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Analysis of variations in band positions for normalization in across-gel denaturing gradient gel electrophoresis

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ABSTRACT

Variation in band position between gels is a well-known problem in denaturing gradient gel electrophoresis (DGGE). However, few reports have evaluated the degree of variation in detail. In this study, we investigated the variation in band positions of DNA samples extracted from soil, normalized using reference positions within marker lanes for DGGE in three organismal (bacterial, fungal, and nematode) conditions. For sample lanes, marker DNA (as a control) and sample DNA were used. The test for normality of distribution showed that the position data of a large percentage of bands were normally distributed but not for certain bands. For the normally-distributed data, their variations [standard deviation of marker bands (SDM) and standard deviation of sample bands (SDS), respectively] were assessed. For all organismal conditions, the degree of within-gel variation were similar between SDMs and SDSs, while between-gel variations in SDSs were larger than those in SDMs. Due to the large effect of between-gel variations, the total variations in SDSs were more varied between sample bands, and the mean variations of all sample bands were higher than those in the markers. We found that the total variation in the fungal and nematode SDSs decreased when the intervals between marker bands were narrowed, suggesting that band interval is important for reducing total variation in normalized band positions. For the non-normally distributed data, the distribution was examined in detail. This study provided detailed information on the variation of band positions, which could help to optimize markers for reducing band position variation, and could aid in the accurate identification of bands in across-gel DGGE analyses.

1. Introduction

Since its invention, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been widely used as a molecular tool to compare the structure and diversity of complex microbial communities and to monitor population dynamics in a wide variety of ecosystems (Muyzer, 1999; Valentin-Vargas et al., 2013). PCR-DGGE that targets small-subunit rDNA has been widely used for community analysis studies of soil bacteria (Ferrari and Hollibaugh, 1999; Nakatsu, 2007; Sekiguchi et al., 2002; Smit et al., 2001; Suzuki et al., 2009), fungi (Anderson and Cairney, 2004; Gomes et al., 2003; Hoshino and Morimoto, 2008; Suzuki et al., 2009) and nematodes (Foucher et al., 2004; Fujii et al., 2009; Okada and Oba, 2008; Waite et al., 2003).

DGGE is useful as an initial step for discriminating among communities because multiple samples can be rapidly and easily screened (Nakatsu et al., 2000; Tsushima, 2014); however, methodological limitations of these technique have also been recognized — for example, difficulty in exactly reproducing gel gradients, band smearing, and ambiguity in band identification (Nakatsu, 2007). Among these limitations, differences in the denaturing gradient between gels is a well-known problem that causes gel-to-gel variations in band positions and poses a major obstacle in experiments where a large number of samples requires simultaneous comparisons across different gels (Ferrari and Hollibaugh, 1999; Ferris et al., 1996; Nakatsu, 2007; Tourlomousis et al., 2010). Standard markers should be included in all gels to monitor gel-to-gel variability, and a sufficient number and diversity of organisms need to be chosen when making DNA reference markers (Nakatsu, 2007). Although one previous study examined the appropriate number of marker bands used for normalization in DGGE (Tourlomousis et al., 2010), the actual intervals between the marker bands were not shown in the report. On the other hand, variations in band position may also occur within an individual gel; in particular, gel distortion (known as the "smiling effect") (Fromin et al., 2002; Valaskova and Baldrian,

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2009) affects the variations in band position within a gel. A previous study clarified that such distortion could be minimized by reducing the temperature differential between the gel solutions and the glass plates when making denaturing gradient gels (Matsushita et al., 2010). However, bands are sometimes distorted at the edges of a gel (Huber and Peduzzi, 2004) even when gel pouring and running conditions are carefully regulated.

In the present study, band position variations were evaluated under previously reported DGGE conditions for communities of soil bacteria, fungi (Bao et al., 2012; Hoshino and Morimoto, 2010; Morimoto and Hoshino, 2008; NIAES, 2010a; Suzuki et al., 2009, 2012), and nematodes (Bao et al., 2012; NIAES, 2010b; Oba and Okada, 2008; Takemoto et al., 2010), where sample bands were normalized by reference markers in across-gel analysis (Tsushima, 2014). We compared within-gel and between-gel variations, in addition to total variations, in the band positions of both marker DNA (as a control) and sample DNA. Furthermore, each-lane variation within a gel was also evaluated between two lane arrangements (16- and 20-well gels). From these results, we discuss the cause of the variations in band position and technique to reduce these variations.

2. Materials and methods

2.1. Soil samples and DNA extraction

The agricultural soils used in this study are listed in Table 1. All soil samples were collected from the surface to a depth of 15 cm. Soil samples were passed through a 2 mm sieve, mixed thoroughly, and stored at −20 °C until DNA extraction. For bacterial and fungal PCR-DGGE, DNA was extracted using a FastDNA SPIN Kit for Soil (Qbiogene Inc., Irvine, CA, USA) and purified using a DNA Clean & Concentrator™-25 (Zymo Research Corp., Orange, CA, USA), as described previously (Hoshino and Morimoto, 2008). For nematode PCR-DGGE, DNA was extracted from 300 nematodes (which had been collected from soil samples according to the modified Baermann Funnel Method) using the Wizard SV Genomic DNA Purification System® (Promega, Madison, WI, USA), as described previously (Okada and Oba, 2008).

2.2. PCR amplification

Bacterial 16S rDNA and fungal 18S rDNA were amplified from soil DNA using KOD-Plus (Toyobo, Osaka, Japan) (Hoshino and Morimoto, 2008; Suzuki et al., 2009). The primer pairs were F984GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and R1378 (5′-CGG TGT GTA CAA GGC CCG GGA ACG-3′) for bacterial 16S rDNA (Heuer et al., 1997), NS1 (5′-GTA GTC ATA TGC TTG TCT-3′) and GCFung (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAT TCC CCG TTA CCC GTT G-3′) for fungi (Hoshino and Morimoto, 2008; May et al., 2001). The PCR reactions (50 μL) contained each primer at a concentration of 0.2 μM (bacteria) or 0.3 μM (fungi), 0.2 mM of each dNTP, 1 mM of MgSO4, 5 μL of 10× PCR buffer, 0.4 μM of bovine serum albumin (Takara Bio, Otsu, Japan), 1 U of KOD-Plus, and 1 μL of template DNA. The thermal profile for the PCR was as follows: an initial 2 min denaturation at 94 °C, followed by 34 cycles (bacteria) or 30 cycles (fungi) of 15 s at 94 °C, 30 s at 55 °C (bacteria) or 50 °C (fungi), and 30 s at 68 °C. Nematode 18S rDNA (Okada and Oba, 2008) were amplified from the extracted nematode DNA using PrimeSTAR HS (Takara Bio Inc., Shiga, Japan). The primer pairs were SSU18A (5′-AAA GAT TAA GCC ATG CAT G-3′) and SSU9R/GC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAG CTG GAA TTA CCG CGG CTG-3′) (Blaxter et al., 1998; Okada and Oba, 2008). The PCR reactions (25 μL) contained each primer at a concentration of 0.5 μM, 0.2 mM of each dNTP, 5 μL of 5× PCR buffer, 0.5 μM of PrimeSTAR HS, and 10 μL of template DNA. The thermal profile for the PCR was as follows: an initial 3 min denaturation at 98 °C, followed by 27 cycles of 10 s at 98 °C, 15 s at 54 °C, and 40 s at 72 °C. The products were purified with a QIAquick PCR Purification Kit (Qiagen, Santa Clarita, CA, USA).

2.3. DGGE

DGGE analysis was performed using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For bacterial 16S rDNA, 6% polyacrylamide gels containing a gradient of 50–70% denaturant were used at a constant voltage of 50 V at 58 °C for 18 h. For fungal 18S rDNA, 7% polyacrylamide gels containing a gradient of 20–45% denaturant were used at a constant voltage of 50 V at 60 °C for 20 h. For nematode 18S rDNA, 6% polyacrylamide gels containing a gradient of 20–50% denaturant were used at a constant voltage of 75 V at 60 °C for 16 h. When making denaturing gradient gels, the temperatures of gel solutions and glass plates were set to 5 °C and 10 °C, respectively (Matsushita et al., 2010), and were left for over 3 h to polymerize. The gels were stained with SYBR Green I (Cambrex Bio Science, Rockland, ME, USA) in 1× TAE for 30 min and then were scanned with a Molecular Imager FX system (Bio-Rad Laboratories).

2.4. Lane occupancy in DGGE gels

We used both 16-well gel and 20-well gels for analysis of marker DNA bands and used 16-well gel for analysis of sample DNA bands (Fig. 1). Because bands tended to be distorted at the edges of a gel (Huber and Peduzzi, 2004), we excluded the outer 2 lanes for 16-well gel and the outer 3 lanes for 20-well gel on both sides of each gel.

Table 1
The agricultural soils used for DNA extraction and PCR-DGGE in this study.

Soil information					Targetting gene in PCR[b]	
Sample	Site	Taxonomy[a]	Cultivated crop	Sampling date		
s1	Tsukuba	Andosol	Cabbage	2007/10/24	Bacterial 16S rDNA	(Sb1)
s2	Toyohashi	Yellow soil	Cabbage	2007/9/19	Bacterial 16S rDNA	
s3	Fukushima	Andosol	Spinach	2008/2/12	Bacterial 16S rDNA	(Sb2)
s4	Shiojiri	Andosol	Celery	2008/7/28	Bacterial 16S rDNA	
s5	Kyoto	Brown lowland soil	Spinach	2007/4/3	Fungal 18S rDNA	(Sf)
s6	Tsukuba	Yellow soil	Sweet corn	2007/9/18	Fungal 18S rDNA	
s7	Nagano	Gray lowland soil	Tomato	2008/8/19	Fungal 18S rDNA	
s8	Tsukuba	Gray lowland soil	Sweet corn	2008/5/16	Nematode 18S rDNA	(Sn)
s9	Tsukuba	Andosol	Sweet corn	2008/5/18	Nematode 18S rDNA	

[a] According to "Classification of cultivated soils in Japan" (Classification Committee of Cultivated Soils, 1995).
[b] Letters in parentheses are designed names of PCR products (sample DNA): Sb1 was a single PCR product amplified from soil DNA extracts from s1; Sb2, Sf and Sn were pooled PCR products amplified from soil DNA extracts from s2–s4, s5–s7, and s8–s9, respectively.