[Short Communication]

Quantitative detection of nematodes by using PCR-DGGE for evaluation of community similarities

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Soil zoologists are trying to estimate the community structure or diversity of animals by developing high throughput molecular tools, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), terminal restriction fragment length polymorphism, and real-time PCR, (Foucher et al., 2004; Gibb et al., 2008; Hamilton et al., 2009; Nelson et al., 2009; Sato and Toyota, 2006; Waite et al., 2003; Wu et al., 2009). An essential process in such trials is the performance evaluation of the tools. One of the approaches is to compare the community profiles obtained with those profiles revealed by morphological analyses based on individual identification (Griffiths et al., 2006; Okada and Oba, 2008; Wu et al., 2009).

Previously, we tried to evaluate the use of PCR-DGGE for nematode community analysis, by comparing the resulting band patterns of DNA fragments with the results of morphological analyses of the nematode samples isolated from the field. We found that the correlation in similarity relationships among communities, i.e., dissimilarity matrices, between the two methods was significant, but not high (r = 0.400-0.603, Okada and Oba, 2008). One of the reasons might be that a single morphological taxon, representing a family in our study, produced two or more DNA bands. In addition, it is possible that two or more DNA bands from different taxa may appear at the same position in a DGGE gel by chance, and seemingly form a single band. These situations might lead us to underestimate the potential of PCR-DGGE for evaluating the community structure of animals. This situation should be inevitable if we are to use nematode samples from the field. We can avoid this problem, however, if we use communities of cultured nematode species established from single individuals, which are expected to produce much simpler band patterns. Here, we re-examined the potential of using PCR-DGGE for detecting similarity relationships among communities of cultured nematodes. First, we examined the quantitiveness of our PCR-DGGE results as a correlation between the band intensity and the abundance or the biomass of each species. Second, we examined the correlation in dissimilarity matrices of communities between the DGGE band patterns and the abundance- or biomass-based community structures. We believe our study can provide useful information for developing molecular tools, not only for nematological analyses, but also for extensive analyses of soil fauna by using PCR-DGGE and other molecular tools.

Materials and Methods

We produced nematode communities consisting of eight species with different phylogenetic origins, feeding habits, and ecological traits to simulate field communities (Table 1). Cultures of Aphelenchoides sp. and Ditylenchus destructor T horne were initiated with single gravid females and those of Pratylenchus penetrans Cobb were started with three males and three females. The other cultures were established with single parthenogenetic females. Acrobeleoides sp. and Oscheius sp. were fed Escherichia coli Migula, P. penetrans was fed Medicago sativa L. callus, and the other nematode species were fed Botrytis cinerea Persoon: Fries. In a preliminary examination, each of the eight species produced a single major band at a different position on DGGE gels, although these major bands were also associated with some minor bands (Fig. 1). After the nematodes were extracted from the cultures, the body length and width of ca. 100 randomly selected individuals present in a half-volume of nematode suspension were measured under a microscope for biomass estimation by Andrássy’s formula (Andrássy, 1956). A dult nematodes were also randomly selected from the other half-volume of suspension and measured. To produce five types of communities with different structures (Table 1), the first nematode suspension, including a variety of developmental stages, was mixed. When there were one to five nematodes of a given species in a community, only adults from the second suspension were hand-picked and added to the mixtures, to minimize biomass variation among replicates.

For each of these communities, we performed PCR-DGGE, as described previously (Okada and Oba, 2008; Takemoto et al., 2010). Briefly, DNA was extracted and purified from nematodes by using the Wizard SV Genomic DNA Purification System® (Promega, Madison, WI, USA) according to the manufacturer’s instructions. By using the nematode DNA as a template, PCR-DGGE was performed with the primers SSU9R/GC (5’-CGC CC GCG CGC CCC CCG CCC GGC CGC CCG CCG CCC CCG GAG CTG GAA 3’). The two authors contributed equally to the present work.

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\[ r = 0.400 - 0.603, \text{Okada and Oba, 2008} \]

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TTA CCG CGG CTT (3') and SSU18A (5'-AAA GAT TAA GCC ATG CAT G-3'), which were originally designed by Blaxter et al. (1998) and modified with a GC clamp by Okada and Oba (2008). The reaction mixtures containing Prime Star Polymerase HS (TaKaRa, Otsu, Japan) were pre-prepared in 25 µl volumes following the manufacturer's recommendations. PCR consisted of 98°C for 3 min, followed by 26 cycles at 98°C for 10 sec, 52°C for 15 sec, and 72°C for 40 sec, with a final 10 min at 72°C. The PCR products were further purified using the Wizard SV PCR Product Purification System™ (Promega, Madison, WI, USA) according to the manufacturer's instructions. DGGE was performed on a DCode™ System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were loaded onto 6% polyacrylamide gels and stained with silver. The gel images were analyzed using the Quantity One™ software (Bio-Rad Laboratories, Hercules, CA, USA).

Table 1: Nematode species used, and structures of experimental communities (G1, G2, G3, G4, G5).

<table>
<thead>
<tr>
<th>Species</th>
<th>Order</th>
<th>ID</th>
<th>Feeding habit</th>
<th>Cp score</th>
<th>Mean (μg)</th>
<th>n</th>
<th>Mean (μg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrobebeloides sp. Rhabditida</td>
<td>Acr B</td>
<td>2</td>
<td>0.04 (0.00)</td>
<td>103</td>
<td>0.09 (0.01)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aphelenchoides sp. Aphelenchida</td>
<td>Aph F</td>
<td>2</td>
<td>0.03 (0.00)</td>
<td>130</td>
<td>0.09 (0.02)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aphelenchus avenae Bastian</td>
<td>Aphelenchida</td>
<td>Aav F</td>
<td>2</td>
<td>0.07 (0.01)</td>
<td>108</td>
<td>0.30 (0.06)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Ditylenchus destructor Thorne</td>
<td>Tylenchida</td>
<td>Dit F/H</td>
<td>2</td>
<td>0.12 (0.01)</td>
<td>105</td>
<td>0.22 (0.01)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Oscheius sp. Rhabditida</td>
<td>Osc B</td>
<td>1</td>
<td>0.07 (0.01)</td>
<td>103</td>
<td>0.28 (0.04)</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pratylenchus penetrans Cobb</td>
<td>Tylenchida</td>
<td>Prt H</td>
<td>3</td>
<td>0.05 (0.00)</td>
<td>109</td>
<td>0.07 (0.01)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Filenchus discrepans Andrássy</td>
<td>Tylenchida</td>
<td>Fil F/H</td>
<td>2</td>
<td>0.02 (0.00)</td>
<td>108</td>
<td>0.14 (0.01)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Tylencholaimus panus Ahamid &amp; Araki</td>
<td>Dorylaimida</td>
<td>Tyl F</td>
<td>4</td>
<td>0.06 (0.00)</td>
<td>108</td>
<td>0.14 (0.01)</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

1 Nematode feeding habits were bacterivorous (B), fungivorous (F) and herbivorous (H).
2 Cp scores indicate r (1) - K (5) strategy (Bongers, 1990).
3 Biomass is an average of randomly selected individuals of juveniles and adults from the nematode cultures.
4 The numbers of replicates for G1, G2, G3, G4 and G5 were 2, 4, 3, 3 and 3, respectively.
5 Correlations between band intensity and nematode abundance or biomass were examined by Kendall's τ.'
gels (acrylamide: bisacrylamide = 37.5:1; denaturant gradient of 20-50%) at 10 µl/well. A molecular marker (Okada and Oba, 2008, currently available as DGGE marker V NIPPON GENE CO., LTD, Tokyo, Japan) was also loaded at the same volume. The gels were electrophoresed at 75 V and 60 ºC for 16 hr in Tris-acetate-EDTA buffer. The gels were then stained with SYBR® Green I (Cambrex, Rockland, ME, USA), scanned, and analyzed by using the Molecular Imager FX software (Bio-Rad).

With the DGGE band patterns obtained, we first examined if the relative intensity of the major band for each species (Fig. 2) was correlated with the relative abundance and relative biomass of that species, by using Kendall’s rank correlation. We then averaged the relative intensities of each band across the replicates of each type of community, to test if the dissimilarity matrix (Bray-Curtis dissimilarity) based on the band intensity correlated well with those based on the nematode-relative abundance and relative biomass, by using Mantel test of Pearson’s correlation. The original data were arcsine-transformed before calculation. We conducted all of the statistical tests with the R software package version 2.8.1 (http://www.r-project.org/).

RESULTS AND DISCUSSION

Within the DGGE band patterns obtained, unidentified bands appeared between Fil and Tyl (indicated with an asterisk in Fig. 2), with intensities that do not seem proportional to those of any nematode band. The bands may be artificial chimera fragments, although we could not sequence them. However, we included the bands in the relative intensity calculations of nematode bands, to avoid overestimation of correlations.

The DGGE bands should have a quantitative nature before the PCR amplification reaches a plateau. Our results generally showed this quantitativeness, i.e., the band intensity of any nematode species examined was significantly correlated with its abundance or biomass, although the correlations were not extremely high for some species (Kendall’s \( r = 0.61-0.92 \), Table 1). In addition, the dissimilarity matrix of band intensity correlated highly and significantly with those of abundance (Pearson’s \( r = 0.991, P = 0.009 \)) and biomass (Pearson’s \( r = 0.984, P = 0.009, \) Mantel test, 999 permutations). These results suggest that PCR-DGGE has the potential to appropriately detect similarity relationships among nematode communities, when the DGGE band intensity is proportional to the nematode abundance or biomass. Pintado et al. (2003) showed that the band intensity is proportional to the number of cells of a bacterial species. Wang et al. (2008) suggested that the most predominant nematode species in a field-collected community produced the most intense DGGE band, although they did not provide the band intensity data. We are the first to show a clear correlation between the band intensity and abundance or biomass of each nematode species, which leads to a high correlation within similarity relationships among communities between DGGE band patterns and nematode abundance or biomass. Another reason for a high correlation in similarity relationships might be that we did not study large-sized nematodes, such as species of Mononchida and Dorylaimida, which might cause an underestimation of the abundance of small to medium-sized nematodes (Griffiths et al., 2006); although, we did use a small sized Dorylaimid species, Tylencholaimus parvus Ahmad Jr Araki. PCR-DGGE may also perform well when the nematode communities examined do not comprise numerous species, which would result in reduced band overlap. For species-rich communities, however, we might be able to reduce the band overlap by, for example, extending the band distribution over two gels with smaller gradients. These properties of PCR-DGGE can be applicable for any types of small soil-dwelling animals.

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LITERATURE CITED


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