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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

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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) (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 Nippostrongylus brailiensis (Travassos, 1914) (Nb), and with those of two species of Heligmosomidae (Heligmosomoidea), Heligmosomoides polygyrus bakeri and Ohbayashinema erbaevae. 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

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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 Trichostrongylinae Durette-Desset and Chabaud, 1993: order Strongylida)\(^7,9-11\). These heligmosomia 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 similar\(^7-11\). The classification of the suborder Trichostrongylinae 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 hosts\(^6,8,17\). Furthermore, many studies have described the members of this heligmosome group as an experimental model of intestinal nematodiasis, using *Nippostrongylus brasiliensis* (Heligmonellidae) or the members represented by *Heligmosomoides* spp. (Heligmosomidae)\(^1\).

Genetic classification or phylogenetic analysis using sequence comparisons has been applied to the suborder Trichostrongylinae\(^5,5\). 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 Strongylida\(^4,5,16\). The ITS regions of ribosomal DNA are now one of the most widely sequenced DNA regions in parasitic nematodes including Trichostrongylinae\(^4,5\). 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*], *Orientostrogylina ezoensis* Tada, 1975 [major host, *Rattus norvegicus*] belonging to the subfamily Nippostrongylinae, and *Lagostrongylus 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)²¹ was isolated from the small intestine of an Apodemus argenteus captured in Ebetu, Hokkaido. Orientostrongylus ezoensis²²,²³,²⁶ was collected from the small intestine of a Rattus norvegicus captured in Sapporo, Hokkaido. Specimens of Lagostrongylus leporis¹⁴,¹⁵ 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°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 report²⁴. PCR amplification was performed in a 25 μl reaction mixture containing PCR buffer (TOYOBO, Osaka, Japan), 2 mM dNTPs, 1.0 U/μl DNA polymerase (TOYOBO, Osaka, Japan), 0.3 μM 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'-GAGAGGACTGCGGACTGCTGTATCG-3' and NC1R (reverse): 5'-AACAACCCTGAACCAGCGT-3' for ITS-1, and NC1 (forward): 5'-ACGTCTGGTGTTGTT-3' and NC2 (reverse): 5'-TTAGTTTCTTTTCCTCGCT-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 et al. (1998)²⁰ and Gasser et al. (1993)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. |
|---|---|---|---|---|---|---|---|
| Species | Accession number of ITS1 | Accession number of ITS2 | Host | Major hosts | Locality | Family | Subfamily |
| Heligmonoides speciosus²³ | AB609321 | AB609320 | Apodemus argenteus | Apodemus speciosus | Hokkaido, Japan | Heligmonellidae | Nippostrongylinae |
| Orientostrongylus ezoensis²³ | AB609319 | AB609318 | Rattus norvegicus | Rattus norvegicus | Hokkaido, Japan | Heligmonellidae | Nippostrongylinae |
| Lagostrongylus leporis²³ | AB610547 | AB610546 | Pentalagus furnessi | Lepus spp. | Kagoshima, Japan | Heligmonellidae | Brevistratiinae/ Heligmonellinae |
| Nippostrongylus brasiliensis | AY332646 | AY333380 | Rattus norvegicus | Rattus norvegicus | France | Heligmonellidae | Nippostrongylinae |
| Carolinensis minutus | AY332645 | AY333379 | Microtus montebeli | Microtus spp. | France | Heligmonellidae | Nippostrongylinae |
| Heligmosomoides polygyrus bakeri | AY332648 | AY333382 | Mus musculus | Apodemus spp. | France | Heligmosomidae |
| Ohbayashinema erbaevae | AY332647 | AY333381 | Ochotona daurica | Ochotona daurica | Buriatia | Heligmosomidae |

a) Sequenced in the present study.
Phylogenetic relationships of Heligmonellidae nematodes

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. brasiliensis 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 the 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. brasiliensis 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 Trichostrongylina, 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
Phylogenetic relationships of Heligmonellidae nematodes

Heligmonellidae nematodes are available only for five species examined in the present study, the ITS sequence data for additional Heligmonellidae species are necessary to test the validity of this branching.

The subfamily assignment of the genus *Lagostrongylus* appears not fully established. On the ground of morphological characteristics, the genus *Lagostrongylus* was initially classified in the subfamily Brevistriatinae\(^{15,17}\). However, Durette-Desset\(^9\) moved the genus into the subfamily Heligmonellinae\(^8\). The *L. leporis* specimen used in the current study was collected from *P. furnessi* in the Amami-ohshima Island, southern Japan. *P. furnessi* has been confined in the Amami-ohshima Island and isolated from other parts of the Japanese Archipelago and the Asian continent for at least one million years. During the period of host isolation, there have been no invasion by other Leporidae hosts or parasitic nematodes\(^{14,15}\). In addition, no significant morphological differences were found among the *L. leporis* specimens collected from different hosts in Japan\(^{14,15}\). Therefore, the present specimen of *L. leporis* is likely to have carried specific genes that have been conserved for a long period.

The results of our present study on previously unsequenced *H. speciosus*, *O. ezoensis* and *L. leporis* proved the usefulness of the ITS-1 and ITS-2 regions to infer relationships within the superfamily Heligmosomoidea. The ITS regions are subject to nucleotide insertions, deletions and point mutations occurring over time, providing appropriate targets for phylogenetic analysis\(^{18}\). Furthermore, the ITS-1 and ITS-2 regions are shorter than other sequences used for genetic classification of bursate nematodes, such as the 28S rRNA, providing another advantage for sequencing and analysis. In order to better understand the relationships within the family Heligmonellidae, other species of this family must be analyzed.

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