**Intraspecific variations and phylogenetic relationships of steinernematids isolated from Japan based on the sequences of the ITS region of the nuclear rRNA gene and the partial mitochondrial COI gene**

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DNA sequences of the internal transcribed spacer (ITS) region of the nuclear rRNA gene and mitochondrial cytochrome oxidase I (COI) gene were used to analyze intraspecific variations and infer phylogenetic relationships of steinernematids isolated from Japan. DNA fragment lengths of the amplified ITS region obtained from 19 Japanese steinernematid isolates ranged from 914–1,067 bp and varied considerably among the species. A few intraspecific variations were detected in Steinernema litorale, Steinernema spp. MY 6 and MY 8, and no variation was observed in S. monticolum and Steinernema sp. MY 5 in the sequences of the ITS region. The fragment length of the amplified partial COI gene was 397 bp for all species. The intraspecific variations of the sequences ranged from 0.3% - 7.8% (0-42 nucleotide differences) for S. monticolum, S. litorale, Steinernema spp. MY 5, MY 6, and MY 8. Sequence comparisons and phylogenetic analyses reveal that the sequence of the COI region can distinguish the isolates within a species but is not as effective as the ITS region in elucidating the relationship among species. Jpn. J. Nematol. 36(1), 11-21 (2006).

Key words: entomopathogenic nematode, Steinernema, phylogeny.

INTRODUCTION

Entomopathogenic nematodes (EPNs) of the genus Steinernema have been globally used as safe biocontrol agents against soil borne insect pests (Ehlers, 2005). In an effort to obtain more efficient agents, nematode surveys have been conducted around the world, and more than 47 species belonging to the genus Steinernema have been described thus far (Stock and Hunt, 2005; Qiu et al., 2005). In Japan, surveys on EPNs have been conducted since the late 1980's, and 12 species of steinernematids have been isolated; six described species (S. carpocapsae, S. feltiae, S. kraussel, S. kushidai, S. litorale, and S. monticolum) and six undescribed ones (Steinernema spp. MY 3, MY 4, MY 5, MY 6, MY 7, and MY 8) (Mamiya and Ogura, 1990; Yoshida et al., 1998; Mamiya et al., 2001; Yoshida, 2003a; Yoshida, 2003b; Yoshida, 2004). Although isolation of S. carpocapsae in Japan has been reported by Mamiya and Ogura (1990), the species has never been reisolated.

The internal transcribed spacer (ITS) region of the rRNA gene on the nuclear genome has proven to be useful for not only identifying but also inferring the phylogenetic relationships of steinernematid species (Hominick, 2002; Nguyen et al., 2001; Spiridonov et al., 2004). Other molec-
ular markers that proved to be useful for discrimination are the mitochondrial cytochrome oxidase II (COII)-16S gene and the nuclear 28S rRNA gene (Szalanski et al., 2000; Stock et al., 2001). However, the COII-16S gene was too variable and the 28S rRNA gene was too conserved to infer phylogenetic relationships of steinernematids at the species level (Adams and Nguyen, 2002). In addition, very few studies have been conducted to detect the intraspecific genetic variations in the steinernematid species using DNA sequences and to analyze the phylogenetic relationship within species (Szalanski et al., 2000; Spiridonov et al., 2004).

Because mitochondrial DNA (mtDNA) evolves fairly rapidly, most of the nucleotide substitutions occur at the mtDNA sites and these substitutions are unlikely to be responsible for morphological alternations, the molecular information contained in mtDNA is the most widely used to study the phylogeny and systematics of various animals (Hillis et al., 1996). Among the mitochondrial genes, the cytochrome oxidase I (COI) region has been used to infer the phylogenetic relationship at the species and subspecies levels and to detect the intraspecific variations in insects (Sperling and Hickey, 1994). However, no research has been conducted to analyze the relationship using sequences of the COI region in entomopathogenic nematodes. In this study, we amplified and sequenced the ITS region of the nuclear rRNA gene and the mitochondrial COI gene to detect the intraspecific genetic variations of the Japanese steinernematid isolates. We also analyzed the phylogenetic relationships among the Japanese isolates using the ITS and COI regions to infer the relationships between and within the species.

MATERIALS AND METHODS

Nematodes and DNA extraction:

Nineteen isolates of 10 available Japanese steinernematid species were used for the analysis (Fig. 1 and Table 1). The nematodes were maintained on last instar larvae of the greater wax moth Galleria mellonella. The infective third stage juveniles emerging from host insect cadavers were washed off and stored in distilled water at 15°C until use.

Nematode DNA was extracted from a first generation adult. The nematode was crushed in 50 µl worm lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.2, 2.5 mM MgCl₂, 0.45% Tween 20, 0.05% gelatin, and 60 µg/ml proteinase K) in a sterilized 0.5 ml microcentrifuge tube. The tube was incubated at 65°C for 1 h, and then heated at 95°C for 15 min. After centrifugation at 4,000 g for 2 min, the supernatant containing nematode DNA was collected and stored at −30°C until use.

Fig. 1. The isolation areas of Japanese steinernematid isolates used in the study. The numbers correspond to the locations mentioned in Table 1.
Direct sequencing of the ITS region and the partial COI gene:

The ITS and COI regions were amplified by PCR in a 50 µl reaction mixture using Takara Ex Taq® (TaKaRa Co., Ltd.) containing 5 µl of 10⁻² PCR buffer, 4 µl of dNTP mixture, 1.5 unit of Ex Taq, 1 µM of each primer, and 2 µl of template DNA solution. To amplify the DNA fragment of the ITS region, we followed the PCR method described in Nguyen et al. (2001). To amplify the DNA fragment of the partial COI region, we used novel primers SCF (5’-TTY TWT CWA AYT CTA GWY TAG ATA TTA T-3’) and SCR (5’-TCA GAY TGR TAA CTR TGA CCA AAW ACR TA-3’) designed from the conserved regions of the COI gene of Caenorhabditis elegans (X54252), A. cariis suum (X54252), and S. carpocapsae (AY591323) (Fig. 2). The PCR condition to amplify the COI gene was as follows; 94°C for 5 min for one initial denaturation, followed by 35 cycles of 94°C for 0.5 min, 48°C for 0.5 min and 72°C for 1 min with a final extension at 72°C for 8 min.

PCR products were separated by elec-
trophoresis on a 1.2% agarose gel. The DNA band was excised, and DNA was extracted from the gel by using the Gel-M Gel Extraction System (VIOGENE). Sequencing reactions were performed by using an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with one of the primers used for PCR (18S and 26S; SCF and SCR) and internal primers, KN58 and KNRV described in Nguyen et al. (2001), under the conditions recommended by the manufacture. Sequencing was performed using an ABI PRISM® 310 Genetic Analyzer.

Phylogenetic analysis:

Multiple-sequence alignments were created with 15.0 of gap opening penalties and 6.66 of gap extension penalties using CLUSTAL X version 1.81 (Thompson et al., 1997). The score of DNA weight matrix were assigned by IUB ambiguity codes. The transition weight was set to 0.5. Published sequence data of Pratylenchus coffeae (AY 561436) and Mansonella ozzardi (AF 228562), A. suum (X54253) and Cooperia oncophora (AY 265417) were used as the outgroup species for the analysis of the ITS and partial CO1 regions, respectively. In order to infer phylogenetic relationships based on the sequences of the COI region, nucleotide sites on third codon positions were excluded to avoid potential biases due to saturation.

The aligned sequence data were analyzed by the neighbor-joining (NJ) and maximum likelihood (ML) method. A software package PHYLIP version 3.573c (Saitou and Nei, 1987) was used to obtain the NJ trees with Kimura 2-parameter model (Kimura, 1980). The resulting NJ trees were evaluated by the bootstrap test with 1,000 replications (Felsenstein, 1985). Software package TREE-PUZZLE 5.2 (Schmidt et al., 2002) was used for the ML analysis with the HKY evolutionary model (Hasegawa et al., 1985). The resulting ML trees were evaluated by 1,000 replications of puzzling step. Trees were represented graphically with the software TreeView version 1. 6. 6 (Roderic D. M. Page, Division of Environmental and Evolutionary Biology, University of Glasgow, Glasgow, UK).

**RESULTS**

Intraspecific variations of the ITS region of the rRNA gene:

The lengths of DNA fragments of the ITS region, excluding primers, ranged from 914 bp to 1,067 bp and were considerably different between species (Table 1). The DNA fragment lengths of Steinernema spp. MY 6 and MY 8 showed slight intraspecies difference because several gaps between the sequences were present and few intraspecific variations were detected (up to 0.3% and 0.7% for Steinernema sp. MY 6 and Steinernema sp. MY 8, respectively). In the case of S. litorale, the length of the amplified DNA fragments for the six isolates was identical (961 bp) and there were only 0–2 bp (0%–0.2%) substitutions. No intraspecific difference was
Table 2. Sequence variation and nucleotide differences of Japanese steinernematid isolates based on the partial COI gene sequences.

| Nematode isolates | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|-------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| S. kushidai       | -  | 11.1| 13.1| 14.4| 10.3| 10.8| 13.4| 14.1| 14.1| 15.6| 15.4| 16.4| 16.1| 16.1| 16.4| 16.6| 16.6| 15.4| 14.1|
| Steinernema sp. MY8 | 44 | -  | 7.8 | 9.1 | 10.1| 12.1| 11.8| 15.4| 15.4| 15.1| 15.6| 15.4| 15.4| 15.6| 16.6| 14.4| 15.6| 14.1|
| 3. Nij           | 52 | 31 | -  | 10.6| 9.8 | 10.3| 10.1| 11.3| 14.4| 14.9| 14.6| 14.4| 14.1| 14.6| 14.9| 16.6| 15.6| 15.6| 15.4|
| Steinernema sp. MY7 | 4. NnOm36 | 57 | 45 | 42 | -  | 10.3| 10.8| 10.6| 10.8| 14.9| 13.6| 13.4| 13.9| 13.6| 13.6| 13.9| 14.9| 15.1| 16.6| 15.4|
| S. monticolum     | 5. SFM | 41 | 36 | 39 | 41 | -  | 1.0 | 7.6 | 7.3 | 11.1| 13.6| 13.4| 14.4| 14.1| 14.1| 14.4| 14.9| 13.6| 14.4| 13.6|
| 6. YnEn94        | 43 | 40 | 41 | 43 | 4  | -  | 8.1 | 7.8 | 11.6| 14.1| 13.9| 14.9| 14.6| 14.6| 14.6| 15.4| 14.1| 14.6| 14.1|
| Steinernema sp. MY5 | 7. YnEn68 | 53 | 48 | 40 | 42 | 30 | 32 | -  | 1.5 | 12.1| 13.4| 13.1| 13.6| 13.4| 13.6| 13.9| 14.1| 13.1| 15.1| 14.6|
| 8. HkBt1392      | 56 | 47 | 46 | 43 | 29 | 31 | 6  | -  | 13.4| 13.9| 13.6| 14.1| 13.9| 14.6| 14.9| 15.4| 13.1| 15.4| 14.4|
| S. litorale      | 10. AiAt199 | 62 | 61 | 59 | 54 | 54 | 54 | 56 | 53 | 55 | 57 | -  | 0.3 | 2.0 | 2.3 | 2.0 | 2.3 | 13.4| 11.8| 16.4| 15.1|
| 11. NFL          | 61 | 60 | 58 | 53 | 53 | 55 | 52 | 54 | 56 | 1  | -  | 2.3 | 2.5 | 2.3 | 2.5 | 13.1| 11.6| 16.1| 14.9|
| 12. CbSr95       | 65 | 62 | 57 | 55 | 57 | 59 | 54 | 56 | 55 | 8  | 9  | -  | 0.3 | 0.5 | 0.8 | 14.4| 12.1| 15.6| 14.9|
| 13. CbWk140      | 64 | 61 | 56 | 54 | 56 | 58 | 53 | 55 | 54 | 9  | 10 | 1  | -  | 0.8 | 1.0 | 14.6| 12.1| 15.6| 14.9|
| 14. HkSF16       | 64 | 61 | 58 | 54 | 56 | 58 | 54 | 58 | 56 | 8  | 9  | 2  | 3  | -  | 0.3 | 14.6| 12.6| 15.9| 15.1|
| 15. IbDg157      | 65 | 62 | 59 | 55 | 57 | 58 | 55 | 59 | 57 | 9  | 10 | 3  | 4  | 1  | -  | 14.9| 12.9| 15.6| 15.4|
| S. krausei       | 16. HkHn22 | 58 | 66 | 66 | 59 | 59 | 61 | 56 | 61 | 52 | 53 | 52 | 57 | 58 | 58 | 59 | -  | 11.3| 13.9| 13.1|
| Steinernema sp. MY3 | 17. Marniya | 66 | 57 | 62 | 60 | 54 | 56 | 52 | 52 | 56 | 47 | 46 | 48 | 48 | 50 | 51 | 45 | -  | 12.3| 11.8|
| Steinernema sp. MY6 | 18. NnSk50-1 | 61 | 62 | 62 | 66 | 57 | 58 | 60 | 61 | 59 | 65 | 64 | 62 | 62 | 63 | 62 | 55 | 47 | -  | 50 |
| 19. NnMt2s       | 56 | 56 | 61 | 61 | 54 | 56 | 58 | 57 | 59 | 60 | 59 | 59 | 59 | 60 | 61 | 54 | 49 | 20 | -  |
detected between the isolates of *S. monticolum* and *Steinernema* sp. MY5.

**Intraspecific variation of the partial COI gene:**

The length of the DNA fragment of the partial COI gene excluding primers was 397 bp for all isolates, but intraspecific and interspecific genetic variations were detected within and among the species (Table 2). There were remarkable intraspecific variations within the species of *Steinernema* sp. MY 6 and *Steinernema* sp. MY 8. Five% (20 bases) and 7.8% (31 bases) intraspecific differences were detected in the sequences of *Steinernema* sp. MY 6 and *Steinernema* sp. MY 8, respectively. The intraspecific nucleotide differences of *S. litorale* were 0.3% - 2.5% (1-14 bases). The intraspecific differences of *S. monticolum* and *Steinernema* sp. MY 5 were 1.0% (4 bases) and 1.5% (6 bases), respectively.

**Phylogenetic analysis of the ITS region of the rRNA gene:**

The NJ and ML phylogenetic tree diagrams showed considerable similarity to each other (Figs. 3 and 4). The monophyly of each species and the relationship between the species were strongly supported by high values in both the trees. Out of the 10 Japanese *Steinernema* species, *Steinernema* spp. MY 7 and MY 8 were basal to other species. The remaining was divided into four subgroups: *S. monticolum* and *Steinernema* sp. MY 5; *S. kushidai*; *S. kraussei*, *Steinernema* sp. MY 3, and *Steinernema* sp. MY 6; and *S. feltiae* and *S. litorale*.

**Phylogenetic analysis of the partial COI gene:**

The NJ and ML analysis revealed that reliable values for clusters were not always high. Even within the same species, these values were slightly different depending on the analytical methods used (Figs. 5 and 6). Although the values were not very high, a cluster of two species of *S. monticolum* and *Steinernema* sp. MY 5 was detected.

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**Fig. 3.** Phylogenetic relationship among Japanese steinernematid species based on the sequences of the ITS region by the neighbor-joining method. Numbers on branches more than 50% indicate the percentage of 1,000 bootstrap replicates. Sequences of *Pratylenchus coffeae* (AY561436) and *Mansonella ozzardi* (AF228562) were used as outgroups (not shown). Bar represents 0.1 substitutions per nucleotide position.
Fig. 4. Phylogenetic relationship among Japanese steinernematid species based on the sequences of the ITS region by the maximum likelihood method. Numbers more than 50% on branches indicate the percentage of 1,000 replications of puzzling step. Sequences of Pratylenchus coffeae (AY 561436) and Mansonella ozzardi (AF 228562) were used as outgroups (not shown). Bar represents 0.1 substitutions per nucleotide position.

Fig. 5. Phylogenetic relationship among Japanese steinernematid species based on the partial COI gene sequences by the neighbor-joining method. Numbers more than 50% on branches indicate the percentage of 1,000 bootstrap replicates. Sequences of Ascaris suum (X54253) and Cooperia oncophora (AY 265417) were used as outgroups (not shown). Bar represents 0.1 substitutions per nucleotide position.
moderately supported in both the trees. Five species of *S. feltiae*, *S. kraussei*, *S. litorale*, and *Steinernema* spp. MY3 and MY6 also consistently formed a cluster in both the analyses. A cluster of six isolates of *S. litorale* were divided into two subgroups in both analyses.

**DISCUSSION**

In this study, we compare the intraspecific variations of steinernematid species isolated from Japan based on the DNA sequences of their ITS and COI regions. Two researches have been conducted to detect intraspecific variation in the steinernematid species using DNA sequences. Szalanski et al. (2000) detected no variation among the three *S. feltiae* strains in case of both rDNA ITS1 and mtDNA 16S-COIII sequences. Spiridonov et al. (2004) detected the intraspecific variability of the *S. feltiae* ITS sequences among the 11 isolates in the range of 0% - 2.4%. They also detected the sequence differences between *S. kraussei* isolates up to 2.8% among the 13 isolates. In the present study, the intraspecific differences of the two isolates of *Steinernema* spp. MY6 and MY8 in the ITS region were 0.3% and 0.7%, respectively. On the other hand, the differences in the COI region of *Steinernema* spp. MY6 and MY8 were 5% and 7.8%, respectively. Very little intraspecific variation (up to 0.2%) was detected in the ITS region of six *S. litorale* isolates, but 0.3% - 2.5% differences were detected in the COI region. No intraspecific difference was detected in the ITS region of *S. monticolum* and *Steinernema* sp. MY5, but 1.0% and 1.5% differences were detected in the COI region, respectively. These results indicate that the intraspecific divergence of the ITS region is less variable than the COI region that was used in this study of steinernematids.

In the phylogenetic analysis that was based
on the sequences of the ITS region, clusters of species and the relationships between the species were supported by high values, and the values were consistent in both the NJ and ML trees. Steinernema litorale, a newly described species that shows morphological similarity to S. feltiae (Yoshida, 2004), appears to be a species closely related to S. feltiae. Steinernema spp. MY3 and MY6 appear to be sister species of S. kraussei. These five species (S. feltiae, S. kraussei, S. litorale, and Steinernema spp. MY3 and MY6) formed a clade in both the trees. The clade “feltiae-kraussei-litorale-MY3-MY6” was also moderately supported in the phylogenetic analyses based on the COI region. Spiridonov et al. (2004) indicated the monophyly of the clade “faltiae-kraussei- oregonense” based on the analysis of the ITS region. Our study based on the COI and ITS region sequences also supported the monophyly and S. litorale, Steinernema spp. MY3 and MY6 as well as S. feltiae and S. kraussei also appear to have diverged from the same ancestral species.

The clade “monticolum-MY5” was strongly supported in the trees based on the analysis of the ITS region and moderately supported by the analysis on the COI region. These results indicate that Steinernema sp. MY5 and S. monticolum are closely related species.

Although the ITS region has proven to be useful to discriminate species and infer phylogenetic relationships between the closely related species, phylogenetic relationships between the clades are not clearly understood when using ITS sequences (Nguyen et al., 2001; Spiridonov et al., 2004). In the present study, both the NJ and ML trees based on the analysis of the ITS region were supported by high values, but another molecular information is necessary to infer the phylogenetic relationships of the clades.

Steinernema litorale is a widely distributed species in Japan (Fig. 1). In the analysis of the COI region, six S. litorale isolates obtained from different places were divided into two subclades. The COI region appears to be useful to detect intraspecific variation. However, these subclades did not show any relationship with regard to their geographical relationship. There is no information on the movement and distribution of these nematodes. In addition, haplotype variation could be occurred within the population. It is difficult to evaluate whether the relationship between the isolates have been accurately inferred or not. Further researches and more information based on population genetics are required to infer the intraspecies relationship of steinernematids.

In this study we demonstrated the availability of the COI region sequences to detect intraspecific variation among steinernematid species. However, the two phylogenetic trees based on partial COI gene sequences by using NJ and ML methods were not similar to each other. This result indicates that the COI region was less useful to infer interspecific phylogenetic relationships than the ITS region for the steinernematid species. Apart from an intensive nematode survey, accumulation of these molecular data will help us understand the evolution and speciation of steinernematids.

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LITERATURE CITED


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