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Author(s)
Yamada, Seitaro; Yoshida, Atushi; Yoshida, Keiko; Kuraishi, Takeshi; Hattori, Shosaku; Kai, Chieko; Nagai, Yumiko; Sakoda, Takeshi; Tatara, Masaya; Abe, Shintaro; Fukumoto, Shin-ichiro

Issue Date
2012-02

DOI
10.14943/jjvr.60.1.15

Doc URL
http://hdl.handle.net/2115/48537

Type
bulletin (article)

File Information
JJVR60-1_3.pdf
Phylogenetic relationships of three species within the family Heligmonellidae (Nematoda; Heligmosomoidea) from Japanese rodents and a lagomorph based on the sequences of ribosomal DNA internal transcribed spacers, ITS-1 and ITS-2

Seitaro Yamada¹, Atushi Yoshida¹, Keiko Yoshida¹, Takeshi Kuraishi², Shosaku Hattori², Chieko Kai², Yumiko Nagai³, Takeshi Sakoda³, Masaya Tatara³, Shintaro Abe³ and Shin-ichiro Fukumoto¹, *

¹Unit of Veterinary Parasitology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan.
²Amami Laboratory of Injurious Animals, the Institute of Medical Science, the University of Tokyo, Setouchi-cho, Ohshima-gun, Kagoshima, 894-1531, Japan
³Amami Wildlife Center, Yamato-son, Ohshima-gun, Kagoshima, 894-3104, Japan

Received for publication, November 29, 2011; accepted, January 13, 2012

Abstract
Nematodes of the family Heligmonellidae (Heligmosomoidea; Trichostrongylina) reside in the digestive tracts of rodents and lagomorphs. Although this family contains large numbers of genera and species, genetic information on the Heligmonellidae is very limited. We collected and isolated adult worms of three species in Japan that belong to the family Heligmonellidae, namely Heligmonoides speciosus (Konno, 1963) Durette-Desset, 1970 (Hs) from Apodemus argenteus, Orientostrongylus ezoensis Tada, 1975 (Oe) from Rattus norvegicus and Lagostrongylus leporis (Schulz, 1931) (Ll) from Pentalagus furnessi, and sequenced the entire internal transcribed spacer regions, ITS-1 and ITS-2 of ribosomal DNA. ITS-1 of Hs, Oe and Ll was 426, 468 and 449 bp in length, and had a G+C content of about 41, 41 and 37 %, respectively. ITS-2 of Hs, Oe and Ll was 297, 319 and 276 bp in length and had a G+C content of about 38, 40 and 28%, respectively. The data of Hs, Oe and Ll were compared with those of two other known species within the family Heligmonellidae, Calorinensis minutus (Dujardin, 1845) (Cm) and Nippostrogylus brasiliensis (Travassos, 1914) (Nb), and with those of two species of Heligmosomidae (Heligmosomoidea), Heligmosoides polygyrus bakeri and Ohbayashinema erbaeva. Phylogenetic analysis placed Hs, Oe and Ll in the same clade with Cm and Nb, forming a Heligmonellidae branch in both ITS-1 and ITS-2, separate from the Heligmosomoidea branch. These results demonstrated that the ITS-1 and ITS-2 sequences are useful for differentiating the
Heligmonellidae nematode species. This study is the first to describe the ITS-1 and ITS-2 sequences of Hs, Oe and Ll.

Key words: Heligmonellidae (Nematoda), ITS, phylogeny, ribosomal DNA.

Introduction

Family Heligmonellidae is one of the bursate parasitic nematode groups belonging to the superfamily Heligmosomoidea Skrjabin and Schikobalova, 1952 (as a tribe; as a family, Durette-Desset and Chabaud, 1977)7,9,11. This family Heligmonellidae constitutes the superfamily Heligmosomoidea with the family Heligmosomidae (originally as a subfamily, Travassos, 1914) Cram, 1927 (suborder Trichostrongyline Durette-Desset and Chabaud, 1993: order Strongylida7,9-11). These heligmosome nematodes reside in digestive tracts of a wide range of rodent and lagomorph hosts, and many new species and genera are still being reported.

Taxonomy of the Heligmonellidae is complex and difficult, not only because of the large number of species involved, but also these nematodes are quite small in size and morphologically very similar7-11. The classification of the suborder Trichostrongyline including the family Heligmonellidae is based on the morphological characteristics of adult worms, specifically, anterior structure, the arrangement of bursal rays and the shape of spicules of male. Also, in this group, the arrangement of longitudinal ridges on the body surface, present in both sexes, is important in identifying the morphological structure as the synlophe. In addition to the above morphological classification, many reports have described systematic analyses of nematode differentiation during their diversification occurring in association with host evolution, as supplementary information to the geographical distribution-based systematic classification of these nematodes and their hosts6,8,17. Furthermore, many studies have described the members of this heligmosome group as an experimental model of intestinal namatodiasis, using Nippostrongylus brasiliensis (Heligmonellidae) or the members represented by Heligmosomoides spp. (Heligmosomidae)1.

Genetic classification or phylogenic analysis using sequence comparisons has been applied to the suborder Trichostrongyline5,9. Internal transcribed spacer (ITS) regions of the ribosomal DNA are useful for elucidating the relationships among congeneric species and closely related genera in many eukaryotes as well as parasitic nematode species including Strongylida4,5,16. The ITS regions of ribosomal DNA are now one of the most widely sequenced DNA regions in parasitic nematodes including Trichostrongyline4,5. However, genetic information reported so far for Heligmonellidae is scant. The ITS sequence of Heligmonellidae nematodes has been reported only for Nippostrongylus brasiliensis (Travassos, 1914) and Carolinensis minutus (Dujardin, 1845)3.

In the present study, we sequenced and analyzed the ITS-1 and ITS-2 regions of Heligmonellidae nematodes collected from the small intestines of mammals in Japan, including Heligmonoides speciosus (Konno, 1958) [major host, Apodemus speciosus], Orientostrongylus ezoensis Tada, 1975 [major host, Rattus norvegicus] belonging to the subfamily Nippostrongylinae, and Lagoestrongylus leporis (Schulz, 1931) [major hosts, Lepus spp. and Pentalagus furnessi] of the subfamily Brevistriatinae. These nematodes and their hosts are indigenous to the Japanese Archipelago or in the far eastern areas of Asia and have species-specific host-parasite relationships. We also compared their ITS-1 and ITS-2 sequences with those previously described for two Heligmonellidae species, N. brasiliensis and C. minutus, and for two species of family Heligmosomidae, Heligmosomoides polygyrus.
bakeri and Ohbayashinema erbaevae, and constructed phylogenetic trees.

Materials and Methods

Parasites: Three species of nematodes collected from rodents and a lagomorph in Japan were examined. Heligmonoides speciosus (Konno, 1958)\textsuperscript{(21)} was isolated from the small intestine of an Apodemus argenteus captured in Ebetu, Hokkaido. Orientostrongylus ezoensis\textsuperscript{(12,13,26)} was collected from the small intestine of a Rattus norvegicus captured in Sapporo, Hokkaido. Specimens of Lagostrongylus leporis\textsuperscript{(14,15)} were isolated from the small intestine of Pentalagus furnessi captured in the Amami-Ohshima Island, Kagoshima. All nematode specimens were washed with physiological saline, then fixed and preserved in 100% ethanol and stored at \(-80^\circ\text{C}\). Information of the studied nematodes is shown in Table 1.

PCR amplification and sequencing: Genomic DNA was extracted and purified from male nematodes of the three species as described in our previous report\textsuperscript{(25)}. PCR amplification was performed in a 25 \(\mu\text{l}\) reaction mixture containing PCR buffer (TOYOBO, Osaka, Japan), 2 mM dNTPs, 1.0 U/\(\mu\text{l}\) DNA polymerase (TOYOBO, Osaka, Japan), 0.3 \(\mu\text{M}\) of each primer, and 50 ng of genomic DNA. The ITS-1 and ITS-2 of each species were amplified using the primers, 18SF3 (forward): 5’-GAGAGGACTGCAGCTGCTGTATCG-3’ and NC1R (reverse): 5’-AACAACCCTGAACCAGCAGT-3’ for ITS-1, and NC1 (forward): 5’-ACGTCTGGTACGGTTGTGTT-3’ and NC2 (reverse): 5’-TTAGTTTCTTTTCCCTCGCT-3’ for ITS-2. 18SF3 was designed from the 18S rDNA sequence of Caenorhabditis elegans (GenBank accession number X03680). NC1R, NC1 and NC2 were as described by Host \textit{et al}. (1998)\textsuperscript{(20)} and Gasser \textit{et al}. (1993)\textsuperscript{(16)}. The reaction condition was as follows: 10 minutes of initial denaturation at 95°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds, at 55°C and 30 seconds at 72°C. Final extension was carried out for 7 minutes at 72°C for both ITS-1 and ITS-2. Obtained PCR products

Table 1. Species used in the present study for comparing nematodes from the two rodents and a lagomorph hosts collected in Japan and known species in the family Heligmonellidae and Heligmosomidae.

<table>
<thead>
<tr>
<th>species</th>
<th>Accession number of ITS1</th>
<th>Accession number of ITS2</th>
<th>Host</th>
<th>Major hosts</th>
<th>Locality</th>
<th>Family</th>
<th>Subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heligmonoides speciosus\textsuperscript{(a)}</td>
<td>AB609321</td>
<td>AB609320</td>
<td>Apodemus argenteus</td>
<td>Apodemus speciosus</td>
<td>Hokkaido, Japan</td>
<td>Heligmonellidae</td>
<td>Nippostrongylinae</td>
</tr>
<tr>
<td>Orientostrongylus ezoensis\textsuperscript{(a)}</td>
<td>AB609319</td>
<td>AB609318</td>
<td>Rattus norvegicus</td>
<td>Rattus norvegicus</td>
<td>Hokkaido, Japan</td>
<td>Heligmonellidae</td>
<td>Nippostrongylinae</td>
</tr>
<tr>
<td>Lagostrongylus leporis\textsuperscript{(a)}</td>
<td>AB610547</td>
<td>AB610546</td>
<td>Pentalagus furnessi</td>
<td>Lepus spp.</td>
<td>Kagoshima, Japan</td>
<td>Heligmonellidae</td>
<td>Breviatriatina/ Heligmonellinae</td>
</tr>
<tr>
<td>Nippostrongylus brasiliensis</td>
<td>AY332646</td>
<td>AY333380</td>
<td>Rattus norvegicus</td>
<td>Rattus norvegicus</td>
<td>France</td>
<td>Heligmonellidae</td>
<td>Nippostrongylinae</td>
</tr>
<tr>
<td>Carolinensis minutus</td>
<td>AY332645</td>
<td>AY333379</td>
<td>Microtus montebeli</td>
<td>Microtus spp.</td>
<td>France</td>
<td>Heligmonellidae</td>
<td>Nippostrongylinae</td>
</tr>
<tr>
<td>Heligmosomoides polygyrus bakeri</td>
<td>AY332648</td>
<td>AY333382</td>
<td>Mus musculus</td>
<td>Apodemus spp.</td>
<td>France</td>
<td>Heligmosomidae</td>
<td></td>
</tr>
<tr>
<td>Ohbayashinema erbaevae</td>
<td>AY332647</td>
<td>AY333381</td>
<td>Ochotona daurica</td>
<td>Ochotona daurica</td>
<td>Buriatia</td>
<td>Heligmosomidae</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{(a)} Sequenced in the present study.
were detected on 2% agarose gel, and purified using Qiaquick™ spin column (Qiagen, Hilden, Germany). The nucleotide sequences were determined on an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems, Foster City, U.S.A.) using the primers 18SF3 or NC1R for ITS-1, and NC1 or NC2 for ITS-2 in separate reactions with a DYE
dynamic ET Terminator Cycle Sequencing Kit (GE Healthcare UK Limited, Little Chalfont, UK).

Sequence analysis: The sequences were aligned using the ClustalW method28. Pairwise sequence comparisons were performed for ITS-1 and ITS-2, either separately or in combination, as previously described2,3. All sequences obtained have been deposited in the GenBank (their accession numbers are in Table 1). Sequence data were aligned with the ITS-1 and ITS-2 sequences for additional four species of N. brasilensis (AY332646 and AY333380), C. minutus (AY332645 and AY333379) and H. polygyrus bakeri (AY332648 and AY333382), O. ezoensis (AY332647 and AY333381). These seven species were determined as an outer group by comparison with the rDNA sequence of Caenorhabditis elegans (X03680).

Phylogenetic trees were constructed by using the neighbor-joining methods of the program MEGA428. Bootstrap values were determined with 1,000 replicates of the data sets.

Results

The ITS-1 and ITS-2 sequences of three species varied in length from 426 to 468 bp and 276 to 320 bp. Length and G+C content of ITS-1 were 426 bp and 41% for H. speciosus, 468 bp and 41% for O. ezoensis, and 449 bp and 37% for L. leporis, respectively. Length and G+C content of ITS-2 were 297 bp and 38% for H. speciosus, 319 bp and 40% for O. ezoensis, and 276 bp and 28% for L. leporis, respectively.

Pairwise comparisons of five species of Heligmonellidae nematodes for the ITS-1 and ITS-2 sequences showed that coincidence rate ranged from 70.9 to 95.0% for ITS-1 and 71.2 to 94.4% for ITS-2. Among five species of Heligmonellidae, H. speciosus and C. minutus were genetically the closest in ITS-1 (91.6%) and ITS-2 (95.0%). N. brasilensis and L. leporis were also close in ITS-1 (84.0%) and ITS-2 (83.9%).

Among seven species of the superfamily Heligmosomoidea, O. erbaevae and H. polygyrus bakeri were genetically the closest in ITS-1 and ITS-2. O. ezoensis and H. polygyrus bakeri were genetically the most distant in the ITS-1, and O. ezoensis and O. erbaevae were genetically the most distant in the ITS-2.

Phylogenetic relationships of seven species of Heligmosomoidea nematodes in the ITS-1 and ITS-2 sequences determined with the neighbor-joining method produced similar results (Fig. 1). Five species of Heligmonellidae constituted a clade, while O. erbaevae and H. polygyrus bakeri branched off other five species of Heligmonellidae and formed a Heligmosomidae clade. Of five Heligmonellidae species, L. leporis and N. brasilensis were clustered into one clade. The other three species of the subfamily Nippostrongylinae were placed into two separate clades, with H. speciosus and C. minutus in one clade and O. ezoensis in the other clade.

Discussion

We sequenced and analyzed the ITS-1 and ITS-2 regions of rDNA of three species of the family Heligmonellidae, H. speciosus, O. ezoensis and L. leporis, and delineated the phylogenetic relationships between seven species of the superfamily Heligmosomoidea, including two additional Heligmonellidae species and two Heligmosomidae species.

The ITS-1 and ITS-2 sequences highly conserved among the Heligmonellidae species (average of five species was 78.1% in ITS-1 and 78.9% in ITS-2, respectively) or Heligmosomidae species (ITS-1: 95.1%, ITS-2: 96.5%).
Our phylogenetic analyses using the ITS-1 and ITS-2 sequences separated seven species of the superfamily Heligmosomoidea into Heligmonellidae and Heligmosomidae branches, consistent with the current family designations. Five Heligmonellidae were placed into two separate clades, one consisting of *H. speciosus* and *C. minutus* and the other containing *O. ezoensis*.

The ITS-1 and ITS-2 sequences have been used for species delineation and phylogenetic analysis of bursate nematodes. Their usefulness has been suggested for the Trichostrongyloidea and Molineoidea. While the D1-D2 domain of 28S rRNA has also been used for phylogenetic analysis of the Trichostrongyloidae, the same task has been accomplished using the ITS-1 and ITS-2 regions. This study was in agreement with the genomics classification of these reports.

However, *L. leporis* (subfamily Heligmonellinae) and *N. brasiliensis* (subfamily Nippostrongylinae) were clustered into one clade. Thus, these results differ from the current subfamily designations within the family Heligmonellidae. The ambiguity about the subfamily assignment of the genus *Lagostrongylus* can also be seen in the phylogenetic trees. The clustering of *L. leporis* and *N. brasiliensis* in one clade resulted from the presence of one branching. This branching that separated *L. leporis* and *N. brasiliensis* from *H. speciosus*, *C. minutus* and *O. ezoensis* had bootstrap values of 100% for ITS-1, whereas that for ITS-2 was a rather low value of 45%. The low bootstrap value indicates a weak support to this branching, suggesting the possibility that the subfamilies Heligmonellinae and Nippostrongylinae belong to one clade. Since the ITS sequences of...
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Acknowledgments

This study was supported in part by the Rakuno Gakuen Frontier Science Fund in 2006–2007 and the research subsidy fund from the Rakuno Gakuen Supporters’ Association in 2009–2010. Dr. M. Kamiya, Professor Emeritus of the Hokkaido University supplied nematode specimens. Dr. T. Homma of Meiji University reviewed and commented on this draft. We are grateful to the member of Unit of the Veterinary Parasitology, Rakuno Gakuen University for their field and laboratory works.

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