Original Paper

Phylogenetic relationship of the pinworms of the genus *Syphacia* from murines of Indonesia and some other regions inferred by molecular analysis

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ABSTRACT

Sequencing of partial cytochrome *c* oxidase subunit 1 gene (*Cox-1*) of mitochondrial DNA and 28S ribosomal RNA gene (rDNA) of nuclear DNA was attempted for pinworms of the genus *Syphacia* from murines of Indonesia in order to compare with those from other regions. *Cox-1* sequence of *Syphacia rifaii* from *Bunomys penitus* of Sulawesi was characteristic by having deletions of three consecutive nucleotides at two loci in addition to numerous substitutions, resulting in very long branch in phylogenetic trees. Nevertheless, both in *Cox-1* and 28S rDNA trees, *S. rifaii* formed a clade with *Syphacia muris*. Three *Syphacia* species parasitic in *Apodemus*, *Syphacia emileromani*, *Syphacia stroma* and *Syphacia agraria*, formed one clade in 28S rDNA phylograms, suggesting co-evolutionary relationship with their hosts. Indonesian examples of *S. muris* were rather diverged from those in the laboratory rats, *Rattus norvegicus*, both in *Cox-1* and 28S rDNA, presumably reflecting the process of geographical dispersal of host *Rattus* spp. with this pinworm over the world.

Keywords : *Syphacia*, nematodes, *Cox-1*, 28S rDNA, co-evolution.

1. INTRODUCTION

The pinworm of genus *Syphacia* Seurat, 1916 (Nematoda: Oxyuridae: Syphaciinae) has cosmopolitan distribution occuring in rodents of the families Cricetidae and Muridae [18]. In Indonesia, where murine fauna shows high diversity and endemicity, 10 species of the genus *Syphacia* belonging to three subgenera were hitherto recorded [1-6, 12, 13, 26]. Eight of them belong to the subgenus *Syphacia*, while the remaining two have specialized characteristics, having been assigned to two new subgenera [5]. Because they seem to have rather strict host specificity, it is of special interest to know their evolutionary relationship with their hosts.

Pinworms of mammals have direct life cycle with anal-oral route of transmission. The simplicity of the life cycle is likely to provide less opportunity to acquire a new host than for other parasites that require a long period in external environment or in intermediate host to become infective. Based on the morphological evidence, *Syphacia* species have been considered to have co-evolutionary relationship with their hosts generally [18, 19]. Meanwhile, analyzed partial sequences of cytochrome *c* oxidase subunit 1 gene of mitochondrial DNA (*Cox-1*) and 28S ribosomal RNA gene (rDNA) of *Syphacia* species of Japanese murines could not demonstrate such a co-evolutionary relationship [22, 23]. It was suggested that co-evolutionary relationship might not be so strict in *Syphacia* and host switching probably occurred during the course of evolution. In this study, we determined partial sequences of *Cox-1* and 28S rDNA of *Syphacia* species collected from Indonesia and some other countries to know phylogenetic relationship of *Syphacia* species among Indonesia and other areas.

2. MATERIALS AND METHODS

2-1. Species examined

Two species of *Syphacia* from murines of Indonesia, i.e. *S. muris* from *Rattus tanezumi* collected on Java and Sumatra Islands and *S. rifaii* from *Bunomys penitus* captured on Sulawesi Island, were subjected for DNA extraction and amplification (Table 1). In addition, two species of *Syphacia*, i.e. *S. obvelata* from a laboratory mouse in Japan and *S. stroma* from a laboratory golden hamster in Czech Republic, were also analyzed. Besides the *Syphacia* representatives, *Syphatineria* sp. from an Indonesian sciurid and *Aspiculuris tetraptera* from a laboratory mouse in Japan were also tested for sequencing (Table 1). All samples were fixed in pure ethanol.

2-2. DNA extraction and amplification

Individual worm was rinsed in phosphate buffer (pH 6.5), and homogenized in a 1.5 mL Eppendorf tube containing 100 μL distilled water using a plastic pestle. Five μL of the homogenized solution was mixed with 50 μL liquid phase of Dexpat™ (Takara Bio. Inc., Otsu, Shiga, Japan) in 200 μL tube, heated at 96 °C for 30 min, and then cooled on ice. Subsequently, 5 μL of the solution was added to the 50 μL PCR mixture, which contained 0.5 μL of KOD-Neo™ polymerase, 5 μL of 10x PCR buffer, 5 μL of 2 mM dNTP, 5 μL of 2 mM $MgSO_4$ (Toyobo Co., Tokyo, Japan) and 0.25 μL each of forward and reverse primers. PCR was performed using a thermal cycler, PC-801 (ASTEC Co., Ltd., Fukuoka, Japan). The primers used for amplification and sequencing were those used previously [9, 16, 17, 23] or newly designed (Table 2).

The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of 98°C for 10 sec, 50 °C for 1 min, 68 °C for 1 min, 30 cycles of 98 °C for 10 sec, 55 °C for 1 min, 68 °C for 1 min, and a post-amplification extension at 68 °C for 7 min for *Cox-1*.

PCR products were mixed with Ez-Vision™ Three DNA Dye (Amresco, Solon, Ohio, USA), electrophoresed in a 1.5% agarose gel plate and visualized using a UV illuminator. Positive bands were dissected and processed using Nucleospin™ column (Machery-Nagel, Düren, Germany), and then ethanol precipitated for further purification. Proper amount of the DNA was subjected to direct sequencing using the BigDye™ Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, California, USA), and purified using CentriSep™ spin column (Princeton Separations Inc., Adelphia, New Jersey, USA). Then sequencing was made in an automated genetic analyzer ABI-PRISM 3130 (Applied Biosystems).

Table 2. Primers used in the present study

Name of primer	Direction	Nucleotide sequence
Partial *Cox-1* gene:		
SyphCoxF1	Forward	5'-GGTCAGTTGTATAATGTTRT-3'
StrCoxAfrF	Forward	5'-GTGGTTTTGGTAATTGAATGGTT-3'
JB3	Forward	5'-TTTTTTGGGCATCCTGAGGTTTAT-3'
MH28R	Reverse	5'-CTAACTACATAAT AAGTATCATG-3'
JB4.5	Reverse	5'-TAAAGAAAGAACATAATGAAAATG-3'
Partial 28S rDNA:		
C1'	Forward	5'-ACCCGCTGAATTTAAGCAT-3'
D2	Reverse	5'-TCCGTGTTTCAAGACGG-3'

Table 1. Pinworms examined in this study with the host, locality and accession numbers of DNA sequences in DDBJ

Species	Host		Locality	Year of collection	DDBJ accession Nos.	
	Family: Subfamily	Species			*Cox-1*	28S rDNA
Syphacia muris	Muridae: Murinae	*Rattus tanezumi*	Java, Indonesia	2012	LC038089	LC038096
Syphacia muris	Muridae: Murinae	*Rattus tanezumi*	Sumatra, Indonesia	2012	LC038090	LC038097
Syphacia rifaii	Muridae: Murinae	*Bunomys penitus*	Sulawesi, Indonesia	2012	LC038087	LC038094
Syphacia rifaii	Muridae: Murinae	*Bunomys penitus*	Sulawesi, Indonesia	2012	LC038088	LC038095
Syphacia obvelata	Muridae: Murinae	*Mus musculus**	Honshu, Japan	2012	LC038086	n.t.**
Syphacia stroma	Muridae: Cricetinae	*Mesocricetus auratus**	Brno, Czech Republic	2013	LC038091	LC038098
Syphatineria sp.	Sciuridae: Sciurinae	*Lariscus hosei*	Kalimantan, Indonesia	2012	LC038092	LC038099
Aspiculuris tetraptera	Muridae: Murinae	*Mus musculus**	Honshu, Japan	2012	LC038093	n.t.

* Experimental animal reared in laboratory.
** n.t.: Not tested.

2-3. Phylogenetic analysis

Sequences determined in the present study and those registered in DNA databases were used for phylogenetic analysis. They were aligned using Clustal W, then analyses were made by neighbor-joining (NJ) and maximum likelihood (ML) methods using MEGA5 (v. 5.2.2) software [25, 27]. Both nucleotide and amino acid sequences translated using invertebrate mitochondrion code were analyzed for *Cox-1*. In NJ analysis of nucleotide sequences, the evolutionary distances were computed using the Kimura's two-parameter method [20]. The bootstrap values were calculated by 1,000 replicates [8]. *Aspiculuris tetraptera* was used as an outgroup species to root tree of *Cox-1*. The nucleotide sequences determined in this study were registered in the DNA Databank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/) with accession numbers LC038086 to LC038099.

3. RESULTS

In all of the Indonesian *Syphacia* materials tested, partial *Cox-1* DNA was successfully amplified and sequenced (Table 1). Using primer sets SyphCoxF1-JB4.5, unambiguous sequence of 749bp of *Cox-1* was obtained for each one sample of *S. rifaii* (LC038087) and *Syphatineria* sp. (LC038092). A shorter sequence (LC038088) corresponding to 106th to 748th positions of LC03087 was obtained using primer set StrCoxAfrF-JB4.5 from another sample of *S. rifaii*. This shorter sequence had one synonymous substitution from C to T at 147th position. The sequence of *S. rifaii* was also confirmed by sequencing using primer sets StrCoxAfrF-MH28R and JB3-JB4.5, which gave 610bp and 366bp, respectively. Meanwhile, *S. muris* samples responded only to the primer set JB3-JB4.5, giving shorter sequences with 395bp. Because of these differences in length of the sequences obtained and limitation of the sequences in the DNA database, phylogenetic analyses were carried out separately on the two datasets covering 618bp (Fig. 1) and 249bp (Fig. 2), respectively.

The striking feature is the peculiarity of *Cox-1* of *S. rifaii* in nucleotide and amino acid sequences. By Clustal W alignment, it was found that *S. rifaii* had deletions of three consecutive nucleotides at two sites causing two amino acid deletions. The genetic distance from other congeners was large, making extraordinarily long branch especially in the NJ tree based on the longer sequences (Fig.1). If outgroup setting was not done, *S. rifaii* diverged at the most basal node in the

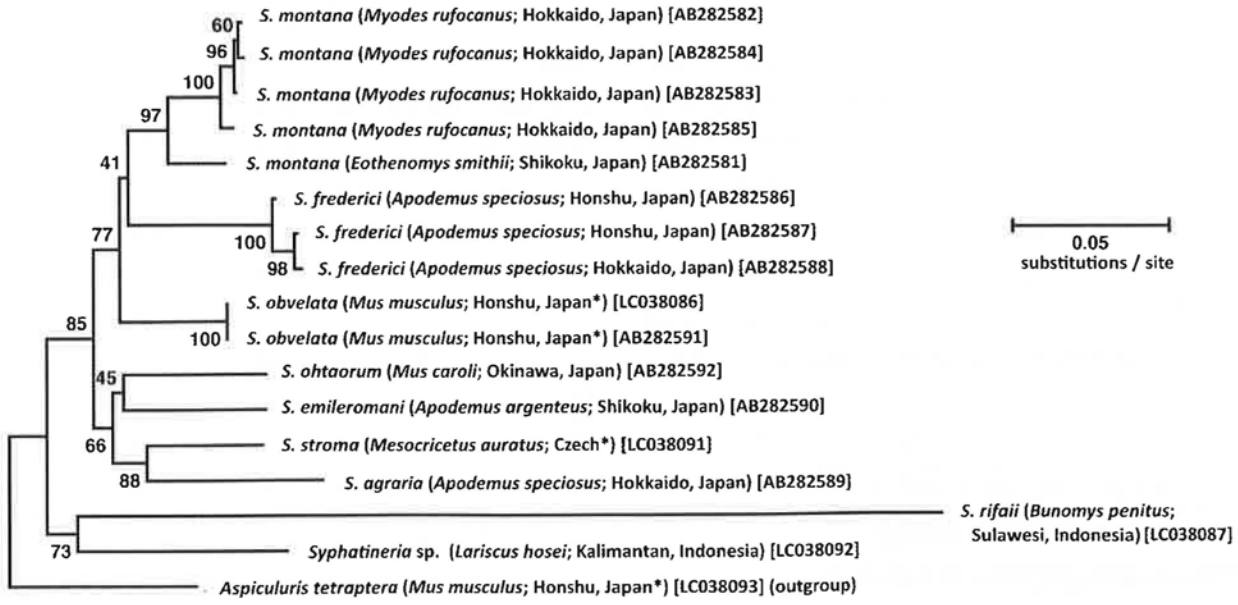

Fig. 1. NJ reconstruction of phylogeny of *Syphacia* spp. based on long nucleotide sequences of *Cox-1*. The optimal tree with the sum of branch length = 0.81201463 is shown. There were a total of 618 positions in the final dataset. Nematode taxon is followed by host and locality in parenthesis and accession number in bracket. Material from laboratory murine is marked with an asterisk.

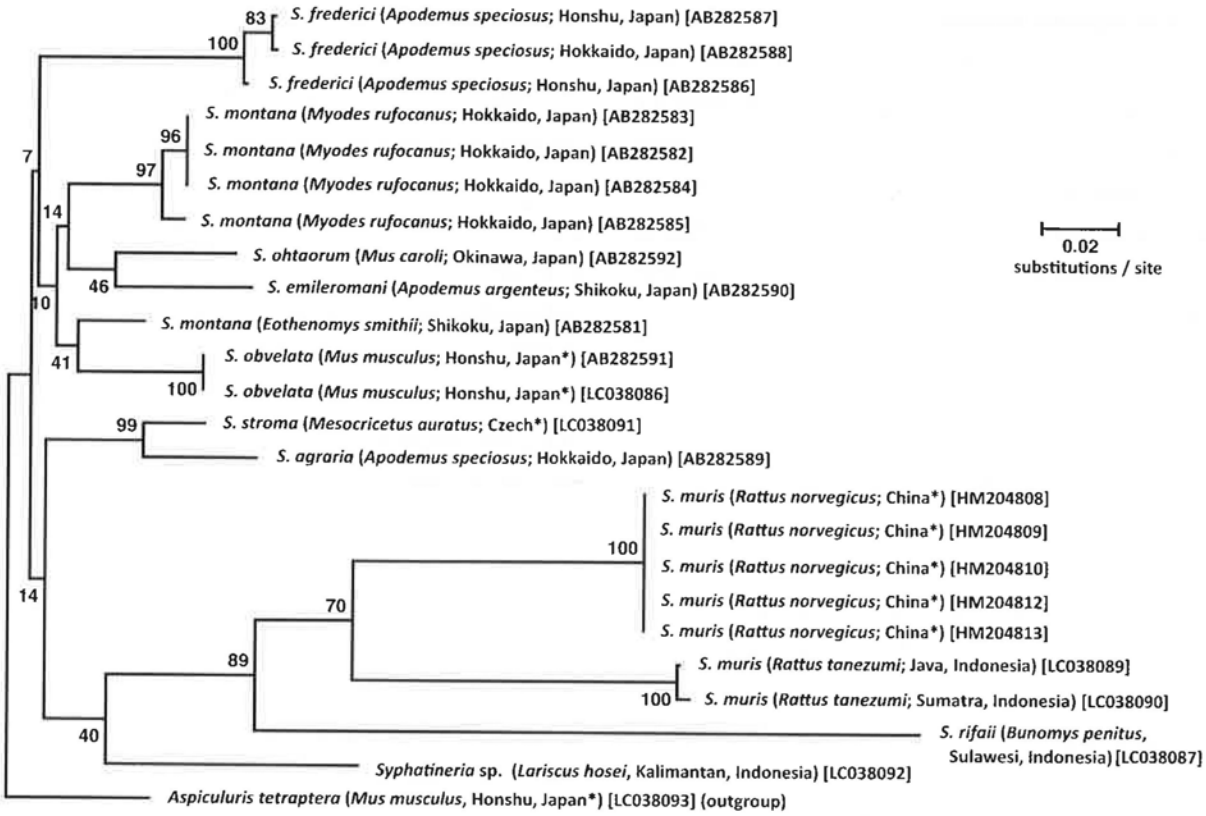

Fig. 2. NJ reconstruction of phylogeny of *Syphacia* spp. based on short nucleotide sequences of *Cox-1*. The optimal tree with the sum of branch length = 0.85148172 is shown. There were a total of 249 positions in the final dataset. Nematode taxon is followed by host and locality in parenthesis and accession number in bracket. Material from laboratory murine is marked with an asterisk.

tree, putting *Aspiculuris* within *Syphacia* spp. (not shown here). When *Aspiculuris* was used as an outgroup, *S. rifaii* and *Syphatineria* sp. formed a clade clearly separated from other species of *Syphacia*. This peculiarity became less prominent but persisted when the analysis was performed based on the short sequences including those of *S. muris* (Fig. 2). *Syphacia rifaii* shared common ancestor with *Syphatineria* sp. In the tree based on the short sequences of *Cox-1*, *S. rifaii* and *S. muris* were close to each other, and they shared a common ancestor with *Syphatineria* sp. though the bootstrap value was not high.

Phylogenetic reconstruction using ML method was attempted for amino acid sequences translated from the long nucleotide sequences of *Cox-1* (Fig. 3). Again, *S. rifaii* showed very curious position by having extraordinary long branch.

Amplification of 28S rDNA was successful for *S. rifaii*, *S. muris* and *Syphatineria* sp. (Table 1). In the NJ tree based on 28S rDNA, bootstrap values were generally high (Fig. 4). Even when outgroup setting was not made, *A. tetraptera* was located most basal. According to this tree, *Syphacia* (*Seuratoxyuris*) *petrusewiczi* from Japanese *Myodes* (syn. *Clethrionomys*) diverged at the most basal node, and then *Syphatineria* sp. was separated. Among the species of the subgenus *Syphacia*, *S. rifaii* and *S. muris* formed a clade, sharing a long branch, diverging from the common ancestor to the other *Syphacia* species. *Syphacia agraria*, *S. stroma* and *S. emileromani*, all parasitic in *Apodemus* spp., are monophyletic. *Syphacia vandenbrueli*, *S. frederici*, *S. obvelata* and *S. montana* formed another monophyletic group. *Syphacia muris* of *R. tanezumi* of Sumatra and Java differed from that of Japan and USA, both were collected from laboratory rats, *Rattus norvegicus*. The same groupings were also found in ML tree based on 28S rDNA (Fig. 5), but *Syphatineria* sp. diverged earlier than *S.* (*Seu.*) *petrusewiczi*.

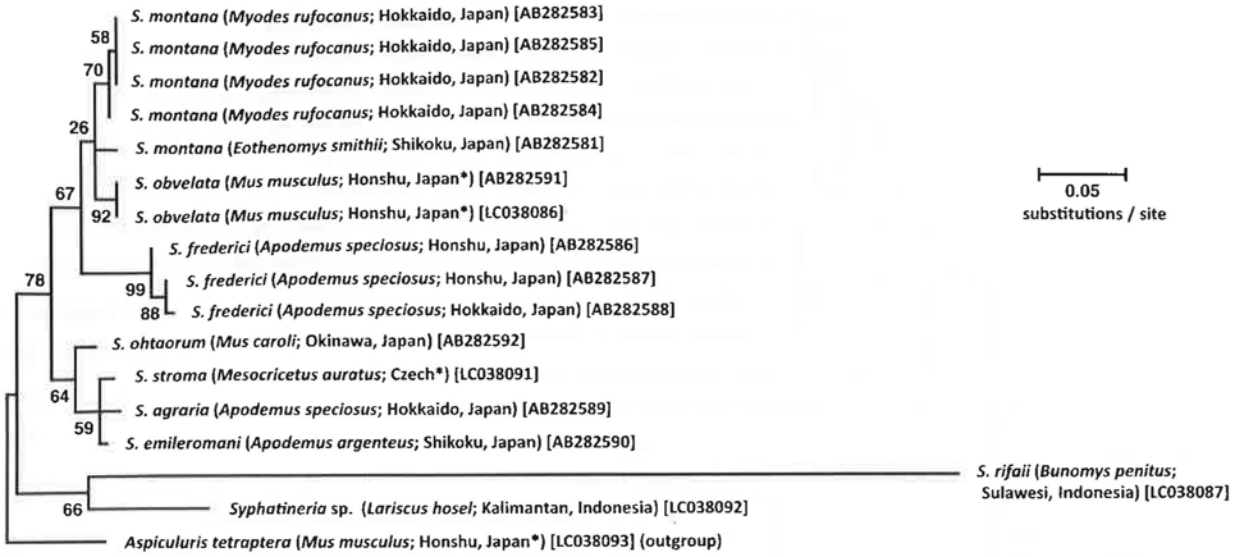

Fig. 3. ML reconstruction of phylogeny of *Syphacia* spp. based on amino acids translated from long nucleotide sequences of mtDNA *Cox-1* using the General Reverse Transcriptional model. The tree with the highest log likelihood (-1382.5027) is shown. There were a total of 206 positions in the final dataset. Nematode taxon is followed by host and locality in parenthesis and accession number in bracket. Material from laboratory murine is marked with an asterisk.

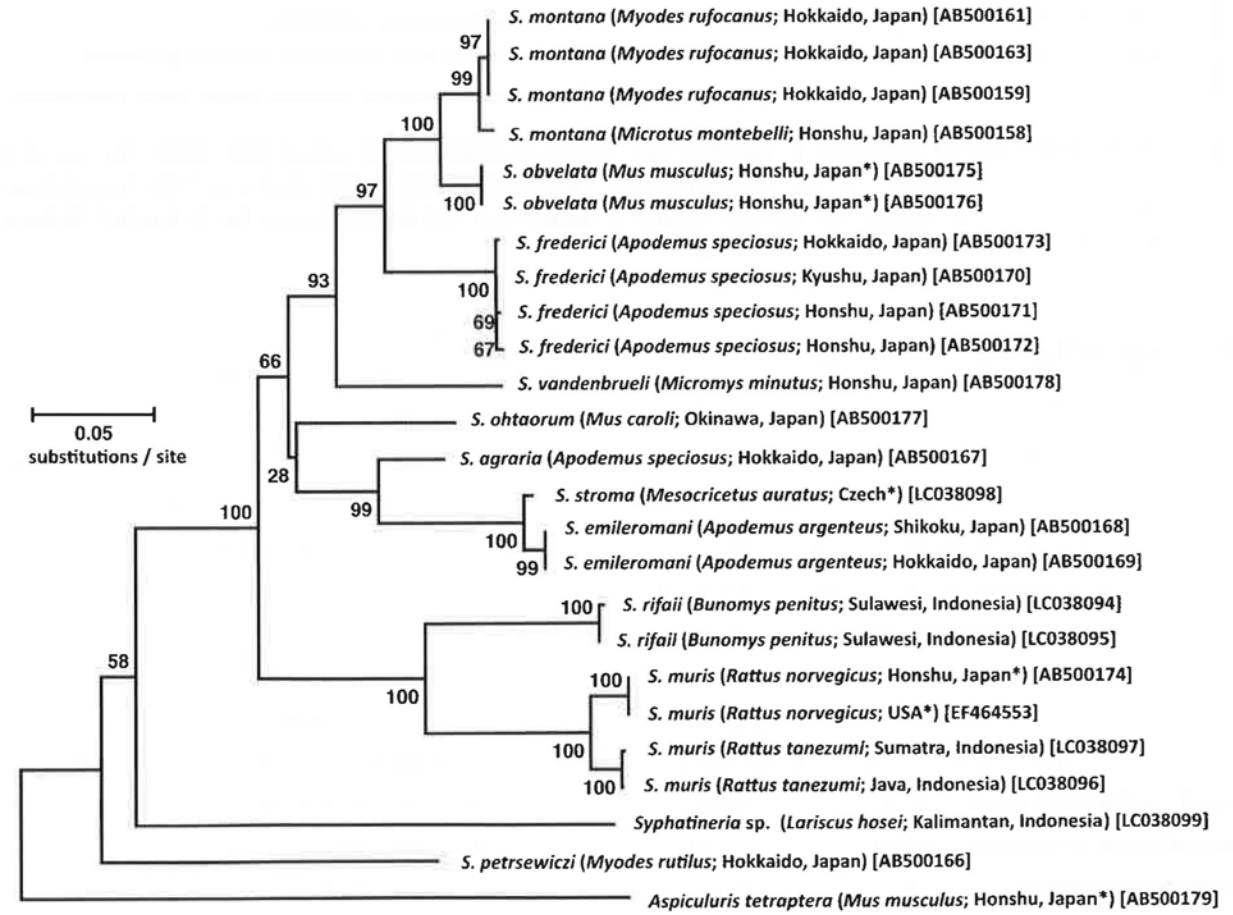

Fig. 4. NJ reconstruction of phylogeny of *Syphacia* spp. based on sequences of partial 28S rDNA. The optimal tree with the sum of branch length = 1.35815348 is shown. There were a total of 689 positions in the final dataset. Nematode taxon is followed by host and locality in parenthesis and accession number in bracket. Material from laboratory murine is marked with an asterisk.

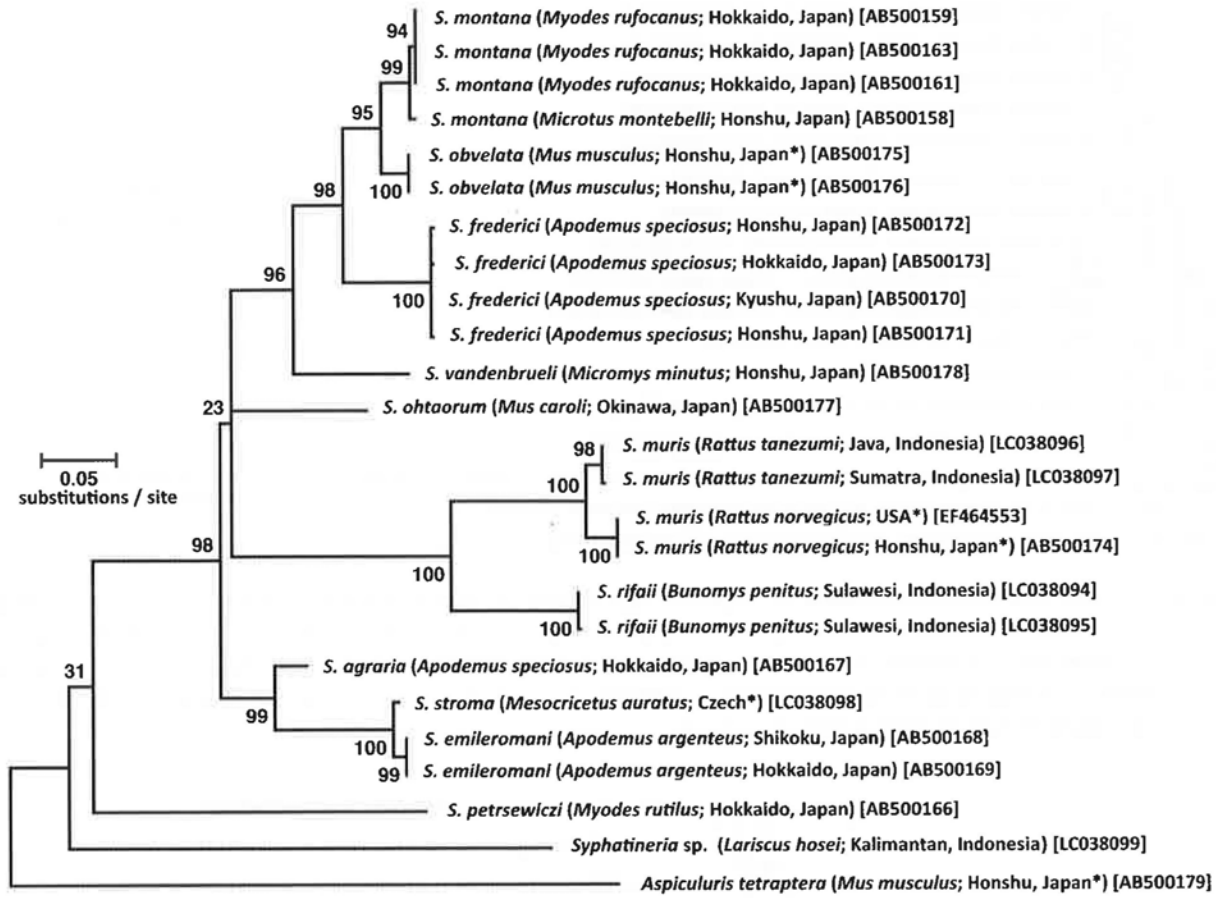

Fig. 5. ML reconstruction of phylogeny of *Syphacia* spp. based on sequences of partial 28S rDNA. The tree with the highest log likelihood (-4716.7483) is shown. There were a total of 689 positions in the final dataset. Nematode taxon is followed by host and locality in parenthesis and accession number in bracket. Material from laboratory murine is marked with an asterisk.

4. DISCUSSION

Syphaciinae arose with glires as hosts in Paleocene, and evolved as Syphaciini in Muroidea in early Eocene [18]. Subsequently, syphaciins were divided into those in murids and sciurids during Eocene, and establishment of the subgenera of *Syphacia* (i.e., *Syphacia*, *Seuratoxyuris* and *Cricetoxyuris*) and genera of syphaciins (including *Syphatineria* and *Syphabulea*) of sciurids occurred in Oligocene. However, the phylogenetic trees based on 28S rDNA may suggest that diversification of murid- and scuirid-parasitic lineages of Syphaciini and diversification of subgenera *Seuratoxyuris* and *Syphacia* occurred in a relatively short geological period.

As shown above, *S. rifaii* and *S. muris* are located close together both in the phylogenetic trees on *Cox-1* and 28S rDNA. This is not unexpected because ancestors of the host genera, *Rattus* and *Bunomys*, are considered to have diverged in early Pliocene while ancestors of *Micromys*, *Mus*, *Apodemus* and Microtines established much earlier, in Miocene [7]. It is also noticeable that individuals of *S. muris* of Java and Sumatra were rather diverged from those in the laboratory rats both in *Cox-1* and 28S rDNA. Ancestor of *S. muris* might be adapted to ancestral *Rattus* probably in Southeast Asia, and then made dispersal to the surrounding areas. Some *Rattus*, i.e., *R. norvegicus* and *R. rattus*, widened distribution over the world. The laboratory rat was domesticated from feral *R. norvegicus* in Europe or North America in middle of 19th century, and then distributed to various laboratories all over the world. *Syphacia muris* of them also has been maintained in the laboratory conditions. Thus, genetic divergence of *S. muris* in 28S rDNA of U.S. and Japan materials was negligible. Also, *Cox-1* of *S. muris* in rats in Chinese laboratories lacked variations (Fig. 2). However, it is apparent that feral *Rattus*

spp. harbored *S. muris* with genetic diversification as suggested by the present study.

The numerous nucleotide and amino acid substitutions found in *Cox-1* of *S. rifaii* are very curious. At first, the sequence obtained was suspected to be a pseudogene. However, repeated amplification and sequencing resulted in the same sequence except for one synonymous substitution, while no other sequence referable to *Cox-1* was obtained. Moreover, the sequence was translatable to amino acid sequence using invertebrate mitochondrial code. Hence, this sequence is regarded as partial *Cox-1* gene. Presumably, the nucleotide substitutions in *Cox-1* of this pinworm have been accumulated during isolation on Sulawesi with host genus *Bunomys*, which is also Sulawesi endemic with seven extant species [21]. Although *S. rifaii* has been known from *B. prolatus* and *B. chrycosomus* besides *B. penitus* [1], only two worms from one host species were subjected to analysis. Further analyses of DNA sequences of this and other endemic species of *Syphacia* are indispensable to have comprehensive understanding of pinworm evolution in Indonesia.

Previous studies suggested that co-evolutionary relationship in *Syphacia* might not be so strict [22, 23]. Their conclusion was based on the fact that both *Mus*-parasitic species and *Apodemus*-parasitic species did not form their own clades but scattered in different clades. It was also known from morphological viewpoint that plural *Syphacia* species parasitic in a murine genus often composed of different lineages. For example, among the *Syphacia* species parasitic in *Mus*, *S. obvelata* was considered to be close to *S. montana*, but clearly differed from *S. ohtaorum* in the cephalic morphology, lateral alae shape and egg surface markings [10, 24]. Among those parasitic in *Apodemus*, *S. frederici* has well-developed and pointed alae in the cervical portion of female and a short tail in male [14, 24], while *S. emileromani*, *S. agraria* and *S. stroma* lack such alae in female and have a long tail in male [10, 24]. The phylogenetic trees presented by Okamoto et al. [22, 23] seemed to congruent with the morphology-based groupings of *Syphacia*.

Syphacia emileromani and *S. stroma*, both were described originally from *Apodemus*, were considered to be related close together [24]. In the phylogenetic trees based on 28S rDNA, *S. agraria*, *S. stroma* and *S. emileromani* formed one clade, suggesting that these species have co-evolved with *Apodemus*. However, host-specificity of *Syphacia* may not be so strict and host-switching may occur especially when hosts become sympatric. Actually, *S. frederici*, *S. emileromani* and *S. agraria* are shared by three *Apodemus* species distributed in Hokkaido, Japan [11]. Moreover, under an artificial condition such as breeding facilities of experimental or pet rodents, *Syphacia* species could be easily transmitted to unfamiliar hosts. It was reported that three species of *Syphacia* infected concomitantly the golden hamsters reared [15]. The present material of *S. stroma* was such worm collected from the golden hamster.

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