest¹². In addition, some sequences of the dominant bands in the DGGE profile were closely related to *Sphingomonas* sp. and *Methylobacterium* sp. (Table 1, bands K and N, respectively), both of which have been isolated frequently from the phyllosphere^{15,16,20}.

The applicability of Direct-DIP was further assessed using DNA samples isolated from the phyllosphere microbial communities of seven different plant species, i.e. Japanese cypress, Chinese fir, Himalayan cedar, Japanese red pine, oak, ginkgo, and cucumber, followed by PCR-DGGE. Again, clear DGGE profiles were observed regardless of the plant species. Variations of banding patterns were observed

Table 1. Identification of major DGGE bands in Fig. 3

Band position	Data base match (accession number) ^a	% similarity	Accession number
A	Uncultured Bacteroidetes bacterium from <i>Parlatoria camelliae</i> (DQ133554)	98	AB325969
B	<i>Cryptomeria fortunei</i> chloroplast (DQ478788)	100	AB325970
C	Uncultured bacterium clone BPH3074 (DQ221667)	99	AB325972
D	Uncultured bacterium clone BPH3074 (DQ221667)	99	AB325973
E	Uncultured bacterium clone BPH3074 (DQ221667)	99	AB325974
F	Uncultured gamma proteobacterium clone 2645 (EF447082)	97	AB325975
G	Uncultured gamma proteobacterium clone 2645 (EF447082)	100	AB325976
H	Uncultured <i>Beijerinckia</i> sp. clone A_A04 (EU513885)	99	AB325977
I	Uncultured <i>Sphingomonas</i> sp. clone C94 (EU503103)	100	AB325978
J	Uncultured <i>Sphingomonas</i> sp. clone C94 (EU503103)	100	AB325979
K	<i>Sphingomonas</i> sp. M60-VN10-2W (AB299584)	100	AB325980
L	Uncultured cyanobacterium clone GASP-MA3W2_D07 (EF663772)	100	AB325981
M	Uncultured alpha proteobacterium clone SMS9.9WL (AY043778)	100	AB325982
N	<i>Methylobacterium</i> sp. XTB-8015 (EU360598)	98	AB325983

^a All sequences are 16S ribosomal RNA genes.

Fig. 4. PCR-DGGE profiles of epiphytic bacteria from the phyllospheres of seven different plant species. Lanes 1–2, 3–4, 5–6, 7–8, 9–10, 11–12, and 13–14 show DGGE profiles of Japanese cypress, Chinese fir, Himalayan cedar, Japanese red pine, oak, ginkgo, and cucumber, respectively. Two lanes for each species represent DGGE profiles from two individual plants.

among individual trees and different plant species (Fig. 4). These results suggest that the quality of DNA isolated by Direct-DIP is sufficient for a PCR-based analysis of the phyllosphere microbial community of a wide variety of plant species.

For the extraction of DNA from the phyllosphere microbial communities, a washing-based method has commonly been used^{12,28}. This method comprises leaf washing with sonication, mechanical disruption of microbial cells with a bead-beater, and total DNA isolation using a commercial kit. The efficiency of Direct-DIP was compared with that of the washing-based method. Japanese cedar leaf-samples (5 g) were subjected to DNA isolation by a conventional method reported by Yang *et al.*²⁸ and Lambais *et al.*¹². Leaf-samples were submerged in sterilized 0.1 M potassium phosphate buffer (pH 7.0) in sterilized polypropylene tubes. The tubes were then sonicated at 50 kHz for 7 min in an Ultrasonic cleaner VS-100 (AS ONE, Osaka, Japan). Microorganisms removed from the leaf-sample were collected by centrifugation (20,000×g, 15 min), and resuspended in 500 µL of potassium phosphate buffer. Total DNA was extracted using the FastDNA kit and a bead-beater (BioSpec Products, Bartlesville, OK), according to the manufacturer's instructions. Because DNA samples obtained by either method may include plant DNA, the quantity of total DNA yielded by the different methods may not be appropriate to assess the efficiency of Direct-DIP. Therefore, we compared epiphytic bacterial diversity by analyzing DGGE patterns of the extracted DNA. DNAs obtained by Direct-DIP and the conventional method were subjected to PCR-DGGE as described above. Digitized images of DGGE profiles were analyzed with the ImageJ 1.37v software package (U.S. National Institutes of Health, Bethesda, MD). The diversity of the epiphytic bacterial community was calculated using the Shannon index (H'),

$$H' = - \sum_{i=1}^S p_i \ln p_i$$

where S is the number of bands and p_i is the ratio of a given band intensity to the total intensity of bands.

We analyzed DNA extracted using the two different methods from leaf samples of four individual Japanese cedar

trees. Although a few bands appeared in the DGGE profiles using conventional extraction, more diverse bands were detected by the Direct-DIP method. Shannon indices (H') of DGGE banding patterns of DNA obtained by Direct-DIP and the conventional method were 2.67 ± 0.214 and 2.35 ± 0.024 (mean $\pm$ SE, $P=0.049$ by Student *t*-test), respectively. A previous study indicated that the leaf-washing method resulted in a low rate of recovery of epiphytic bacteria⁷. Therefore, DNA isolated by the washing-based method may not reflect intact epiphytic microbial community structures. The higher diversity indices in DGGE profiles suggested that the Direct-DIP method extracts more intact epiphytic microbial communities because of the effect of benzyl chloride washing. The conventional method also requires both an expensive kit and a bead-beater, and these steps may limit the number of samples that can be analyzed. Another method for DNA isolation from the phyllosphere is enzyme-based, treating plant leaves with lysozyme, proteinase K and SDS¹⁰. It requires freezing-thawing of the leaves, triplicate incubations, and chloroform purification, and thus is generally laborious and time-consuming. The Direct-DIP method described in this study does not require mechanical disruption, expensive commercial kits, freezing-thawing, or lytic enzymes. Furthermore, DNA with sufficient purity for a PCR-based community analysis can be obtained in less than 90 minutes. Overall, the Direct-DIP method presented in this study should assist studies of metagenomic DNA from phyllosphere microbial communities.

In summary, the advantages of Direct-DIP are as follows: (i) it is a fast, simple and cost-effective procedure for isolating DNA from phyllosphere microbial communities, (ii) DNA can be obtained from the phyllosphere microbial communities of a wide variety of plant species, and (iii) the purity of the isolated DNA is sufficient for PCR-based analyses since PCR-inhibitors are eliminated during the purification step.

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