2.2. Isolation and identification of Y. pseudotuberculosis

The samples (liver and spleen) collected from the dead monkeys were homogenized or suspended in phosphate-buffered saline (PBS: 7.2), and 10-fold serial dilutions of the suspension were plated on irgasan-novobiocin (IN) agar plates (Fukushima et al., 1990). These PBS suspensions were incubated at 4 °C for 3 weeks and then subcultured on IN agar plate after alkali (KOH) treatment (Aulisio et al., 1980). The plates were incubated at 25 °C for 48 h. Colonies morphologically similar to those of *Yersinia* spp. were subcultured on trypticase soy agar (TSA) (BBL, Sparks, MD, USA) and submitted for biochemical examination for identification, as described elsewhere (Wauters et al., 1988).

2.3. Serotyping

Serotyping of *Y. pseudotuberculosis* isolated from the monkeys was performed by slide agglutination with a commercial rabbit anti-*Y. pseudotuberculosis* sera set (Denka-Seiken Co., Tokyo, Japan), and with the rabbit immune sera made in our laboratory. Additional serotyping was performed by PCR as described by Bogdanovich et al. (2003).

2.4. PCR detection of virulence genes

Six sets of primers, designed in Table 2, were used for detection of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. The *virF* and *irp2* genes were used as the markers for the presence of pYV and HPI, respectively. Chromosomal DNA for PCR was isolated with a wizard genomic DNA purification kit (Promega Co., Madison, WI, USA) following the manufacturer's instructions. PCRs were performed in 50 μl volumes containing 5 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer, 3 mM $MgCl_2$, 0.1 μM of each primer, and 0.5 U of Taq DNA polymerase (Promega Co., Madison, WI, USA). The PCR amplifications were carried out at 94 °C for 5 min as an initial denaturation step and then subjected to 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C for detection of *virF*, *inv*, *ypmA* and *irp2*, or at 52 °C for detection of *ypmB*, or at 49 °C for *ypmC* (Table 2), 1 min at 72 °C, followed by a final 5 min extension step at 72 °C. Amplifications were performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan). Ten microliters of the PCR amplification products were subjected to electrophoresis in a 1.5% agarose gel. A 1-kb PLUS DNA ladder (Invitrogen Co., Carlsbad, CA, USA) was used as a DNA size marker. The gels were stained with ethidium bromide for 10 min, and photographed under UV light.

3. Results

3.1. Serotyping of Y. pseudotuberculosis strains

By slide agglutination, 7 (38.9%) strains of the 18 were serotype 4b, 7 (38.9%) were serotype 1b, and

Table 2
Primers for PCR detection of virulence genes

Virulence factor	Taget gene	Sequence (5′–3′)	Annealing temperature (°C)	Size of product (bp)	Reference
pYV	*virF*	TCATGGCAGAACAGCAGTCAG, ACTCATCTTACCATTAAGAAG	55	590	Wren and Tabaqchali (1990)
Inv	*inv*	TAAGGGTACTATCGCGGCGGA, CGTGAAATTAACCGTCACACT	55	295	Nakajima et al. (1992)
YPMa	*ypmA*	CACTTTTCTCTGGAGTAGCG, GATGTTTCAGAGCTATTGTT	55	350	Ito et al. (1995)
YPMb	*ypmB*	TTTCTGTCATTACTGACATTA, TTTCTGTCATTACTGACATTA	52	453	Ramamurthy et al. (1997)
YPMc	*ypmA* and *ypmC*	ACACTTTTCTCTGGAGTAGCG, ACAGGACATTTCGTCA	49	418	Carnoy and Simonet (1999)
HPI	*irp2*	AAGGATTCGCTGTTACCGGAC, TCGTCGGGCAGCGTTTCTTCT	55	280	Schubert et al. (1998)

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there was one each of serotypes 2b, 3, 6 and 7. *Y. pseudotuberculosis* serotype 7 has not been isolated from clinical samples in humans or in animals, and thus PCR-based serotyping was used to eliminate any doubt about the serotype of strain NP030601, which was identified as serotype 7 by the slide agglutination. The PCR result of strain NP030601 matched with the above condition for serotype 7 (data not shown), eliminating any doubt about the serotype of this strain. The results of the PCR-based serotyping of the other 17 strains also matched with those of the slide agglutination (data not shown). All the *Y. pseudotuberculosis* strains isolated from the monkeys who died in the same outbreak were of the same serotype of strains chosen for analysis in this study.

3.2. Detection of virulence genes in Y. pseudotuberculosis strains

All strains were *inv* and *virF* positive, and 16 (88.9%) of the 18 strains were *ypmA* positive by PCR. Of the 2 *ypmA* negative strains, one was serotype 4b, and another was serotype 3. On the other hand, all strains were *ypmB*, *ypmC* and *irp2* negative (Table 3).

Table 3
Characteristics of *Y. pseudotuberculosis* isolated from breeding monkeys

No.	Virulence genes						Serotype
	virF	*inv*	*ypm*			*irp2*	
			ypmA	*ypmB*	*ypmC*		
1	+	+	+	−	−	−	4b
2	+	+	+	−	−	−	4b
3	+	+	+	−	−	−	4b
4	+	+	+	−	−	−	4b
5	+	+	+	−	−	−	1b
6	+	+	+	−	−	−	4b
7	+	+	+	−	−	−	4b
8	+	+	+	−	−	−	1b
9	+	+	+	−	−	−	6
10	+	+	+	−	−	−	1b
11	+	+	+	−	−	−	2b
12	+	+	−	−	−	−	4b
13	+	+	+	−	−	−	7
14	+	+	+	−	−	−	1b
15	+	+	+	−	−	−	1b
16	+	+	−	−	−	−	3
17	+	+	+	−	−	−	1b
18	+	+	+	−	−	−	1b

+: PCR positive; −: PCR negative.

4. Discussion

In the present study, the predominant serotypes of *Y. pseudotuberculosis* isolated from dead monkeys were serotypes 1b and 4b. In Japan, these serotypes have also been the predominant serotypes isolated from clinical samples, for example, of human patients, and the majority of the strains of these serotypes are highly pathogenic, with the *ypmA* (Fukushima et al., 2001). In the present study, almost all of the strains isolated from dead monkeys also had *ypmA* genes. It is known that the presence of the *ypmA* is pretty much limited to the Far East (Japan, Korea and Far-Eastern Russia), and also that it exacerbates the toxicity of *Y. pseudotuberculosis* in systemic infection in mice (Fukushima et al., 2001). Moreover, it has been reported that the clinical signs of *Y. pseudotuberculosis* infection found in the Far East include not only fever, gastroenteric symptoms, and mesenteric lymphadenitis, which are the main symptoms in Europe, but also a variety of systemic manifestations such as rash, desquamation, erythema nodosum and arthritis (Sato et al., 1983). In zoological gardens in Japan, a variety of primates are bred, including monkey species from South America, Southeast Asia or Africa, listed in Table 1, as well as the Japanese macaque (*Macaca fuscata*). It has been noted that monkeys from those regions, where the presence of *Y. pseudotuberculosis* with the *ypm* gene has not been identified, frequently die when infections with this pathogen occur, while there has been little mortality of Japanese macaques due to *Y. pseudotuberculosis* infection (Kageyama et al., 2002). Because of the persistent exposure of the Japanese macaque to *Y. pseudotuberculosis* with the *ypm* gene from ancient times they may have acquired resistance to that pathogen, unlike the imported monkeys. Thus, YPM seems to be the main cause of the high mortality of the monkeys imported from abroad.

This is the first report of isolation of *Y. pseudotuberculosis* serotype 7 from a clinical sample anywhere in the world. This serotype has been isolated from dogs, raccoon dogs, moles, wild mice and water. However, there have been no reports about *Y. pseudotuberculosis* serotype 7 isolated from samples of primate origin. Pathological analysis of the squirrel monkey, from which the serotype 7 were isolated, showed swelling of the spleen and liver and multiple

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white abscesses in the spleen, and the PCR analysis demonstrated that the strain of serotype 7 also harbored pYV and *ypmA* genes. These results possibly suggest that the strain serotype 7 isolated in the present study has the same degree of pathogenicity as the other pathogenic serotypes. Therefore, we should pay attention to the possibility of humans and other animal species infected by serotype 7.

Many monkey species kept at zoological gardens are formally recognized as "threatened" by The World Conservation Union (IUCN), and their deaths pose a serious loss to the zoological gardens involved. Thus, preventive measures against *Y. pseudotuberculosis* infection in breeding monkeys should be established as soon as possible. However, most breeding monkeys kept at zoological gardens are maintained in outdoor cages or enclosures for exhibition. These conditions lead to the exposure of the monkeys to animals living in the wild, such as birds and rodents, and as *Y. pseudotuberculosis* is widely distributed in wild animals, the probability of transmission of this pathogen from those animals is very high. Moreover, it is very difficult to completely prevent wild animals from invading the cages of the monkeys, and thus the foods and water provided for the monkeys can easily become contaminated. Therefore, development of effective vaccines is important for preventing pathogenic *Y. pseudotuberculosis* infection in breeding monkeys.

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Tuberculosis as a zoonosis from a veterinary perspective

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Abstract

Tuberculosis is an important disease among many zoonoses, because both *Mycobacterium tuberculosis* and *Mycobacterium bovis*, which are the major causes of tuberculosis, are highly pathogenic, infect many animal species and thus are likely to be the source of infection in humans. In particular, monkeys are highly susceptible to these bacteria and are important spreaders. Recently, two outbreaks of *M. tuberculosis* occurred in four different kinds of monkeys and humans were also infected with the disease in Japan. In zoos, tuberculosis was reported not only in monkeys, but also in several different kinds of animals, including elephants. Pets such as dogs and cats are believed to be generally less susceptible to *M. tuberculosis*, but in this article we introduce a case of infection from man to dog by close contact. Japan is one of the few countries that have been able to control *M. bovis* infection. In other countries, however, cases of bovine tuberculosis and human *M. bovis* infection have been reported, and thus further attention is still required in the future.

Keywords: Zoonosis; *Mycobacterium tuberculosis*; *Mycobacterium bovis*; Monkey; Elephant; Dog

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Résumé

La tuberculose, parmi de nombreuses zoonoses, est une maladie importante, parce que ses deux causes principales, *Mycobacterium tuberculosis* et *Mycobacterium bovis*, sont toutes les deux très pathogéniques et infectent beaucoup d'espèces, ce qui les rend susceptibles d'infecter aussi les humains. Les singes, en particulier, sont facilement atteints par l'infection de ces bactéries, dont ils deviennent ainsi des propagateurs importants. Récemment, au Japon, il y a eu deux cas d'infection répandue de *M. tuberculosis*, qui se trouvait chez des singes de quatre espèces et aussi chez des humains. Dans les jardins zoologiques, l'infection a été rapportée non seulement chez les singes, mais aussi chez des animaux de plusieurs espèces, y compris les éléphants. On croyait que les chiens et les chats domestiques étaient moins susceptibles à l'infection *M. tuberculosis*, mais nous présentons ici le cas d'une infection transmise par un homme à un chien avec lequel il était en contact prochain. Le Japon est l'un des rare pays qui ont pu contrôler l'infection *M. bovis*. Dans la plupart des pays, des cas de tubercule bovine ont été rapportés de même que les cas d'infection *M. bovis* chex les humains, ce qui porte à croire que ce sujet mérite encore de l'attention future.

Mots clés: zoonose; *Mycobacterium tuberculosis*; *Mycobacterium bovis*; singe; elephant; chien

1. Introduction

Tuberculosis is a major emerging disease in humans and is now the leading cause of death in adults worldwide. According to WHO estimates, 2 billion people, about one-third of the world's population, are infected with tuberculosis. In 2003, about 8.8 million people were estimated to have developed tuberculosis (incidence rate 140 per 100,000 population), and 1.7 million people (mortality rate, 28 per 100,000 population) died of tuberculosis, with 99% of them being concentrated in developing countries, particularly Asia and Africa [1]. This situation is believed to be closely associated with the spread of HIV in developing countries, in addition to the poor sanitary and living conditions due to poverty and to delay in action against tuberculosis [1,2]. In contrast, the incidence rate of tuberculosis is low in developed western countries (7 per 100,000 population). In Japan, however, the tuberculosis incidence rate had steadily decreased until the 1970s, but the decrease slowed down and then in late 1990s showed a temporary upsurge, with the number of new tuberculous patients reaching 39,384 (incidence rate 31.0) in 2000. In 2005, with 28,319 patients (incidence rate, 22.2), Japan is still classified as a country of intermediate-level tuberculosis epidemic [3].

The pathogen that causes tuberculosis, which is a hazard to public health, is the (highly pathogenic) *Mycobacterium tuberculosis* complex (tubercle bacillus), which comprises *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium canetti* [4]. Of these five species, *M. tuberculosis* and *M. bovis* are most highly pathogenic. *M. tuberculosis* is prevalent all over the world and is the cause of almost all cases of mycobacteriosis in Japan.

Humans are the only reservoir hosts for *M. tuberculosis*. The human-to-human infection cycle rotates; however, tubercle bacilli have a wide host range and *M. tuberculosis* has been detected in fish, reptiles, birds, and mammals including marine animals. Naturally, the first contamination of these animals with *M. tuberculosis* is caused by humans, and then infection occurs among animals, which become the source of infection in humans. Therefore, in this report, we describe tuberculosis, a zoonosis, particularly *M. tuberculosis* and *M. bovis* infections, from a veterinary perspective.

2. Tuberculosis in monkeys

Table 1 shows that different animal species have different degrees of susceptibility to tubercle bacilli and various frequency of open tuberculosis according to the animal species. From a public health print of view, the role of each animal differs according to its species. Animals belonging to the high-score group (Group 1), especially monkeys, are important sources of infection with tuberculosis in terms of susceptibility and transmission. Infection risks differ among different species of monkeys. Old-world monkeys are important from the viewpoint of public health, because they are by far the most susceptible to both *M. tuberculosis* and *M. bovis* and are likely to be unrestrained [5].

In Japan, tuberculosis in monkeys has been reported in zoos. Between 1960 and 1995, *M. tuberculosis* infection occurred in pig-tailed macaques, Taiwan macaques, orangutans, and chimpanzees [6,7, private communication]. In this report, we present the two outbreaks of *M. tuberculosis* infection that occurred recently. In an exhibition facility housing 17 Japanese macaques in the Kansai area, two monkeys that died in July and October 2004 were diagnosed with *M. tuberculosis* disease. The rest of the monkeys that were housed with these two monkeys were also found positive with tuberculosis skin test, and thus were euthanized [8].

The other outbreak involved three species of monkeys infected in succession (private communication) in the one facility. In this facility, two reptiles (a Malay gavial [*Tomistoma schlegelii*] and a spectacled caiman [*Caiman crocodilus*]) died in December 2000 and in February 2001 before the outbreak of tuberculosis in monkeys. Both these animals had disseminated lesions in the organs and numerous acid-fast bacteria in the lesions. In February 2001, a tuberculin-positive chimpanzee died. This animal had suppurative granulomatous inflammation with infiltration of multinucleated giant cells in the liver, and a small number of acid-fast bacteria in the lesions. In October 2003, an old Asian elephant died in the same facility. This animal had lung abscesses, and histopathological examination revealed acid-fast bacteria in the lesions. Although all these animals had acid-fast bacteria in common in their lesions, the bacterial species were not identified, because the bacteria were not cultured. PCR of paraffin sections of the lesions of the two different reptiles, however, revealed a band specific to *M. tuberculosis* complex when the IS1-2 (123 bp) primer was used, although no band was noted when the TB1-2 (320 bp) primer was used. In October 2003, when the Asian elephant died, the first of three prosimians

Table 1
Relative mycobacteria susceptibilities and spread

Group	Species 1	No. of bacilli in lesions[a]	Species 2	Susceptibility to infection with three types of tubercle bacilli[a]			Spread
				Bovine	Human	Avian	
1	Primitive humans #1	1		5	5	1	5
	Monkeys	2	Great apes	3	2	3	5
			Asian monkeys	5	5	2	
			African monkeys	4	4	2	
			South American monkeys	2	2	2	
	Guinea pigs	1		5	5	2	1
	Rabbits	2		1	5	4	1
	Mice	3		1	5	4	1
2	Modern humans #2	1		2	2	1	5
	Elephants	3		3	3	1	?
	Cattle	1		1	4	1	5
	Goats	1		1	4	2	1
	Pigs	1		2	4	2	1
3	Chickens	4		1	1	3	4
4	Horses, etc.	3		1	2	1	1
5A	Dogs	2		2	2	0	0
5B	Cats	3		1	4	2	1
	Ferrets	5		1	5	2	0
5C	Hamsters	4		5	5	1	0

The maximum value for each feature in this table is 5. The values for spread represent the degree of case with which tuberculosis spreads naturally between members of any one species. #1: aboriginal people, #2: contemporary human.

[a]The rating scale is as follows: 1, not likely; 2, rare, 3, occasional; 4, common; 5, classic [5,16].

(red ruffed lemurs [*Varecia variegata rubra*]) died of tuberculosis. The remaining two developed the disease in succession by May 2004, as a result of which one died and the other one was euthanized. In addition, four out of nine old-world monkeys (Abyssinian colobus monkeys [*Colobus guereza*]) and eight new-world monkeys (tufted capuchin monkeys [*Cebus apella*]), both of which shared part of the animal facility with the red ruffed lemurs, developed tuberculosis from January 2004 and died or had a positive tuberculosis skin test and thus were euthanized. The acid-fast bacilli isolated from each monkey were identified as *M. tuberculosis*, and were found to be of the same strain belonging to the Beijing family, which is prevalent in the Far East. Subsequently, four out of ten workers, including two veterinarians who performed necropsy on the monkeys, were found to be infected with tuberculosis

(QuantiFERON-TB® Gold positive), and one of the veterinarians developed the disease. The *M. tuberculosis* isolated from this patient was identical to the bacterium isolated from the monkeys.

The type of lesion and amount of bacteria in the lesion varied depending on the species of monkey. The prosimians, in particular, presented suppurative changes, including suppurative pneumonia, lung abscesses, cervical lymph node abscesses, and pyonephritis, with acid-fast bacteria forming a large mass in the lesion. The exudate from the lymph node abscesses on the body surface which self-destructed contained large amounts of bacteria, detected positive on smear.

To detect tuberculosis in live monkeys, a tuberculin skin test, culture for acid-fast bacteria using gastric lavage fluid and/or feces, and chest radiography examination are carried out.

Tuberculosis in monkeys probably arises from the following two situations. One is the case where imported monkeys that were already infected with tuberculosis develop the disease after being imported. In Japan, this case is represented by an orangutan imported from Indonesia [private communication] which had been taking care by Indonesian staff with tuberculosis. However, of the 10,462 laboratory monkeys from 10 consignments imported between 2000 and 2004, none were reported to be positive for tuberculin skin test [7]. In Japan, the import of pet monkeys was completely banned in June 2004, and for exhibition monkeys, tuberculosis testing is obligatory, and thus it is unlikely that imported animals will be the source of infection.

The other situation is where infection occurs within the confines of the country. In this case, animals are generally infected from a human spreader of tubercle bacilli. This manner of transmission seems to be more likely in Japan, a country of intermediate-level tuberculosis epidemic. The original source of infection could not be identified in either of the two institutions referred to, because there was no introduction of an animal that could be the source of infection, nor were there any tuberculous patients among the zoo staff.

3. Tuberculosis in elephants and other exhibition animals

In Japan, infection with *M. tuberculosis* was reported in Asian elephants and polar bears as early as 1962 [6,7] and in Malayan tapirs (*Tapirus indicus*) in 1991 [7] and 2004 (private communication).

We describe here tuberculosis in elephants, which very common, and a problem not only in the country of origin, but also in Europe and America [9–15]. Susceptibility to *M. tuberculosis* depends on the species of elephant. Asian elephants are more susceptible to *M. tuberculosis* (susceptibility score, 4) than African elephants (susceptibility score, 1) and their level of risk to humans is 4 [5,16]. In Sweden there was an outbreak of tuberculosis in Asian elephants, which became the source of infection in giraffes [10]. In the US, eight out of 379 elephants in one report died of tuberculosis [9]. There have also been cases in which handlers were infected with tuberculosis from Asian elephants [9,11]. Therefore, in the US, the culture and

PCR of trunk wash is officially carried out regularly to detect tuberculosis in elephants in captivity [15,17]. In one report 12 of 118 elephants (10.1%) were found positive for tubercle bacilli by culture of trunk wash samples [12], while another report stated that 3.3% of the elephants in captivity in North America have active disease [5]. The fact that Asian elephants have a much higher carrier rate of *M. tuberculosis* and a much higher incidence rate of the disease than African elephants is attributed to both greater susceptibility and greater risk of exposure to *M. tuberculosis* in Asian than in African elephants [5]. Asian countries originally have a high level of tuberculosis prevalence in the human population [1] and elephants are raised in close contact with humans, who are reservoirs. These factors are considered to result in the high infection rates in the elephant population. Particularly in Thailand, the number of tuberculous patients is increasing with increased incidence of HIV [1]. Therefore, a further increase in the infection rate among animals and particularly elephants is feared [18].

In Thailand, periodic tuberculosis screenings are performed on elephants, but positive elephants unlikely to receive treatment partly because of high medical costs [18]. In Japan, where about 120 elephants are in captivity, tuberculosis tests are not performed.

Tapirs are less susceptible to *M. tuberculosis* complex than elephants but are slightly more susceptible to *M. bovis* than to *M. tuberculosis* [1]. In Japan, *M. tuberculosis* infection occurred in four Malayan tapirs (*T. indicus*) spanning three generations within one pedigree (private communication).

4. Tuberculosis in pets

Public health risks from dogs and cats are classified as group 5, as shown in Table 1, because these animals are less susceptible to *M. tuberculosis* and, moreover, are not likely to be spreaders. Even dogs, which are more susceptible than cats, have a low incidence of tuberculosis [19]. Most cases of canine tuberculosis are transmitted by human reservoirs; dog-to-dog transmission is very rare [20]. Therefore, the incidence of canine tuberculosis is closely related to the incidence of human tuberculosis. In one study, *M. tuberculosis* was isolated from 75% of dogs with tuberculosis, and 88% or more of these dogs were known to have contact with patients with active tuberculosis [20]. The incidence is also higher in urban areas, where human patients are concentrated, than in the suburbs [19,21]. In addition, from the 1930s to the 1950s, in Europe and America, the incidence of canine tuberculosis was between 0.1% and 4.6% among dogs necropsied [20,21], but now there are hardly any cases of canine tuberculosis with a decrease in the number of human tuberculous patients. In Japan, only four cases have been reported, in 1954 [22]. In 2004, however, canine tuberculosis was reported in US [23] and we have presented a case that occurred in Japan (private communication).

The affected dog was a 3-year-and-8-month-old miniature dachshund. In April 2003, one of the owner's family developed tuberculosis, was isolated in a hospital, and was discharged in July after receiving treatment. As the dog developed a

respiratory symptom (wet cough) in December, it was brought to a veterinary clinic. Since it did not respond to treatment and a family member had open tuberculosis, the dog's pharyngeal swab and bronchial lavage fluid were cultured, and *M. tuberculosis* was isolated. The dog was euthanized and necropsied in January the following year. The RFLP patterns of bacteria isolated from the owner (Fig. 1), those isolated from the dog before its death, and those isolated from its organs collected during necropsy were completely identical. Considering the time course, it was thus concluded that the disease was transmitted from human to dog.

This case shows that although dogs are only weakly susceptible to *M. tuberculosis*, they may be infected if they come in close contact with a source of infection (e.g. human). The dog did not have any findings suggesting immunosuppression.

On the other hand, dogs can very rarely be the source of infection in humans. In the present case there is a possibility that the dog might have been the source of infection, because *M. tuberculosis* was also isolated from the dog's pharyngeal swab. Afterwards, the health of veterinary staff involved in the treatment or necropsy of the dog was investigated. It was found that the person who necropsied the dog had a strongly positive tuberculin reaction and a positive QuantiFERON test. These findings suggested infection with tuberculosis during necropsy. Extreme care must be exercised in the necropsy of animals infected with tuberculosis, as in the aforementioned cases of tuberculosis in monkeys.

5. *M. bovis* infection

M. bovis is an important species from the viewpoint of public health for the following reasons: it is the second most pathogenic mycobacterium, following *M. tuberculosis*; it has a wider host range and thus infects more varied animal species, including ruminants, its original host, as shown in Table 1; many of the animals it affects, which can become sources of infection, are in the human living environment [5,24].

Human infection with *M. bovis* is mostly caused by the intake of contaminated milk or dairy products. Transmission by direct contact or droplet transmission is also possible among high-risk people, such as veterinarians and animal keepers, who are in frequent contact with animals. Unlike *M. tuberculosis*, however, it is considered that *M. bovis* does not transmit easily from human to human or by air [5], expect in the case of carriers with lung lesions [25]. Therefore, the public health risks of *M. bovis* should be reduced if it is controlled sufficiently in affected animals such as cattle.

In Japan, dairy cattle receive a tuberculin skin test under a bovine tuberculosis eradication project established in 1901 and the Animal Infectious Diseases Control Law enacted in 1951, and cattle found positive are culled. The number of cases has been reduced to 0–2 per year since 2000, although there were as many as 100 or more cases per year in the 1980s, a relatively large-scale outbreak also involving deer occurred between 1992 and 1993, and an outbreak among beef cattle in 1999 (Table 2). *M. bovis* as such, however, was not detected in any of the culled

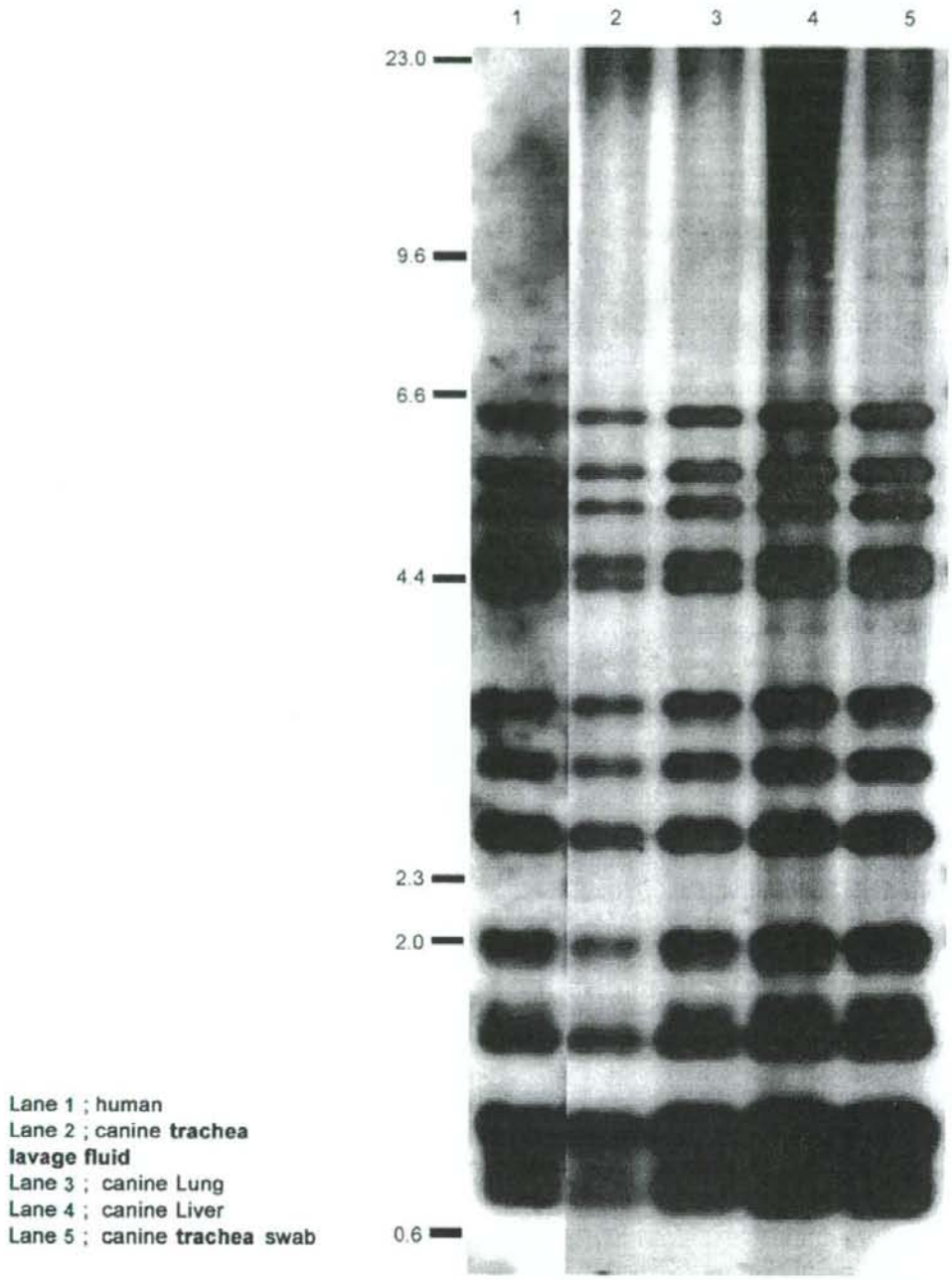

Fig. 1. *Mycobacterium tuberculosis* restriction fragment length polymorphism pattern using IS6110.

Table 2
Changes in population of cattle with positive TB skin test in Japan

Year	No.	Year	No.	Year	No.
1980	120	1989	35	1998	1
1981	121	1990	32	1999	37
1982	45	1991	33	2000	2
1983	35	1992	195	2001	0
1984	18	1993	203	2002	1
1985	32	1994	10	2003	1
1986	45	1995	9	2004	1
1987	89	1996	8	2005	1
1988	40	1997	2		

tuberculin-positive cattle, which are so-called reactors with no visible lesions, and thus they are very unlikely to transmit *M. bovis* to humans. There have been no reports of isolation of *M. bovis* from wild animals in Japan. Cases of *M. bovis* were reported between 1954 and 1976 in rhinoceroses, camels, giraffes, goats, and raccoon dogs in Japanese zoos [6,7], but there have been no such cases since then.

Japan, however, is one of the few countries that have been able to control *M. bovis*. Cases of human *M. bovis* infection are reported worldwide. In Asia, *M. bovis* infection has been occurring in Korea and Taiwan [1].

One of the reasons *M. bovis* is difficult to control in livestock even in developed countries is that wild animals are contaminated with this bacterial species. As described earlier, many animals are highly susceptible to *M. bovis* and thus are potential sources of infection. In fact, bovine *M. bovis* is transmitted from badgers in the UK, from wild pigs in Australia, and from opossums in New Zealand [24].

M. bovis is very well controlled in Japan. To maintain the high level of control of *M. bovis* in Japan the culling of tuberculin-positive cattle should be continued and the introduction of *M. bovis* infected animals from other countries which can contaminate wild animals with *M. bovis* should be prevented. In particular, in the case of imported animals, a certificate showing that the animal is free of *M. bovis* infection should be requested according to the Office International des Ēpizooties (OIE) guidelines.

6. Conclusions

Thus far, 700 or more different zoonoses have been identified. Among them, tuberculosis is especially important because of the large numbers of human patients and of animals susceptible to this disease. In Japan, tuberculosis in livestock is controlled by the Animal Infectious Diseases Control and has been virtually eradicated in dairy cattle. Therefore, there is a very low risk to humans. Human tuberculosis is controlled by the Tuberculosis Prevention Law, but Japan is still a

country of intermediate-level tuberculosis epidemic. Under the present circumstances, incidences of tuberculosis have occurred in pet animals and monkeys, causing a public health problem, yet neither of the relevant laws applies to these animal species. In Japan at present, revision of the Infectious Diseases Control Law is under discussion for more effective control of tuberculosis transmitted from animals.

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A NEW RHABDITOID NEMATODE SPECIES IN ASIAN SCIURIDS, DISTINCT FROM *STRONGYLOIDES ROBUSTUS* IN NORTH AMERICAN SCIURIDS

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ABSTRACT: *Strongyloides callosciureus* n. sp. (Nematoda: Rhabditoidea), from Asian sciurids, is described based on morphology, morphometry, and the small and large subunit (SSU/LSU) ribosomal RNA gene (rDNA) sequences. This new species was collected from Pallas's squirrels (*Callosciurus erythraeus*) in the central part of mainland Japan (Honshu), which were originally introduced from Taiwan some decades ago, and plantain squirrels (*Callosciurus notatus*) imported from Malaysia as personal pets. For comparison, *Strongyloides robustus* Chandler, 1942 was collected from American red squirrels (*Tamiasciurus hudsonicus*) and southern flying squirrels (*Glaucomys volans*) imported from the United States as personal pets. The parasitic females found in North American and Asian sciurids shared some key morphological features such as the ovary running spirally around the gut, and the shapes of the stoma in the apical view and the tail. However, morphometric features of parasitic females in North American and Asian sciurids differed significantly from each other; the former was larger than the latter, and the relative position of the vulva to the whole body length from the mouth was different. The SSU/LSU rDNA sequences supported the division of sciurid *Strongyloides* isolates by geographical distribution of the host and morphological features, leading us to propose the erection of new species.

Strongyloides robustus Chandler, 1942 has been recorded from diverse sciurid species (*Sciurus* spp., *Tamiasciurus* spp., *Glaucomys* spp., *Spermophilus* spp., and *Tamias* spp.) in North America (Bartlett, 1995), and is the only sciurid *Strongyloides* species described to date (Chandler, 1942; Speare, 1989; Bartlett, 1995). Although species of *Strongyloides* generally exhibit both homogonic and heterogonic development in their life cycles (Schad, 1989; Viney, 1994; Dorris et al., 2002), *S. robustus* is considered to be exceptional because it has no free-living generation. Specifically, it does not undergo a heterogonic development in which male and female adults appear (Bartlett, 1995). Because species of *Strongyloides* have only parthenogenetic females in the parasitic phase, *S. robustus* should be characterized mainly by the morphology of parasitic females.

Little information is available on the prevalence and morphology of *Strongyloides* species in Asian sciurids (Matsudate et al., 2003). However, *Strongyloides* sp. females were the prominent parasites found during a parasitological survey of feral Pallas's squirrels (*Callosciurus erythraeus*) introduced to the Japan (Honshu and Kyushu) some decades ago. Similarly, we found that *Strongyloides* sp. females were common in imported pet squirrels such as plantain squirrels (*Callosciurus notatus*) from Malaysia, and American red squirrels (*Tamiasciurus hudsonicus*) and southern flying squirrels (*Glaucomys volans*) from the United States.

In the present study, we characterized the morphology and the small and large subunit (SSU/LSU) ribosomal RNA gene (rDNA) sequences of these sciurid *Strongyloides* sp. nematodes, and erected a new species for isolates of Asian sciurid origin.

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MATERIALS AND METHODS

Examined animals

Introduced Pallas's squirrels were collected at Hamamatsu City (34°42′–34°43′N, 137°40′–137°42′E; 34 females and 54 males) and Izu peninsula (34°40′–34°55′N, 139°00′–139°10′E; 16 females and 6 males) in the central part of mainland Japan (Honshu) with permission from the Shizuoka prefectural office (Table I). These squirrels were originally introduced from Taiwan as zoo or park squirrels in the 1930s–1990s, then became feral and expanded their geographic ranges, particularly in the central part of mainland Japan (Tamura et al., 1989; Tamura, 1999; Tamura and Miyamoto, 2005). Pet squirrels were purchased from 4 pet animal dealers for a cooperative survey of zoonotic (viral, bacterial, fungal, protozoal, and helminthic) agents in imported pet rodents, and necropsied in the School of Veterinary Medicine, Azabu University, within several days or a few weeks after importation. These included 9 species of Sciuridae, with 264 in total number as shown in Table I.

Parasitological examination

Frozen or raw digestive tracts (from the stomach to the rectum) of feral Pallas's squirrels were sent to the parasitology laboratory, opened longitudinally, and immersed in physiological saline. After leaving the materials for a few hours, the mucosa was carefully scraped. Then, these materials were washed repeatedly by simple sedimentation in physiological saline. Sediments were checked thoroughly using a dissection microscope, and the collected parasites were fixed in 10% neutral-buffered formalin. Rectal feces of imported pet squirrels were transported in cool conditions (4 C) to the parasitology laboratory, and a direct wet smear of each fecal sample was examined. When *Strongyloides* sp. eggs were found in the feces, formalin-fixed intestines of specified pet squirrels were examined to collect adult parasites. Cecal and rectal contents of Pallas's squirrels, plantain squirrels, American red squirrels, and southern flying squirrels were cultured to collect filariform larvae using the petri-dish fecal culture method with an unglazed tile.

Morphological examination

Morphological examinations were performed using light microscopy according to the method established by Little (1966a, 1966b) and Speare (1989), and figures were drawn with the aid of a camera lucida. Measurements were made using these figures. For scanning electron microscopy (SEM), nematodes preserved in 10% neutral-buffered formalin were immersed in 2.5% glutaraldehyde in 0.2M Na_2HPO_4–NaH_2PO_4-buffered solution (PB), pH 7.8, overnight, washed 3 times in PB, and postfixed in 1% (weight/volume) osmium tetroxide in PB for 1 hr. After washing 3 times in PB, specimens were dehydrated through a graded ethanol series, immersed in warmed *t*-butyl-alcohol, and cooled at 4 C for 2 hr. Then, specimens were freeze-dried, mounted on stubs, sputter-coated with gold–palladium at 300 Å, and examined using a SEM (Model JSM-6100; JEOL) at an accelerating voltage of 10 kV.

TABLE I. Squirrels examined in the present study, along with the prevalence and abundance of *Strongyloides* adults.

Squirrel host	Place of origin	Date of necropsy	Animal dealer	Number of animals*	Prevalence*	Intensity†	Isolate name
(1) Feral alien squirrels							
Pallas's squirrels (*Callosciurus erythraeus*)	Hamamatsu, Japan	16 February 2004 to 25 December 2004	—	34F 54M	50% (17F) 63% (34M)	1–9 (2.0)‡ 1–201 (6.6)‡	HAMA
	Izu peninsula, Japan	26 January 2005 to 20 March 2005	—	16F 6M	100% (16F) 100% (6M)	2–83 (11.6)‡ 28–86 (51.1)‡	IZU
(2) Imported pet squirrels							
Plantain squirrels (*Callosciurus notatus*)	Malaysia	12 July 2004 4 June 2005	I I	20 (12F; 8M) 10 (5F; 5M)	53%	1–10 (3.6)§	BR
Eurasian red squirrels (*Sciurus vulgaris*)	China	1 June 2003 2 July 2005	O O	10 (8F; 2M) 10 (10M)	0%	—	—
Columbian ground squirrels (*Spermophilus columbianus*)	United States	1 June 2003 27 June 2004 2 July 2005	I I I	10 (5F; 5M) 10 (6F; 4M) 10 (1F; 9M)	0%	—	—
Richardson's ground squirrels (*Spermophilus richardsonii*)	United States	25 May 2003 15 June 2003 27 June 2004 4 June 2005	I I I I	10 (4F; 6M) 10 (4F; 6M) 10 (6F; 4M) 10 (3F; 7M)	0%	—	—
Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*)	United States	14 July 2003	I	10 (8F; 2M)	0%	—	—
Siberian chipmunks (*Tamias sibiricus*)	China	15 June 2003 14 July 2003 27 June 2004 16 April 2005 15 April 2006 10 June 2006	I R I R A A	10 (10M) 10 (6F; 4M) 19 (9F; 10M) 10 (4F; 6M) 20 (9F; 11M) 10 (8F; 2M)	0%	—	—
American red squirrels (*Tamiasciurus hudsonicus*)	United States	27 June 2004	I	19 (10F; 9M)	79%	1–11 (3.5)§	AA
Southern flying squirrels (*Glaucomys volans*)	United States	16 April 2005	R	10 (10M)	90%	1–76 (12.2)§	AM
Russian flying squirrels (*Pteromys volans*)	China	15 June 2003 12 July 2003 2 July 2005	I R O	10 (6F; 4M) 6 (6M) 10 (1F; 9M)	0%	—	—

* F, female; M, male.
† Range (geomean).
‡ Statistically significant differences in worm recovery between female and male squirrels in either Hamamatsu City or Izu peninsula.
§ Worm recovery was successful in only some of the squirrels with positive fecal test for eggs, because parts of the intestine had been used for histological examinations. Therefore, this value should be referred to as minimum numbers of detectable worms.

Experimental infection

Inbred BALB/c mice (7- to 8-mo-old), and Mongolian jirds (*Meriones unguiculatus*) (5- to 7-wk-old) were bred in the Institute for Animal Experiments, Hirosaki University School of Medicine. All animal experiments were performed according to the Guidelines on Animal Experimentation as set out by Hirosaki University. An appropriate number of filariform larvae suspended in water were injected subcutaneously into the mice and jirds. Some mice were immunosuppressed by subcutaneous injection with 0.25 ml of Depo-Medrol® sterile aqueous suspension (5 mg of methylprednisolone acetate; Pfizer Japan, Yoyogi, Tokyo) at the time of infection. A direct wet smear of each rodent was examined daily after 1 wk PI to determine the prepatent period. After being killed and necropsied, the small intestines of mice and jirds were examined for recovery of parasitic females as described above. Collected parasites were fixed in 70% alcohol or 10% neutral-buffered formalin. Simultaneously, cecal contents and colonic and rectal feces were cultured separately on unglazed tiles in petri dishes containing water for the recovery of filariform larvae as described above. Because plantain squirrels and American red squirrels were frequently infected with *Brevistriata* sp. (prevalence, 12/30; intensity, at least 1–44, with a mean of 4.2), and *Citellinema* sp. (16/19; at least 1–159, with a mean of 12.4), respectively, fecal culture from experimentally infected jirds eliminated their contamination, facilitating pure extraction of *Strongyloides* sp. DNA.

Furthermore, to determine essential morphometric differences of parasitic females of North American and Asian isolates, approximately 350 filariform larvae of each isolate were injected s.c. into 2 Mongolian jirds (11-wk-old), and killed at 27 day PI. Parasitic females collected from the small intestine were fixed in 10% neutral-buffered formalin and measured as described above.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Molecular genetic characterization of SSU/LSU rDNA was conducted using pooled filariform larvae obtained from the culture of cecal contents and intestinal feces from infected jirds. DNA extraction, PCR, and nucleotide sequencing were performed as described in our previous work (Sato et al., 2006). Direct sequencing was made for most PCR amplicons. Exceptionally, amplicons including internal transcribed spacer (ITS) regions were inserted into the plasmid vector pCR®2.1 (Invitrogen Co., Carlsbad, California), and transformed into *Escherichia coli* TOP10 (Invitrogen) according to the instructions of the manufacturer. Following propagation and plasmid purification, inserts (from 3 independent clones per amplicon) were sequenced using the vector universal primers M13 reverse and forward primers. Sequences were assembled manually with aid of the CLUSTRAL W multiple alignment program (Thompson et al., 1994). The SSU/LSU rDNA construction of the sciurid *Strongyloides* sp. isolates was determined following Sato et

TABLE II. Specimen information and DDBJ/EMBL/GenBank accession numbers for *Strongyloides* spp. and related nematodes used in phylogenetic analysis.

Species	Isolate	Animal source	Locality of collection	Accession number	Reference
(1) 18S rDNA					
Strongyloides callosciureus n. sp.	HAMA	Pallas's squirrels (*Callosciurus erythraeus*)?*	Japan (feral, but alien squirrel)	AB272229	The present study
	IZU	Pallas's squirrels (*Callosciurus erythraeus*)	Japan (feral, but alien squirrel)	AB272230	The present study
	BR	Plantain squirrels (*Callosciurus notatus*)	Japan (imported as pets from Malaysia)	AB272231	The present study
Strongyloides robustus	AM	Southern flying squirrels (*Glaucomys volans*)	Japan (imported as pets from U.S.A.)	AB272232	The present study
	AA	American red squirrels (*Tamiasciurus hudsonicus*)	Japan (imported as pets from U.S.A.)	AB272233	The present study
Strongyloides stercoralis		Dog	Laboratory isolate in U.S.A.	AF279916	Dorris et al. (2002)
		?	?	AJ417023	Dorris et al. (2002)
Strongyloides procyonis		Raccoon (*Procyon lotor*)	Japan (feral, but alien mammal)	AB205054	Sato et al. (2006)
		Japanese badger (*Meles meles anakuma*)	Japan	AB272234	Our unpublished data
Strongyloides ratti		?	?	U81581	—
Strongyloides fuelleborni fuelleborni		Japanese monkey (*Macaca fuscata fuscata*) in captivity	Japan	AB272235	Our unpublished data
Strongyloides cebus		Squirrel monkeys (*Saimiri sciureus*) in captivity	Japan	AB272236	Our unpublished data
Parastrongyloides trichosuri		Common brushtail possum (*Trichosurus vulpecula*)	Australia	AJ417024	Dorris et al. (2002)
Rhabditophanes sp. KR3021		Free-living	Canada (Vancouver Island)	AF202151	Dorris et al. (2002)
(2) 28S rDNA					
Strongyloides callosciureus n. sp.	HAMA	See above	See above	AB272229	The present study
	IZU	See above	See above	AB272230	The present study
	BR	See above	See above	AB272231	The present study
Strongyloides robustus	AM	See above	See above	AB272232	The present study
	AA	See above	See above	AB272233	The present study
Strongyloides stercoralis	Ssdog4	Dog	?	SSU39489	—
	Ssh1	Human	Southeast Asia	SSU38855	—
	AL3	?	?	AY294186	—
	AE-894	Human	Probably Asia	DQ145661	Nadler et al. (2006)
Strongyloides procyonis		Raccoon (*Procyon lotor*)	Japan (feral, but alien mammal)	AB205054	Sato et al. (2006)
Strongyloides ratti	SR3	?	?	SRU39490	—
Strongyloides fuelleborni		See above	See above	AB272235	Our unpublished data
	Sf7	Rhesus monkey (*Macaca mulatta*) in captivity	?	U42595	—
Rhabditophanes sp. KR3021		Free-living	Canada (Vancouver Island)	DQ145655	Nadler et al. (2006)

* After Oshida et al. (2007), it is uncertain at present whether the host for this isolate was Pallas's squirrels or Finlayson's squirrels (*Callosciurus finlaysonii*). (See Discussion section of the present study).

al. (2006). The newly obtained nucleotide sequence of rDNA from sciurid *Strongyloides* sp. isolates was deposited in DDBJ/EMBL/GenBank (accession numbers AB272229–AB272233).

Phylogenetic analysis

For phylogenetic analyses, newly obtained 18S and 28S rDNA sequences of 5 sciurid *Strongyloides* sp. isolates and related species obtained from DDBJ/EMBL/GenBank (Table II) were aligned using the CLUSTRAL W multiple alignment program, with subsequent manual adjustment. For each alignment, regions considered to be poorly aligned and characters with a gap in any sequence were excluded from subsequent analyses. Three alignments were constructed for rDNA analyses: (1) for 18S rDNA, 1097- to 1120-bp-long 18S rDNA sequences of 14 isolates/species, corresponding to the *Strongyloides procyonis* 18S sequence (DDBJ/EMBL/GenBank AB205054) between the 119th and 1,238th base positions were initially aligned. Bases at positions 221, 222, 253, 254, 478 479, 614, 624, 642-646, 654–661, 669–675, 691,

731–733, 774–777, 791, 792, 1,129, and 1,130 were judged to be poorly aligned, then were excluded; 1,081 characters, of which 39 (19 among *Strongyloides* spp., and 4 among sciurid *Strongyloides* sp. isolates) were parsimony-informative were included in subsequent analyses; (2) for 28S rDNA, 788- to 827-bp-long 28S rDNA sequences of 14 isolates/species, corresponding to the *S. procyonis* 28S sequence (DDBJ/EMBL/GenBank AB205054) between the 82nd and 900th base positions were initially aligned. Bases at positions 124, 125, 183–185, 241, 451, 452, 490, 491, 516, 520, 531, 533, 537, 567–590, 598–646, 804–815, 846, and 847 were judged to be poorly aligned, then were excluded; 716 characters, of which 64 (58 among *Strongyloides* spp., and 10 among sciurid *Strongyloides* sp. isolates) were parsimony-informative were included in subsequent analyses; and (3) sequences of 18S and 28S rDNA of each species were concatenated to increase the informative characters for phylogenetic analyses. Phylogenetic and molecular evolutionary analyses of aforementioned alignments (1)–(3) were conducted using neighbor-joining (NJ), minimum evolution (ME), and maximum parsimony (MP) methods as implemented in the program MEGA, version 3.1 (Kumar et al., 2004). For all 3 methods, 1,000 bootstrap replicates were calculated.

Statistical analysis

Differences between 2 groups were examined for significance using Student's *t*-test. A P value of less than 0.05 denoted statistical significance.

RESULTS

Prevalence and morphology

In feral Pallas's squirrels from Hamamatsu and Izu, *Strongyloides* sp. nematodes were highly prevalent (Table I). The mean numbers of collected worms from female and male squirrels were significantly different; male squirrels had more parasites than females, regardless of the month that hosts were trapped in. *Strongyloides* sp. eggs were found in 16 of 30 plantain squirrels, 15 of 19 American red squirrels, and 9 of 10 southern flying squirrels, whereas other imported pet squirrels were negative for this nematode.

Strongyloides sp. females from Pallas's squirrels (named HAMA and IZU isolates after their collection sites), plantain squirrels (BR isolate after Japanese host name), American red squirrels (similarly, AA isolate), and southern flying squirrels (similarly, AM isolate) had cephalic extremity with an indented, X-shaped mouth, ovaries running spirally around the gut, and bluntly rounded tails (Figs. 1, 2). SEM examinations of the anterior end of sciurid *Strongyloides* sp. parasitic females disclosed for the first time a unique morphological arrangement and the presence of 4 linguiform projections connecting the oral rim with the oral base (Fig. 3). This morphological character was seen in both North American and Asian isolates of sciurid *Strongyloides* sp. females, although these 2 had different body sizes. Comparisons of actual and relative lengths of different features of these *Strongyloides* sp. isolates from the natural hosts and experimental hosts are summarized in Tables III and

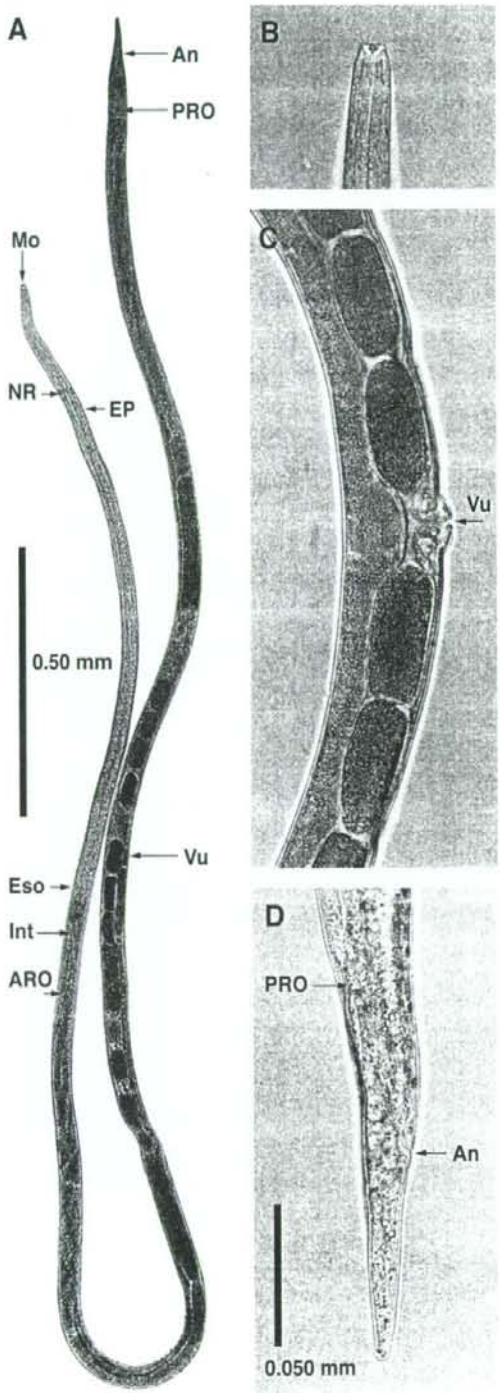

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FIGURE 1. Parasitic female of *Strongyloides callosciureus* n. sp. of Asian sciurid origin (HAMA isolate). (**A**) Whole view, (**B**) lateral view of the anterior end, (**C**) lateral view of the vulva, and (**D**) lateral view of the posterior end). Photographs B–D are at the same magnification. Abbreviations: An, anus; ARO, anterior reflexion of the ovary; EP, excretory pore; Eso, posterior end of esophagus, connecting to the intestine; Int, intestine; Mo, mouth; NR, nerve ring; PRO, posterior reflexion of the ovary; and Vu, vulva.

TABLE III. Comparison of actual and relative lengths of different features of *Strongyloides* isolates from American and Asian squirrels.*

Specimen group:	A	B	C (AA isolate)	D (AM isolate)	C vs. D†
Parasite species:	*Strongyloides robustus*	*Strongyloides robustus*	*Strongyloides robustus*	*Strongyloides robustus*	
Number of examined worms:	(n = 11)	(n = 9)	(n = 8)	(n = 8)	
Host:	*Tamiasciurus hudsonicus*	*Tamiasciurus hudsonicus*	*Tamiasciurus hudsonicus*	*Glaucomys volans*	
Reference:	Bartlett, 1995	Bartlett, 1995	The present study	The present study	
Body length	5.5–6.5 (6.0)	6.4–8.0 (7.3)	5.3–6.5 (6.0)	7.2–8.5 (7.6)	SSD
Body width at end of esophagus	0.052–0.058 (0.056)	0.056–0.062 (0.058)	0.056–0.074 (0.062)	0.049–0.064 (0.059)	
Body width at vulva	0.056–0.064 (0.061)	0.056–0.065 (0.062)	0.056–0.071 (0.065)	0.060–0.073 (0.066)	
Esophagus, length	1.14–1.40 (1.29)	1.45–1.70 (1.56)	0.99–1.31 (1.23)	1.09–1.35 (1.21)	
Esophagus, % of total body length	18.1–23.3 (21.5)	18.8–25.0 (21.4)	18.9–22.6 (20.5)	14.9–17.1 (16.0)	(Distinct)
Nerve ring, from anterior end	—	—	0.186–0.231 (0.210)	0.202–0.283 (0.250)	SSD
Excretory pore, from anterior end	—	—	0.223–0.289 (0.255)	0.217–0.305 (0.276)	
Vulva, from anterior end	3.3–3.9 (3.6)	3.7–5.0 (4.5)	3.3–4.0 (3.7)	4.1–5.0 (4.6)	SSD
Vulva, % of body length from anterior end	58.3–63.6 (60.0)	57.8–64.8 (61.6)	60.0–63.9 (61.6)	55.7–66.5 (60.5)	
Extent of anterior branch of ovary‡	0.168–0.348 (0.238)	0.132–0.348 (0.244)	0.143–0.263 (0.190)	0.165–0.415 (0.321)	SSD
Extent of posterior branch of ovary§	0.160–0.428 (0.279)	0.160–0.384 (0.279)	0.280–0.337 (0.314)	0.146–0.426 (0.254)	
Tail length	0.090–0.116 (0.101)	0.088–0.110 (0.096)	0.097–0.131 (0.111)	0.106–0.126 (0.117)	
Egg size	0.045–0.072 (0.058; n = 15) by 0.033–0.042 (0.036)		0.050–0.064 (0.055 ± 0.004; n = 25) by 0.029–0.033 (0.031 ± 0.001)		

* Values are expressed as range with mean or mean ± SD in parentheses; —, no data.
† SSD, statistically significant difference; blank cell, no statistically significant difference; (distinct), no overlap of range values between 2 groups.
‡ Distance from the posterior end of the esophagus.
§ Distance from the anus.

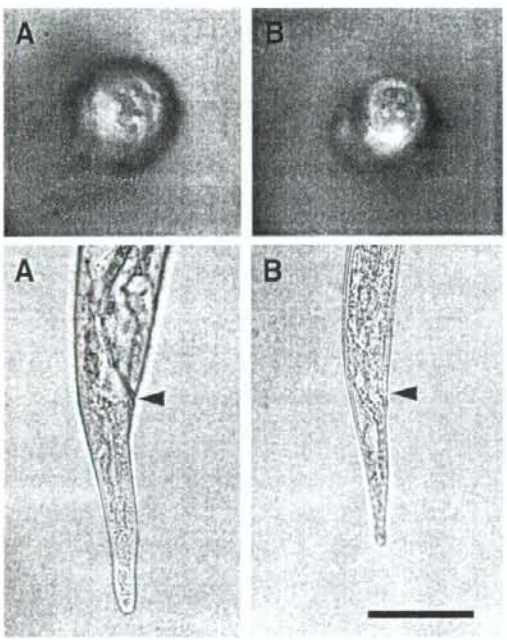

FIGURE 2. En face view (upper photographs) and tail (lower photographs) of *Strongyloides robustus* of North American sciurid origin (**A**; AA isolate), and *Strongyloides callosciureus* n. sp. of Asian sciurid origin (**B**; HAMA isolate). To image en face view of *S. robustus* precisely, refer to a drawing by Bartlett (1995) (Fig. 3d in that article). Scale bar = 0.020 mm for top photographs; and 0.050 mm for bottom photographs. Arrowheads indicate the anus.

IV, respectively. Actual measurements of body width and tail length, as well as the relative position of the vulva to the whole body length, could divide sciurid *Strongyloides* sp. isolates into 2 groups, i.e., isolates of North American origin and those of Asian origin.

Culture and infectivity to laboratory rodents

Culture of cecal contents and/or feces from Pallas's squirrels, plantain squirrels, American red squirrels, and southern flying squirrels successfully produced filariform larvae. Emergence of these larvae in the water continued for approximately 1 mo,

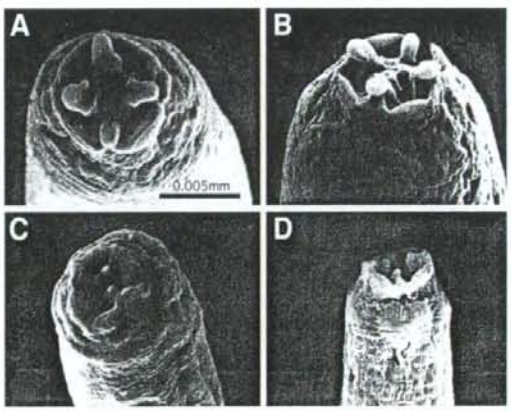

FIGURE 3. Scanning electron microscopic views of the anterior end of *Strongyloides* sp. isolates (**A**, AA isolate; **B**, AM isolate; **C**, BR isolate; and **D**, IZU isolate). Note the presence of 4 linguiform projections connecting the oral rim with the oral base in all isolates. All micrographs are at the same magnification.