Phylogenetic analyses of Japanese steinernematid nematodes and their associating Xenorhabdus bacteria

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The phylogenetic relationships of ten Japanese steinernematid species and their symbiotic Xenorhabdus bacteria were analyzed. Based on the sequences of the ITS region of the rRNA gene, Japanese steinernematids were categorized into three clades: ‘affine-intermedium’ including Steinernema sp. MY7; "karii-glaseri" including Steinernema sp. MY8; and "monticolum-kushidaifeltiae-kraussei" including Steinernema sp. MY5, S. monticolum, S. kushidai, S. litorale, S. feltiae, Steinernema sp. MY3, Steinernema sp. MY 6, and S. kraussei. In the phylogenetic analysis of the 16S rRNA gene of their symbiotic Xenorhabdus bacteria, six out of ten Xenorhabdus bacteria isolated from Japanese steinernematids formed a cluster with X. bovienii, but the other four bacteria isolated from Steinernema sp. MY8, S. kushidai, S. monticolum and Steinernema sp. MY5 did not. The phylogenies of the nematodes and their associating bacteria were partially related to each other, with a few exceptions. Jpn. J. Nematol. 36 (2), 75-85 (2006).

Key words: ITS, 16S, phylogeny, Steinernema, Xenorhabdus.

INTRODUCTION

Entomopathogenic nematodes (EPNs) in the genera Steinernema and Heterorhabditis have great potential as biological control agents against insect pests (Ehlers, 2005). In an effort to seek out more efficient agents, nematode surveys have been conducted around the world, resulting in the discovery of more than 40 steinernematid and 10 heterorhabditid species (Adams et al., 2006). Because a ubiquitous finding throughout the world has been that steinernematids are recovered more frequently than heterorhabditids, it appears that steinernematids are distributed more widely in the soil than heterorhabditids (Hominick, 2002).

Description of nematodes is basically founded on morphological characters, which are not readily applicable to nematode identification primarily because of overlapping morphometrics and similar morphology (Poinar, 1990; Hominick, 2002). In order to overcome the difficulty of morphology-based identifications, DNA sequences of ribosomal RNA (rRNA) genes have been used to identify steinernematids (Szalanski et al., 2000; Nguyen et al., 2001; Stock et al., 2001; Spiridonov et al., 2004).

Steinernema species have symbiotic association with bacteria in the genus Xenorhabdus (Enterobacteriaceae). Ten species have been described in the genus Xenorhabdus thus far (Somvanshi et al., 2006). Taxonomic studies have been conducted on these bacteria to clarify phenotypic and/or genotypic traits (Boemare, 2002; Lengyel et al., 2005; Somvanshi et al., 2006). Although the bacteria clearly differ from each other in their phenotypes and genotypes,
the information on the phylogenetic relationship among them have been limited (Rainey et al., 1995; Liu et al., 1997, 2001; Lengyel et al., 2005; Somvanshi et al., 2006). In addition, phenotypic and genotypic characters of Xenorhabdus bacteria isolated from newly described steinernematids have not been reported.

Surveys of EPNs have been conducted in Japan since the late 1980's. Six described species (S. carpocapsae, S. feltiae, S. kraussei, S. kushidai, S. litorale and S. monticolum) and six undescribed putative species (Steinernema spp. MY3, MY4, MY5, MY6, MY7 and MY8) have been isolated thus far (Mamiya and Ogura, 1990; Yoshida et al., 1998; Mamiya et al., 2001; Yoshida, 2003a; Yoshida, 2004). Isolation of S. carpocapsae in Japan has been reported by Mamiya and Ogura (1990), but the species has never been reisolated. The putative species were categorized by their morphological differences and PCR-RFLP patterns (Yoshida et al., 1998; Yoshida 2003b), but the phylogenetic relationships among Japanese steinernematids and within the genus Steinernema have not yet been analyzed. In addition, there is no information on the symbiotic bacteria associated with the Japanese steinernematids except for Xenorhabdus japonica isolated from S. kushidai (Nishimura et al., 1994; Suzuki et al., 1996).

In this study we sequenced ITS region of the rRNA gene of Japanese steinernematids, and analyzed the phylogenetic relationships of them within the genus Steinernema. The 16S rRNA gene sequences of symbiotic bacteria isolated from Japanese steinernematids were also analyzed to elucidate the phylogenetic relationship between the Steinernema species and their symbiotic bacteria.

**MATERIALS AND METHODS**

Nematode isolates:

Five described Japanese steinernematid species and five putative species were used for phylogenetic analyses (Fig. 1 and Table 1). The nematodes were maintained on last instar larvae of the greater wax moth, Galleria mellonella (Lepidoptera: Pyralidae). Infective juveniles emerging from host insect cadavers were washed off and stored in distilled water at 15 °C until use.

Nematode DNA extraction, PCR and direct sequencing:

A single adult female 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) modified from Joyce et al. (1994) in a sterilized 0.5 ml microcentrifuge tube. The tube was incubated at 65 °C for 1 h and then at 95 °C incubation 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.

The ITS region of the rRNA gene corresponding to the nucleotide positions 2,524 - 3,773 of the sequence of the rDNA tandem unit from...
Caenorhabditis elegans (GenBank accession number X03680) was amplified by PCR. The total volume of the PCR mixture was 50 µl using Takara ExTaq® (Takara Co., Ltd.), containing 5 µl of 10⁻⁶ 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 gene, we used the primer set of 18S (5'-TTG ATT ACG TCC CTG CCC TTT-3') and 26S (5'-TTT CAC TCG CCG TTA CTA AGG-3') described in Vrain et al. (1992). The reaction mixture was heated to 94 °C for 5 min for one initial denaturation, followed by 35 cycles of 94 °C for 0.5 min, 50 °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 electrophoresis on a 1.2% agarose gel. The DNA band was excised and 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) and internal primers, KN58 (5'-GTA TGT TTT GGT GAA GGT C-3') and KNRV (5'-CAC GCT CAT ACA ACT GCT C-3'), described in Nguyen et al. (2001), under the conditions recommended by the manufacture. Double-stranded sequencing of PCR products was carried out by ABI Prism® 310 Genetic Analyzer. The DNA sequences were deposited in the DNA Data Bank of Japan (DDBJ), accession number: AB243435-AB243444.

Isolation of symbiotic bacteria:

Symbiotic bacteria were isolated from surface-sterilized infective juveniles. Infective juveniles were immersed in 0.1% merthiolate solution for 2 h, washed three times in sterile saline and crushed with the end of a Pipetman (Gilson) tip in a small amount of sterile saline to release the bacteria from the nematode intestine. About 0.5 ml of LB broth was added to the suspension and the suspension was spread on an NBTA plate (Akhurst, 1980). Single colonies were successively extracted and streaked on a new NBTA plate until no contamination was identified. Bacterial colonies maintained on the NBTA plates were used for further study.

Bacterial DNA extraction, PCR and direct sequencing of 16S rRNA gene:

Bacterial DNA was prepared as follows. Cells obtained from 5ml of a 24 h bacterial culture were lysed in TKE buffer (0.1 M Tris-HCl, 0.1 M KCl and 20 mM EDTA-Na, pH 8.0) con-

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Table 1. List of Japanese steinernematid nematodes examined and fragment sizes of ITS region on rRNA gene.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Location</th>
<th>Site No.</th>
<th>Reference</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steinernema sp. MY7</td>
<td>NnOm36</td>
<td>Omachi, Nagano</td>
<td>□</td>
<td>Yoshida et al., 1998</td>
<td>1,067</td>
</tr>
<tr>
<td>Steinernema sp. MY8</td>
<td>Ikwj136</td>
<td>Wajima, Ishikawa</td>
<td>□</td>
<td>Yoshida 𝑐</td>
<td>997</td>
</tr>
<tr>
<td>Steinernema sp. MY5</td>
<td>YnEn68</td>
<td>Enzan, Yamanashi</td>
<td>□</td>
<td>Yoshida et al., 1998</td>
<td>914</td>
</tr>
<tr>
<td>S. monticolum</td>
<td>YnEn94</td>
<td>Enzan, Yamanashi</td>
<td>□</td>
<td>Yoshida et al., 1998</td>
<td>916</td>
</tr>
<tr>
<td>S. kushidai</td>
<td>Hamakita</td>
<td>Hamakita, Shizuoka</td>
<td>□</td>
<td>Ogura ｃ</td>
<td>990</td>
</tr>
<tr>
<td>S. litorale</td>
<td>AiA199</td>
<td>Atsumi, Aichi</td>
<td>□</td>
<td>Yoshida, 2004</td>
<td>961</td>
</tr>
<tr>
<td>S. feltiae</td>
<td>HkEr36</td>
<td>Erino, Hokkaido</td>
<td>□</td>
<td>Yoshida, 2003a</td>
<td>970</td>
</tr>
<tr>
<td>Steinernema sp. MY3</td>
<td>Mamiya</td>
<td>Hachioji, Tokyo</td>
<td>□</td>
<td>Mamiya ｃ</td>
<td>980</td>
</tr>
<tr>
<td>Steinernema sp. MY6</td>
<td>NnM12s</td>
<td>Matsumoto, Nagano</td>
<td>□</td>
<td>Yoshida et al., 1998</td>
<td>980</td>
</tr>
<tr>
<td>S. kraussei</td>
<td>HkHm22</td>
<td>Hamatonbetsu, Hokkaido</td>
<td>□</td>
<td>Yoshida, 2003a</td>
<td>977</td>
</tr>
</tbody>
</table>

𝑎 Numbers are corresponding to location in figure 1.  
𝑏 Sizes of the DNA fragment of the ITS region amplified by PCR.  
𝑐 These isolates are identified following the methods described in Yoshida (2003b).
taining 0.1 mg lysozyme and 10 µg RNase A at 37 °C for 20 min. After addition of 0.25 ml of 10% sarkosyl to the bacterial lysate, DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA was dissolved in 500 µl TE buffer.

PCR was performed to amplify bacterial 16S rDNA corresponding to the bases 5,016,978 - 5,018,473 of Escherichia coli O157 genome (GenBank accession number BA000007) fragment for sequencing. The total volume of the PCR mixture was 100 µl using Takara ExTa®q® (Takara Co., Ltd.), containing 10 µl of 10x PCR buffer, 8 µl of dNTP mixture, 2.5 unit µl of Ex Taq, 1 µM of each primer, and 2 µl of template DNA solution. To amplify the gene, we used the primer set of 16S-F (5’-GAA GAG TTT GAT CAT GGC TC-3’) and 16S-R (5’-AAG GAG GTG ATC CAG CCG CA-3’) (Fischer-Le Saux et al., 1999). The reaction mixtures were heated to 95 °C for 10 min for one initial denaturation, followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 8 min. PCR products were purified and used for direct sequencing following the aforementioned methods. In addition to 16S-F and 16S-R primers, six internal primers described in Fischer-Le Saux et al. (1999) were used to sequence both strands of the DNA fragment. The DNA sequences were deposited in the DDBJ, accession number: AB243425-AB243434.

RESULTS

Nematode phylogeny:

The fragment sizes of the ITS region of the rRNA gene for the steinernematids examined varied in the range from 914 bp to 1,067 bp (Table 1).

Based on the phylogenetic analyses of the ITS region, ten Japanese steinernematid species were categorized into three clades: "affine-intermedium"; "karii-glaseri"; and "monticolum-kushidai-feltiae-kraussei" (Fig. 2). Two putative species, Steinernema spp. MY7 and MY8 were categorized into the "affine-intermedium" and "karii-glaseri" respectively. These clusters were supported by high bootstrap numbers. Eight out of ten species, Steinernema sp. MY5, S. monticolum, S. kushidai, S. litorale, S. feltiae, Steinernema sp. MY3, Steinernema sp. MY6 and S. kraussei, were included in the third clade. The species in this clade were further divided into at least three subclades; "MY5-monticolum", "litorale-feltiae" and "MY3-MY6-kraussei".

Bacterial phylogeny:

The length of the 16S rRNA gene fragments amplified from Xenorhabdus bacteria was 1,497 bp. These sequences showed less than 3.7% dif-
ferences among the isolates (Table 2). The sequences of bacteria isolated from *S. monticolum* and *Steinernema* sp. MY5 were identical, and those from *S. feltiae*, *S. kraussei*, *S. litorale*, *Steinernema* spp. MY3, MY6 and MY7 showed less than 0.5% differences to each other.

In the phylogenetic analysis of the 16S rDNA sequences, ten bacterial isolates associating with Japanese steinernematids were categorized tentatively into four different clades (Fig. 3). Bacterial isolates from *S. feltiae*, *S. kraussei*, *S. litorale*, *Steinernema* spp. MY3, MY6 and MY7 clustered together with *X. bovienii*, and were categorized into "bovienii" group. On the other hand, the bacteria isolated from *Steinernema* sp. MY8, *S. kushidai*, *S. monticolum*

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Fig. 2. Phylogenetic relationship of Japanese steinernematids based on the sequences of the ITS region. Tree was reconstructed by the maximum-likelihood method. Japanese isolates are shown as bold-faced and underlined. Sequences of Pratylenchus coffeae (AY561436) and *Mansonella ozzardi* (AF228562) were used as outgroup species (not shown). Numbers in parentheses indicate the GenBank accession number. Numbers more than 50% at branch-point indicate in percent how often the corresponding cluster was found among the 1,000 intermediate trees. Bar represents 0.1 nucleotide substitutions per site.
Table 2. Sequence divergence of Japanese Xenorhabdus isolates for 1,497 bp of 16S rRNA gene sequences.

<table>
<thead>
<tr>
<th>Xenorhabdus species and isolates</th>
<th>Site No.</th>
<th>Host nematode species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Xenorhabdus sp. IKWJ136</td>
<td>56</td>
<td>Steinernema sp. MY8</td>
<td>3.7</td>
<td>3.1</td>
<td>3.1</td>
<td>3.2</td>
<td>3.2</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>2. X. japonica Hamakita</td>
<td>56</td>
<td>S. kushidai</td>
<td>2.7</td>
<td>2.7</td>
<td>3.1</td>
<td>3.1</td>
<td>3.0</td>
<td>2.7</td>
<td>3.1</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Xenorhabdus sp. YnEn94</td>
<td>46</td>
<td>S. monticolum</td>
<td>0</td>
<td>2.8</td>
<td>2.8</td>
<td>2.7</td>
<td>2.7</td>
<td>2.9</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Xenorhabdus sp. YnEn68</td>
<td>46</td>
<td>Steinernema sp. MY5</td>
<td>0</td>
<td>2.8</td>
<td>2.8</td>
<td>2.7</td>
<td>2.7</td>
<td>2.9</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. X. bovienii HkEr36</td>
<td>48</td>
<td>S. feltiae</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. X. bovienii HkHm22</td>
<td>48</td>
<td>S. kraussei</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Xenorhabdus sp. AiAt199</td>
<td>47</td>
<td>S. litorale</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<td>3</td>
<td></td>
</tr>
<tr>
<td>8. Xenorhabdus sp. Mamiya</td>
<td>46</td>
<td>Steinernema sp. MY3</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>9. Xenorhabdus sp. NnMt2s</td>
<td>47</td>
<td>Steinernema sp. MY6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td></td>
</tr>
<tr>
<td>10. Xenorhabdus sp. NnOm36</td>
<td>46</td>
<td>Steinernema sp. MY7</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td></td>
</tr>
</tbody>
</table>

Above diagonal indicates the percentage of nucleotide variation and below diagonal indicates the number of nucleotide differences. * Numbers are corresponding to location in figure 1.

Fig. 3. Phylogenetic relationship of Xenorhabdus bacteria associated with Japanese steinernematids based on the 16S rRNA gene sequences. The tree was reconstructed by maximum-likelihood method. Bacteria isolated from Japanese steinernematids are shown as bold-faced and underlined. The 16S rRNA gene sequences of Photorhabdus temperata Meg1 (Z76750) and Photorhabdus luminescens subsp. akhurstii W14 (A Y 278642) were used as outgroup species (not shown). Host nematode and the GenBank accession numbers are indicated in parentheses. Numbers more than 50% at branch-point indicate in percent how often the corresponding cluster was found among the 1,000 intermediate trees. Bar represents 0.01 substitutions per site.
and Steinernema sp. MY5 showed 2.7-3.2% differences (40-48 nucleotide differences) to the bacteria in the "bovienii" group, and did not form a cluster with them (Fig. 3 and Table 2).

The bacterium isolated from Steinernema sp. MY8 clustered with X. poinarii and X. indica, and this cluster was moderately supported. Xenorhabdus japonica which is known to be a symbiont of S. kushidai, and the bacterial isolates from both S. monticolum and Steinernema sp. MY5 did not form a cluster with another known Xenorhabdus species.

**DISCUSSION**

In this study we sequenced the ITS region of the rRNA gene and analyzed the phylogenetic relationships of ten Steinernema species isolated from Japan. We also sequenced and analyzed the 16S rRNA gene of Xenorhabdus bacteria isolated from the ten Japanese steinernematids to infer the phylogenetic relationships among known Xenorhabdus species and co-speciation of nematode-bacterium complexes.

Steinernema sp. MY7 was categorized into the "affine-intermedium" clade. This categorization was also supported morphologically (Yoshida et al., 1998). The symbiotic bacteria from S. affine and S. intermedium are known to be X. bovienii (Boemare, 2002), and the bacterium isolated from Steinernema sp. MY7 appears to be X. bovienii because of the high similarity of the 16S rRNA gene sequences. These findings indicate that the members of the "affine-intermedium" clade associate with X. bovienii.

Steinernema sp. MY8 falls into the "karii-glaseri" clade. The infective juvenile of this nematode species is relatively large and morphologically similar to that of S. glaseri (Yoshida, 2003b). In the 16S rDNA analysis, the symbiotic bacteria of Steinernema sp. MY8 form a cluster with X. poinarii, which associates with S. glaseri. In addition, the symbiont of Steinernema sp. MY8 did not absorb bromothymole blue on a NBTA plate (data not shown), which is a typical character for X. poinarii. These results suggest a close relationship between the nematodes and the bacteria in these groups and a co-speciation of the nematode-bacterium complex.

Out of the ten Japanese Steinernema species, eight were categorized in the "monticolum-kushidai-feltiae-kraussei" clade. Among the species in this group, nematodes in the sub-clades of "litorale-feltiae" and "MY3-MY6-kraussei" appear to be associated with X. bovienii because the symbiotic bacteria of the two sub-clades show high similarities to X. bovienii in their 16S rDNA sequences. This close phylogenetic relationship and the fact that these nematodes associate with X. bovienii indicate that the nematodes in these sub-clades originated from a common ancestral species that associates with X. bovienii.

Steinernema kushidai was a sister taxon to the "litorale-feltiae" and "MY3-MY6-kraussei" sub-clades but was deeply branched. Based on the 28S rDNA sequences, Stock et al. (2001) have also indicated S. kushidai as a sister taxon to the "feltiae-kraussei" group. The nematodes Steinernema sp. MY5 and S. monticolum formed a cluster together and were recognized as sister taxa to the "kushidai", "litorale-feltiae", and "MY3-MY6-kraussei" by the strong value supports (Fig. 2). Although S. monticolum is categorized into the group S. carpocapsae + S. scepterisci + S. siamkayai in the 28S rDNA analysis (Stock et al. 2001), phylograms of Spiridonov et al. (2004) indicate S. monticolum as a sister taxon of the "feltiae-kraussei-oregonense" group based on the ITS1-5.8S-ITS2 region of rDNA and morphometric features. Although many of the nematode species in this clade appeared to associate with X. bovienii, the three species, S. kushidai, S. monticolum, and Steinernema sp. MY5 did not. Further study on the nematodes in
Fig. 4. Tanglegram comparison of the phylogenetic trees of steinernematid species and their associating Xenorhabdus bacteria. Both nematode and bacterial trees were modified from the phylograms described in the figures 2 and 3, respectively. Japanese isolates are shown as bold-faced and underlined. The dashed lines indicate the nematode-bacteria associations, and host nematode species are indicated in parentheses in the bacterial tree.

this clade and their symbiotic bacteria is necessary to elucidate the Steinernema - Xenorhabdus associations.

The results of the present study indicate that the phylogenies of the steinernematid nematodes and their symbiotic bacteria are related to each other, but there are a few exceptions: the phylogenetic relationship between Steinernema sp. MY7 and their symbiotic bacteria (Fig. 4). These discrepancies could be due to non-appropriate molecular markers used in the present study for analyses and using novel markers may solve this problem. However, there remains the possibility that a heterogeneous infection has induced a change in the nematode/bacterium combination over the course of evolution. In order to demonstrate a convergent evolution of Steinernema - Xenorhabdus symbiosis, further comparative studies are required on the systematics, taxonomy, and phylogeny of described and non-described steinernematid nematodes and symbiotic bacteria around the world.

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