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Effect of Storage Temperature on Soil Nematode Community Structures as Revealed by PCR-DGGE

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Abstract: The optimal duration and conditions for storage of soils collected for nematode community analyses are unknown. To study this issue, three types of soils with different geographical origins from the subarctic to cool-temperate Japan were kept at three temperature levels (5, 10, and 20°C) for up to 8 wk following collection. During the storage period, nematode population density was measured, and community structure was assessed by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE). No significant changes in the population density or diversity of nematodes (Shannon-Wiener Diversity Index) were observed during storage compared to initial states, except that density in an andosol collected from Tsukuba, Central Japan decreased significantly after 28 d of storage at 5°C. However, a regression analysis showed a declining trend in nematode density in the latter half of the storage period when soils were stored at 5 or 20°C, depending on the geographic origin of the soil. These results indicate that soils can be stored for 14 d at 5–20°C, with 10°C as optimal. This is the first study to experimentally determine the optimal preservation conditions for nematode assemblages in soils that are to be analyzed using PCR-DGGE.

Key words: biodiversity, ecology, method, soil fauna.

Nematode community analyses are valuable tools for soil-based assessments of physical and chemical disturbances and ecosystem development (Bongers, 1990; Bongers and Bongers, 1998; Neher, 2001; Yeates, 2003). However, before such analyses, nematodes are usually extracted from soil, fixed appropriately, and mounted on slides, which takes considerable time and labor. Alternatively, nematode ecologists are increasingly employing molecular-based techniques such as PCR-DGGE to evaluate the diversity of nematode communities (Waite et al., 2003; Cook et al., 2005; Okada and Oba, 2008; Sato et al., 2009).

Researchers inevitably store soil samples for days or weeks after collection from the field. It is generally recommended to store soil samples at cool temperatures of 5 or 10°C (Hooper, 1986; Hunt and De Ley, 1996; Oba and Okada, 2008) or room temperature (Mizukubo, 2004) until nematode extraction. However, these guidelines are anecdotal, and no published data substantiate them. Some researchers have conducted detailed studies (Barker et al., 1969), but they concentrated on plant-parasitic nematodes; information on the nematode assemblage, including free-living species, was not reported. Cool and room temperatures seem to be equally reasonable and dubious for the preservation of soil nematode fauna and community structure. Cool temperatures may reduce the metabolism of nematodes and allow them to survive longer without remarkable changes in community structure, but a significant shift to cool temperatures may be lethal to some species. To address this issue, reasonable data on the succession of nematode communities during storage are needed.

Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) is an increasingly employed technique used to profile the community structure of nematodes (Waite et al., 2003; Cook et al., 2005; Okada and Oba, 2008; Sato et al., 2009) and other small organisms (Hatamoto et al., 2008; Yu et al., 2008). During DGGE, PCR products migrate through increasingly higher concentrations of chemical denaturants in the gel. Upon reaching a threshold denaturant concentration, the double-stranded PCR product begins to denature, and its migration rate decreases significantly. Different PCR products have specific threshold denaturant concentrations depending on their DNA sequences, even if they are of the same length (Muyzer 1993), thus generating an electrophoretic fingerprint for a community. PCR-DGGE with universal primers amplifying a portion of the nematode small subunit ribosomal DNA (18S rDNA) is thus useful for estimating community diversity, as the number of unique bands in the electrophoretic fingerprint correlates to species richness. Although banding patterns obtained by DGGE do not indicate taxonomic placement of operational taxonomic units (OTUs), they provide enough information to assess changes in community structure between samples or along a time sequence. Moreover, denaturation conditions and primers can be modified to maximize the separation of DGGE bands from different genera or species of nematodes (Okada and Oba, 2008). PCR-DGGE is an effective tool for nematode community analysis that circumvents labor-intensive analysis using microscopy. The present study used PCR-DGGE to 1) investigate the changes in indigenous nematode community structure during soil storage and 2) determine the optimal temperature and storage period.

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Materials and Methods

Soil sampling and storage: Three andosol soils of different origins (HOK, TAK, and NIA) were used in this study. NIA was collected from a soybean field at the National Institute for Agro-Environmental Sciences, Tsukuba, Japan, in mid October 2008. HOK was collected in a soybean field at a branch of the Hokkaido Agricultural Research Center, Memuro, Japan, in early November 2008. TAK was collected in a deciduous forest, Takahagi, Japan, in early October 2008. The climate of Sapporo is subarctic, and that of Takahagi and Tsukuba is cool-temperate. The physico-chemical properties of the soils are shown in Table 1. The HOK and NIA soils were sieved (5 mm) and mixed thoroughly, and 80-g aliquots were sealed in small polyethylene bags. TAK was similarly processed, except that 30-g aliquots were packed. The bags were held in a foam polystyrene box to avoid desiccation and stored at 5, 10, or 20°C. Herein, soils that underwent a storage period are referred to as aged soils, while those that had not yet undergone storage are referred to as unaged soils.

Nematode extraction and preparation of DNA templates: Three bags of soil for each soil × temperature × period treatment were arbitrarily selected and recovered from the boxes after 0, 2, 4, 7, 14, and 28 d of storage. Additional samples were similarly taken from TAK after 56 d of storage. The entire content of each recovered bag was directed to Baermann trays (Whitehead and Hemming, 1965), and nematodes were extracted for 48 h at room temperature, approximately 25°C. The nematodes were counted under a stereomicroscope, and DNA was extracted and purified from 300 nematodes per soil sample using the Wizard SV genomic DNA Purification System® (Promega, Madison, WI, USA) according to the manufacturer's instructions, generally following Okada and Oba (2008). The arbitrarily selected sample DNA concentration was 3.0 ± 0.9 ng/μl (mean ± SD; measured with a NanoDrop (ND-1000) spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA).

PCR-DGGE: Using the nematode DNA templates, PCR-DGGE was performed with the primers SSU9R/GC (5′- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAG CTG GAA TTA CCG CGG CTG-3′) and SSU18A (5′-AAA GAT TAA GCC ATG CAT G-3′), which were originally designed by Blaxter et al. (1998) and modified with GC clamp by Okada and Oba (2008). Reaction mixtures containing Prime Star Polymerase HS (TaKaRa, Otsu, Japan) were prepared in 25-μl volumes following the manufacturer's recommended composition. PCR was carried out using the following thermal cycles: 98°C for 3 min, 26 cycles at 98°C for 10 s, 52 for 15 s, 72°C for 40 s, and 72°C for 10 min. PCR products were loaded onto 2% agarose gels, electrophoresed in TAE at 100V for 20 min, stained with 2 mg/l ethidium bromide, and visualized under UV light to confirm that their lengths were approximately 610 bp. The products were quantified using a spectrophotometer and diluted to 10 ng/μl with nuclease-free water after purification using the Wizard SV PCR Product Purification System™ (Promega, Madison, WI, USA) according to the manufacturers' instructions. DGGE was performed using the DCode™ System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were loaded onto 6% (w/v) polyacrylamide gels (acrylamide: bisacrylamide = 37.5:1; denaturant gradient of 20–50% for HOK and NIA and 28–43% for TAK) at 10 μl/well and dissolved by electrophoresis at 75 V and 60°C for 16 h in 1× Tris-acetate-EDTA (TAE) buffer. Gels were stained with SYBR® Green I (Cambrex, Rockland, ME, USA), scanned, and analyzed using the Molecular Imager FX software (Bio-Rad). Two bands were determined to be of the same OTU if they had the same mobility in the gels. A molecular marker (DGGE Marker V; Nippon Gene, Tokyo, Japan) was used to compare mobility between gels. The OTUs from each of the three soils were sequentially numbered in ascending order along with corresponding DGGE mobility. Sample bands that appeared above the fourth most upper marker bands were omitted because the majority of those are not nematodes, i.e., alveolates, rotifers, and dipterans (Okada and Oba, 2008). The band intensity obtained from DGGE was thereafter used as an alternative measure of the relative abundance of each OTU. Band intensity is correlated to the abundance of specific DNA fragments in a DNA template mixture, and is expected to be correlated with the population size or biomass of focal OTUs. For example,

Table 1. The properties of the soils used in this study.

Soil	NIA	HOK	TAK
Sampling site	Tsukuba, Ibaraki	Sapporo, Hokkaido	Takahagi, Ibaraki
Soil type	Andosol	Andosol	Andosol
Soil texture	Silt loam	Sandy loam	Silt loam
Humus content	Few	Few	Abundant
Bulk density	0.77	0.83	0.49
pH	6.8	5.6	4.5
Truog-P [mg/100 g]	3.2	6.7	1.2
CEC [me / 100 g]	31.920	19.9	59.2
Total N [%]	0.351	0.231	1.41

the relative intensity of DGGE bands has been used for quantifying bacterial cell numbers in defined mixed cultures (Pintado et al., 2003). A semi-quantitative PCR-DGGE analysis also was performed for nematodes in our previous paper (Okada and Oba, 2008), although band intensity does not necessarily equal conventional measures of abundance due to different copy numbers of target genes for PCR amplification per genome, different PCR efficiency, overlapping bands of different species, and duplicated bands from single species.

Sequencing and homology search: Small pieces (ca. 5 × 1 × 1 mm) of gels corresponding to some of the major DGGE bands were excised with a disposable surgical blade. Each piece was immersed in 100 µl of sterilized distilled water, frozen, and thawed at 95°C for 5 min. The thawed solution (2 µl) was used as a template for the PCR, as described above, except that the number of cycles was 25. PCR products (2 µl) were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and used as templates for the sequencing reaction with the BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies, Carlsbad, CA, USA) along with each of the primers, following the manufacturer's instructions. Sequence data were collected with an ABI 3100 genetic analyzer (Life Technologies). The homology of the partial sequences of the 18S rRNA gene was obtained via searches of a public database using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on).

Statistical analyses: The Shannon-Wiener's diversity index (H′) was calculated for each sample. By definition,

$$H' = -\sum_{i=1}^{S} P_i \cdot \log_2 P_i, \quad (1)$$

where S is the number of OTUs and P_i is the relative abundance (relative intensity of the band in DGGE) of the i-th OTU in the sample.

A two-factor ANOVA was used to examine the effects of temperature, period, and their interaction on the $\log[x+1]$-transformed number of nematodes per g dry soil and the H′ value for aged samples of each soil. Data from unaged samples were omitted because of the lack of the three-level temperature treatment. When the period × temperature interaction was significant, the Tukey-Kramer test was performed for each dataset split by day to clarify the accumulated effect of temperature on each day. When the interaction was not significant and the effect of period itself was significant, the Tukey-Kramer test was applied to compare the mean differences in the H′ value and densities between differently aged samples across temperatures. Dunnett's test was conducted to compare the mean differences in H′ values and log-transformed nematode densities between aged and unaged samples for each temperature for each soil. The data of log-transformed nematode density split by sampling site and storage temperature was fitted to polynomial functions (up to second order) of storage period. Akaike's information criterion (AIC) was used for the selection of models, including the order of the functions. All statistical analyses were performed with the R software package version 2.10.1 (R Development Core Team 2009) available at http://www.r-project.org./.

Results

From NIA, 18 OTUs were distinguished (Figs. 1, 2), the most dominant of which represented more than 50% of total abundance, based on relative band intensity. Nematode population density was 96 g^{-1} at the beginning of storage, but changed with temperature and the period × temperature interaction (ANOVA, P= 0.020 and P = 0.004, respectively; Fig. 3A). Nematode density decreased in time when soils were stored at 5°C, and was 62 g^{-1} at the end of 28 d of storage: the data for the change in density at 5°C were fitted with a downward-sloping convex curve ($y = -0.1555x^2 + 0.0557$, R^2 = 0.64, $P < 0.001$). However, nematode density remained high when the soils were stored at 20°C, and on day 14 was significantly higher (112 g^{-1}) than that of samples stored at 10°C (79 g^{-1}; Tukey-Kramer test, P = 0.030). There were no significant differences in nematode density between aged and unaged samples for any temperatures (Dunnett test, $P > 0.072$). There were no significant regressions between nematode density and storage period at 10 or 20°C. Mean H′ values ranged from 1.25 (4 d of storage at 20°C) to 2.72 (4 d of storage at 5°C) (Fig. 3B), and were affected by storage period (ANOVA, P = 0.015) and the period × temperature interaction (ANOVA, P = 0.049). The single effect of temperature on H′ values was marginally significant (ANOVA, P = 0.053), with the samples stored at 5°C being most diverse. There were no significant differences in H′ values between aged and unaged samples for any temperatures (Dunnett's test, P = 0.060). The change in H′ value at 5°C was fitted with a convex curve ($y = -0.0024x^2 + 0.0737x + 1.7369$, R^2 = 0.34, P = 0.045). There were no significant regressions between H′ value and storage period at 10 or 20°C. The Tukey-Kramer test confirmed that the samples stored at 20°C for 4 d were less diverse than those stored at 5°C for the same period (P = 0.018).

Twenty OTUs were distinguished from HOK (Fig. 2), and the most dominant represented about 40% of total abundance. Nematode population density was 31 g^{-1} at the beginning of storage, but changed with temperature, period, and their interaction (Fig. 4, ANOVA, $P <$ 0.001, P = 0.005, and $P < 0.001$, respectively). Density at 5°C decreased ($y = -0.0004x^2 + 0.0054x + 1.4879$, R^2 = 0.76, $P < 0.001$) to 21 g^{-1} after 28 d of storage, significantly lower than that at 10°C and 20°C (Tukey-Kramer test, P = 0.021 and P = 0.005, respectively) and that of unaged samples (Dunnett's test, P = 0.010). There were no other significant differences in nematode density

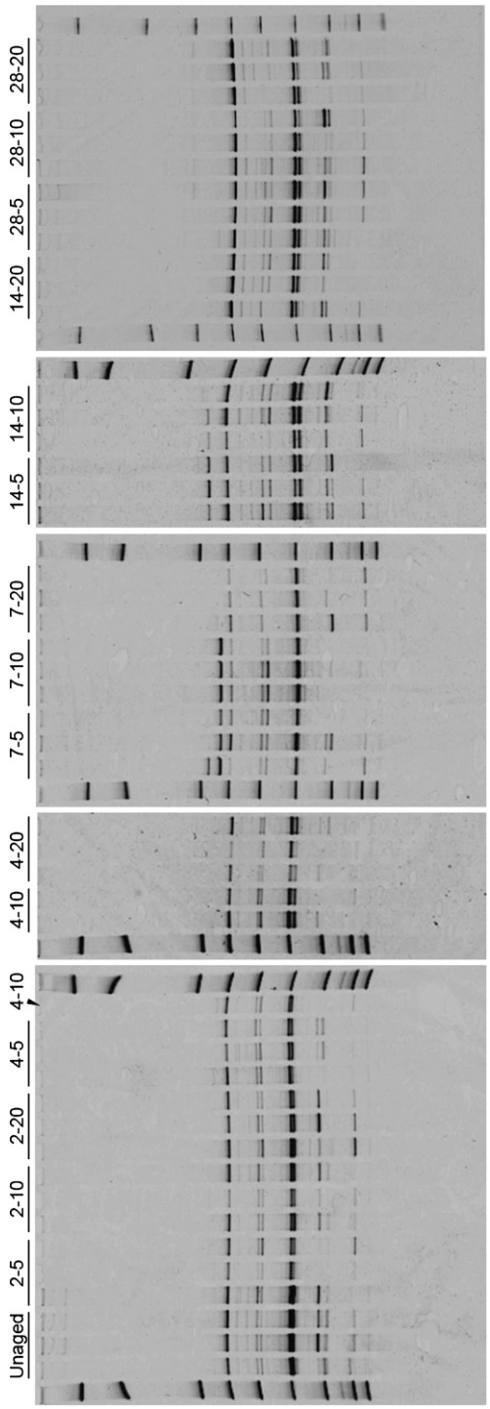

FIG. 1. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) gel images. Nematode communities in an andosol from a soybean field in Tsukuba, eastern Japan, (NIA) are shown as examples. The combination of two digits indicated with bars and an arrowhead on the lane indicates the soil storage period (d) and temperature (°C), respectively. Lanes without notification are those loaded with 10-μl aliquots of a molecular marker (DGGE Marker V; Nippon Gene, Tokyo, Japan).

between aged and unaged samples for any temperatures (Dunnett's test, $P = 0.096$). The change in nematode density at 10°C was fitted with a moderately upward-sloping, moderately concave curve for 10°C ($y = -0.0001x^2 + 1.501$, $R^2 = 0.38$, $P = 0.006$). Density

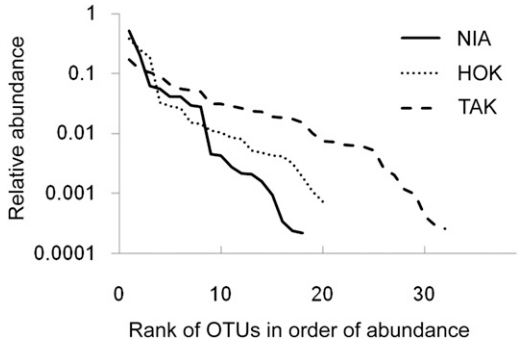

FIG. 2. Rank-abundance curves of nematode communities in soils collected from three different localities in the subarctic and cool-temperate Japan. Horizontal axis represents the rank of each OTU in order of relative abundance in each soil. Vertical axis represents the relative abundance of each OTU. NIA: andosol from a soybean field in Tsukuba, eastern Japan; HOK: andosol from a soybean filed in Sapporo, northern Japan; TAK: andosol from a deciduous forest in Takahagi, eastern Japan.

increased when samples were stored at 20°C, with a maximum of 56 g^{-1} on day 14; these data were fitted with a convex upward-sloping curve ($y = -0.0007x^2 + 0.0266x + 1.4600$, $R^2 = 0.47$, $P = 0.009$). Mean H′ values ranged from 1.81 (2 d of storage at 5°C) to 2.72 (4 d of storage at 5°C) (Fig. 4B), and were affected similarly

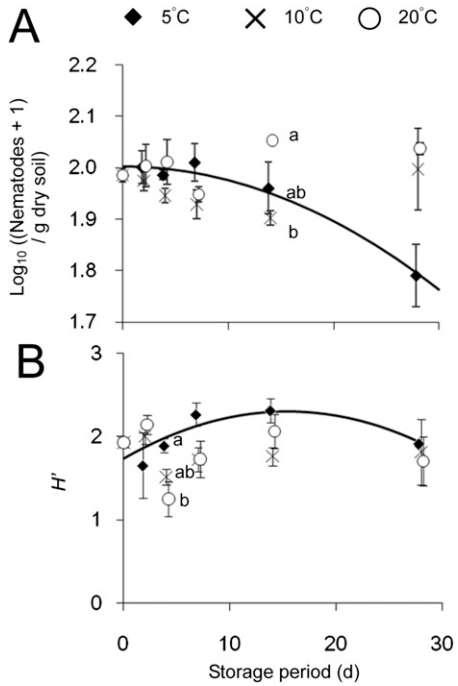

FIG. 3. Changes in nematode communities in NIA during storage at 5, 10, and 20°C. Data are shown as average and standard error for each temperature × period treatment. Data points with the same lowercase letters are not significantly different (Tukey-Kramer test, $P < 0.05$, among temperatures within single storage periods, when the period × temperature interaction was significant). The most predictive and significant ($P < 0.05$) regression models are indicated with solid lines. A: nematode density g^{-1} dry soil; B: Shannon-Wiener's diversity index (H′). NIA: andosol from a soybean field in Tsukuba, eastern Japan.

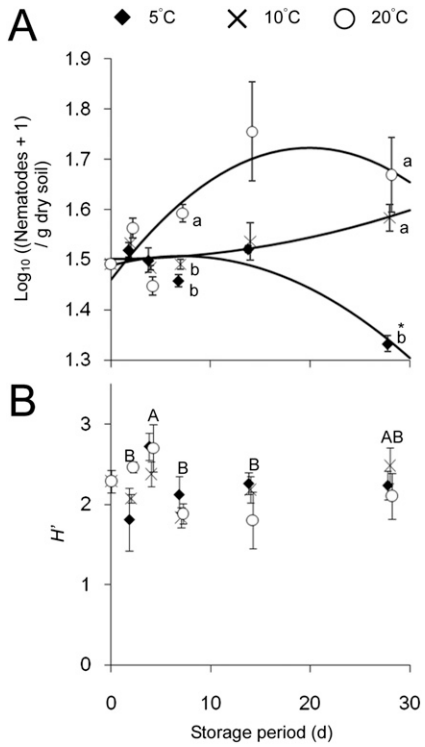

FIG. 4. Changes in nematode communities in HOK during storage at 5, 10, and 20°C. Data are shown as average and standard error for each temperature–period treatment. Data points with the same lowercase or uppercase letters are not significantly different (Tukey-Kramer test, $P < 0.05$, lowercase: among temperatures within single storage periods when period × temperature interaction was significant; uppercase: among storage periods when only the period effect was significant). Asterisks indicate a significant difference from unaged samples (Dunnett's test, $P < 0.05$). The most predictive and significant regression models ($P < 0.05$) are indicated with solid lines. A: nematode density g^{-1} dry soil; B: Shannon-Wiener's diversity index (H′). HOK: andosol from a soybean filed in Sapporo, northern Japan.

only by storage period (ANOVA, $P = 0.001$), with H′ values of 4-d-old samples being larger than all others, with the exception of 28-d-old ones (Tukey-Kramer test, $P < 0.05$). There were no significant differences in H′ values between aged and unaged samples for any temperatures (Dunnett's test, $P = 0.310$). There was no significant regression between H′ value and storage period after the data of the three temperature levels were pooled together.

Thirty-two OTUs were identified from TAK, (Fig. 2), and the most dominant represented 17% of total abundance. The nematode population density was 437 g^{-1} at the beginning of storage, but changed with temperature, period, and their interaction (ANOVA, $P = 0.020$, $P < 0.001$, and $P = 0.005$, respectively). There were no significant differences in nematode density between aged and unaged samples for any temperatures (Dunnett's test, $P = 0.157$). Density at 20°C temporarily increased to 523 g^{-1} after 7 d of storage, with a significant difference from soils stored at 5°C and 10°C (Tukey-Kramer test, $P = 0.003$ and $P = 0.005$, respectively), but decreased to 331 g^{-1} by day 56. The change in nematode density at 20°C was fitted with a downward-sloping convex curve ($y = -0.0004x^2 + 0.0054x + 1.4879$, $R^2 = 0.76$, $P < 0.001$). There were no indications that temperatures could affect the proportion of OTUs in TAK nematode assemblages during the first two days of storage. Mean H′ values ranged from 2.28 (14 d of storage at 10°C) to 4.06 (28 d of storage at 10°C) (Fig. 5B), and were affected only by storage period (ANOVA, $P < 0.001$), with the mean H′ value of 28-d-old samples being larger than those of 4- and 14-d-old samples (Tukey-Kramer test, $P < 0.05$). There were no significant differences in H′ value between aged and unaged samples for any temperatures (Dunnett's test, $P > 0.240$). There was no significant regression between H′ value and storage period after the data of the three temperature levels were pooled together.

Three, six, and six major DGGE bands were sequenced for HOK, NIA, and TAK, respectively (Table 2, Fig. 6). All were nematodes except for one stramenopile (band D in Fig. 6). Band D electrophoresed more slowly than the fourth-uppermost marker band, and, thus, was omitted from community analysis in this study.

DISCUSSION

Our PCR-DGGE analyses have provided the first data on the effects of various storage conditions on soil nematode community structure. If indigenous nematode communities contain species that are easily killed or whose fecundity is affected by temperature shifts, their DGGE patterns should vary according to storage temperature after a short period of time following collection. In fact, NIA soils stored for 4 d at 5°C had higher nematode diversities, due to the decreased proportion of the most abundant species (data not shown), than those stored at 20°C (Fig. 3B). However, nematode diversity was maintained in HOK stored at 5°C for 4 d. These results indicate that temperature shifts may kill or cause infertility in certain nematode species, which may skew diversity estimates of nematode assemblages via decreases in abundant species; however, this may have a reverse effect on communities by reducing less-abundant species. There was no consistent effect of temperature on the diversity index, so we cannot make suggestions about preferable temperature conditions in this line.

The nematode density in NIA soil decreased at 5°C, suggesting susceptibility to cool temperatures. Similar results have been obtained for several plant-parasitic nematodes (Baker et al. 1969). Regression analyses indicated that nematode density also tended to decrease in the late stage of storage at 20°C for HOK and TAK soils. Baker et al. (1969) reported that the recovery rate of several plant-parasitic nematodes from soil was lower when stored at 30 or 36°C for a few weeks. They suggested that the low recovery rate might reflect a loss of soil moisture, which may have led to low survival.

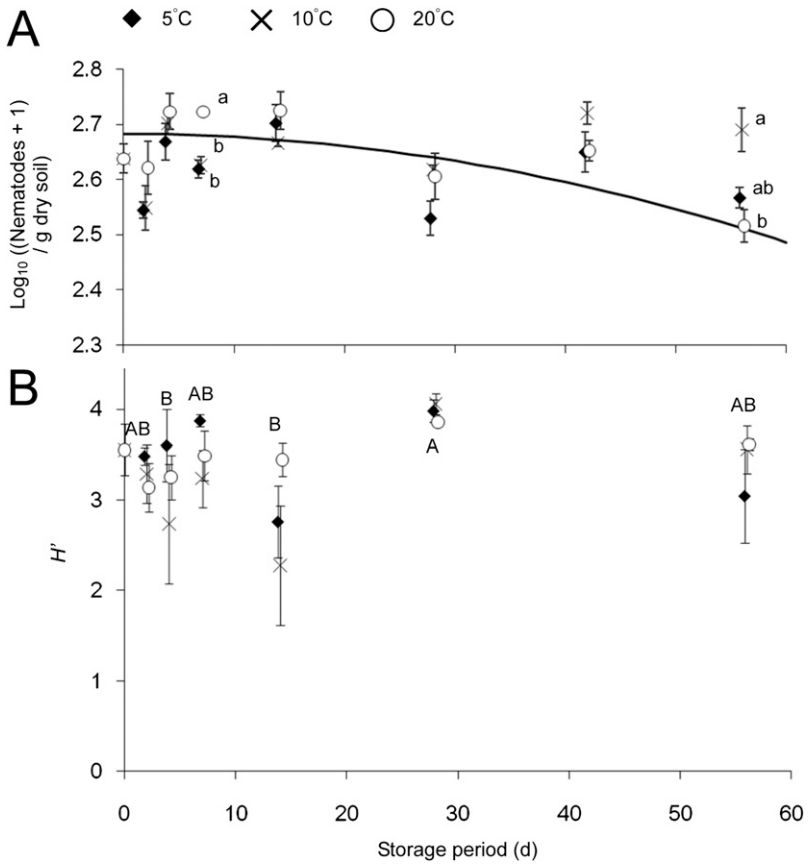

FIG. 5. Changes in nematode communities in TAK during storage at 5, 10, and 20°C. Data are shown as average and standard error for each temperature–period treatment. Data points with the same lowercase or uppercase letters are not significantly different (Tukey-Kramer test, $P < 0.05$, lowercase: among temperatures within single storage periods when period × temperature interaction was significant; uppercase: among storage periods when only period effect was significant). The most predictive and significant ($P < 0.05$) regression models are indicated with a solid line. A: nematode density g^{-1} dry soil; B: Shannon-Wiener's diversity index (H′). TAK: andosol from a deciduous forest in Takahagi, eastern Japan.

Such soil desiccation was not found in the present study (data not shown) and thus cannot explain our results. In the case of the pinewood nematode *Bursaphelenchus xylophilus*, nematodes treated at a warm temperature of 25°C in starvation were less reproductive than those treated at 20°C in a laboratory test (Takemoto, 2008). This outcome might have also been due to exhaustion because a temperature of 25°C itself had no adverse effect on the reproduction of the nematode (Mamiya, 1975). From these observations, we consider that a

TABLE 2. Closest relatives for the denaturing gradient gel electrophoresis bands with the greatest similarities in the BLAST database search.

Band in Figure 6	Family	Species	Accession No.	Similarity (%)	Length (bp)
A	Cephalobidae	*Acrobeloides thornei*	EU543175	100	514
B	Pratylenchidae	*Pratylenchus penetrans*	EU669925	99	514
C	Rhabditidae	*Rhabditis* sp.	HQ130504	100	441
D	Chromulinaceae (stramenopile)	*Spumella*-like flagellate	EF043285	99	493
E	Cephalobidae	*Acrobeloides thornei*	EU543175	99	469
F	Aphelenchidae	*Aphelenchus avenae*	AY284639	100	469
G	Rhabditidae	*Oscheius* sp.	HQ130503	100	488
H	Pratylenchidae	*Pratylenchus penetrans*	EU669925	99	484
I	Rhabditidae	*Rhabditis* sp.	EU196007	100	469
J	Cephalobidae	*Acrobeloides thornei*	EU543175	100	425
K	Rhabditidae	*Oscheius tipulae*	HQ130502	100	455
L	Pratylenchidae	*Pratylenchus penetrans*	EU130807	96	438
M	Microlaimidae	*Prodesmodora circulata*	AY284720	97	279
N	Prismatolaimidae	*Prismatolaimus* cf. *dolichurus*	AY284727	98	482
O	Plectidae	*Plectus aquatilis*	GQ892827	94	411

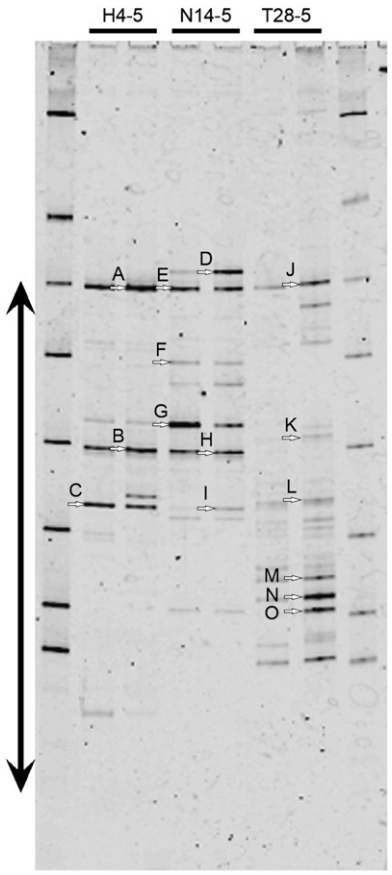

FIG. 6. An image of the denaturing gradient gel electrophoresis bands examined for nucleotide sequence. Two of the three replicated samples were examined for HOK soil stored at 5°C for 4 d (H4-5), NIA at 5°C for 14 d (N14-5), and TAK at 5°C for 28 d (T28-5). The denaturation gradient of the gel was 28–43%. Note that the two uppermost marker bands overlapped with this denaturation gradient. Horizontal white arrows indicate the bands whose sequences was determined partially or completely. Letters on the white arrows correspond to those in Table 2. A vertical arrow denotes the range of nematode bands. NIA: andosol from a soybean field in Tsukuba, eastern Japan; HOK: andosol from a soybean filed in Sapporo, northern Japan; TAK: andosol from a deciduous forest in Takahagi, eastern Japan.

negative, long-term effect of temperature on nematode density is plausible. Soils should not be stored at 5 or 20°C for long periods. Different responses of nematode communities to temperatures among the soils in the present study may be attributed to the different climatic conditions of the soil sampling sites: mean air temperatures in the month just before soil sampling were 25.0°C, 12.9°C, and 20.5°C for NIA, HOK, and TAK, respectively (census data from the Automated Meteorological Data Acquisition System, provided by the Japan Meteorological Agency, based on stations nearest to the sites, with altitude correction).

In some cases, storage period was the only factor that affected diversity, with fluctuation being common among the different storage temperatures (e.g., 4-d-old samples in HOK; Fig. 4B). These results suggest that the differences in nematode communities that can be attributed to different storage temperatures were often much smaller than were their temporal fluctuations. At the same time, we cannot completely exclude the possibility that sample handling could have confounded the effect of storage period. More attention is needed to the standardization of sample handling procedures and environmental conditions (e.g., room temperature during nematode extraction) across experimentation.

Oba and Okada (2008) suggested that soils should be stored at 10°C for less than 3 wk, but not at cool temperatures below 4°C. Lazarova et al. (2004) stored soil samples at 4°C until nematode extraction. Hunt and De Ley (1996) mentioned that soils could be stored at 5–10°C for a few weeks without serious problems, although it would be far better to extract the nematodes as soon as possible after soil sampling. These authors commonly recommended soil storage at cool, but not freezing, temperatures. In the present study, nematode density and diversity did not change significantly during 14 d of storage compared to their initial states, irrespective of storage temperature. However, populations tended to change in all three soils stored at 20°C. Considering also that populations declined in NIA and HOK soils after 28 d of storage at 5°C, a storage temperature of 10°C may be more acceptable than other temperatures. In conclusion, we suggest that soils collected in the cool-temperate zone, such as in this study, can be stored for 2 wk at 5-20°C, and most desirably at 10°C. It should be noted that the present study used only andosol, which is common in Japan but not globally; thus, further study is needed on other soil types.

Recently, nematode DNA was extracted from soils that were compressed until the bodies of the nematodes burst (Goto et al., 2009). They succeeded in estimating the densities of *Heterodera glycines* in the soils by real-time PCR with the DNA extracted. The quantity and quality of the DNA template of *Pratylenchus penetrans* were not affected by soil storage for months at room temperature after air-drying of the soils (Sato et al., 2010). Air-drying followed by compaction may be an alternative method of storage for community analysis of nematodes on a molecular basis. However, further studies are needed to assess the applicability of this method to other species and assemblages of nematodes. Until then, soil storage at approximately 5–10°C may continue to be a better way to preserve the structure of nematode communities.

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