

Microbial Diversity in Milled Rice as Revealed by Ribosomal Intergenic Spacer Analysis

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Microbial diversity in milled rice was examined using culture-independent methods. Environmental DNA samples were extracted from commercial products of milled rice, and the intergenic spacer regions between the small and large subunit ribosomal RNA genes were amplified by ribosomal intergenic spacer analysis (RISA). The results indicated the presence of microbial communities in milled rice and subsequent sequencing of RISA amplicons has identified 17 unique microbial sequences. These include several sequences highly similar to known plant-associated microbes, including *Pseudomonas fluorescens*, *Xanthomonas sacchari*, and *Pseudozyma antarctica*, as well as to several uncultured bacteria. In addition, one sequence shows significant similarity to the sequence of *Fusarium graminearum*, a potent pathogenic fungus in foodstuffs that produces trichothecene mycotoxins. The potential presence of a pathogenic *Fusarium* fungus in milled rice was also suggested by a diagnostic PCR procedure that detects the genes involved in the production of trichothecene mycotoxins.

Key words: *Fusarium graminearum*, microbial community analysis, ribosomal intergenic spacer analysis, rice, trichothecene mycotoxin

Modern molecular diagnostic methods, particularly PCR-based techniques, have become indispensable to microbial examinations of agronomic products for clinical, forensic, and other purposes. While most of these methods have focused on the analysis or detection of specific groups of organisms, many of which can be targeted by PCR, less effort has been made regarding the investigation of non-culturable microorganisms or of unexpected contamination by microbes which have either accidentally or latently come into contact with foodstuffs. However, it is known that the population and/or diversity of non-pathogenic culturable microbes can affect the activities of pathogenic culturable microbes¹⁰, and can also change the properties of agro-

nomic products such as grain quality during storage²⁷. However, current advances in environmental microbiology have now revealed that the majority of microbes in nature are in fact non-culturable with current techniques, and have therefore not been subjected to conventional microbiological examinations³.

Both the quality and yield of template DNAs are crucial for a successful PCR analysis. However, the common components of biological materials such as fats, polysaccharides, polyphenols and other secondary metabolites, are known to act as PCR inhibitors^{11,40}. In addition, it is generally quite difficult to amplify target sequences using DNA preparations from agricultural commodities, foodstuffs, or animal feeds, since high levels of non-target DNA can also act as a PCR inhibitor²⁶. Moreover, microbial spores may represent a significant fraction of any given biomass sample, and this often poses a technical challenge to efficient DNA extraction⁴⁴.

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Previously, we found that a DNA extraction method used in soil microbiology was sufficiently robust to both detect and monitor environmental microbes from a range of biological materials, including foodstuffs²⁰). As for a detection method, the use of multicopy target sequences (rRNA gene regions) for ribosomal intergenic spacer analysis (RISA) enhances the sensitivity of the PCR assay since it reduces the amount of DNA template necessary for PCR amplification. Previously, we have shown that RISA can be used to generate profiles of plant-associated microbial communities from diverse agronomic products without any noticeable bias caused by the presence of excess amounts of plant DNA^{19,20–23}). Our previous study also suggests that RISA can detect not only conventional microbes, but also a wide range of unpredicted microbes including potential pathogens and non-culturable organisms. Thus, RISA has proved to be useful for revealing microbial community structures as unique fingerprinting signatures in agronomic products.

Among agronomic products, rice is one of the most important globally, and microbial examinations of rice products have been undertaken in a number of reports^{4,28,43,48}). Meanwhile, little is known about microbial community structures in commercial rice products such as milled rice.

In this study, we have examined the feasibility of using the culture-independent methodology in the microbiological examination of milled rice. The results of these analyses suggest the presence of microbial communities in commercial products of milled rice. In addition, sequencing of RISA amplicons has revealed the presence of one fungal DNA sequence showing high similarity to the sequence of a *Fusarium graminearum* strain which produces trichothecene mycotoxins. This was subsequently confirmed by the use of diagnostic PCR to detect the genes for the biosynthesis of type B trichothecene mycotoxins.

Materials and Methods

Materials and sampling

Products of milled rice were purchased from local commercial vendors. Sampling of all products was carried out in triplicate by extracting DNA from each of three packages for separate products. The characteristics of the samples used in the present study are summarized in Table 1.

DNA extraction

Milled rice (0.5 g) was transferred into 2-ml screw-capped tubes, which contained a mixture of ceramic and silica particles to disrupt microbial cells by bead-beating. The

Table 1. Milled rice samples used in the present study

Sample name	Cultivar	Production Year	Production site ^a
R-1	Akitakomachi	2004	Akita
R-2	Akitakomachi	2004	Ibaraki
R-3	Fusaotome	2005	Chiba
R-4	Goropikari	2004	Gunma
R-5	Hitomebore	2005	Okinawa
R-6	Hitomebore	2004	Ibaraki
R-7	Hitomebore	2004	Iwate
R-8	Hitomebore	2004	Miyagi
R-9	Hitomebore	2005	Tochigi
R-10	Hitomebore	2004	Fukushima
R-11	Koshihikari	2004	Fukushima
R-12	Koshihikari	2005	Chiba
R-13	Koshihikari	2004	Fukui
R-14	Kirara397	2005	Hokkaido
R-15	Kinuhikari	2004	Ibaraki
R-16	Koshihikari	2004	Ibaraki
R-17	Koshihikari	2004	Ibaraki
R-18	Koshihikari	2004	Ishikawa
R-19	Koshiibuki	2005	Niigata
R-20	Koshihikari	2004	Ibaraki
R-21	Koshihikari	2005	Tochigi
R-22	Koshihikari	2004	Niigata
R-23	Koshihikari	2004	Niigata
R-24	Koshihikari	2004	Niigata
R-25	Milky queen	2004	Fukushima
R-26	Milky queen	2004	Ibaraki
R-27	Nanatuboshi	2004	Hokkaido
R-28	Sasanishiki	2004	Miyagi

^a Prefecture.

samples were then subjected to DNA extraction using a 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.

For the evaluation of the efficiency of DNA extraction and the detection sensitivity of RISA, one of our milled rice samples (R-2) was spiked either with cells of *Escherichia coli* strain DH5α or spores of *Fusarium graminearum* s. str. (*F. graminearum*-complex lineage 7) strain AFG03-011C. The preparation of cells of *E. coli* or spores of *F. graminearum* s. str. was carried out as described in our previous study¹⁹). The numbers of *E. coli* cells and *F. graminearum* spores were determined by direct microscopic

examination, and then adjusted to 10^5 cells/ml and 10^6 spores/ml, respectively. Different cell densities were then prepared by serial dilutions prior to spiking the seeds. Two sets of adjusted cell densities, with subsequent serial dilutions, were independently prepared as duplicates. Subsequently, the milled rice (0.5 g) was inoculated with 100- μ l cell suspensions containing up to 10^4 cells and 10^5 spores of *E. coli* and *F. graminearum*, respectively.

Ribosomal intergenic spacer analysis (RISA)

RISA was carried out as described in our previous study²², except that the annealing temperature was set at 55°C for both bacterial and fungal RISAs. Briefly, the primer sets used in the present study were ITSf/ITSReub, which targeted the end of the 16S rRNA gene and beginning of the 23S rRNA gene for bacterial RISA⁷, and 1406f/3126T, which targeted the end of the 18S rRNA gene and beginning of the 28S rRNA gene for the fungal RISA¹⁸ (Table 2). Each PCR mixture (final volume, 50 μ l) contained 5 μ l of 10 X buffer, 10 μ g of BSA, 0.5 μ M of each primer, 200 μ M of dNTPs, 4U of Ex *Taq* HS DNA polymerase (Takarabio, Otsu, Japan), and 8 ng of DNA extract. For fluorescence detection, the 5' end of the forward primer was labeled with 6-carboxyrhodamine (Sigma Genosys Japan, Ishikari, Japan). The amplification program for both bacterial and fungal RISAs consisted of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final extension step of 7 min at 72°C. PCR products (3 μ l) were then mixed with 2.5 μ l 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 2,000 V in 1 X TBE for 2.5 h. Following the electrophoresis, digital fingerprinting images were obtained using a fluorescent scanner (Molecular Imager FX, BIO-RAD Laboratories, Inc., Hercules, CA, USA).

Molecular cloning and sequencing of RISA amplicons

The cloning and sequencing were carried out according to methods reported previously²⁰. In order to ensure the homogeneity of the sequences within an excised band, eight sequences were analyzed for each amplicon. All sequence data in the present study have been deposited in the DDBJ/EMBL/GenBank data library, and the sequences were aligned to public database entries using the BLASTN subroutine²¹.

Standard diagnostic PCR-based detection of the genes responsible for the biosynthesis of type B trichothecene mycotoxins

Using environmental DNA templates extracted from the surfaces of milled rice, standard PCR-based detection was performed for the *F. graminearum*-complex (former group 2) and for the genes involved in the production of the type B trichothecene mycotoxins. The PCR primers employed are listed in Table 2. Each PCR mixture (final volume, 50 μ l) contained 5 μ l of 10 X buffer, 10 μ g of BSA, 0.5 μ M of each primer, 200 μ M of dNTPs, 4U of Ex *Taq* HS DNA polymerase (Takarabio), and 8 ng of template DNA extract,

Table 2. Primer sets used in the present study

Primer name	Sequence	Sizes (bp)	Ref.
ITSf	GTC GTA ACA AGG TAG CCG TA	— ^a	(7)
ITSReub	GCC AAG GCA TCC ACC		
1406f	TGY ACA CAC CGC CCG T	—	(18)
3126T	ATA TGC TTA AGT TCA GCG GGT		
UBC85F ₄₁₀	GCA GGG TTT GAA TCC GAG AC	332	(45)
UBCR ₄₁₀	AGA ATG GAG CTA CCA ACG GC		
Tr5F	AGC GAC TAC AGG CTT CCC TC	544	(12)
Tr5R	AAA CCA TCC AGT TCT CCA TCT G		
Tri13NIV	CCA AAT CCG AAA ACC GCA G	312	(8)
Tri13R	TTG AAA GCT CCA ATG TCG TG		
ToxP1	GCC GTG GGG RTA AAA GTC AAA	300/360 ^b	(30)
ToxP2	TGA CAA GTC CGG TCG CAC TAG CA		

^a Variable sizes for amplicons.

^b Expected sizes of amplicons for DON and NIV respectively.

Table 3. Sequence similarities between PCR products amplified from milled rice using RISA and known microbial sequences

Clone name (ID) ^a	Length (bp)	DB No. ^b	Closest relative	Similarity (%)
Bacterial RISA				
B3 (AB236007) (a) ^c	577	AY582364	<i>Pseudomonas fluorescens</i>	97.2
B4 (AB236008) (b)	529	AF209762	<i>Xanthomonas sacchari</i>	98.2
B5 (AB236009) (c)	528	AY485410	Rice phyllosphere bacterium	85.2
B6 (AB236010) (d)	464	AY485405	Rice phyllosphere bacterium	82.5
B7 (AB236011) (e)	455	AY485410	Rice phyllosphere bacterium	99.1
B8 (AB236012) (f)	442	AJ010485	<i>Erwinia amylovora</i>	84.5
B9 (AB236013) (g)	439	AJ010485	<i>Erwinia amylovora</i>	81.3
B10 (AB236014) (h)	410	DQ011254	Uncultured bacterium	86.5
B11 (AB236015) (i)	398	AF536451	<i>Nocardia globerula</i>	81.4
B12 (AB236016) (j)	388	AF536451	<i>Nocardia globerula</i>	90.6
B13 (AB236017) (k)	379	AF536451	<i>Nocardia globerula</i>	88.5
B17 (AB236021) (l)	327	AY728162	<i>Staphylococcus</i> sp.	85.1
B18 (AB236022) (m)	302	AF124217	Uncultured bacterium	56.9
Fungal RISA				
F1 (AB235999) (n)	868	AF294698	<i>Pseudozyma antarctica</i>	100.0
F2 (AB236000) (o)	661	AB106649	<i>Cordyceps brongniartii</i>	99.1
F3 (AB236001) (p)	637	AB250414	<i>Fusarium graminearum</i>	99.7
F4 (AB236002) (q)	539	AB158652	<i>Pichia burtonii</i>	98.5

^a DDBJ accession number of PCR clones reported in the present study.

^b The accession number of sequences of the closest relative found in Genbank.

^c Letters in parentheses indicate the corresponding letters in Figure 2.

except for the primer set UBC85F₄₁₀/UBC85R₄₁₀, for which 4U of *Taq* Gold (Applied Biosystems, CA, USA) and 20 ng of template DNA were used. For the UBC85F₄₁₀/UBC85R₄₁₀ primer set⁴⁵, the amplification program consisted of 10 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final extension step of 10 min at 72°C. For the Tr5F/Tr5R primer set¹², the program used was 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 70°C, and 15 sec at 72°C, and a final extension step of 7 min at 72°C. For the Tri13NIV/Tri13R primer set⁸, the program consisted of 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 65°C, and 15 sec at 72°C, and a final extension step of 7 min at 72°C. For the ToxP1/ToxP2 primer set³⁰, the program consisted of 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 62.5°C, and a final extension step of 7 min at 72°C. PCR amplifications were carried out using a Gene Amp[®] PCR System (Applied Biosystems), except for the UBC85F₄₁₀/UBC85R₄₁₀ primers for which we used TAKARA 480 (Takarabio). All PCR products were analyzed on 1.5% agarose gels in 0.5 X TBE.

Quantification of trichothecene mycotoxins and isolation of Fusarium fungi from milled rice

Each sample of milled rice (500 g) was ground into a powder and mixed thoroughly, and 5-g aliquots were then used for the quantification of trichothecene mycotoxins (Deoxynivalenol, nivalenol, T-2 toxin, and HT-2 toxin) using ELISA. The ELISA was carried out on our behalf by Kyowa Medex Co. Ltd. (Chuo-ku, Tokyo, Japan).

Colony counts were carried out using Komada medium³³. Fifty grains of non-sterilized milled rice were placed on Komada medium for each sample, and then incubated for one month at 25°C.

Results

The efficiency of DNA extraction and the sensitivity of RISA for milled rice samples

Prior to the investigation of microbial diversity in milled rice, the efficiency of the DNA extraction method and the sensitivity of the subsequent RISA were examined by spiking milled rice samples with known concentrations of both *F. graminearum* s. str. spore and *E. coli* cell suspensions.

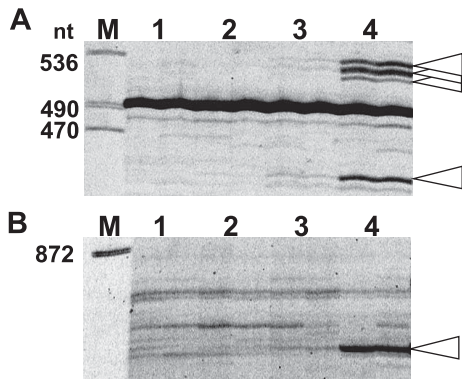


Fig. 1. RISA profiles showing detection sensitivity based on known numbers of *E. coli* cells (A) or *Fusarium graminearum* spores (B). Milled rice was spiked with the numbers of cells/spores indicated for each lane and subjected to DNA extraction, followed by RISA. (A) Bacterial RISA: lane M, GeneScan-2500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

The R-2 commercial rice sample was used for these spiking experiments to simplify the RISA profiles. As shown in Figure 1, the presence of 10^3 to 10^4 spores of *F. graminearum* s. str. could be clearly visualized by RISA. The locations of the amplicons derived from both the *E. coli* and *F. graminearum* contaminants in these profiles were confirmed by comparison with the RISA profiles of DNA directly prepared from these microbes (data not shown). The multiple fragments derived from *E. coli* most likely reflect the intragenomic variation in ribosomal rRNA gene

operons, as reported for several bacteria including *E. coli*³⁵.

Microbial community structures on the surface of milled rice

Whereas the overall profiles showed a high degree of similarity across all samples, several unique bands were found in each sample via both the bacterial and fungal RISA (Fig. 2). In general, the fungal RISA profiles were more variable.

Molecular characterization of the amplicons generated by RISA

We cloned and sequenced the amplicons generated by RISA profiling and found that they were all of microbial origin. A number of amplicons from the bacterial RISA experiments could be successfully cloned, and approximately 50% of these clones showed sequence similarity to plant-associating bacteria. Clone B3 (band a in Fig. 2) showed 97.2% similarity to *Pseudomonas fluorescens*, a beneficial organism that contributes to plant protection¹⁸. Three clones, B11, B12, and B13 (bands i, j, and k in Fig. 2), all of which were unique to product R-13, showed high levels of homology to *Nocardia globerula*, a potential biological control agent for post-harvest disease in potato¹³. Clone B4 (band b in Fig. 2) showed 98.2% similarity to *Xanthomonas sacchari*, a pathogen in sugar cane⁴⁹. Two clones, B8 and B9 (bands f and g in Fig. 2), showed 84.5% and 81.3% similarities, respectively, to *Erwinia amylovora*, the causal agent of fire blight in apples²⁴. Three clones, B5, B6, and B7 (bands c, d, and e in Fig. 2), showed 80% to 90% similarities to rice phyllosphere bacteria reported previously (accession no. AY485405 and AY485410).

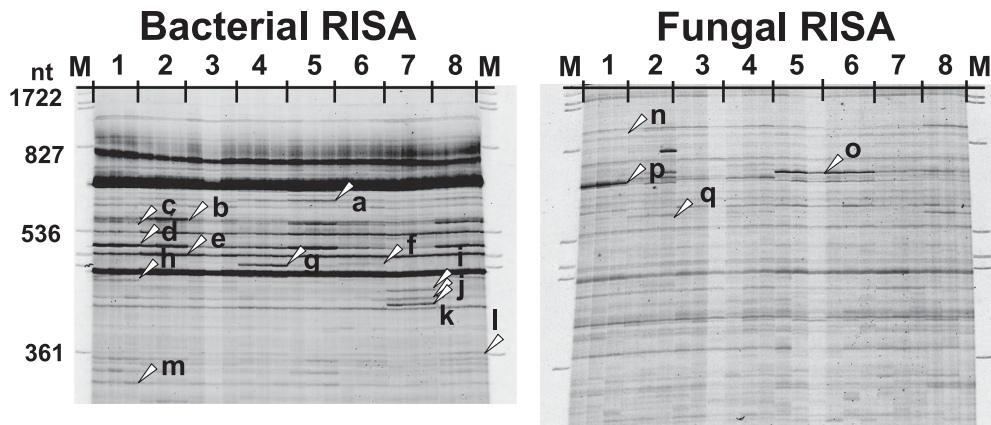


Fig. 2. RISA profiles showing the complexity of the microbial community structures on the surface of milled rice. (A) Bacterial RISA (B) Fungal RISA: lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lane 1, R-1; lane 2, R-3; lane 3, R-4; lane 4, R-5; lane 5, R-7; lane 6, R-12; lane 7, R-13; lane 8, R-15. Triplicate results for each species are shown. Numbers indicate marker fragment lengths.

Unlike in the bacterial RISA, we encountered difficulties in cloning a number of low intensity amplicons from fungal RISA gels. As a result of this, only four unique fungal amplicons could be sequenced and these clones showed similarities to known sequences deposited in the current databases. Clones F1 and F4 (bands n and q in Fig. 2) showed 100% and 98.5% similarities to *Pseudozyma antarctica* and *Pichia burtonii*, respectively, both of which are common environmental yeasts found in plants and food materials^{1,34}). In addition, clone F2 (band o in Fig. 2) showed 99.1% similarity to *Cordyceps brongniartii*. Interestingly, the anamorph of this fungus has been reported as one of the strongest pathogens for Scarabaeid insects¹⁵), but no previous association with rice has been reported. Clone F3 (band p in Fig. 2) showed 99.7% similarity to *F. graminearum*, one of the major causal agents of Fusarium head blight (scab) and a pathogen that has generated global concern among cereal producers due to the risk associated with its production of trichothecene mycotoxins⁸).

Standard diagnostic PCR-based detection of Fusarium graminearum and genes for the synthesis of type B trichothecene mycotoxins

In order to confirm our sequencing results for the *F. graminearum*-like fungus, we attempted to identify this pathogen using standard PCR protocols reported by others. After the PCR conditions were refined using environmental DNA from a subset of samples, several primer sets were selected for analysis. We were subsequently able to detect specific PCR products for both the *F. graminearum*-complex and for a subset of genes involved in the biosynthesis of type B trichothecene mycotoxins. The UBC85F₄₁₀/UBC85R₄₁₀ primer set was developed for the detection of a genomic region specific to the group of fungi in the *F. graminearum*-complex. The Tr5F/Tr5R primer set targets the *Tri5* gene which encodes trichodiene synthase, an enzyme that catalyzes the key step in the biosynthetic pathway of all trichothecene mycotoxins. Hence, the *Tri5* gene would be present in all fungal species producing trichothecene mycotoxins. The primer set Tri13NIV/Tir13R was developed specifically to detect the *Tri13* gene of the NIV chemotype⁸). This gene encodes C-4 monooxygenase, and is thought to convert deoxynivalenol (DON) to nivalenol (NIV)²⁹). The ToxP1/ToxP2 primer set was developed based on the intergenic sequence between the *Tri5* and *Tri6* genes³⁰).

Comparable and stable positive signals were observed in three samples (R-1, R-8, and R-25) for all of the primer sets tested (Table 4). The PCR results differed between produc-

tion sites and cultivars, but all of the rice tested was cultivated in the Tohoku region which is the northeast part of Japan. While positive signals were observed more frequently (36 subsamples in 18 samples) for the UBC85F₄₁₀/UBC85R₄₁₀ primers (UBC in Table 4) than the other primer sets, no signals were detected with this primer set in sample R-3 which was shown to be positive for at least one of the sub samples with other primer sets to detect the genes involved in mycotoxin biosynthesis.

Quantification of trichothecene mycotoxins and isolation of Fusarium fungi from milled rice

No mycotoxin or fungal growth was detected in the milled rice samples under any of the conditions.

Discussion

The yield and purity of the DNA isolated from foodstuffs are crucial determining the success of PCR-based microbial examinations, and a number of improvements to DNA extraction procedures have been reported^{5,9,14,25}). However, most of these methods require a series of extraction and purification steps and an appreciable quantity of starting material. As a result, they are not of practical use in the routine microbial examination of food commodities. For the purification of extracted DNA, commercial kits have also been widely used^{9,16,25,37,46}). However, in our experience, these kits are not sufficiently robust to obtain high quality DNA from polysaccharide-rich materials, such as milled rice, and yield poor results during microbial community analyses (data not shown).

In contrast, as shown here and in previous studies, an extraction method developed for soil DNA facilitates the simple and rapid preparation of microbial DNA directly from various foodstuffs with less than one gram of starting material. In addition, the current findings suggest that RISA is sufficiently sensitive to detect either fungi or bacteria that are present in quantities of less than 10³ spores or 10³ cells in the case of milled rice, respectively, without the need for culture enrichment prior to DNA extraction and PCR amplification.

Recently, Morisaki and his colleagues^{31,39}) have deleted the presence of both epiphytic and endophytic bacterial communities in rice seeds using conventional culture methods, and have also identified several taxonomic groups of bacteria in rice seeds. Interestingly, most of these bacterial groups were not found in the present study. However, since there are several differences in experimental design between the two studies, such as the status of the materials and the

Table 4. PCR detection of biosynthetic genes for trichothecene mycotoxins in milled rice

Sample name	UBC ^a	Tr5 ^b	Tri13 ^c NIV	ToxP1/P2 ^d		Sample name	UBC	Tr5	Tri13 NIV	ToxP1/P2	
				DON	NIV					DON	NIV
R-1	+ ^e	- ^f	+	+	+	R-15	+	-	-	-	-
	+	+	+	+	+		-	-	-	-	+
	+	+	+	-	+		-	+	-	-	-
R-2	+	-	-	-	-	R-16	-	-	-	+-	-
	-	-	-	-	-		+	-	-	-	+
	+	-	-	-	-		-	-	-	-	+
R-3	-	+	+	-	+	R-17	-	-	-	-	-
	-	+	-	-	-		-	-	-	-	-
	-	+	-	-	+		+	-	-	-	-
R-4	-	-	-	-	-	R-18	-	-	-	-	-
	-	-	-	-	-		-	-	-	-	-
	-	-	-	-	+		-	-	-	-	-
R-5	+	-	-	-	-	R-19	-	-	-	-	-
	+	-	-	+- ^g	-		+	-	-	-	-
	-	-	-	-	-		-	-	-	-	-
R-6	+	-	-	-	-	R-20	-	-	-	-	+
	+	-	+	-	-		-	-	-	-	-
	-	-	-	-	+-		+	+	-	-	-
R-7	+	-	+	-	-	R-21	+	-	+-	-	-
	-	-	+	-	-		-	-	-	-	-
	+	-	-	-	-		-	-	-	-	+-
R-8	+	+	+	-	+	R-22	-	-	-	-	-
	+	+	+	+	-		-	-	-	-	-
	+	+	+	+	+		+	-	-	-	-
R-9	-	-	+	-	-	R-23	-	-	-	-	-
	-	-	-	-	-		+	-	+-	-	-
	-	-	+	-	-		+	-	-	-	-
R-10	+	-	-	-	-	R-24	+	-	-	-	-
	+	-	-	-	-		-	-	-	-	-
	-	-	-	-	-		-	+	-	-	-
R-11	-	-	-	-	-	R-25	+	+	+	-	+
	-	-	-	-	+-		+	+	+	-	+
	+	+	+	-	+		+	+	+	-	-
R-12	+	-	-	-	-	R-26	-	-	-	-	-
	+	-	-	-	+-		-	-	-	-	-
	-	-	-	-	-		-	-	-	-	-
R-13	+	-	-	-	-	R-27	-	-	-	-	-
	-	-	+	-	-		-	-	-	-	-
	+	-	-	+	-		-	-	-	-	-
R-14	+	+	-	-	-	R-28	-	-	-	+	+
	+	-	-	-	+-		-	+	+	-	-
	-	-	-	-	-		-	+	-	-	-

^a UBC85F410/UBCR410. ^b Tr5F/Tr5R. ^c Tri13NIV/Tri13R. ^d ToxP1/ToxP2.

^e Predicted size of PCR product. ^f no product. ^g ambiguous result.

primer sets employed, direct comparisons of the data are difficult. Meanwhile, our results suggest that molecular diagnoses for the microbes in cereal grains are possible with less than one gram of material at the end-product level, including milled rice which is generally considered to be a difficult substance for molecular biological analyses.

Our data also raise the possibility that the microbes identified by RISA may persist during both the milling process and domestic storage, since we prepared our materials from commercially available products. As shown previously in a study of the bacterial contamination of commercial milled rice by Bainotti and Parra⁴⁾, these microbes may tolerate several types of environmental stress such as high temperature and low moisture during the milling process and domestic storage. Moreover, micro flora in cereals that includes unculturable microbes may be responsible for altering grain quality during storage.

It is potentially very significant that we detected *F. graminearum*-like sequences in milled rice as this may indicate a possible risk in terms of food security for commercial rice products. *F. graminearum* is known to produce highly toxic secondary metabolites known as mycotoxins^{32,36)}. It has also been reported that culturable *Fusarium* species can grow and produce toxins in cereals not only in the field but also after harvesting under unfavorable storage conditions³⁸⁾. In order to confirm the presence of DNA derived from a pathogenic *Fusarium* fungus in our milled rice samples, a series of PCR amplifications were conducted using a number of primer sets specific for these fungal genomes and also for genes involved in the production of trichothecene mycotoxins. Interestingly, although the ToxP1/ToxP2 primer set has been reported to distinguish between DON and NIV chemotypes based on differences in the sizes of their PCR products, most of the positive signals obtained with this primer set were for the NIV chemotype. In this study, only a fraction of the samples tested by PCR were found to be positive for the genes responsible for the production of mycotoxins. However, some caution should be exercised when interpreting such negative results, since the absence of a PCR product does not necessarily confirm the absence of the pathogen being tested.

While the detection of *F. graminearum*-like rDNA and genes responsible for the biosynthesis of trichothecene mycotoxins may indicate a risk of contamination by type B trichothecene mycotoxins, including nivalenol and its relatives⁶⁾, no mycotoxin or fungal growth was found in milled rice samples in the present study. This discrepancy may have several explanations. First, while the fungi in rice grains could be killed during the milling process, the DNA

of dead cells may persist in the milled rice. Second, the fungi may enter a state where they are viable but not culturable in the milled rice. Third, although Komada's selective medium which is selective for *Fusarium* species was used, difficulty in the recovery of *Fusarium* fungi with this medium has been reported even for plants heavily infected with *F. graminearum*⁴²⁾.

Recently, Goswami and Kistler¹⁷⁾ have demonstrated that *F. graminearum*-complexes are capable of infecting rice grain under greenhouse conditions, and causing clear symptoms. However, no production of trichothecene mycotoxin was detectable in infected rice florets despite the severe symptoms in their report¹⁷⁾. In contrast, contamination by DON and NIV in rice grain has been reported in Japan, although levels were low and so far no contamination by these toxins has been detected after the milling process^{47,50)}. Because of the significance of *Fusarium* head blight to food safety, the combination of molecular approaches employed in the present study could be useful for an initial survey of the potential contamination of rice grains by mycotoxigenic fungi due to its rapidity and simplicity as for diagnostic analyses.

In conclusion, our strategy combining a method for extracting DNA from soil and a subsequent RISA could provide useful information regarding microbial community structures, including both unculturable and culturable microbes. In addition, because of the potential significance and hazards related to trichothecene mycotoxins, continuous monitoring of contaminating pathogenic fungi or measurements of toxin contents in milled rice products are highly desirable. RNA-based molecular diagnostics may need to be undertaken to complement the monitoring of the biological activities of such pathogenic microbes in plants⁴¹⁾ or in agronomic products⁴⁶⁾. The molecular methods described in the present study are useful and applicable to such analysis in different cereals and related agronomic products.

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