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FULL PAPER

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 Yamada[1)], Atushi Yoshida[1)], Keiko Yoshida[1)], Takeshi Kuraishi[2)], Shosaku Hattori[2)], Chieko Kai[2)], Yumiko Nagai[3)], Takeshi Sakoda[3)], Masaya Tatara[3)], Shintaro Abe[3)] and Shin-ichiro Fukumoto[1, *)]

[1)]Unit of Veterinary Parasitology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069–8501, Japan.

[2)]Amami Laboratory of Injurious Animals, the Institute of Medical Science, the University of Tokyo, Setouchi-cho, Ohshima-gun, Kagoshima, 894–1531, Japan

[3)]Amami Wildlife Center, Yamato-son, Ohshima-gun, Kagoshima, 894–3104, Japan

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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) 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), *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

*Corresponding author: Shin-ichiro Fukumoto, Unit of Veterinary Parasitology, Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069–8501, Japan
Phone: +81–11–388–4747. Fax: +81–11–387–5890. E-mail: fukumoto@rakuno.ac.jp

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 Trichostrongylina Durette-Desset and Chabaud, 1993: order Strongylida)[7,9–11]. 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 similar[7–11]. The classification of the suborder Trichostrongylina 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 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 Trichostrongylina[3,5]. 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 Trichostrongylina[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*], *Orientostrongylus 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 ezoensis*[12,13,26] was collected from the small intestine of a *Rattus norvegicus* captured in Sapporo, Hokkaido. Specimens of *Lagostrongylus leporis*[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°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[24]. 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'-AACAACCCTGAACCAGA CGT-3' for ITS-1, and NC1 (forward): 5'-ACGTCT GGTTCAGGGTTGTT-3' and NC2 (reverse): 5'-TT AGTTTCTTTTCCTCCGCT-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 speciosus[a]	AB609321	AB609320	*Apodemus argenteus*	*Apodemus speciosus*	Hokkaido, Japan	Heligmonellidae	Nippostrongylinae
Orientostrongylus ezoensis[a]	AB609319	AB609318	*Rattus norvegicus*	*Rattus norvegicus*	Hokkaido, Japan	Heligmonellidae	Nippostrongylinae
Lagostrongylus leporis[a]	AB610547	AB610546	*Pentalagus furnessi*	*Lepus* spp.	Kagoshima, Japan	Heligmonellidae	Brevistriatinae/ 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.

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 DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare UK Limited, Little Chalfont, UK).

Sequence analysis: The sequences were aligned using the ClustalW method[28]. Pairwise sequence comparisons were performed for ITS-1 and ITS-2, either separately or in combination, as previously described[2,3]. 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. brasiliensis* (AY332646 and AY333380), *C. minutus* (AY332645 and AY333379) and *H. polygyrus bakeri* (AY332648 and AY333382), *O. erbaevae* (AY332647 and AY333381). These seven species were determined as an outer group by comparison with the rDNA sequence of *Caenorhabditis elegans* (X03680).

Phylogenic trees were constructed by using the neighbor-jointing methods of the program MEGA4[28]. 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. 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%).

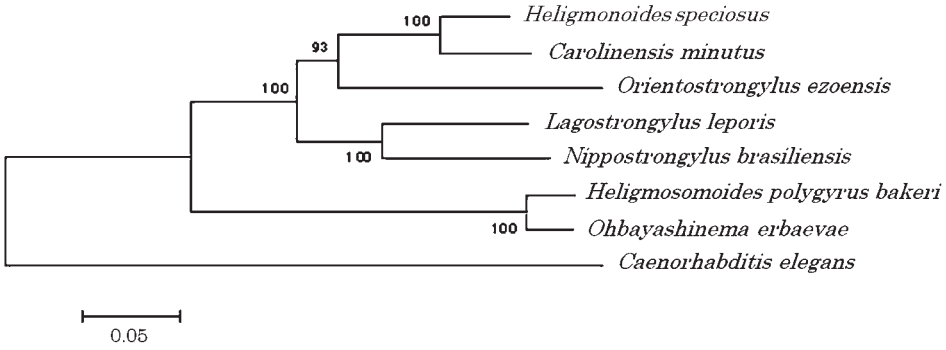

Fig. 1A. Phylogenetic relationships in the ITS-1 sequences of the five species of Heligmonellidae nematodes by the neighbor-joining method. Values on the tree nodes are bootstrap proportions (%). The scale bar represents divergence of 0.05.

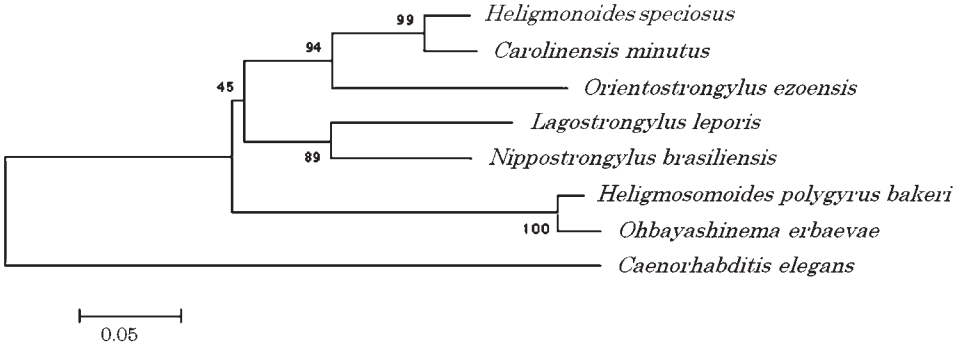

Fig. 2B. Phylogenetic relationships in the ITS-2 sequences of the five species of Heligmonellidae nematodes by the neighbor-joining method. Values on the tree nodes are bootstrap proportions (%). The scale bar represents divergence of 0.05.

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[4,19,20,24] and Molineoidea[2,22,23]. While the D1-D2 domain of 28S rRNA has also been used for phylogenetic analysis of the Trichostrongylina[5], the same task has been accomplished using the ITS-1 and ITS-2 regions[3]. 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

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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References

1) Anderson, R. C. 2000. The superfamily Trichostrongyloidea. In: *Nematode parasites of vertebrates. Their development and transmission. (2 nd edit.)*, CAB International, Oxford. UK, pp. 81–128.
2) Audebert, F., Durette-Desset, M.-C and Chilton, N. B. 2000. Internal transcribed spacer rDNA can be used to infer the phylogenetic relationships of species within the genus *Nematodirus* (Nematoda: Molineoidea). *Int. J .Parasitol*., **30**: 187–191.
3) Audebert, F., Chilton, N., Justine J.-L., Gaullut C., Tillier, A. and Durrete-Desset, M.-C. 2005. Additional molecular evidence to support a sister taxon relationship between Heligmosomoidea and Molineoidea nematodes (Trichostrongylina). *Parasitol. Res*., **96**: 343–346.
4) Chilton, N. B., Newton, L. A., Beveridge, I. and Gasser, R. B. 2001. Evolutionary relationships of Trichostrongyloid nematodes (Strongylida) inferred from ribosomal DNA sequence data. *Mol. Phyol. Evol*., **19**: 367–386.
5) de Bellocq, J. G., Ferte, H., Depaquit, J., Justine, J.-L., Tillier, A., and Durette-Desset, M.-C. 2001. Phylogeny of the Trichostrongylina (Nematoda) inferred from 28S rDNA sequences. *Mol. Phylo. Evol*., **19**: 430–442.
6) Durette-Desset, M.-C. 1971. Essai de classification des nématodes héligmosomes. Correlations avec la paléobiogéographie des hôtes. *Mémoire de la Muséum National de la*

Histoire Naturel de Paris (A), **69**: 1–126.
7) Durette-Desset, M.-C.1983. CIH keys to the nematode parasites of vertebrates. Keys to the genera of the Superfamily Trichostrongyloidea, Commonwealth Agricultural Bureau, Farnham Royal, UK (1983), pp. 1–68.
8) Durette-Desset, M.-C. 1985. Trichostrongyloid nematodes and their vertebrate hosts: Reconstruction of the phylogeny of a parasitic group. *Adv. Parasitol*., **24**: 239–306.
9) Durette-Desset, M.-C. 2008. Heligmonellidae and Heligmosomidae. In *Biology Index*. (coordinator). Home page of College of Agriculture & Life Sciences, Texas A & M University.
10) Durette-Desset, M.-C. and Chabaud, A. G. 1993. Nomenclature des Strongylida au-dessus du groupe-famille. *Ann. Parasitol. hum. comp*., **68**: 111–112.
11) Durette-Desset, M.-C., Beveridge, I. and Spratt, D. M. 1994. The origins and evolutionary expansion of the Strongylida (Nematoda). *Int. J. Parasitol*., **24**: 1139–1165.
12) Fukumoto, S.-I. 1979. Life history of *Orientostrongyius ezoensis* Tada, 1975 (Nematoda: Heligmonellidae). *Jpn. J. Parasitol*., **28**: 465–471 (in Japanese with English summary).
13) Fukumoto, S. and Ohbayashi, M. 1985. Variations of synlophe of *Orientostrongylus ezoensis* Tada, 1975 (Nematoda: Heligmonellidae) among different populations in Japan. *Jpn. J. Vet Res*., **33**: 27–43
14) Fukumoto, S., Kamiya, M. and Ohbayashi M. 1986a. Morphological study of the synlophe of the intestinal nematode, *Heligmonella leporis* (Schulz, 1931) Durette-Desset, 1971. (Heligmonellidae: Trichostrongyloidea) from lagomorphs in Japan. *Jpn. J. Vet. Res.*, **34**: 203–217.
15) Fukumoto, S., Kamiya, M. and Ohbayashi, M. 1986b. A new genus of the intestinal nematode, *Lagostrongylus* (Heligmonellidae) from lagomorphs: a review of related genera of subfamilies Heligmonellinae and Brevistriatinae. *Jpn.J. Vet. Res*., **34**: 241–250.
16) Gasser, R. B., Chilton, N. B., Hoste, H. and Beveridge, I. 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Res*., **21**: 2525–2526.
17) Gibbons, L. M. 2010. Keys to the nematode parasites of vertebrates. Supplementary volume. CAB International, p416, Oxford. UK
18) Hillis, D. M. and Dixon, M. T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Quart. Rev. Biol*., **66**: 411–453.
19) Hoste, H., Chilton, N. B., Gasser R. B., Beveridge, I.1995. Differences in the second internal transcribed spacer (ribosomal DNA) between five species of *Trichostrongylus* (Nematoda: Trichostrongylidae). *Int. J. Parasitol*., **25**: 75–80.
20) Hoste, H., Chilton, N. B., Beveridge, I., Gasser, R. B. 1998. A comparison of the first internal transcribed spacer of ribosomal DNA in seven species of *Trichostrongylus* (Nematoda: Trichostrongylidae). *Int. J. Parasitol*., **28**: 1251–1260.
21) Konno, S. 1958. On *Longistriata speciosa* n. sp. from *Apodemus speciosus*. Medicine and Biology, **48**: 56–58 (in Japanese, translated title).
22) Nadler, S. A., Hoberg, E. P., Hudspeth, D. S. S., Rickard, L. G. 2000. Relationships of *Nematodirus* species and *Nematodirus battus* isolates (Nematoda: Trichostrongyloidea) based on nuclear ribosomal DNA sequences. *J. Parasitol*., **86**: 588–601.
23) Newton, L. A., Chilton, N. B., Beveridge, I., Gasser, R. B. 1998. Differences in the second internal transcribed spacer of four species of *Nematodirus* (Nematoda: Molineidae). *Int. J. Parasitol*., **28**: 337–341.
24) Mochizuki, R., Endoh, D., Onuma, M. and Fukumoto, S.-I. 2006. PCR-based species-specific amplification of ITS of *Mecistocirrus digitatus* and its application in identification of GI nematode eggs in bovine feces. *J. Vet. Med. Sci*., **68:** 345–351.
25) Stevenson, L. A., Chilton, N. B., Gasser, R. B. 1995. Differentiation of *Haemonchus placei* from *H. contortus* (Nematoda: Trichostrongylidae) by the ribosomal DNA second internal transcribed spacer. *Int. J. Parasitol*., **25**: 483–488.
26) Tada, Y. 1975.*Orientostrongylus ezoensis* n. sp. (Nematoda: Heligmosomidae) from the brown rat, *Rattus norvegicus* Berkenhout. *Jpn.J.Vet. Res*., **23**: 41–44.
27) Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Bio. Evol*., **24**: 1596–1599.
28) Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**: 4673–4680.