Microbes Environ. Vol. 21, No. 2, 112–121, 2006
http://wwwsoc.nii.ac.jp/jsme2/

Community Analysis of Seed-Associated Microbes in Forage Crops using Culture-Independent Methods

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(Received January 5, 2006—Accepted March 2, 2006)

Microbial diversity in forage crop seeds was examined using culture-independent methods. Environmental DNA samples were extracted from the surface of the seeds, and the intergenic spacer region between small subunit and large subunit RNA genes was then amplified by ribosomal intergenic spacer analysis (RISA) for the profiling of microbial community structures. The results suggested the presence of stable microbial communities in seeds and sequencing of the RISA amplicons identified a total of 33 unique microbial sequences in 1.6 g of seed material from Italian ryegrass (*Lolium multiflorum*) and timothy (*Phleum pretense*). These included several sequences showing high similarity to known plant-associated microbes, such as *Pseudomonas fluorescens* and *Clavibacter michiganensis*, the *Cladosporium* and *Dioszegia* group of fungi, and also several uncultured bacteria.

Key words: environmental DNA, forage crop seed, Italian ryegrass, ribosomal intergenic spacer analysis, timothy

Introduction

Seed-associated microbial communities are considered to be an important environmental factor affecting seed performance and subsequent plant growth. Among the microbes present in seeds, plant pathogens have been thoroughly investigated due to their obvious deleterious effects on crop production[43]. In addition, beneficial bacteria have been artificially inoculated in seeds as either biological control agents or plant growth promoters[7,44]. Under laboratory conditions, beneficial bacteria such as *Pseudomonas fluorescens* and *P. putida* have been shown to adhere to seeds and then to establish colonies in the rhizosphere[10,15]. It has also been reported that seed germination can be promoted by the presence of certain fungi such as those of the genera *Rhizoctonia* and *Fusarium*[42,46]. The subsequent proliferation of these pathogenic or beneficial microbes in the phytosphere, however, may largely rely on interactions with other environmental organisms. For example, the application of biological control agents for plant protection often results in unstable effects during actual cultivation, despite the indication of promising results in the laboratory[44]. Furthermore, inconsistent results have often been observed between the laboratory and the field during the assessment of seed vigor[19]. Among the potential causes of these phenomena, seed-associated microbes are thought to be one of the most important[6,9,11,33,34].

Current advances in environmental microbiology have now revealed that the majority of microbes in nature are unculturable and have therefore not been subjected to conventional microbiological examinations[1]. Moreover, based upon culture-independent methodologies, it is now evident that these unculturable microbes play indispensable roles in the sustainability of diverse ecosystems[41]. Hence, seed-associated unculturable microbes may also have a significant ecological and economic impact upon plants.

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To date, there have been several reports of community-based analyses of seed-associated microbes, but these studies were limited in their scope by low detection sensitivities in the profiling of microbial communities, and in the coamplification of either organelle or nuclear RNA genes of plant DNA[11,34]. In a previous report from our laboratory[21], we found that the culture-independent methodologies employed in soil microbiology are sufficiently robust to both detect and monitor environmental microbes from a range of biological materials, including agronomic products. In addition, we have also shown that ribosomal intergenic spacer analysis (RISA), which amplifies the intergenic spacer region between the small and large subunit RNA genes in microbial genomes, can generate profiles of microbial communities in the rhizosphere of chrysanthemum without a noticeable bias caused by the presence of plant DNA[23]. Furthermore, RISA is considered simpler than other techniques for analyzing microbial communities, such as denatured gradient gel analysis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP). This is principally because it does not require special conditions or pretreatments for electrophoresis and simply detects the variation in length between ribosomal intergenic spacer regions among microbes. By taking advantage of a robust soil DNA extraction method and RISA for studying microbial communities, we have examined seed-associated microbial diversity in the present study. Our findings demonstrate the potential usefulness of these culture-independent methodologies for evaluating microbial diversity and for monitoring specific microbes in seeds.

Materials and Methods

Seed materials

Seed products of the Italian ryegrass (*Lolium multiflorum*) cultivars "Waseyutaka" and "Excellent", timothy (*Phleum pretense*) cultivars "Akkeshi" and "Kiritappu", and alfalfa (*Medicago sativa*) cultivar "Hisawakaba", a product of red clover (*Trifolium pretense*), a product of white clover (*Trifolium repens*), and a product of milk-vetch (*Astrugalus sinicus*) were obtained commercially.

DNA extraction

Seed samples (0.4 g) were transferred into 2-ml screw-capped tubes, which contained a mixture of ceramic and silica particles to disrupt microbial cells by bead-beating. The samples were then subjected to DNA extraction for analyzing the microbial community using the FastDNA SPIN Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol, except that 40 μl of skim milk solution (100 mg/ml) was added to the extraction buffer before bead-beating and the washing step was repeated three times. Each DNA sample was eluted in a final volume of 50 μl. One sample was collected from each package for either duplicate or triplicate analyses, except for Italian ryegrass seeds from which three samples were collected from each package in order to examine the stability of both inter- and intra-cultivar microbial communities.

For the evaluation of the efficiency of DNA extraction and the detection sensitivity of ribosomal intergenic spacer analysis (RISA), the seeds of timothy were spiked with the spores of *Fusarium graminearum* strain AFG03-011C or cells of *Escherichia coli* strain DH5α. *F. graminearum* strain AFG03-011C was used as an isolate derived from a rice plant and has been identified based on morphology and a molecular analysis of histone H3 and reductase regions (Laboratory of Plant Protection, Akita Prefectural University). For the conidiation of *F. graminearum*, fungi were grown on oatmeal sucrose agar (5% oatmeal, 1.5% sucrose and 1.5% agar) plates at 25°C for 14 days, and the plates were placed under a fluorescent lamp (FL20SBR, NEC Lighting, Ltd., Tokyo, Japan) for 7 days after removal of the aerial mycelia with a sterilized brush. The conidia that formed were then brushed off and suspended in sterilized water. *E. coli* was purchased from a commercial source (Toyobo, Tokyo, Japan), and the cells were suspended in sterilized water after low speed centrifugation. The numbers of the spores of *F. graminearum* and cells of *E. coli* were counted by direct microscopic examination, and then adjusted to 10^6 spores/ml and 10^5 cells/ml, respectively. Other cell densities were prepared by serial dilution prior to spiking the seeds. Two sets of adjusted cell densities, followed by serial dilutions, were independently prepared as duplicates. Subsequently, timothy seeds (0.4 g) were inoculated with 100-μl cell suspensions containing up to 10^5 spores and 10^4 cells of *F. graminearum* and *E. coli*, respectively.

Ribosomal intergenic spacer analysis

RISA was carried out as previously described by Ikeda *et al.*[22]. Briefly, the primer sets used in the present study were 1406f/3126T, which target the end of the 18S rRNA gene and beginning of the 28S rRNA gene for fungal RISA[20], and ITSF/ITSReub which target the end of the 16S rRNA gene and beginning of the 23S rRNA gene for bacterial RISA[8]. Each PCR mixture (final volume, 50 μl) contained 5 μl of 10 X buffer, 10 μg of BSA, 0.5 μM of each primer, 200 μM of dNTPs, 4U of Ex *Taq* HS DNA polymerase (TaKaRa, Kyoto, Japan), and 8 ng of DNA extract. For flu-

orescence detection, the 5' end of the forward primer was labeled with 6-carboxyrhodamine (Sigma Genosys Japan, Hokkaido, Japan). The amplification program consisted of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, and a final extension step of 7 min at 72°C. PCR products (3 µl) were then mixed with 2.5 µl of loading dye (95% formamide, 10 mM EDTA, pH 8.0 and 0.1% bromophenol blue). After incubation at 80°C for 3 min, these samples were chilled on ice for 5 min, and directly loaded onto 5% polyacrylamide gels (19:1 ratio of acrylamide to bisacrylamide, 0.4 mm thick, 40 cm long) containing 7.7 M urea and 0.5 X TBE. Electrophoresis was conducted at a constant voltage of 2000 V in 1 X TBE for 2.5 h.

Digital image analysis

Following gel electrophoresis, digital fingerprinting images were obtained using a fluorescent scanner (Molecular Imager FX, BIO-RAD Laboratories, Inc., Hercules, CA, USA). The RISA banding patterns were then analyzed with Bio-Rad Quantity One™ software (BIO-RAD Laboratories, Inc.) to assess any similarities (Dice coefficient of similarity) between the lanes. Lane background subtraction was carried out with a rolling disk size of 50 and band detection was performed using the default parameters, with the exception of the sensitivity setting of 700. The range of the DNA band size for data collection was 238–1722 nt. Similarities were then calculated using the band position information (both the presence and absence of bands).

Molecular cloning and sequencing analysis of RISA amplicons

The cloning and sequencing of amplicons from the RISA profiles were carried out according to a previous report from our laboratory[21]. All sequence data in the present study have been deposited in the DDBJ/EMBL/GenBank data library, and were aligned to public database entries using the BLASTN subroutine[3].

Standard diagnostic PCR-based detection using environmental DNA extracted from seeds

Using environmental DNA templates, extracted from the surface of imported seeds, the standard PCR-based detection protocols for *Pseudomonas fluorescens* and *Erwinia amylovora* were conducted using the primer sets reported by Scarpellini *et al.*[38] and Maes *et al.*[31], respectively. Ex *Taq* HS DNA polymerase (TaKaRa) was used as described for RISA. The thermal cycler program was as follows: initial denaturation for 2 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C; and a final extension time of 7 min at 72°C. In order to increase the specificity of the primers, the annealing temperature was increased to 65°C in the case of the primer set for *E. amylovora*. PCR products were analyzed on 1.5% agarose gels in 0.5 X TBE.

Results

The efficiency of DNA extraction and the sensitivity of RISA

Prior to investigating microbial diversity in forage crop seeds, the efficiency of the DNA extraction and sensitivity of the subsequent RISA experiments were examined by spiking timothy seeds with known concentrations of spore and cell suspensions of *F. graminearam* and *E. coli*, respectively. One cultivar of timothy was used for these spiking experiments for simplicity. As shown in Fig. 1, the presence of 10^4 to 10^5 spores of *F. graminearam* and of 10^3 to 10^4 *E. coli* cells could be clearly visualized by RISA. The locations of amplicons derived from *F. graminearam* and *E. coli* in the profiles were confirmed by comparison with the profiles of RISA using DNA directly prepared from spores of *F. graminearam* or cells of *E. coli* in the absence of seeds. The three fragments derived from *E. coli* most likely reflect the

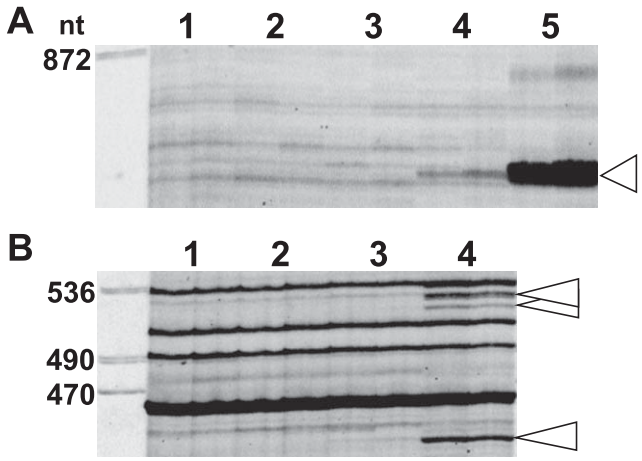

Fig. 1. RISA profiles showing the detection sensitivity to known numbers of *Fusarium graminearum* spores (A) or *E. coli* cells (B). Timothy seeds were spiked with the numbers of spores/cells indicated for each lane and subjected to DNA extraction, followed by RISA. Duplicate results are shown. (A) Fungal RISA: lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lanes 1–5, from 10–10^5 spores in tenfold increments. (B) Bacterial RISA: lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lanes 1–4, from 10–10^4 cells in tenfold increments. Numbers indicate marker fragment lengths.

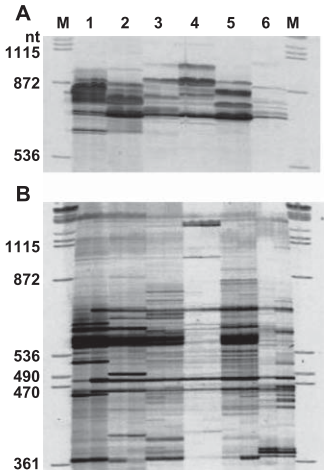

Fig. 2. RISA profiles showing the complexity of the microbial community structures on the surface of forage crop seeds. (A) Fungal RISA (B) Bacterial RISA: lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lane 1, Italian ryegrass (*Lolium multiflorum*); lane 2, timothy (*Phleum pretense*); lane 3, alfalfa (*Medicago sativa*); lane 4, red clover (*Trifolium pretense*); lane 5, white clover (*Trifolium repens*); lane 6, milk-vetch (*Astrugalus sinicus*). Duplicate results for each species are shown. Numbers indicate marker fragment lengths.

intragenomic variation in ribosomal rRNA gene operons, as reported for several bacteria including *E. coli*[32].

Microbial community structures on the surface of forage crop seeds revealed by RISA

The overall profiles generated by both fungal and bacterial RISA suggested the presence of unique microbial community structures in the seed samples from each plant species under study (Fig. 2). The profiles of fungal RISA showed relatively simple fingerprinting patterns, comprising a few dominant bands in each of the samples examined. In contrast, the bacterial RISA profiles showed both a higher degree of complexity and variability between samples. In order to evaluate the stability of the microbial community structures in seed samples, two cultivars of Italian ryegrass were extensively examined. The subsequent fungal and bacterial RISA profiles of these specimens revealed significant levels of intra-cultivar stability, in comparison with the inter-cultivar similarities (Fig. 3). The fungal RISA profiles of these two cultivars again showed more stable fingerprinting patterns than the bacterial RISA pattern, regardless of the differences between the samples. The bacterial RISA profiles showed a higher degree of both complexity and variability but also revealed specific patterns for each cultivar, in addition to some stable banding patterns across each of the samples examined. Moreover, the intra-cultivar similarity was shown to be statistically higher than the inter-cultivar similarity for both fungal and bacterial RISA (Table 1).

Molecular characterization of the amplicons generated by RISA

Seeds of Italian ryegrass and timothy, both of which are widely used in Japan in terms of both cultivation areas and seed quantities, were chosen in order to identify the amplicons generated by RISA. DNA bands that were stably detected in triplicate samples were then selected for cloning and sequencing (Fig. 4). The subsequent sequencing analysis of the cloned amplicons revealed that 33 out of 36 DNA bands were derived from unique seed-associated microbes, and that only three clones were derived from plant DNA. Among the fungal RISA clones, five were identified as eukaryotic microbial sequences, including one protozoan sequence, from Italian ryegrass and six were obtained from timothy. Three PCR clones were found to be derived from nuclear ribosomal genes of plants. From the bacterial RISA amplicons, 13 PCR clones from Italian ryegrass and nine from timothy were identified as bacterial sequences. In addition, none of the PCR clones were found to have been derived from ribosomal genes of plant organelles. BLAST searches in the public databases revealed that the PCR clones derived from both fungal and bacterial RISA consisted of a taxonomically broad range of microorganisms (Table 2).

Most of our fungal PCR clones showed high similarity to known fungal sequences. Five of these clones (IF2, IF4, IF5, TF1 and TF4) showed high similarity (95.1 to 99.6%) to yeast species belonging to the *Dioszegia* and *Cryptococcus* genera. These genera are commonly found as environmental microbes[35]. In addition, clone TF2 showed 96.9% similarity to an environmental strain of *Rhodotorula* sp., which has also been characterized as a cosmopolitan organism[16]. Clone TF3 was almost identical (99.8% similarity) in sequence to *Epicoccum* sp., which has been reported as a group of epiphytic fungi that mainly inhabits the leaves of grass plants[27].

In addition to these clones, members of the *Cladosporium* genus were also found to have an association with the seeds of both Italian ryegrass and timothy. Clone IF3 showed 98.6% similarity to *Cladosporium oxysporum*, which is a known fungal pathogen in both animals[36] and plants[45]. Clone TF6-1 was excised from the same band location as clone TF6-2, with only a 1 bp difference in length, and the sequence similarity between these two clones was found to be 90.1%. Interestingly, clone TF6-1 showed 98.5% homol-

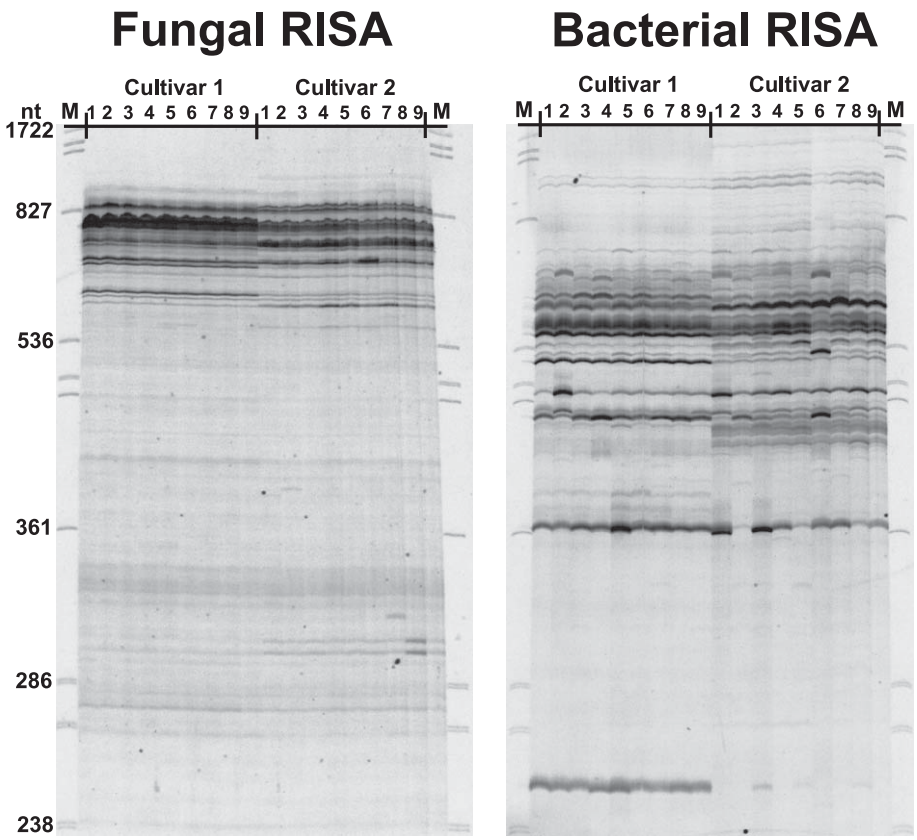

Fig. 3. RISA profiles showing the stability of the microbial community structures in the seeds of Italian ryegrass. Lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lane 1–3, 4–6 and 7–9 represent three replicates that were prepared from one of three packages for two Italian ryegrass cultivars. All replicates were independently subjected to DNA extraction and RISA. Numbers indicate marker fragment lengths.

Table 1. Similarity among the RISA profiles for Italian ryegrass seeds

	Inter-cultivar similarity (%)[c]	Intra-cultivar similarity (%)[d]	
		cultivar 1	cultivar 2
F-RISA[a]	65.7±4.9	84.4±5.5*	88.2±4.9*
B-RISA[b]	56.2±5.6	78.6±8.3*	70.0±9.9*

[a] Fungal RISA.
[b] Bacterial RISA.
[c] Mean±S.D. of the average inter-cultivar similarity.
[d] Mean±S.D. of the average intra-cultivar similarity.
* Statistically significant differences between the inter- and intra-cultivar similarity at the 0.01 level measured using the U-test.

ogy to *C. herbarum*, which is recognized as a major fungal allergen[2] and has also been reported as a plant pathogen[4]. There were four additional clones (clones IF1, IF6, IF7 and TF5) that contained non-fungal sequences derived from both plants and from a species of protozoan, which were likely to have been amplified due to the low specificity of the fungal primers employed.

As expected from the fingerprinting patterns, we identified a greater diversity of sequences from the bacterial RISA clones, compared to the fungal RISA amplicons. As reported for analyzing microbial communities in various environments, several clones in the present study showed similarity to uncultured bacteria (clones IB4, IB7, TB4-2 and TB7), or had a low level of similarity to known bacterial species (IB8, IB9-2, IB10, TB2-2 and TB4-1). Interestingly, most of clones in the present study showed no similarity to bacterial pathogens of either Italian ryegrass or timothy, but showed similarity to a wide range of other bacteria, including several important pathogens of other crop species. As anticipated, several clones showed a high degree of similarity to epiphytic bacteria such as *Aeromicrobium fastidiosum* and *Curtobacterium plantarum. C. plantarum* has been reported as a ubiquitous epiphytic bacterium and is known to be transmitted via soybean and corn seed[14]. Three clones,

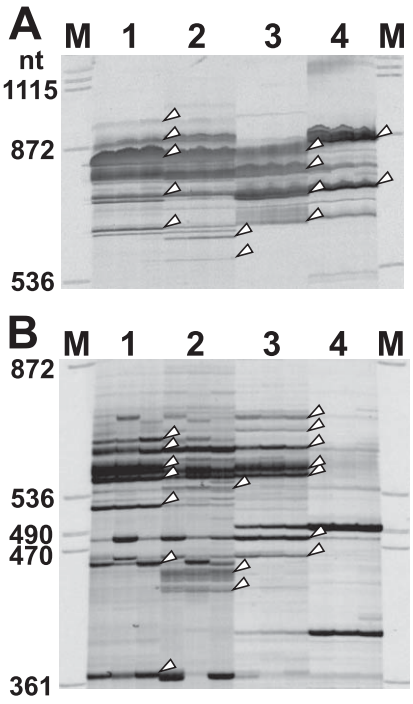

Fig. 4. RISA profiles showing inter- and intra-species variability among microbial communities on the surface of Italian ryegrass and timothy seeds. (A) Fungal RISA (B) Bacterial RISA: lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lanes 1–2 and lanes 3–4 indicate two different cultivars of Italian ryegrass and timothy, respectively. Triplicate results for each cultivar are shown. Numbers indicate marker fragment lengths and arrowheads indicate the subsequently cloned DNA bands in the present study.

(clones IB2 TB2-1 and TB3) showed high similarity to *P. fluorescens*, which has been identified as a beneficial organism that contributes to plant protection[18]. However, there have been no previous reports of the seed transmission of this bacterium.

In addition to the clones showing a high degree of similarity to mutually beneficial bacteria, several clones showed extensive similarity to known crop pathogens. For example, clone IB1 showed 99.6% similarity to *Pseudomonas syringae* pv. *garcae*, which has been reported as a causal agent of both bacterial blight in Kenyan coffee plantations and halo blight in coffee crops from Brazil[26]. Clone IB5 had 96.3% homology to *Clavibacter michiganensis* subsp. *michiganensis*, which is one of the principal bacterial pathogens in tomato and causes bacterial wilt and canker[17]. It has also been well established that seed transmission is an important pathway in the life cycle of this pathogen[24]. However, little has been documented regarding the possible seed transmission of *C. michiganensis* in non-host plants, such as forage crops. Moreover, clone IB6-1 had 91% sequence similarity to *Erwinia pyrifoliae*, which has been shown to cause blight in the Asian pear (*Pyrus pyrifolia*)[28]. In addition, clone TB6 had a relatively low level of similarity (83%) to *E. amylovora*, which is the causal agent of fire blight in apples[25]. Clone TB5 showed 85.7% similarity to *Pseudomonas cichorii*, which is infectious to a wide range of plant species, including Italian ryegrass[40].

Standard diagnostic PCR-based detection using environmental DNA extracted from seeds

In order to confirm the results of sequencing of our cloned RISA amplicons, we also assayed some of the sequences for their putative identity using standard PCR protocols previously reported by other groups. As a result, we were able to detect the products of expected size reported for *P. fluorescens*[38] and *E. amylovora*[31] (Fig. 5). On the other hand, we could not detect a positive signal for *E. amylovora* using a PCR that targeted pEA29[5,30], a whole marker plasmid for *E. amylovora*, or other genomic regions such as *rlsB* for levan synthesis[13] and unknown genomic regions[39]. Diagnostic PCR-based detection methods for other pathogenic bacteria such as *E. pyrifoliae*[29] and *C. michiganensis*[12,37] were also conducted, but no positive signals were obtained.

Discussion

Normander and Prosser[34] first attempted to analyze seed-associated microbes using culture-independent methodologies out of the general concern for an effective biological control for barley seeds. Subsequently, Dent *et al.*[11] demonstrated the evaluation of microbial communities, associated with the seeds of sugar beet, with regard to overall seed vigor. In the former study, however, the authors failed to detect any meaningful profiles by direct use of denaturing gradient gel electrophoresis (DGGE) of seed material. In the latter study, the analyses were limited by contaminating co-amplification of organelles and nuclear ribosomal genes. Moreover, the DGGE profiles were of poor visual quality and the detection sensitivity of the experiments appeared to be considerably low.

In contrast, we applied both fungal and bacterial RISAs to the profiling of the community structures of seed-associated microbes, in combination with a DNA extraction method originally developed for soils. It is significant that the DNA extraction and RISA employed in the present study are relatively simple techniques compared with the methodologies employed in the reports described above. Moreover,

Table 2. Sequence similarity between the cloned RISA amplicons from forage crop seeds and known microbes

Clone name (ID)[a]	Length (bp)	DB No.[b]	Closest relative	Similarity (%)
Fungal RISA clone for Italian ryegrass				
IF1 (AB222610)	779	Y09814	*Festuca pratensis*	99.0
IF2 (AB222611)	732	AF444354	*Cryptococcus chernovii*	99.2
IF3 (AB222612)	643	L25432	*Cladosporium oxysporum*	98.6
IF4 (AB222613)	609	AB049614	*Dioszegia hungarica*	95.1
IF5 (AB222614)	568	AJ581068	*Dioszegia crocea*	99.6
IF6 (AB222615)	523	AF223570	*Spathidium amphoriforme*	82.8
IF7 (AB222616)	853	AJ491193	*Begonia fenicis*	75.6
Bacterial RISA clone for Italian ryegrass				
IB1 (AB222617)	585	AY342172	*Pseudomonas syringae* pv. *garcae*	99.6
IB2 (AB222618)	567	AF134705	*Pseudomonas fluorescens*	88.9
IB3-1 (AB222619)	539	AJ783972	*Ralstonia pickettii*	63.3
IB3-2 (AB222620)	532	AF479766	*Dechloromonas* sp.	65.4
IB4 (AB222621)	525	AY172146	*Candidas* Competibacter phosphatis	61.3
IB5 (AB222622)	479	U09380	*Clavibacter michiganensis* subsp. *michiganensis*	96.3
IB6-1 (AB222623)	420	AY509612	*Erwinia pyrifoliae*	91.0
IB6-2 (AB222624)	419	CP000029	*Staphylococcus epidermidis*	70.4
IB7 (AB222625)	331	AF124217	*Uncultured eubacterium*	51.8
IB8 (AB222626)	507	AF198379	*Aeromonas caviae*	67.2
IB9-1 (AB222627)	406	AF017509	*Aeromicrobium fastidiosum*	94.4
IB9-2 (AB222628)	407	AF127587	*Pseudomonas fluorescens*	57.5
IB10 (AB222629)	392	AF265258	*Rodococcus* sp.	58.3
Fungal RISA clone for timothy				
TF1 (AB222630)	733	AF444354	*Cryptococcus chernovii*	99.5
TF2 (AB222631)	690	AB025982	*Rhodotorula* sp.	96.9
TF3 (AB222632)	636	AJ279463	*Epicoccum* sp.	99.8
TF4 (AB222633)	583	AB049614	*Dioszegia hungarica*	99.4
TF5 (AB222634)	775	AY686669	*Poa cita voucher*	95.7
TF6-1 (AB222635)	644	L25431	*Cladosporium herbarum*	98.5
TF6-2 (AB222636)	643	L25432	*Cladosporium oxysporum*	89.6
Bacterial RISA clone for timothy				
TB1 (AB222637)	627	AY191511	*Curtobacterium plantarum*	92.3
TB2-1 (AB222638)	600	AY582371	*Pseudomonas fluorescens*	92.1
TB2-2 (AB222639)	596	AY713456	*Acidovorax avenae*	62.0
TB3 (AB222640)	568	AF134705	*Pseudomonas fluorescens*	87.9
TB4-1 (AB222641)	541	AJ783972	*Ralstonia pickettii*	68.1
TB4-2 (AB222642)	545	AY485407	*Rice phyllosphere bacterium*	86.2
TB5 (AB222643)	523	AJ279242	*Pseudomonas cichorii*	85.7
TB6 (AB222644)	443	AJ010485	*Erwinia amylovora*	83.0
TB7 (AB222645)	424	AY485410	*Rice phyllosphere bacterium*	81.3

[a] DDBJ accession number of the PCR clones reported in this study.
[b] The accession number of the Genbank sequences corresponding to the closest relative.

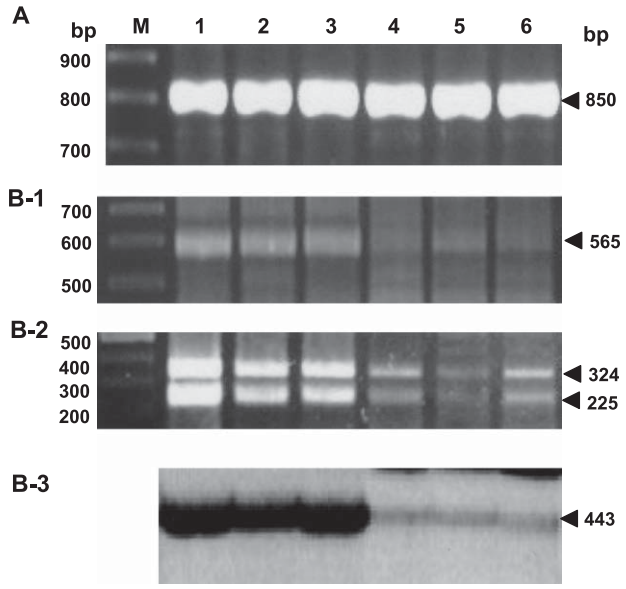

Fig. 5. Standard diagnostic PCR-based detection of environmental DNA samples extracted from seeds using primer sets for *Pseudomonas fluorescens* and *Erwinia amylovora*. (A) Detection of *P. fluorescens* in Italian ryegrass (B) Detection of *E. amylovora* in timothy: lane M, molecular size makers; lanes 1 to 3 and 4 to 6 indicate triplicate results for two cultivars of Italian ryegrass (A) and timothy (B), respectively. Panel A shows the expected size of the PCR product for *P. fluorescens* reported by Scarpellini *et al.*[38]. Panels B-1 and B-2 show the expected PCR products and their components digested with *Bsp* 1286I, for *E. amylovora* as reported by Maes *et al.*[31]. Panel B-3 indicates the bacterial RISA amplicons from the present study that show a high degree of similarity to *E. amylovora*. Triplicate results for the corresponding samples are shown in panels B-1, B-2 and B-3. Numbers and arrowheads indicate marker fragment lengths and the expected sizes of the PCR products and their components digested with *Bsp* 1286I, respectively.

our current findings suggest that RISA could be sufficiently sensitive to detect fungi or bacteria at quantities of less than 10^4 spores or 10^3 cells, respectively, without the need for culture enrichment prior to DNA extraction and PCR amplification. Dent *et al.*[11] had previously employed culture enrichment by seed imbibition for 16 hours, followed by tissue fractionation prior to DNA extraction. However, this procedure should be avoided when evaluating microbial diversity because it can change the ratio of individual species in a population, particularly in the case of bacteria. Because environmental samples often contain inhibitors of various enzymatic reactions, including PCR, the purity of the environmental DNA and the success of the subsequent analyses are highly dependant upon the DNA extraction method employed. As shown by our previous study[21] and by our current experiments, an extraction method that has been developed for soil DNA facilitates the simple and rapid preparation of environmental microbial DNA directly from diverse biological materials.

Ribosomal gene regions are currently in use to analyze microbial communities in order to take advantage of the accumulation of such sequences in the public databases. In most cases, DNA fingerprinting techniques such as DGGE are employed to visualize microbial community structures. However, as reported by Dent *et al.*[11], the use of DGGE to generate profiles for analyzing microbial communities often results in poor resolution, particularly when these data are generated using environmental DNA associated with the phytosphere. In addition, nested primer sets are often used to amplify bacterial and fungal communities to avoid biased amplification of plant DNA. These factors can severely interfere with microbial signals, particularly in the case of low-abundance organisms. In contrast, the fungal RISA profiles generated in the present study were largely unaffected by the presence of plant DNA under the conditions used, and no noticeable bias was observed in the case of bacterial RISA. This demonstrates the robustness of the RISA method for examining microbial diversity for various biological samples. Our results also suggest that forage crop seeds sustain stable and unique microbial structures, indicating that seeds can be considered to be a special niche for certain groups of microbes, including unculturable organisms (Figs. 2 and 3).

Sequencing revealed that our cloned RISA amplicons were representative of the microbial sequences from a taxonomically wide range of fungal and bacterial species. Unexpectedly, all of the fungal clones and nearly half of the bacterial clones isolated in the present study showed high sequence similarity to known species, most of which have been identified as plant-associated microbes. However, there are also several bacterial sequences showing low levels of similarity to known species. To date, uncultured bacteria have not been taken into account during the evaluation of seed performance, including seed vigor, for establishing seedlings and assessing the efficiency of biological controls in seed inoculations. However, these organisms may well have a significant impact upon the growth properties of plants and upon the activities of known beneficial/pathogenic microbes.

Clone TB6 may be a closely related species but is unlikely to be a subtype of *E. amylovora* since the sequence similarity (83%) to *E. amylovora* is lower than the similarity for the corresponding regions (86.3%) between *E. amylovora* and *E. pyrifoliae*. In addition, we could not detect positive signals for the pEA29 plasmid and other genomic regions

using a previously reported diagnostic PCR test for *E. amylovora*. These results therefore indicate that some caution should be taken when molecular diagnostic techniques such as PCR are applied to the detection of animal or plant pathogens in environmental samples. Thus, molecular diagnostic techniques may give misleading results regardless of their accuracy and reliability under laboratory conditions. This is particularly pertinent if the specificity and reliability of the method has not been fully evaluated for the assessment of microbial diversity in natural environmental samples, such as seeds and soil. On the other hand, our current results also show that the examination of seed-associated microbes can be considerably simplified by employing culture-independent methodologies, which we demonstrate to be potentially very useful as a preliminary diagnostic test for seed-related plant pathogens.

In conclusion, culture-independent methodologies could be efficient tools for the evaluation of microbial diversity in seeds. The present results generated by RISA, and by the subsequent cloning and sequencing of the amplicons, suggest that seeds sustain a particular profile of microbes, including unculturable bacteria, on their surface as a specific niche. The culture-independent methodologies adopted in the present study also provide an efficient way to routinely monitor certain groups of seed-associated microbes, such as biological control agents or plant pathogens, in seeds. Further analysis of seed-associated microbes should therefore be undertaken to provide a better understanding of the ecological roles of microbial communities in seeds.

Acknowledgements

This work was partially supported by the Japan Society for the Promotion of Sciences, a biosafety program of the Innovative Development Project.

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