Technical Report on the PCR-DGGE Analysis of Soil Nematode Community

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National Institute for Agro-Environmental Sciences

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1. Preparation of soil samples

【Collection of soil samples from a local agricultural field】

In order to ensure that the samples represent the entire field that is to be investigated, collect soil samples*[1] to the depth of approximately 15 cm from multiple locations by using an auger, soil probe, etc. Mix each sample thoroughly in a plastic bag. Avoid exposing the samples to high temperatures, and bring them to the lab under room temperature*[2].

*[1] Avoid sample collection immediately after a rainfall since it may be difficult to extract nematodes from wet soil.

[2] To prevent nematodes from being damaged by heavy weight, do not pile the plastic bags containing the soil samples. Place the bags in shade before they are brought indoors. Refer to other textbooks such as “Experimental Technique in Nematology”[1)] or “Methods of soil analysis”*[4)] for methods of collecting soil samples for nematode analysis.

【Sieving】

Pass the soil through a sieve with a mesh size of approximately 5 mm to remove gravel, plant roots, etc.*[3]. Chip any soil mass with hands, wearing gloves. Remove all soil masses that are too hard to chip, together with gravel etc. After mixing the soil, transfer 300 g of each soil sample to a relatively large plastic bag (No. 15, width 30 cm × length 45 cm × thickness 0.03 mm) for analysis of nematodes. Store the samples at 10°C before and after sieving, and perform nematode extraction as described in the next section within 3 weeks of soil sample collection.

*[3] Since nematodes are damaged by the pressure of heavy weight, do not place the sieve on the sieved soil, but place it on the collection vessel during sieving.

2. Extraction of nematodes from soil and pretreatment of nematode samples

Extract nematodes using the Baermann tray method (see figure below). Compared to the Baermann funnel method, the Baermann tray method uses an instrument that is simpler and has higher collection efficiency.

【Materials required】

-Stainless steel strainer (kitchen use, diameter approximately 15 cm)

-Tray (plastic pot tray for gardening, diameter approximately 15 cm)

-JK Wiper® (Kimberly-Clark)

-Round paper plate (diameter approximately 18 cm)

-Instruments for collecting nematode suspension (glass beakers of approximately 100 ml and 500 ml, 50-ml centrifuging tube, etc. These should be large enough to hold the suspension and the water used to rinse the tray and funnel.)

-Wash bottle

-Distilled water

-Room in which temperature can be maintained at approximately 25°C

-Refrigerators with temperatures set at approximately 5°C and 10°C

【Preparation】

(1) Wear gloves before assembling the instrument. Cut double sheets of JK Wiper®. Ensure that the sheets have a diameter approximately 15 mm greater than the outer diameter of the strainer, and place them inside the strainer.

(2) Set the room temperature to 25°C.

(3) Use soil samples collected within 3 weeks and stored at 10°C. Mix a soil sample in a plastic bag immediately before use*4.

*4 Take care not to mix too vigorously since doing so will kill the nematodes.

(4) Put the strainer on the tray containing a small amount (approximately 200–300 ml) of distilled water, and flood the wiper with water. Add some more water and remove large air bubbles.

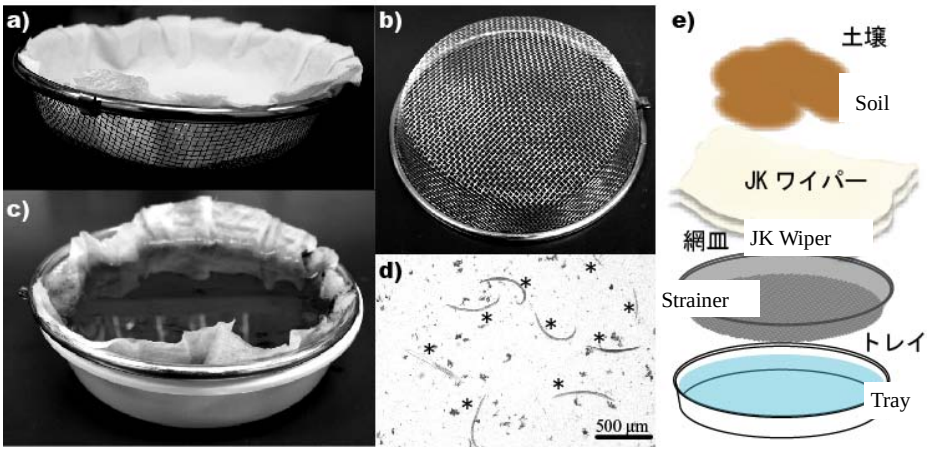


Fig. Example of Baermann tray: a) Strainer set with JK Wiper®, b) Strainer (bottom view), c) Nematode extraction, d) Extracted nematodes (*), e) Pattern diagram.

【Procedure】

(1) Sprinkle 80 g of mixed virgin soil over the wiper, and add distilled water to the tray by using the wash bottle*[5]. Spread the soil over the tray using a spatula etc.*[6] Add more water to the tray until the soil sample is completely submerged.

*[5] Avoid pouring distilled water directly on the soil.

*[6] Take care not to mix the soil more than necessary since doing so will make it difficult to release the nematodes.

(2) Ensure that the tray and the strainer have nearly leveled off and the entire soil sample has been submerged, and then fold the edge of the wiper toward the inside (to prevent water from leaking out of the tray). Cover the strainer with a paper plate.

(3) Allow it to stand still for 48 h, and then collect the nematode samples according to the following procedure:

1) Lift the strainer and immediately remove the content without waiting for the water to drain*[7].

*[7] Take care not to let the dripping water enter another tray.

2) Inject about 70% of the water in the tray into the collection vessel. Rinse the tray contents by stirring with the remaining water, and pour it into the same collection vessel.

3) Add new distilled water to the tray, rinse again, and then pour it into the collection

vessel. Repeat the same operation, and then store the vessel at 5°C. This will be used as a sample.

(4) Let the collection vessel stand still until the nematodes settle out, and concentrate the nematode suspension by removing the clear part of the supernatant. Repeat this operation until the suspension is concentrated enough to transfer into a 15–50 ml centrifuge tube. Settling of nematodes is achieved by letting the centrifuge tube stand still for half a day or overnight (depending on the amount of suspension). The samples can be stored at 5°C, but the following operation should be performed as soon as possible.

(5) Estimation of the nematode concentration. Dilute or concentrate the sample suspension as necessary, add Tween 20 (final concentration 0.05%) to prevent nematodes from adhering to plastic products, stir the sample suspension*[8], and then take a subsample*[9] to count the nematodes using a stereomicroscope. Repeat this operation at least twice per sample, and estimate the average nematode concentration in the sample.

*[8] Do not vigorously pipette the sample suspension or stir the suspension by shaking the tube, because this will damage the nematodes. It is recommended that the tube be rotated gently so that the sample suspension is whirled.

*[9] If an auto-pipette is used to collect the sample suspension, use a pipette tip with a large head diameter (approximately 1–2 mm) or one with the tip of the head cut off so that large-sized nematodes can pass through it.

(6) Removal of protozoans, enchytraeid worms, etc. After Step (5), allow the nematodes in the sample to settle out by letting the sample stand still for a night (at 5°C) or using some other method. Then, return the sample to normal temperature to release the protozoans, and remove them by taking away the supernatant by suction. Transfer the sample (reduced to approximately 5 ml) to a petri dish etc., and remove the enchytraeid worms and large debris under a microscope.

(7) Collect the sample in a 10–15 ml centrifuge tube. To collect as many of the remaining nematodes as possible, rinse the petri dish (used in Step 6) with approximately 5 ml of distilled water containing Tween 20, and transfer the rinse solution to the centrifuge tube.

(8) Repeat the centrifugation (5,800 ×g, 5 min) and concentration steps until 5–20 ml of the suspension is left. Transfer an aliquot of the suspension containing approximately 300 nematodes to a 1.5-ml tube using a pipette, and then centrifuge (5,800 ×*g*, 5 min). To concentrate the nematode suspension, gently pipette the supernatant until approximately 40 µl is left. If DNA extraction is not performed immediately, store the sample at -20°C or lower. If possible, store a part of the sample for morphological identification. Kill the nematodes by heating, add TAF solution (a mixture of 40 ml triethanolamine, 140 ml 40% formalin, and 820 ml distilled water) to an equal amount of nematode suspension, and after stabilization, store it at room temperature.

3. DNA extraction

Use crushing tubes with optimized crushing performance to extract DNA from nematodes. Use a commercially available kit for DNA purification.

【Materials required】

- Tubes for bead mill homogenization

  Prepare samples in 2-ml microtubes (Model 72.693, Assist Co., Ltd.,) containing 0.1 g of glass beads (diameter 0.1 mm; Model GB-05, Tomy Seiko Co., Ltd., , sterilized) and 4 g of zirconia-silica beads (diameter 1.2 mm; Model ZS12-0001, BioMedical Science Co., Ltd., sterilized).

-1.5-ml microtubes (sterilized)

-20% (w/v) skim milk

  Even after separating the nematodes using the tray, small soil particles are left in the sample. In this case, add skim milk to enable DNA extraction. After dissolving the skim milk (for culture substrate) in ultrapure water, heat-treat the solution at 115°C for 5 min using an autoclave to prevent invasion of DNA. The product is colored slightly brown, but it is still useful for DNA extraction. Pour approximately 2 ml of the product into screw-capped tubes, and store the tubes at -20°C.

-Wizard® SV Genomic DNA Purification System (Promega, model A2360)

-Nuclei Lysis Solution (Promega, model A7941. Purchase this if the amount included with the

kit is not enough.)

-Special grade 95% ethanol (Add to the wash solution included in the above kit.)

-Cell breaker [FastPrep 100A (BIO101), Bead-Beater (Central Science Co.), etc.]

【Extraction procedure】

(1) Add 200 μl of Nuclei Lysis Solution (NLS, included in the Promega Wizard® SV Genomic DNA Purification System) to the nematode suspension, containing approximately 300 nematodes, in a 1.5-ml tube, and transfer to a crushing tube using a micropipette. Then, rinse the 1.5-ml tube with 200 μl of NLS, and pour the suspension into the crushing tube.

(2) Add 50 μl of 20% skim milk and 50 μl of 0.5 M EDTA (included in the kit). After stirring well, freeze it at -80°C for 15 min or longer.

(3) Set the tube on FastPrep 100A, and shake at 6.5 m/s for 155 s (45 s × 3 times, 20 s × 1 time).

(4) Centrifuge at 13,000 ×*g* for 1 min to remove air bubbles.

(5) Add 500 μl of Wizard SV Lysis Buffer, included in the kit, and stir.

(6) Centrifuge at 13,000 ×*g* for 1 min.

(7) Set an SV membrane column on a new 2-ml tube (included in the kit), add 800 μl of the supernatant of Step (6) to the column, and centrifuge at 13,000 ×*g* for 3 min.

(8) Discard the eluate in the 2-ml tube using a decanter or pipette.

(9) Add 650 μl of wash solution containing ethanol , and centrifuge at 13,000 ×*g* for 1 min.

(10) Repeat Steps (8) and (9) four times, ending at Step (8), and then centrifuge at 13,000 ×*g* for 3 min to completely remove the liquid remaining in the membrane.

(11) Transfer the membrane column to a new 1.5-ml microtube.

(12) Add 250 μl of Nuclease-Free Water (included in the kit), let it stand still for 1 min, and then centrifuge at 13,000 ×*g* for 1 min.

(13) Add another 250-μl aliquot of Nuclease-Free Water, and centrifuge at 13,000 ×*g* for 3 min.

(14) Discard the membrane column, cap the 1.5-ml tube, and store at -20°C in a freezer.

4. PCR

Perform polymerase chain reaction (PCR) using the following primer set, which targets the anterior part of the *18S rRNA* gene. This primer set*[2), 3)] can be used to amplify DNA from all kinds of nematodes, but it also amplifies DNA from filamentous fungi, protozoans, and some enchytraeids. However, since nematodes are usually dominant in the samples obtained by the Baermann method, the probability of appearance of bands from other organisms is low when denaturing gradient gel electrophoresis (DGGE) is performed.

Forward primer: SSU18A

5′-aaa gat taa gcc atg cat g-3′

Reverse primer: SSU9R/GC

5′-cgc ccg ccg cgc ccc gcg ccc ggc ccg ccg ccc ccg ccc gag ctg gaa tta ccg cgg ctg-3′

The underlined segment indicates a GC clamp.

【PCR conditions】

Use Takara Bio Inc. “Prime® STAR HS” under the following conditions:

- The composition of the reaction mixture (Total volume, 25 μl)

	Amount	Final concentration
Sterile distilled water	5.5 μl	
5× Buffer	5.0 μl	1×
dNTPs (2.5 mM)	2.0 μl	0.2 mM

SSU18A (20 μM)	0.6 μl	0.5 μM
SSU9R/GC (20 μM)	0.6 μl	0.5 μM
Prime Star HS	0.25 μl	2.5 U/100 μM
Template DNA	10.0 μl	

- Reaction cycle

98°C (3 min) → [98°C (10 s), 54°C (15 s), 72°C (40 s)] × 27 → 72°C (10 min)

【Detection and purification of PCR product】

Perform agarose gel electrophoresis using a 5-μl aliquot of the PCR product to confirm that a product of the expected size (approximately 590 bp) has been obtained. Purify the remaining product using a commercially available PCR product purification kit, and then measure the DNA concentration. If DGGE is not performed immediately, store the purified product at -20°C or lower.

5. DGGE

Use the Bio-Rad DCode™ system for DGGE. The denaturant gel composition (see Table) and electrophoresis conditions should be appropriately changed for nematode analysis, but the fundamental procedure for casting the gel slabs, electrophoresis, and staining is the same as that for microbial analysis. Refer to the "Technical Report on the PCR-DGGE Analysis of Bacterial and Fungal Soil Communities" for details. The electrophoresis conditions for nematodes are as follows: gel concentration, 6%; denaturing concentration gradient, 20%–50% at 60°C and 75 V for 16 h. A DGGE marker optimized for the analysis conditions specified in this manual is available from Nippon Gene Co., Ltd. (DGGE Marker V, model 319-06941).

Table Composition of gel stock solutions for DGGE for nematode analysis*[10]

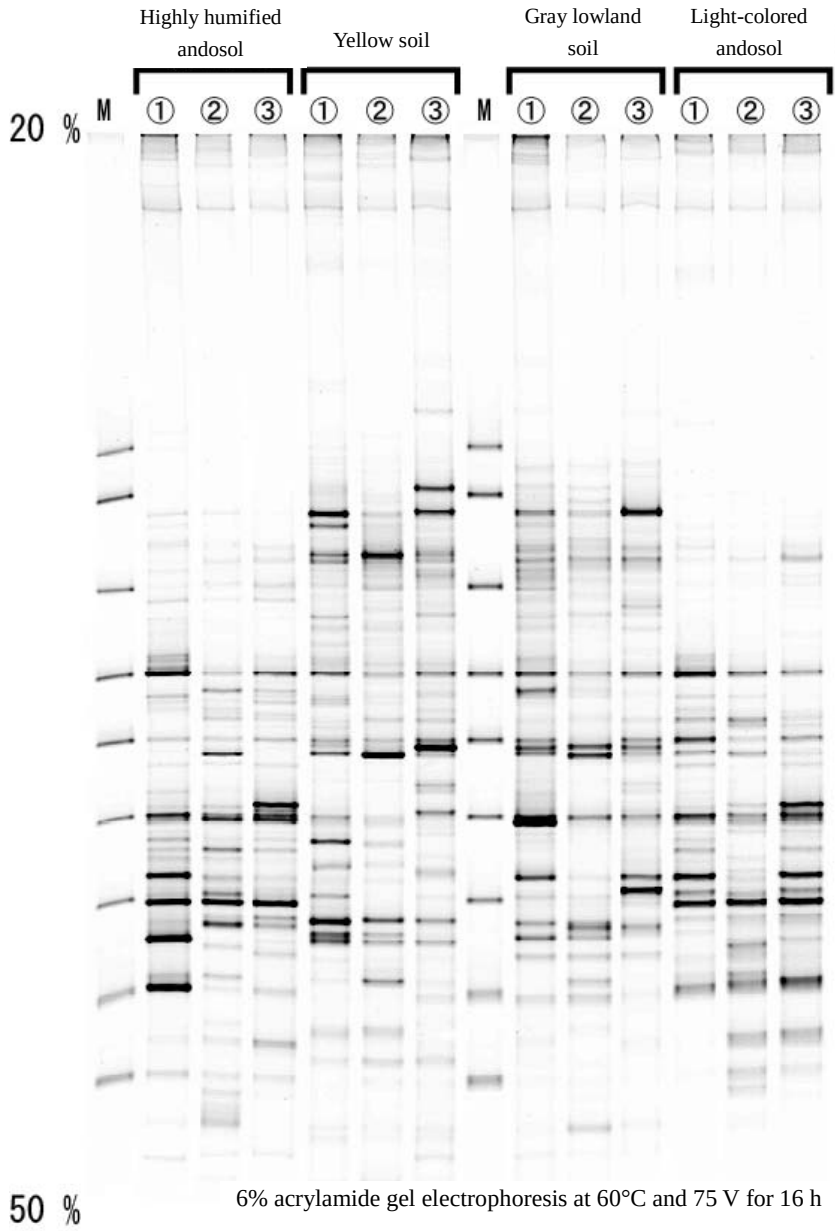
Composition	20% denatured	50% denatured
40% acrylamide/bis 37.5:1	15 ml	15 ml
50× TAE	2 ml	2 ml
Formamide (deionized)	8 ml	20 ml
Urea	8.4 g	21 g
Ultrapure water	up to 100 ml	up to 100 ml

*[10] 100% denaturation concentration is equivalent to a combination of 7 M urea and 40% formamide.

6. Examples

【DGGE analysis of nematode community】

The soil samples were provided by Dr. Kazunari Nagaoka of the National Agricultural Research Center. The numbers indicate the difference in fertilization management. After extracting nematode DNA and purifying the PCR product of *18S rRNA* gene following the instructions in this manual, an amount equivalent to 100 ng of each sample (20-well comb was used) was electrophoresed. The product was first stained with SYBR® Green I, and then imaged using Pharos FX (Bio-Rad). M is a DGGE marker for nematodes.



6% acrylamide gel electrophoresis at 60°C and 75 V for 16 h

Generally, the DNA bands of the nematodes in the soil of a tilled field tend to appear at or lower than the position of the 4th band of the marker.

7. Troubleshooting

This section describes problems related exclusively to the PCR-DGGE analysis of nematodes. For more general problems related to PCR and DGGE, refer to the "Technical Report on the PCR-DGGE Analysis of Bacterial and Fungal Soil Communities."

【Problems related to nematode extraction】

- The number of nematodes is less.
  - → The extraction efficiency may decrease if the soil used was collected immediately after a rainfall. Collect soil samples at another time. The extraction efficiency also decreases when heavy clay soil, such as soil from a paddy field, is used. In this case, use more trays (i.e., more amount of soil) for each sample, or use the density gradient centrifugation method*[1), 4)] etc.
- The nematode densities vary between the same samples.
  - → Put soil on the wiper inside the strainer in advance, and then submerge the strainer in the tray of water. In this procedure, there is little disintegration of soil aggregate, thereby making nematode release from soil smooth and stabilizing the extraction efficiency. However, care must be taken since it is not possible to confirm whether there are any air bubbles under the strainer.

【Problems related to DNA extraction】

- DNA cannot be obtained.
  - → Nematodes may have been lost during the concentration process. Collect some part of the waste solution and check whether a large number of nematodes are present by using a stereomicroscope.
  - → The shaking intensity and duration of the process of crushing the nematodes by using a cell breaker may not be optimal. Check the settings of the equipment, and observe the residue after the sample was applied to the column by using a stereomicroscope to confirm that the nematode bodies were crushed. If the crush is incomplete, extend the treatment time or increase the shaking intensity. However, take care not to crush the nematode bodies too much since doing so will fragment DNA.

【Problems related to DGGE】

- Bands appear as a smear.
  - → The DNA of nematodes that belong to the genus *Caenorhabditis* and the genus *Diploscapter* do not form bands but appear as a smear under the current experiment system. To confirm the existence of these nematodes, it is necessary to perform specific PCR etc.
- The movement of the marker bands is irregular.
  - → Since the second band from the top may show Irregular movement, care must be taken when comparing different gels.

8. References

1) Edited by the Japanese Nematological Society (2004) Experimental Technique in Nematology, Japanese Nematological Society, p. 247, Tsukuba (in Japanese).
2) Blaxter M, Ley P, Gareys J, Liu L, Scheldeman P, Vierstraete A, Vanfleteren J, Mackey L, Dorris M, Frisse L, Vida J and Thomas K (1998) A molecular evolutionary framework for the phylum Nematoda. Nature, 392, 71-75.
3) Okada H and Oba H (2008) Comparison of nematode community similarities assessed by polymerase chain reaction–denaturing gradient gel electrophoresis (DGGE) and by morphological identification. Nematology, 10, 689-700.
4) Ingham RE (1994) Nematodes. p. 459–490. *In* R.W. Weaver et al. (ed.) Methods of soil analysis. Part 2. SSSA Book Series 5. SSSA, Madison, WI.