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Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis Analysis of Bacterial Community Structure in the Food, Intestines, and Feces of Earthworms

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The bacterial communities in the food, intestines, and feces of earthworms were investigated by PCR-denaturing gradient gel electrophoresis (DGGE). In this study, PCR-DGGE was optimized by testing 6 universal primer sets for microbial 16S rRNA in 6 pure culture strains of intestinal microbes in earthworms. One primer set effectively amplified 16S rRNA from bacterial populations that were found in the food, intestines, and feces of earthworms. Compared with the reference markers from the pure culture strains, the resulting DGGE profiles contained 28 unique DNA fragments. The dominant microorganisms in the food, intestines, and feces of earthworms included *Rhodobacterales bacterium*, Fusobacteria, *Ferrimonas marina*, *Aeromonas popoffii*, and soil bacteria. Other strains, such as *Acinetobacter*, *Clostridium*, and *Veillonella*, as well as rumen bacteria and uncultured bacteria also were present. These results demonstrated that PCR-DGGE analysis can be used to elucidate bacterial diversity and identify unculturable microorganisms.

Keywords: denaturing gradient gel electrophoresis, bacterial community, 16S rRNA, earthworms

Earthworms live in humid habitats, such as soil, manure, swamps, lakes, forests, caves, and seashores. In soil, they have many important roles, including mixing and aerating the soil, bringing minerals closer to the soil surface, decomposing plant and animal matter, and promoting the growth of beneficial microbes in the soil (Edwards and Lofty, 1972). In addition, earthworms can emit much larger amounts of nitrous oxide (N_2O) than soil. Denitrification (i.e., the reduction of nitrate or nitrite to a nitrogenous gas) is often facilitated by ingested soil microorganisms (Horn *et al*., 2005). For example, *Eisenia fetida* is a common vermicomposting earthworm in agricultural ecosystems. Since its feces are a rich source of plant nutrients, they can be used as manure. However, little is known about the bacteria that digest organic matter in the intestines of earthworms.

Until recently, microorganisms have been identified by traditional methods, including culturing and distinctive morphological and biochemical characteristics. However, less than 1% of all bacteria can be cultured (Rondon *et al*., 1999; Schabereiter-Gurtner *et al*., 2001; Clegg *et al*., 2003). Moreover, cultures may be time-consuming and may not accurately reflect the natural population dynamics of microbial communities due to artificial environmental conditions in the laboratory (e.g., temperature, pH, light) (Liu *et al*., 1997). In contrast, molecular methods that are based on the recovery of bacterial 16S rRNA from samples are more efficient and provide accurate information about the diversity of the microbial community (Gurtner *et al*., 2000). For example, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), a molecular fingerprinting technique that differentiates microbes on the basis of differences in the melting behavior of PCR amplified fragments in denaturing gradient gels, is one of the most widely used techniques for microbe analysis (Muyzer *et al*., 1993). Recently, PCR-DGGE has been used to assess the diversity of microbial communities in horse feces (Endo *et al*., 2009), human intestines (Nam *et al*., 2008), manure (Leung and Topp, 2001), paddy field soil (Watanabe *et al*., 2004), groundwater (Cho *et al*., 2003), sea sponges (Li *et al*., 2007), sawfly (Zahner *et al*., 2008), probiotic products (Temmerman *et al*., 2003), red wine (Spano *et al*., 2007), raw milk (Cocolin *et al*., 2002), cheese (Cocolin *et al*., 2004), fermented soybean paste (Kim *et al*., 2010), and fermented sausages (Rantsiou *et al*., 2005).

The aims of this study were to determine the efficiency of 16S rRNA primers that are used in PCR-DGGE analysis to profile bacterial populations and define the bacterial community structure in the food, intestines, and feces of earthworms.

Materials and Methods

Bacterial strains and growth conditions

The bacteria strains that were used in this study were *Clostridium subterminale* (ATCC 25774), *Clostridium butyricum* (KCCM 35433), *Staphylococcus epidemicus* (KCCM 40416), *Klebsiella oxytoca* (KCCM 11418), *Pseudomonas pseudoalcaligenes* subsp. *pseudoalcaligenes* (KCCM 12539), and *Streptomyces setonill* (KCCM 40359). All strains were obtained from the American Type Culture Collection (ATCC) or the Korean Culture Center of Microorganisms (KCCM), and then cultured in Brain Heart Infusion medium (Difco, USA) for 48 h at 37°C.

Sample collection and preparation

E. fetida was collected from an earthworm farm in Eumsung, Korea, and cattle manure was gathered from a commercial farm in Wonju, Korea. The earthworms were fed a 1:1 mixture (w/w) of cattle manure

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Table 1. Polymerase chain reaction primers

Primer[a,c]	Sequence (5′→3′)	T_m (°C)	Reference
27F	AGA GTT TGA TCM[b] TGG CTC AG	50.5	Lane *et al.* (1985)
341F	CCT ACG GGA GGC AGC AG	53.6	Jung *et al*. (2005)
518R	ATT ACC GCG GCT GCT GG	56.7	Jung *et al*. (2005)
519R	GW[b]A TTA CCG CGG CK[b]G CTG	61.4	Lane *et al.* (1985)
907R	CCG TCA ATT CM[b]T TTR[b] AGT TT	47.8	Lane *et al.* (1985)
926F	AAA CTY[b] AAA K[b]GA ATT GAC GG	45.6	Lane *et al.* (1985)
1392R	ACG GGC GGT GTG TR[b]C	49.3	Lane *et al.* (1985)
1492R	TAC GGY[b] TAC CTT GTT ACG ACT T	52.1	Lane *et al.* (1985)

[a] F (forward) and R (reverse) indicate the relative orientation of the primers to the 16S rRNA sequence.
[b] Modified bases: M=C:A; W=A:T; Y=C:T; K=G:T; R=A:G. All modified bases were used in a 1:1 ratio.
[c] A 40 nucleotide GC-rich sequence (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') was appended to the 5' end of the primers as described by Walter *et al*. (2000).

and sawdust that was composted for 5 weeks and cultured on composted cattle manure at 20°C with 70% relative humidity. Fecal samples were collected immediately after defecation from the top of the manure and stored at -20°C. Food samples were collected from composted cattle manure that had not been cultured with earthworms. The intestines of the earthworms were surgically resected.

The samples of food, intestine, or feces (about 1 g) were suspended in 9 ml of a sterile solution that contained 0.85% NaCl and 0.01% Tween 80. Aliquots (1 ml) of this suspension were centrifuged at 4,000×g for 10 min at 4°C, and then the pellets were washed with sterile phosphate buffered saline (pH 7.0) before extracting the bacterial DNA.

DNA extraction

To extract the bacterial DNA, cells were harvested from the cell culture by centrifugation at 5,000×g for 10 min at 4°C. Then, DNA was extracted from the cell pellet by using the DNeasy Tissue kit (QIAGEN, USA) according to the manufacturer's directions. The concentration and purity of the extracted DNA were determined by using a biophotometer (Eppendorf, Germany).

Polymerase chain reaction

Six universal PCR primer sets were used to amplify the 16S rRNA sequences (Tables 1 and 2). The 20 μl PCR reactions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.5 units of Taq polymerase, 0.4 mM of each dNTP; 20 pmol of each primer, 20 μg/ml template DNA, and sterile water. The PCR was performed in a Mastercycler thermal cycler (Eppendorf) with the following conditions: an initial denaturation at 95°C for 5 min; 30, 45, or 60 cycles of denaturation at 94°C for 45 sec, annealing for 45 sec, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The melting temperatures (T_m) for the primers are shown in Table 1. The PCR products were electrophoresed on 2% agarose gels with 1× TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.0), stained with ethidium bromide (0.5 μg/ml), and then visualized with an ultraviolet transilluminator (Korea Bio-Tech Co., Korea).

Table 2. Specificity of universal primer sets for 16S rRNA in various strains

Primer Sets	Pure culture strains					
	1	2	3	4	5	6
$27F^{GC}$-907R	-	v	v	-	v	v
$27F^{GC}$-1392R	-	v	v	-	v	v
$27F^{GC}$-1492R	-	v	v	-	v	v
$27F^{GC}$-519R	-	v	+	-	v	v
$926F^{GC}$-1392R	+	v	+	+	+	+
$341F^{GC}$-518R	+	+	+	+	+	+

1, *C. subterminale*; 2, *C. butyricum*; 3, *S. epidemicus*; 4, *K. oxytoca*; 5, *P. pseudoalcaligenes* subsp. *pseudoalcaligenes*; 6, *S. setonill*.
+, amplification; -, no amplification; v, variable amplification

Denaturing gradient gel electrophoresis analysis

The DGGE analysis was performed with a DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA). The 35-60% denaturing gradient gels were prepared by combining urea with formamide (Sigma Chemical Co., USA) and 8% (w/v) poly-

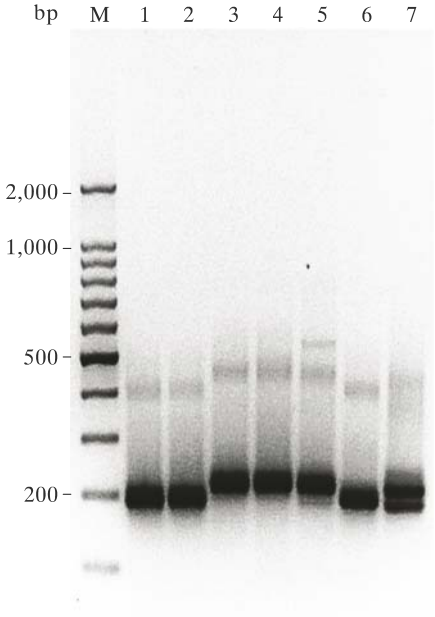

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products of 16S rRNA from pure culture strains. Lanes: M, 100 bp ladder marker; 1, *C. subterminale*; 2, *C. butyricum*; 3, *S. epidemicus*; 4, *K. oxytoca*; 5, *P. pseudoalcaligenes* subsp. *pseudoalcaligenes*; 6, *S. setonill*; 7, Mixture of equal amounts of products from lanes 1-6.

acrylamide [Acryl/Bis 29:1, 40% (w/v); Amresco, USA]. The electrophoresis was performed in 0.5× TAE buffer at 20 V for 30 min, and then at 60 V for 12 h at 60℃. Subsequently, the gels were stained for 20 min in TAE buffer containing a 1:10,000 dilution of GreenStar Nucleic Acid Staining dye (Bioneer Co., Korea) and visualized with a UV transilluminator (Korea Bio-Tech Co.). The intensity of the bands on the DGGE gel was analyzed by Gel-Pro Analyzer software (Media Cybernetics Inc., USA). The reference marker was composed of a mixture of equal amounts of the amplicons that were obtained from the dominant bacterial species in the intestines of earthworms, namely, *C. subterminale*, *C. butyricum*, *S. epidemicus*, *K. oxytoca*, *P. pseudoalcaligenes* subsp. *pseudoalcaligenes*, and *S. setonill*.

Sequence and phylogenetic analysis

The bands corresponding to the 16S rRNA were excised from the DGGE gel, and then the DNA was extracted by passive diffusion in 50 μl sterilized water overnight at 4°C. Subsequently, the DNA was used as the template for PCR with the 341F (without the GC clamp) and 518R primers. The PCR products were purified by using a QIAquick PCR purification kit (QIAGEN Inc., USA), and then sequenced. DNA sequencing was performed on an ABI 377 Genetic Analyzer (Applied Biosystems, USA). Finally, the sequences were aligned with the GenBank reference sequences for 16S rRNA (http://www.ncbi.nlm.nih.gov) by using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the bacteria samples. A phylogenetic tree was constructed by using the ClustalW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2) with 1,000 replications of the neighborhood-joining method. MEGA 4.0 was used to assess the phylogenetic tree (Tamura *et al*., 2007).

Results and Discussion

Selection of primer sets for PCR-DGGE analysis

Previously, Kim *et al*. (2004) and Shin *et al*. (2004) reported that *C. subterminale*, *C. butyricum*, *S. epidemicus*, *K. oxytoca*, *P. pseudoalcaligenes* subsp. *pseudoalcaligenes*, and *S. setonill* were the dominant bacterial species in the intestines of earthworms under aerobic and anaerobic conditions. As a result, the specificity and efficiency of 6 universal primer sets for 16S rRNA from these 6 pure culture strains were evaluated to determine the optimum PCR-DGGE analysis conditions in this study. Although all of these primer sets produced 16S

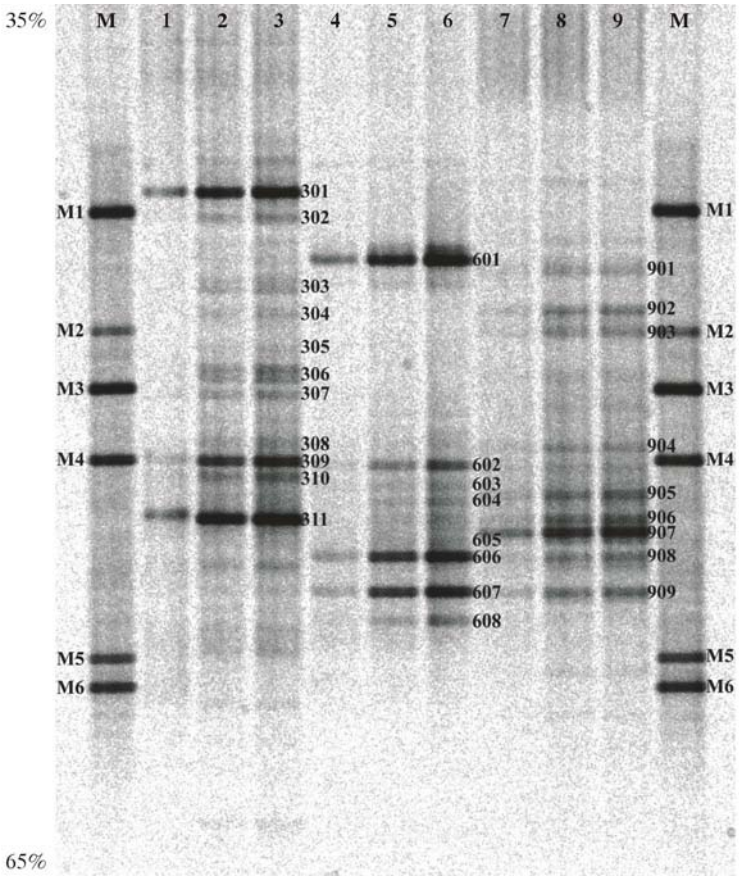

Fig. 2. Effect of the number of PCR cycles on polymerase chain reaction-denaturing gradient gel electrophoresis analysis of 16S rRNA from food, intestine, and feces samples of earthworms. M, reference ladder marker that consists of equal amounts of 16S rRNA amplicons from 6 pure culture strains: M1, *Staphylococcus epidemicus*; M2, *Pseudomonas pseudoalcaligenes* subsp. *pseudoalcaligenes*; M3, *Clostridium subterminale*; M4, *Clostridium butyricum*; M5, *Klebsiella oxytoca*; and M6, *Streptomyces setonill*. Lanes 1, 4, and 7: 30 cycles; lanes 2, 5, and 8: 45 cycles; lanes 3, 6, and 9: 60 cycles. 16S rRNA amplicons from the food (lanes 1-3), intestines (lanes 4-6), and feces (lanes 7-9) of earthworms. The denaturing gradient ranges from 35-60%.

rRNA amplicons from some strains, only 1 set, 341F^{GC}-518R, reliably produced amplicons from all 6 strains (Table 2). As a result, this primer set was selected to identify intestinal microbes in earthworms on the basis of their 16S rRNA fingerprints. This finding is consistent with a previous study that showed that universal PCR primers can be used to detect almost all known bacteria (Osorio *et al*., 1999). Although Watanabe *et al*. (2004) reported that the average size of amplicons (600 bp) may be too long to be resolved with DGGE, the size of the 341F^{GC}-518R amplicon is more suited to this technique because it amplified approximately 240 base pairs (bp) of the V3 variable region of 16S rRNA (Fig. 1). The inefficiency of the other primer sets may have been due to factors that could hamper PCR amplification, such as the size of the genomic DNA, copy number of the 16S rRNA gene, or strength of the interaction between the primers and template.

Construction of DGGE reference ladder marker

The DGGE reference ladder marker consisted of 6 DNA fragments that corresponded to the 16S rRNA amplicons that were characteristic of the 6 pure culture strains. The unique 240-bp 16S rRNA fragments were PCR amplified by using the 341F^{GC}-518R primer set (Fig. 1) and these bands were clearly separated on a 35-65% denaturing gradient gel (Fig. 2 lane M). Moreover, the relatively large difference in migration distance between amplicons from 2 species in the *Clostridium* genus demonstrated that DGGE analysis can clearly resolve different 16S rRNA fragments. This reference ladder marker made it possible to easily identity each band in the DGGE profiles of sample microbes, as described by Endo *et al.* (2009). Similarly, Temmerman *et al*. (2003) and Theunissen *et al*. (2005) compared DGGE profiles to reference markers from *Lactobacilli* and *Bifidobacterium* species. Previous studies also have shown that using DGGE with a reference ladder marker can detect bacterial strains in samples quickly and reliably. For example, Fasoli *et al*. (2003) and Ji *et al*. (2004) used DGGE with a reference ladder marker to rapidly detect probiotic bacteria and bacterial pathogens.

PCR-DGGE analysis of bacterial community structure

The results from the PCR-DGGE analysis of 16S rRNA from the food, intestines, and feces of earthworms were used to identify the bacterial community structures in these samples. The banding patterns on the DGGE gels showed good resolution of different bacterial community structures in the samples (Fig. 2). This result is consistent with the high resolving power of DGGE; in fact, this technique can separate 16S rRNA sequences with only single base pair differences (Miller *et al*., 1999). The 16S rRNA clone library was determined by sequence analysis of the DGGE gel bands and comparison of the band positions with the reference ladder marker. Altogether, 11 bacterial 16S rRNA clones were sequenced

Table 3. Microorganisms identified in the food, intestines, and feces of earthworms by polymerase chain reaction-denaturing gradient gel electrophoresis analysis

Sample source	Band No.	16S rRNA sequence results	GenBank accession no.	BLAST homology (%)	Band intensity (%)
Food	301	Uncultured Bacteroidetes bacterium	DQ811911	97	14.4
	302	Uncultured Clostridiaceae bacterium	AB192022	98	2.5
	303	Uncultured bacterium	AB205760	99	1.4
	304	Uncultured bacterium	DQ154266	98	1.1
	305	*Veillonella* sp.	DQ087189	95	1.1
	306	Uncultured *Lactobacillus* sp.	AM117177	99	2.5
	307	*Clostridium disporicum*	DQ855943	97	2.0
	308	Uncultured rumen bacterium	AB270263	97	2.8
	309	*Clostridium* sp.	AY695835	97	10.7
	310	Uncultured bacterium	DQ904773	99	4.1
	311	Uncultured Rhodobacterales bacterium	DQ857223	98	18.1
Intestines	601	Uncultured Fusobacteria bacterium	DQ211504	99	20.5
	602	*Clostridium* sp.	AY695835	97	5.2
	603	Uncultured *Streptobacillus* sp.	DQ914526	98	2.6
	604	*Aeromonas* sp.	AF539665	97	2.5
	605	*Gamma proteobacterium*	AB274781	99	1.2
	606	*Ferrimonas marina*	AB193751	97	14.6
	607	*Aeromonas popoffii*	DQ178598	97	13.5
	608	Uncultured earthworm cast bacterium	AY154631	98	2.9
Feces	901	Uncultured soil bacterium	DQ642735	99	3.0
	902	Uncultured bacterium	AF091516	98	3.6
	903	*Pseudomonas* sp.	AM409194	96	3.9
	904	*Acinetobacter bouvetii*	AF509827	99	3.3
	905	Uncultured Chloroflexi bacterium	AY542256	97	7.0
	906	Uncultured Rhodobacterales bacterium	DQ857223	98	6.4
	907	Soil bacterium	EF105549	98	18.9
	908	*Ferrimonas marina*	AB193751	97	4.2
	909	*Aeromonas popoffii*	AF472506	97	5.4

Fig. 3. Phylogenetic tree of 16S rRNA sequences identified from bands in the denaturing gradient gel electrophoresis profile. Sequences that were determined in this study are shown in bold, and GenBank accession no. are shown in parentheses for all reference sequences. The scale bar represents the Jukes-Cantor evolutionary distance.

from the food of earthworms, 8 from their intestines, and 9 from their feces. Since the intensity of bands is proportional to the relative concentration of genomic DNA in a sample, the highest intensity bands represent the dominant population (Watanabe *et al*., 2004). In the food, intestines, and feces of earthworms, the dominant populations were characterized by 3 distinct bands (301, 309, and 311), 3 distinct bands (601, 606, and 607), and 1 distinct band (907), respectively (Fig. 2). In addition, the DGGE gel contained other groups of bands, namely, 304 and 902; M4, 309 and 602; 311 and 906; 606 and 908; 607 and 909; and 903 and M2, which corresponded to other bacterial populations in the samples. Furthermore, Fig. 2 also shows that as the number of PCR cycles increased, the intensity of the DGGE bands increased slightly. Thus, PCR-DGGE can differentiate between 16S rRNA sequences from different bacteria on the basis of specific banding patterns due to the differential denaturing characteristics of these sequences.

Identification of bacteria in samples

The 16S rRNA sequences allowed the identification of the species that were represented by the DGGE bands (Table 3) and their phylogenetic relationships are shown in Fig. 3. The 16S rRNA sequences of the 304 and 902 fragments (Fig. 2) were 98% similar to an unculturable bacterium. Similarly, the sequences of the M4 marker and the 309 and 602 fragments were 97% similar to *Clostridium* sp. while those of the 311 and 906 fragments were 98% similar to uncultured Rhodobacterales bacteria. Likewise, the sequences of the 606 and 908 fragments were 97% similar to *Ferrimonas marina* and those of the 607 and 909 fragments were 97% similar to *Aeromonas popoffii*. In addition, the 903 fragment and the M2 marker were 96% similar to *Pseudomonas* sp. This bacterial diversity in the food, intestines, and feces of earthworms is consistent with previous studies. For example, Kim *et al*. (2004) reported that many different microorganisms, including *Aeromonas*, *Agromyces*, *Bosea*, *Gordonia*, *Klebsiella*, *Microbacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Tsukamurella*, *Streptomyces*, and *Bacillus*, were isolated from the intestinal tracts of *E. fetida* by traditional culture-based methods. Shin *et al*. (2004) also found that earthworm intestines contain *Clostridium*, *Staphylococcus*, and *Propionibacterium* under anaerobic conditions. In addition, Horn *et al*. (2005) reported that gut wall-associated microorganisms are responsible for the degradation of organic matter in the earthworm gut and the capacity of earthworms to emit N_2O. Enterobacteriaceae-related species and other γ-proteobacteria, including the H_2-producing fermenter *Paenibacillus terrae* and Clostridiaceae-related species, have been isolated from the gut of the earthworm *Aporrectodea caliginosa* (Ihssen *et al*., 2003; Horn *et al*., 2005). Furthermore, Horn *et al.* (2006) showed that species that are related to *Bradyrhizobium*, *Flavobacterium*, *Dechloromonas*, *Brucella*, *Sinorhizobium*, *Pseudomonas*, *Ralstonia*, and *Paracoccus* are found in earthworm gut contents and are involved in denitrification. Indeed, such microorganisms are abundant in the gut of earthworms (Ihssen *et al*., 2003). It is likely that some of these microorganisms are ingested by earthworms as part of their diet.

In conclusion, this study showed that PCR-DGGE analysis of 16S rRNA can be used to elucidate bacterial diversity and identify unculturable microorganisms. Among the 6 universal primer sets that were tested, 1 set that amplified the V3 region of 16S rRNA from 6 pure culture strains was sufficient to define the bacterial community structure in the food, intestines, and feces of earthworms. In addition, by using a reference ladder marker, PCR-DGGE can provide valuable information about the bacterial communities, such as the relative abundance of species.

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