TABLE III. Extended.

| E (BR isolate) *Strongyloides callosciureus* n. sp. (n = 4) *Callosciurus notatus* The present study | C vs. D† | F (HAMA isolate) *Strongyloides callosciureus* n. sp. (n = 6) *Callosciurus erythraeus* The present study | C vs. F† | E vs. F† | G (IZU isolate) *Strongyloides callosciureus* n. sp. (n = 8) *Callosciurus erythraeus* The present study | C vs. G† | E vs. G† | F vs. G† |
|---|---|---|---|---|---|---|---|---|
| 5.0–6.2 (5.8) | | 3.4–6.0 (4.2) | SSD | SSD | 4.8–6.3 (5.6) | SSD | | SSD |
| 0.046–0.049 (0.047) | SSD | 0.034–0.054 (0.041) | SSD | | 0.038–0.042 (0.040) | SSD | SSD | |
| 0.051–0.057 (0.055) | SSD | 0.040–0.066 (0.051) | SSD | | 0.043–0.048 (0.045) | SSD | SSD | |
| 0.89–1.12 (0.99) | SSD | 0.80–1.14 (0.94) | SSD | | 0.84–1.09 (0.96) | SSD | | |
| 14.9–18.5 (17.0) | (Distinct) | 19.1–25.9 (22.9) | | (Distinct) | 15.1–22.5 (17.1) | (Distinct) | | (Distinct) |
| 0.131–0.211 (0.184) | | 0.189–0.211 (0.199) | | | 0.160–0.204 (0.189) | SSD | | |
| 0.149–0.286 (0.226) | | 0.209–0.254 (0.235) | | | 0.193–0.244 (0.223) | SSD | | |
| 3.4–4.1 (3.9) | | 2.3–4.1 (2.9) | SSD | SSD | 3.3–4.4 (3.9) | | | SSD |
| 65.1–67.9 (67.0) | (Distinct) | 67.2–69.5 (68.5) | (Distinct) | | 66.5–71.2 (68.5) | (Distinct) | | |
| 0.040–0.160 (0.086) | SSD | 0.074–0.231 (0.153) | | | 0.080–0.308 (0.162) | | | |
| 0.131–0.234 (0.171) | SSD | 0.106–0.189 (0.161) | SSD | | 0.042–0.139 (0.086) | SSD | SSD | SSD |
| 0.080–0.097 (0.089) | SSD | 0.057–0.103 (0.077) | SSD | | 0.060–0.095 (0.074) | SSD | SSD | |
| 0.048–0.062 (0.056 ± 0.004; n = 16) by 0.027–0.038 (0.032 ± 0.003) | | 0.050–0.066 (0.056 ± 0.004; n = 24) by 0.028–0.036 (0.032 ± 0.002) | | | — | | | |

although a free-living generation could not be found in these cultures. Mongolian jirds inoculated subcutaneously with filariform larvae (200–320 in number) of each isolate became patent at 10 day PI, and up to 25 parasitic females were collected from them when animals were killed at approximately 1 mo PI. Four female BALB/c mice were treated with prednisolone at infection with 320 filariform larvae of the HAMA isolate, and 4 other female mice and 5 male mice were kept as nontreated, infected controls. A single parasitic female was recovered from a prednisolone-treated mouse at 13 days PI, whereas no parasites were recovered from the other 12 mice. After these observations, jirds were used to maintain the parasites in the laboratory and prepare fecal cultures to produce filariform larvae. Egg sizes of all *Strongyloides* sp. isolates were identical, when direct fecal smears of infected jirds at 17 day PI were analyzed (Table III). Fecal cultures from 1 jird infected with the IZU isolate and 3 jirds infected with the AM isolate, killed at 194 and175 days PI, respectively, produced filariform larvae in all examined samples, demonstrating an extraordinarily persistent infection of jirds with sciurid *Strongyloides* sp. isolates.

### rDNA sequences and phylogenetic analyses

The SSU/LSU rDNA construction of *Strongyloides* sp. isolates from North American and Asian sciurids were as follows: 1,732-bp and 1,728/1,729-bp 18S rDNA; 162/163-bp and 142–145-bp ITS1; 155-bp 5.8S for all; 185-bp and 180/181-bp ITS2; and more than 3,257-bp and 3,248–3,250-bp 28S rDNA, respectively (Table V). The identity of available whole-length sequences between the 2 isolates (AM and AA) from North America sciurids was 99.8%, that between the 2 isolates (HAMA and IZU) from Pallas's squirrels was 99.7%, and that

TABLE IV. Comparison of actual and relative lengths of different features of sciurid *Strongyloides* isolates in Mongolian jirds.*

| Isolate name: | AM | | IZU | | Statistics† |
|---|---|---|---|---|---|
| Parasite species: | *Strongyloides robustus* | | *Strongyloides callosciureus* n. sp. | | |
| Number of examined worms: | (n = 6) | | (n = 6) | | |
| Original host: | *Tamiasciurus hudsonicus* | | *Callosciurus notatus* | | |
| Body length | 5.7 ± 0.34 | (5.2–6.1) | 4.7 ± 0.23 | (4.3–4.9) | SSD |
| Body width at end of esophagus | 0.047 ± 0.006 | (0.039–0.055) | 0.039 ± 0.004 | (0.035–0.045) | SSD |
| Body width at vulva | 0.055 ± 0.004 | (0.050–0.060) | 0.045 ± 0.005 | (0.038–0.050) | SSD |
| Esophagus, length | 1.04 ± 0.07 | (0.92–1.12) | 0.95 ± 0.10 | (0.79–1.03) | |
| Esophagus, % of total body length | 18.5 ± 1.6 | (16.8–21.2) | 20.4 ± 2.7 | (16.6–24.2) | |
| Vulva, from anterior end | 3.5 ± 0.2 | (3.2–3.8) | 3.2 ± 0.2 | (2.9–3.4) | SSD |
| Vulva, % of body length from anterior end | 62.5 ± 0.6 | (61.8–63.6) | 69.2 ± 1.2 | (67.4–70.9) | (Distinct) |
| Extent of anterior branch of ovary‡ | 0.32 ± 0.11 | (0.19–0.45) | 0.15 ± 0.06 | (0.04–0.21) | SSD |
| Extent of posterior branch of ovary§ | 0.22 ± 0.04 | (0.16–0.26) | 0.11 ± 0.05 | (0.03–0.17) | SSD |
| Tail length | 0.112 ± 0.018 | (0.088–0.138) | 0.079 ± 0.013 | (0.066–0.102) | SSD |

* Worm recovery from 2 each of Mongolian jirds were 28 and 11 parasitic females for AM isolate and 9 and 3 for IZU isolate. Values are expressed as mean ± SD, with range in parentheses.
† 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.

between Pallas's squirrels (HAMA or IZU) and plantain squirrels (BR) was 99.2–99.3%. The identity of available whole-length sequences between the North American and Asian sciurid isolates ranged from 98.0 to 98.4%. The bases at several positions in 18S, ITS1, ITS2, and 28S rDNA were different between isolates of American and Asian sciurid origin (Table V), although the number of examined isolates was limited, and the bases at multiple positions were variable regardless of the geographical origin of isolates.

Phylogenetic trees of either 18S rDNA or 28S rDNA sequences, containing 1,081 and 716 characters, respectively, showed different topologies for some *Strongyloides* spp., including sciurid isolates regardless of the method of analysis, i.e., NJ, ME, or MP methods (data not shown). To see more precisely the molecular relationship of sciurid *Strongyloides* sp. isolates, concatenated sequences of 18S and 28S rDNA for each isolate or species were analyzed again by 3 methods described above. The resulting phylogenetic trees by the 3 methods were identical, separating Asian sciurid isolates (HAMA, IZU, and BR) from American sciurid isolates (AA and AM) with 100% bootstrap support (Fig. 4). Results of phylogenetic analyses indicated that, in addition to morphological separation, *Strongyloides* sp. isolates of North American and Asian sciurid origins could be distinct species, even if they were closely related with each other.

## DESCRIPTION

### *Strongyloides callosciureus* n. sp.

(Figs. 1, 2B, 3C, 3D)

*General (measurements of holotype described here, and those of paratypes shown in Table III):* Slender nematode, gradually tapering at anterior region, and abruptly tapering at tail (Fig. 1); 6.25 mm in length, and 0.039 mm and 0.048 mm in width at end of esophagus and vulva, respectively. Body wall thin, cuticle finely striated. Circumoral elevation without lobes. Stoma shallow, and slightly indented X-shaped in the apical view (Figs. 2B, 3C, 3D). Muscular esophagus 0.159 mm in length and glandular esophagus 0.78 mm in length; total length of esophagus corresponding to 15% of total body length. Nerve ring and excretory pore at 0.185 mm and 0.227 mm from mouth. Vulva at 4.05 mm from anterior end, corresponding to 64.7 % along body length from mouth. Lips of vulva were prominent. Both anterior and posterior branches of ovaries were spiraling around the intestine; anterior with two-and-a-half and posterior with 2 spirals. Extent of anterior branch of ovary 0.115 mm from posterior end of esophagus, and that of posterior branch of ovary 0.039 mm from anus. Uteri containing eggs in single row. Tail digitiform and bluntly rounded, 0.070 mm in length. Eggs, 0.054–0.066 mm (0.056 mm average) by 0.027–0.038 mm (0.032 mm average), containing tadpole-stage embryos in freshly passed fecal pellets of experimentally infected jirds.

#### Taxonomic summary

*Type host: Callosciurus erythraeus* (Pallas, 1779).

*Other hosts: Callosciurus notatus* (Boddaert, 1785), and probably *Callosciurus finlaysonii* (Horsfield, 1824) (see below).

*Site of infection:* Duodenum.

*Prevalence:* 100%.

*Intensity:* 2–86.

*Type locality:* Izu peninsula, Shizuoka, Japan (34°40′–34°55′N, 139°00′–139°10′E). These squirrels were originally introduced from Taiwan several decades ago (Tamura and Miyamoto, 2005).

*Specimens deposited:* The National Science Museum, Tokyo: holotype and paratypes from the identical host (NSMT-As 3033, IZU isolate); other paratypes (NSMT-As 3034–3053, IZU isolate; NSMT-As 3054–3059; HAMA isolate; and NSMT-As 3060–3066, BR isolate); and referring specimens of *S. robustus* (NSMT-As 3067–3071, AA isolate; and NSMT-As 3072–3078, AM isolate).

TABLE V. Nucleotide differences in the rDNA of *Strongyloides* isolates from North American (AM and AA) and Asian (HAMA, IZU and BR) sciurids.

| Name of isolate | Number of base pairs included* | Position of base change† | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 177 | 182 | 190 | 216 | 219 | 221 | 222 | 243 | 1,294 | 1,302 | 1,303 | 1,306 | 1,308 | 1,624 | 1,627 | 1,630 | 1,643 | | | | | | | |
| 1) 18S | | C | G | A | A | A | A | T | C | A | A | T | A | T | C | A | A | T | | | | | | | |
| AM | 1,732 | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | | | | | | | |
| AA | (1,616) | • | • | • | • | — | T | • | • | G | — | • | • | — | • | G | G | C | | | | | | | |
| HAMA | (1,642) | T | • | C | T | — | T | • | • | G | — | • | • | — | • | G | G | C | | | | | | | |
| IZU | 1,728 | T | A | • | T | — | • | A | T | G | — | • | • | — | • | G | G | C | | | | | | | |
| BR | 1,729 | T | • | C | T | — | T | • | • | • | — | — | T | T | T | G | G | C | | | | | | | |
| 2) ITS1 | | (9/10) | 17 | 21 | 22 | 23 | 26 | 27 | 28 | 29 | 65 | 73 | 88 | 99 | 100 | 123 | 131 | 132 | 133 | 134 | | | | | |
| AM | 162 | — | C | T | A | A | T | T | T | T | T | A | G | C | T | A | T | T | T | T | | | | | |
| AA | 163 | • | • | • | • | • | • | • | • | • | • | • | • | C/T | • | • | • | • | • | • | | | | | |
| HAMA | 145 | • | A | — | — | • | • | C | A | C | — | C | A | T | A | T | A | C | C | C | | | | | |
| IZU | 142 | • | A | — | — | • | — | C | A | • | — | C | A | T | • | T | — | • | • | • | | | | | |
| BR | 143 | T | A | — | — | — | C | A | C | • | — | C | A | T | • | T | — | • | • | • | | | | | |
| | | 137 | 141 | 142 | 143 | 144 | 145 | 146 | 147 | 148 | 149 | 150 | 151 | 152 | 153 | 154 | 155 | 156 | 157 | | | | | |
| AM | | T | T | T | T | T | A | A | A | A | T | A | A | A | A | A | A | T | T | | | | | | |
| AA | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | | | | | | |
| HAMA | | A | • | — | — | — | • | • | — | — | — | — | — | — | — | — | — | — | — | | | | | | |
| IZU | | A | • | • | — | — | • | • | — | — | — | — | — | — | — | — | — | — | — | | | | | | |
| BR | | A | C | • | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | | | | | | |

TABLE V. Continued.

| Name of isolate | Number of base pairs included* | Position of base change† | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3) 5.8S | | | | | | | | | | | | | | | | | | | | |
| AM | 155 | | | | | | | | | | | | | | | | | | | |
| AA | (23) | | | | | | | | | | | | | | | | | | | |
| HAMA | 155 | | | | | | | | | | | | | | | | | | | |
| IZU | 155 | | | | | | | | | | | | | | | | | | | |
| BR | 155 | | | | | | | | | | | | | | | | | | | |
| 4) ITS2 | | **28** | 30 | 31 | 32 | 33 | **40** | 41 | 51 | 58 | (66/67) | **67** | 80 | **(92/93)** | **97** | 134 | **140** | **141** | 155 | | |
| AM | 185 | **T** | T | T | T | A | **A/T** | T | A | G | — | **T** | T | — | **A/G** | T | **T** | T | T | | |
| AA | (0) | | | | | | | | | | | | | | | | | | | |
| HAMA | 181 | • | C | — | — | — | **T** | — | G | T | A | • | C | **T** | **A** | A | — | — | A | | |
| IZU | 180 | • | C | — | — | — | **T** | — | G | T | A | • | C | • | **A** | A | — | — | A | | |
| BR | 181 | **C** | C | — | — | — | **T** | — | G | T | A | — | C | • | **A** | A | **A** | **A** | A | | |
| 5) 28S | | 124 | 130 | **134** | **135** | 137 | **172** | **499** | 516 | **547** | **554** | **572** | 573 | 574 | 575 | 608 | **609** | 633 | 643 | 644 |
| AM | >3,257 | A | A | **C** | **A** | T | **A** | **T** | A | **A** | **T** | **T** | T | T | T | C | **A** | A | T | **T** |
| AA | >(1,255) | • | • | • | • | • | • | • | • | • | • | • | • | | | | | | | |
| HAMA | >3,248 | G | T | • | **T** | C | **G** | **C** | G | **T** | • | • | — | — | — | T | • | T | — | — |
| IZU | >3,248 | G | T | • | • | C | **G** | **C** | G | **T** | • | • | — | — | — | T | • | T | — | — |
| BR | >3,250 | G | T | **T** | **G** | C | • | • | G | • | **C** | — | — | — | — | T | **G** | T | — | — |
| | | 645 | 652 | 671 | 776 | **815** | 858 | **935** | **1,046** | **1,062** | **1,066** | **1,153** | **1,472** | 1,512 | 1,789 | 1,797 | 1,924 | **2,156** | 2,158 | 2,171 |
| AM | | A | G | A | A | **A** | G | **C** | **A** | **T** | **A** | **T** | A | T | A | A | A | **A** | A | A |
| AA | | | • | • | • | **C** | • | **G** | **G** | A | **G** | • | | | | | | | | |
| HAMA | | — | A | G | G | • | T | • | • | A | • | **C** | **G** | C | G | C | G | • | G | G |
| IZU | | — | A | G | G | • | T | • | • | • | • | • | • | C | G | C | G | • | G | G |
| BR | | — | A | G | G | • | T | • | • | A | • | • | **G** | C | G | C | G | **T** | G | G |
| | | **(2,263/ 2,264)** | **2,281** | 2,602 | **2,603** | **2,606** | 2,676 | 2,677 | **2,683** | **2,685** | 2,686 | **2,717** | **2,720** | **(2,727/ 2,728)** | **2,728** | **(2,732/ 2,733)** | **2,145** | | | |
| AM | | — | **A** | A | **T** | **A** | A | A | **A** | A | A | **T** | **A** | — | **A** | — | **T** | | | |
| AA | | | | | | | | | | | | | | | | | | | | |
| HAMA | | **G** | • | T | — | **T** | — | — | **T** | — | T | • | • | • | • | • | A/C/G/T | | | |
| IZU | | • | • | T | — | • | — | — | **T** | • | T | • | • | • | • | • | • | | | |
| BR | | **G** | **T** | T | • | • | — | — | • | — | T | **A** | **T** | **T** | **G** | **G** | A/C/G/T | | | |

* When the respective regions were incompletely read, the number of sequenced nucleotides is shown in parentheses.

† Position numbers correspond to the respective region of published rDNA sequence of *Strongyloides robustus* (AM isolate; accession number AB272232) (•, identical with AM isolate; —, deletion; blank, no data). The position of insertion is expressed as adjoining position numbers in parentheses; for example, (66/67). Positions of bases different even within isolates from the same continents are in bold type, whereas possible base positions showing intercontinental differences are in regular type.

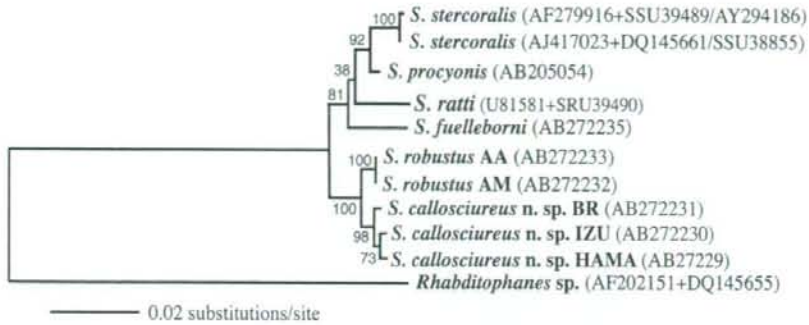


FIGURE 4. Neighbor-joining distance tree for *Strongyloides* sp. isolates and *Rhabditophanes* sp. KR3021 as an out-group. This phylogenetic analysis was conducted using concatenated sequences of 18S and 28S rDNA without alignment ambiguity (parsimony-informative characters: 103 for all, 77 for *Strongyloides* spp., and 14 for sciurid *Strongyloides* spp. isolates). Bootstrap supports are shown near nodes.

*Etymology:* The species was named after the generic name of the type host.

### Remarks

Actual measurements of body width and tail length of *S. callosciureus* n. sp. were smaller than those of *S. robustus*. In addition, the vulva of *S. callosciureus* n. sp. was situated more posteriorly than that of *S. robustus*; this feature was consistent, appearing to be the most reliable character for separating these 2 species by light microscopy. The rDNA sequence of the new species was deposited in DDBJ/EMBL/GenBank (accession numbers AB272229, HAMA isolate; AB272230, IZU isolate; and AB272231, BR isolate), exhibiting a definite critical difference from *S. robustus* (accession numbers AB272232 and AB272233).

## DISCUSSION

The key features of the parasitic female used to distinguish species of the *Strongyloides* are the shape of the stoma in the apical view, the type of ovary, the shape of the tail, and the number of lobes on the circumoral elevation (Speare, 1989). Referring to these morphological points, all isolates collected from North American and Asian sciurids in the present study closely resembled each other. However, actual measurements of body width and a tail length, as well as the relative position of the vulva to the whole body length, could divide sciurid *Strongyloides* isolates into those of North American and Asian origins. Although the body size of the North American isolates was generally larger than that of the Asian isolates, some differences in actual measurements could not be ascribed to proportional differences related to the body size. The relative length of the esophagus against the body length fell into 2 spectra regardless of having the identical morphological features mentioned above and the same geographical distribution of the host, so that the feature may be regarded as of no significance in the morphological differentiation when the molecular analysis supports this view. The same relative ratios of the esophagus to the body length were observed by Bartlett (1995) in *S. robustus* specimens recovered from the same host species, i.e., *T. hudsonicus*, and preserved in the same fixative.

The SSU/LSU rDNA sequences of the sciurid *Strongyloides* isolates were determined for the first time in this study. Regardless of the unknown collection sites of the hosts, 2 North American isolates genetically resembled each other. Similarly, 3 Asian isolates genetically resembled each other. When 2 isolates from Pallas's squirrels were genetically compared, there were a few, but substantial, differences (21 base positions of 5371–5454-bp length) in rDNA nucleotide sequences, reflected in a well-supported branching of the 2 in the phylogenetic tree (Fig. 4). This may be ascribed, however, to the difference in the original geographical source of the host in Taiwan (Tamura and Miyamoto, 2005). Interestingly, Oshida et al. (2006) demonstrated 4 main mitochondrial DNA (mtDNA) phylogroups of Pallas's squirrels associated with geography of Taiwan (northern, western, southern, and eastern regions) and suggested isolation of these populations from one another by mountain ranges during the Pleistocene glaciation. More recently, Oshida et al. (2007) demonstrated that so-called "Pallas's squirrels" caught in Hamamatsu City were separated into 2 mtDNA phylogroups; one group was in a cluster with Pallas's squirrels distributed in the eastern part of Taiwan, whereas the other group did not cluster with any phylogroups of Pallas's squirrels in Taiwan. They suggested that alien non-Pallas's squirrels distributed in Hamamatsu City could be Finlayson's squirrels (*Callosciurus finlaysonii*) distributed originally in Thailand, Cambodia, Laos, and Vietnam (Hoffmann et al., 1993). Simultaneously, Oshida et al. (2007) reported that alien squirrels caught in the Izu peninsula were closely related to the Taiwanese population of Pallas's squirrels, but were in an independent cluster to the 4 main mtDNA phylogroups mentioned above. Alternatively, it is possible that a Japanese isolate of sciurid *Strongyloides* nematodes was generated by alien *Callosciurus* squirrels, which are expanding their population in Japan after their introduction several decades ago. At present, we have no molecular data for *Strongyloides* nematodes in Japanese sciurids. To assess all these possibilities, collection of more sciurid *Strongyloides* isolates in Asia should be made in the future.

Anderson et al. (1998) and Blouin (2002) consider that even ITS sequence data may not be as useful as mtDNA for identifying potential cryptic species (morphologically similar, but genetically distinct) from small numbers of individuals, because mtDNA evolves very quickly in nematodes, and quickly reaches reciprocal monophyly between even very closely related species. From this viewpoint, our analysis of nuclear rDNA sequences might be insufficient to identify cryptic *Strongyloides*

species from sciurid hosts in the same continent, or the history of colonization by an exotic parasite (Criscione et al., 2005). Because substantial genetic differences exist even in rDNA, including all structural parts such as 18S, ITS, and 28S, between North American and Asian sciurid *Strongyloides* spp. isolates (Table V), in addition to geographical separation of the definitive host and morphological differences, we erected *S. callosciureus* n. sp. for isolates of Asian sciurid origin, and reserve *S. robustus* Chandler, 1942 for isolates of North American sciurid origin.

Our understanding of the phylogeographical relationships of terrestrial squirrels has advanced in the last few years (Mercer and Roth, 2003; Steppan et al., 2004; Oshida et al., 2006, 2007). Dorris et al. (2002) found, however, no support for strong coevolution between the host and *Strongyloides* spp., and instead suggested an explosive recent radiation and host colonization or switching in this parasite based on the genetic similarity within the genus. Furthermore, they indicated that there is little molecular correlation with morphological features of *Strongyloides* spp. and thus the molecular phylogeny of *Strongyloides* spp. differs from that predicted from morphological and ecological criteria alone. It should be noted, however, that Dorris et al. (2002) compared only 329- to 332-bp-long 18S rDNA at the 5′ end of 10 *Strongyloides* spp. Becasue this region of 18S rDNA or even the total length of 18S rDNA sequence has few variations and few base substitutions, it seems valuable to reexamine their conclusion using other parts of rDNA sequences or mtDNA sequences.

As shown in the present study, laboratory maintenance and propagation of sciurid *Strongyloides* isolates is possible using Mongolian jirds as a laboratory host, as in other *Strongyloides* spp. (Horii et al., 1992; Tsuji et al., 1993; Nolan et al., 1993). This facilitates laboratory maintenance and analyses of sciurid *Strongyloides* spp. Accumulation of biologic and genetic features of sciurid *Strongyloides* sp. isolates as well as other members of the genus in the future may elucidate more clearly the taxonomic and evolutional relationships of the sciurid *Strongyloides* isolates of different geographical origins.

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# Helminths Collected From Imported Pet Murids, with Special Reference to Concomitant Infection of the Golden Hamsters with Three Pinworm Species of the Genus *Syphacia* (Nematoda: Oxyuridae)

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ABSTRACT: A total of 210 individuals of 13 species belonging to 4 subfamilies of Muridae imported into Japan as pets were examined; 5 species of *Syphacia* (Nematoda: Oxyuridae), *Aspiculuris tetraptera* (Nematoda: Heteroxynematidae), and *Rodentolepis nana* (Cestoidea: Hymenolepididae) were collected. Concurrent infection with 3 pinworm species, *Syphacia mesocriceti*, *Syphacia stroma*, and *Syphacia peromysci*, was recorded for the first time in the golden hamster, *Mesocricetus auratus*. *Syphacia mesocriceti* was also identified in the desert hamster, *Phodopus roborovskii*, and *S. peromysci* was recovered from the fat-tailed gerbil, *Pachyuromys duprasi*, and the Cairo spiny mouse, *Acomys cahirinus*. From the pygmy mouse, *Mus minutoides*, an undetermined species closely resembling *Syphacia megaloon* and *Syphacia ohtaorum*, both parasitic in *Mus* spp., was collected. Females of another undetermined *Syphacia* sp. were observed in the greater Egyptian gerbil, *Gerbillus pyramidum*. All of the host–*Syphacia* associations, except *S. mesocriceti* in the golden hamsters, were recorded for the first time. It is suggested that overlapping breeding situations provided the opportunity for host switching by the pinworms.

Rearing of exotic pet animals has gained worldwide popularity. In Japan, various pet mammals were imported freely from foreign countries until 1 September 2005, when New Notification System for the Importation of Animals was enforced by the Ministry of Health, Labor and Welfare of the Japanese Government. Because quarantine was not previously obligatory for these mammals, there was concern that zoonotic pathogens may have been introduced with them. To determine the prevalence of zoonotic pathogens in various imported rodents, a cooperative survey by veterinary virologists, bacteriologists, parasitologists, and pathologists was conducted from 2003 to 2006. Viral and bacterial agents detected have been reported elsewhere (Une et al., 2004; Masuzawa et al., 2006). Herein, the helminth parasites that were recovered are reported.

Imported murid rodents were purchased from 5 animal brokers in Tokyo. The origin of export countries was not specified in detail, but apparently included the Czech Republic and the Netherlands. The rodents were killed by chloroform inhalation, and their viscera were excised and fixed in 10% neutral-buffered formalin solution. After removing various portions of the viscera for histologic examination and recovery of protozoans, the remaining alimentary canals were subjected to helminthological assessment. The gastrointestinal tracts were cut open and examined using stereomicroscopy for helminths. Parasitic worms collected were preserved in 10% neutral-buffered formalin solution. For light microscopy, parasites were cleared in a glycerol–alcohol solution by evaporating alcohol and then were mounted on glass slides with 50% aqueous glycerol solution. Intensity data for each species were based on adult-stage worms, as species identification of larval forms was not possible. Eggs excised from gravid females were also observed using a scanning electron microscope (SEM). Voucher specimens were deposited in the National Science Museum, Tokyo, Japan.

A total of 210 individuals of 13 species belonging to 4 subfamilies of Muridae were examined. Five species of *Syphacia* Seurat, 1916 (Nematoda: Oxyuridae) were collected in 6 murid species from 4 of the 5 dealers (Table I). In addition to *Syphacia* spp., *Aspiculuris tetraptera* (Nitzsch, 1821) (Nematoda: Heteroxynematidae) and *Rodentolepis nana* (Siebold, 1852) (Cestoidea: Hymenolepididae) were found in the hamsters (Table I). Prevalence and intensity of infections are reported in Table I. Among the 5 *Syphacia* spp., 4 have not previously been recorded from pet rodents. Concomitant infection of more than 1 *Syphacia* spp. was found in 2 groups of the golden hamsters: 1 group was infected with 2 species and the other group was infected with 3 species. Brief comments are made below for each *Syphacia* sp. collected.

*Syphacia mesocriceti* Quentin, 1971, was found in 17 of 20 *M. auratus*, with intensity up to 168 (Table I). The worms were mostly female; the number of males in a host ranged from 1 to 5. This pinworm was also found in *P. roborovskii*, with low intensity (Table I). *Syphacia mesocriceti* is characterized by having cephalic papillae projecting dorsally and ventrally from amphids (Quentin, 1971). It has been classified in the subgenus *Cricetoxyuris* Hugot, 1988 (Hugot, 1988). Morphology and measurements of the present worms are identical to those of *S. mesocriceti* reported previously (Quentin, 1971; Dick et al., 1973; Hasegawa, 1981). This species was first observed in a golden hamster in captivity in Alaska (Quentin, 1971). Since then, it has been identified in this host kept as pets and as experimental animals in various parts of the world (Dick et al., 1973; Hasegawa, 1981; Pinto et al., 2001). *Phodopus roborovskii* is recorded here for the first time as the host of *S. mesocriceti*.

*Syphacia stroma* (Linstow, 1884) was recovered from *M. auratus*, concomitantly with *S. mesocriceti* in both groups of the golden hamsters examined, with an intensity that ranged from 3 to 99 (Table I). *Syphacia stroma* has been known as a pinworm of *Apodemus* spp. in Europe (Quentin, 1971; Tenora and Mészáros, 1975; Genov, 1984). It belongs to the group IX of Quentin (1971), along with *Syphacia emileromani* Chabaud et al., 1963. Pinworms of this group are characterized by an oval cephalic plate separated clearly from the cervical region by a groove in which the cephalic papillae are located, along with 4 well-discernible labial papillae and a stout male tail (Quentin, 1971). The characteristics and measurements of the present worms were identical to those of previously described *S. stroma*, although the males have a somewhat narrower body (1.53–1.75 mm long and 91–115 μm wide at mid-body) and an accessory piece of gubernaculum that lacks faint cuticular creases (Quentin, 1971). The surface features of the eggs are also identical to those of *S. stroma* (Barus et al., 1979). *Mesocricetus auratus* is recorded as a new host of *S. stroma*.

*Syphacia peromysci* Harkema, 1936, was found in *M. auratus*, the fat-tailed gerbil, *Pachyuromys duprasi*, and the Cairo spiny mouse, *Acomys cahirinus* (Table I). Intensity of infection was highest (>500) in *P. duprasi*; it ranged from 2 to 5 in *M. auratus*, and a single female was collected in *A. cahirinus* (Table I). *Syphacia peromysci* is a typical member of the subgenus *Seuratoxyuris* Hugot, 1988, in having well-developed cervical alae with internal supports and prominent deirids in females, and a short tail and an accessory piece of gubernaculum with cuticular ornamentations in males (Hugot, 1988). It closely resembles *Syphacia petrusewiczi* Bernard, 1966 and *Syphacia rauschi* Quentin, 1969 in having a laterally elongated cephalic plate. *Syphacia petrusewiczi* and *S. rauschi* are parasitic in voles, *Clethrionomys* spp., in the Palaearctic Region and Alaska, respectively, whereas *S. peromysci* is known from the deer mouse, *Peromyscus maniculatus*, in North America (Bernard, 1966; Quentin, 1969, 1971; Quentin and Kinsella, 1972; Jancév, 1973; Tenora and Mészáros, 1975; Quentin and Gran, 1977; Genov, 1984; Hasegawa et al., 1994). The body dimensions and morphology of the present worms are almost identical to those of *S. peromysci* (Quentin and Kinsella, 1972). *Syphacia minuta* Greenberg, 1969, has been described in *A. cahirinus* in Israel. The present specimens are readily distinguishable from *S. minuta* by having larger females (3.13–4.11 mm long vs. 1.74–2.85 mm long). Males in the present study have a longer spicule (55–70 μm long vs. 44–53 μm), whereas the body length is comparable (0.80–1.04 mm vs. 0.68–1.01 mm) to that described by Greenberg (1969). The present 3 rodents are recorded as new hosts for *S. peromysci*.

*Syphacia* sp. 1 was recorded from the pygmy mouse *M. minutoides*, with low intensity (Table I). The present species is included in group VIII of Quentin (1971) as having a well-developed cephalic vesicle, a

TABLE I. Prevalence of helminth parasites in pet murids imported into Japan.

| Rodent host | No. hosts examined | Date of necropsy | Helminths found | % Prevalence and intensity (range [mean]) | NSMT accession No. | Dealer |
|---|---|---|---|---|---|---|
| Arvicolinae | | | | | | |
| *Lagurus lagurus* | 9 (6M, 3F*) | 15 IV 2006 | —† | | | A |
| Cricetinae | | | | | | |
| *Mesocricetus auratus* | 10 (6M, 4F) | 10 VI 2006 | *Syphacia mesocriceti* | 70 (1–10 [6.2]) | As 3093 | B |
| | | | *Syphacia stroma* | 80 (3–99 [19.9]) | As 3095 | |
| | | | *Syphacia peromysci* | 80 (2–5 [3.4]) | As 3096 | |
| | | | *Rodentolepis nana* | 40 (1–6 [3.0]) | Pl 5611 | |
| | 10 (5M, 5F) | 10 VI 2006 | *S. mesocriceti* | 100 (7–168 [86.6]) | | A |
| | | | *S. stroma* | 10 (47 [47]) | | |
| | | | *R. nana* | 50 (2–13 [8.2]) | | |
| *Phodopus roborovskii* | 10 (7M, 3F) | 10 VI 2006 | *S. mesocriceti* | 30 (1–4 [2.0]) | As 3094 | A |
| | | | *Aspiculuris tetraptera* | 30 (1 [1]) | As 3495 | |
| *Phodopus sungorus* | 20 (10M, 10F) | 15 IV 2006 | *A. tetraptera* | 90 (1–82 [14.4]) | As 3496 | A |
| | | | *R. nana* | 60 (1–227 [22.5]) | Pl 5612 | |
| | 10 (3M, 7F) | 10 VI 2006 | *A. tetraptera* | 100 (2–157 [48.5]) | | B |
| | | | *R. nana* | 40 (1–30 [8.8]) | | |
| Murinae | | | | | | |
| *Acomys cahirinus* | 20 (12M, 8F) | 11 X 2004 | *S. peromysci* | 15 (1 [1]) | As 3098 | C |
| | 9 (6M, 3F) | 2 IV 2005 | — | | | D |
| *Acomys russatus* | 13 (6M, 7F) | 2 IV 2005 | — | | | D |
| *Lemniscomys barbarus* | 11 (8M, 3F) | 2 VII 2005 | — | | | E |
| *Mus minutoides* | 20 (15M, 5F) | 11 X 2004 | *Syphacia* sp. 1 | 60 (1–7 [3.2]) | As 3099 | E |
| Gerbillinae | | | | | | |
| *Gerbillus pyramidum* | 10 (7M, 3F) | 4 VI 2005 | *Syphacia* sp. 2 | 60 (1–8 [2.4]) | As 3100 | A |
| *Meriones tristrani* | 4 (4M) | 11 X 2004 | — | | | C |
| *Pachyuromys duprasi* | 5 (5F) | 13 VII 2003 | — | | | D |
| | 8 (8M) | 2 IV 2005 | — | | | D |
| | 10 (6M, 4F) | 4 VI 2005 | *S. peromysci* | 50 (1–34 [11.2]) | As 3097 | A |
| | 10 (5M, 5F) | 15 IV 2006 | *S. peromysci* | 90 (6–>500 [>100]) | | A |
| *Psammomys obesus* | 11 (1M, 10F) | 2 IV 2005 | — | | | D |
| *Sekeetamys calurus* | 10 (4M, 6F) | 4 VI 2005 | — | | | A |

* M, male; F, female.
† Negative.

laterally elongated cephalic plate, and a facial mask that includes cephalic papillae and amphids that are laterally placed. Among the members of this group, *S. megaloon* Quentin, 1966, and *S. ohtaorum* Hasegawa, 1991, resemble the present worms by having a stout body and a short tail in females (Quentin, 1966; Hasegawa, 1991). *Syphacia megaloon* was originally described from *M. minutoides*, the same host species seen in the present worms, and *Mus setulosus* of Zaire (Congo). However, the worms in the present study are distinguished from *S. megaloon* by having more eggs (about 100 vs. 20–30) of much smaller size (123–132 × 35–42 μm vs. 150 × 60 μm) as described by Quentin (1966, 1971). The present males are differentiated from *S. ohtaorum* by having a spicule constricted in the proximal 1/4 position, whereas the spicule in the latter species is narrowed near the middle. Worms in the present study also possess a shorter tail, which is 115–116 μm long, and a shorter body length of 0.89–1.35 mm versus 150–186 μm long in worms with a body length of 1.21–1.45 mm, as described by Hasegawa (1991). Because only a few worms were available for observation, proposition of a new taxon is withheld.

Only females of *Syphacia* sp. 2 were collected from the greater Egyptian gerbil, *G. pyramidum*, and with low intensity (Table I). This nematode is tentatively assigned to *Syphacia* because of the absence of males. Pinworms from *Gerbillus campestris* of Tunisia have been identified as *Syphacia nigeriana* Baylis, 1928. However, the eggs of the present females are thinner than those of *S. nigeriana* (104–110 × 25–30 μm vs. 70–116 × 30–38 μm; Quentin, 1971).

*Syphacia* spp. nematodes are usually host-specific and have a tendency to co-evolve with their hosts since the environmental phase in their monoxenous life cycle is much reduced or absent (Hugot, 1988). Consequently, mixed infections in the present golden hamsters with 3 *Syphacia* spp. are of special interest. *Syphacia mesocriceti* has been known as being host-specific for golden hamsters, although it has not been reported in the feral hamster. *Syphacia stroma* is parasitic in *Apodemus* spp. in the Palaearctic region, and *S. peromysci* has been recorded only in North America (Hugot, 1988); thus, they were certainly acquired by hamsters under captive conditions. Likewise, the fat-tailed gerbil may have become infected with *S. peromysci* during captivity because this rodent is endemic to the north Sahara region. Apparently, pet breeders' facilities provide suitable conditions for host capture by pinworms, which may seldom occur under natural conditions.

Pinto et al. (2001) reported mixed infections of golden hamsters with 2 pinworm species, *S. mesocriceti* and *Syphacia criceti* Quentin, 1969. However, the identification of the latter pinworm is questionable. In *S. criceti*, the tail of the male narrows suddenly and its length is almost twice that of the spicule (Quentin, 1971), whereas the male referred to as *S. criceti* sensu by Pinto et al. (2001) has a gradually tapered tail and the length is far greater than twice the length of the spicule. These features for *S. criceti* Pinto et al., 2001, are similar to those of *S. stroma* collected in the present survey. The shape of gubernaculum, as depicted by Pinto et al. (2001), is also identical to that of the present *S. stroma*.

Therefore, it is strongly suggested that *S. stroma* infection of golden hamsters is global.

*Syphacia* sp. 1 from *M. minutoides* is surmised to have been maintained in this host from its original capture in Africa, because it resembles *S. megaloon* and *S. ohtaorum*, both parasitic in *Mus* spp. *Syphacia megaloon* is distributed in Africa and *S. ohtaorum* is distributed in Japan, Inner Mongolia, China, and Nepal (Hasegawa, 1991; H. Hasegawa, unpubl. obs.). Presumably, this pinworm lineage has co-evolved with *Mus* spp. and is dispersed in a wide geographic range from the Far East to Africa. It is necessary to determine the intraspecific variations that might have been formed during dispersal. For this purpose, DNA sequence analysis may be advantageous. For example, Okamoto et al. (2007) recently analyzed nucleotide sequences of *cox1* gene of *Syphacia* spp. and found that *S. ohtaorum* is far different from *Syphacia obvelata* (Rudolphi, 1802), another pinworm of *Mus* spp. If a similar analysis is applied to all of the present species, the geographic and host origins of the *Syphacia* spp. in the pet murids may be elucidated.

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**Laboratory and Epidemiology Communications**

# *Salmonella enterica* Serotype Typhimurium Infection Causing Mortality in Eurasian Tree Sparrows (*Passer montanus*) in Hokkaido

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From December, 2005, dead sparrows were observed frequently in Hokkaido, Japan. By July 28, 2006, 1,517 cases had been reported (Table 1). They were observed in a wide area of Hokkaido, primarily in Asahikawa City (Hokkaido Government publication). Several organizations investigated these sparrow deaths, but the cause has not yet been determined. We conducted a study in which 15 dead sparrows found in the area of extensive mortality were examined pathologically and microbiologically. Based on the results, we inferred that mortality was due to an epidemic caused by *Salmonella enterica* serotype Typhimurium DT40. It was the first time this serotype has been identified in Japan. This report outlines our study and findings.

We examined 15 dead sparrows found in various locations in Hokkaido: Noboribetsu City (two sparrows), Asahikawa City (six), Otaru City (three) and Sapporo City (four). Of these, the eight sparrows from Noboribetsu and Asahikawa had been decomposing for some time, so they were immediately frozen for preservation. A standard pathological examination was conducted on all sparrows. The crop, liver, spleen and intestine were tested for general bacteria and *Salmonella*. The strains identified as *Salmonella* were isolated and their serotypes and phage types were determined. Drug susceptibility testing and genetic testing by means of pulsed-field gel electrophoresis (PFGE) (Fig.1) and random amplified polymorphic DNA (RAPD) analysis were also conducted.

Ingluvitis, splenomegaly, hepatomegaly and the formation of white nodules were observed in the two Noboribetsu sparrows, one Asahikawa sparrow, the three Otaru sparrows and two Sapporo sparrows (Fig. 2). A characteristic finding in these birds was bacterial and necrotic ingluvitis. Colonies of Gram-negative bacilli and necrotic lesions were observed

Table 1. Number of sparrow deaths by subprefecture in Hokkaido

| Branch office | No. of incidents reported | No. of carcasses |
|---|---|---|
| Ishikari | 153 | 404 |
| Osima | 12 | 7 |
| Hiyama | 3 | 3 |
| Siribesi | 43 | 70 |
| Sorati | 23 | 51 |
| Kamikawa | 203 | 717 |
| Rumoi | 13 | 35 |
| Souya | 5 | 5 |
| Abasiri | 7 | 10 |
| Iburi | 104 | 190 |
| Hidaka | 1 | 1 |
| Tokati | 10 | 13 |
| Kusiro | 14 | 9 |
| Nemuro | 2 | 2 |
| Total | 593 | 1,517 |

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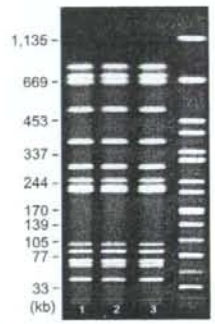


Fig. 1. *Xba*I-digested PFGE patterns of *Salmonella* Typhimurium isolated from sparrow crop samples. Lane 1, Noboribetsu 1; lane 2, Noboribetsu 2; lane 3, Asahikawa; lane 4, a molecular size marker (*S.* Braenderup H9812 digested with *Xba*I).

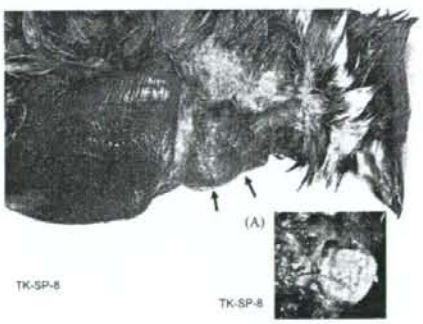


Fig. 2. Sparrow with necrosis of crop indicated by arrowheads. (A) A cutaway picture of the crop. The wall of the crop was extensively enlarged.

in visceral organs. These bacteria were found positive in an immunostaining assay using *Salmonella* O4 antiserum. *Salmonella* were isolated from organs in all sparrows except five from Asahikawa, and the strain was identified as Typhimurium. The isolated *S.* Typhimurium showed a very weak reaction in a catalase test and was negative for citrate utilization. The phage type was DT40, and the antibiotic resistance profiles and PFGE and RAPD patterns were almost the same in all isolates, suggesting a single origin.

Extensive mortality due to *S.* Typhimurium in wild birds such as finches and house sparrows (*Passer domesticus*) has been reported in Europe, North America and New Zealand, and *S.* Typhimurium is attracting attention as the cause of drastic reductions in the populations of these species (1-5).

Hepatomegaly, splenomegaly and the formation of white nodules have been observed frequently in finches infected with *S.* Typhimurium, with a pathognomonic finding of ingluvitis (4,6,11). These findings match our own. Even though the population we analyzed was small, we inferred that *S.* Typhimurium caused the extensive mortality in sparrows in Hokkaido, especially as many of the abovementioned epizootics also occurred in winter.

In New Zealand, there was a mass outbreak of *S.* Typhimurium infection in humans at the same time as mortality in house sparrows was observed, and house sparrows were thought to be the source of the infection in humans (4,6).

In addition, death due to *S.* Typhimurium originating from house sparrows was observed in ducks and quails; hence, there is concern that it could spread to livestock. *S.* Typhimurium infection requires extreme vigilance from the viewpoints of public health, animal health and the preservation of species. The phage type of *S.* Typhimurium isolated in this study was DT40, which has been significantly associated with mortality in Norway, North America and the United Kingdom (1,2,7-9). DT40 had not been detected previously in animals or humans in Japan (10, Izumiya, H., unpublished data), and it is not known when or how it entered this country.

We would like to thank Dr. Hisashi Ade who collected and supplied materials.

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ORIGINAL PAPER

# *Trypanosoma* (*Herpetosoma*) *kuseli* sp. n. (Protozoa: Kinetoplastida) in Siberian flying squirrels (*Pteromys volans*)

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**Abstract** All trypanosome species classified in the subgenus *Herpetosoma* in sciurid hosts have been recorded from ground and tree squirrels to date, but not from any flying squirrels. We describe in this paper a novel trypanosome species, *Trypanosoma* (*Herpetosoma*) *kuseli* sp. n., from Siberian flying squirrels (*Pteromys volans*) imported from China, and compare it with *T.* (*H.*) *otospermophili* in Richardson's ground squirrels (*Spermophilus richardsonii*) and Columbian ground squirrels (*Spermophilus columbianus*) from the USA. Due to a short free flagellum, the new species appeared stumpy compared with *T. otospermophili* (length of free flagellum 7.0±0.8 μm, total length 32.1± 0.8 μm, $n$=13 and length of free flagellum 15.5±1.6 μm, total length 35.9±1.0 μm, $n$=13, respectively). Another conspicuous morphological feature of the new species was an anteriorly positioned kinetoplast, found approximately at the midpoint between the nucleus and the posterior end. These characters have not been recorded from any squirrel *Herpetosoma* trypanosome species. Comparison of the nucleotide sequences of the small and large subunit rRNA genes indicated that *T. kuseli* sp. n. was more homologous to *T. otospermophili* than murid *Herpetosoma* species, such as *T. grosi*, *T. lewisi*, *T. musculi*, *T. microti* and *T. evotomys*.

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## Introduction

Trypanosomes classified in the subgenus *Herpetosoma* contain approximately 50 named and several unnamed species, and more than two thirds of them are recorded from mammals of the order Rodentia, particularly the families Muridae and Sciuridae (Hoare 1972; Riedel 1975). All *Herpetosoma* species known in sciurids have been reported from ground and tree squirrels to date but not from any flying squirrels (Hilton and Mahrt 1972; Hoare 1972; Riedel 1975). The Sciuridae family is divided into two subfamilies, Sciurinae and Petauristinae (Hoffman et al. 1993), and *Herpetosoma* trypanosomes have only been recorded from the former subfamily. Our previous study (Sato et al. 2005) reported the detection of *Herpetosoma* trypanosomes from flying squirrels [Siberian flying squirrel, *Pteromys volans* (Linnaeus 1758)] for the first time, as well as the nucleotide sequences of its small (SSU) and large (LSU) subunit ribosomal RNA genes (rDNA). However, the morphological characters of this species were not reported.

In this study, we describe the species mentioned above as a novel trypanosome species, *Trypanosoma* (*Herpetosoma*) *kuseli* sp. n., and compare it with other squirrel *Herpetosoma* trypanosomes, in particular *T.* (*H.*) *otospermophili* (Wellman et Wherry 1910) from American ground squirrels.

In addition, we carried out an experimental infection of laboratory jirds (*Meriones unguiculatus*) with the latter species according to our previous studies (Sato et al. 2003–2005), which demonstrated high usability of jirds as experimental hosts for *Herpetosoma* trypanosomes of field mice, i.e. *Trypanosoma grosi*.

## Materials and methods

### Parasitological survey

A cooperative survey of zoonotic agents in imported rodents as personal pets in Japan was conducted between 2003 and 2006 by veterinary virologists, bacteriologists, parasitologists and pathologists. The rodents that were examined included nine species of sciurids as follows: 17 female and 13 male plantain squirrels (*Callosciurus notatus*) necropsied on either 11 July 2004 (20 animals) or 4 June 2005 (10); 8 female and 12 male Eurasian red squirrels (*Sciurus vulgaris*) necropsied on either 31 May 2003 (10) or 2 July 2005 (10); 12 female and 18 male Columbian ground squirrels (*Spermophilus columbianus*) necropsied on either 31 May 2003 (10), 26 June 2004 (10) or 2 July 2005 (10); 17 female and 23 male Richardson's ground squirrels (*Spermophilus richardsonii*) necropsied on either 24 May 2003 (10), 14 June 2003 (10), 26 June 2004 (10) or 4 June 2005 (10); 8 female and 2 male 13-lined ground squirrels (*Spermophilus tridecemlineatus*) necropsied on 13 July 2003; 36 female and 43 male Siberian chipmunks (*Tamias sibiricus*) necropsied on either 14 June 2003 (10), 13 July 2003 (10), 26 June 2004 (19), 16 April 2005 (10), 15 April 2006 (20) or 10 June 2006 (10); 10 female and 9 male American red squirrels (*Tamiasciurus hudsonicus*) necropsied on 26 June 2004; 10 male southern flying squirrels (*Graucomys volans*) necropsied on 16 April 2005; and 7 female and 19 male Siberian flying squirrels necropsied on either 14 June 2003 (10), 11 July 2003 (6) or 2 July 2005 (10). All these squirrels were purchased from two pet-animal brokers and necropsied in the School of Veterinary Medicine, Azabu University within several days or a few weeks after importation. Blood was collected by cardiac puncture from each squirrel, and serum and cellular components were separated by centrifugation for 10 min at 3,000 rpm at ambient temperature. Simultaneously, a thin smear of blood was prepared from each squirrel, which was fixed in methanol then stained with Giemsa's solution. The screening of trypanosomes on thin smears of blood was conducted under a light microscope at a magnification of 400×. After the first year of the survey, in addition to the microscopic examination of stained blood smears, direct examination of blood samples diluted in physiological saline and buffy coats of centrifuged blood samples in hematocrit capillary tubes was carried out under an inverted microscope to detect suitable samples for isolating trypanosomes for subsequent culture.

### Morphological examination

Positive blood films with trypanosomes were observed under a light microscope at a magnification of 1,000×. Arbitrarily selected, but undistorted, well-stained trypanosomes, 13 in number for each host species, were photographed at this magnification, transformed into photographs of Adobe® Photoshop® v. 5.0, then printed at a higher magnification. Measurements were conducted on printed photographs, and a digital curvimeter type S (Uchida-Yoko, Chuoh-ku, Tokyo, Japan) was used when required. Measurements (expressed in μm) included total length, width at the widest point, distance between the posterior end and the middle kinetoplast (PK), distance between the kinetoplast and the middle of the nucleus (KN), distance between the middle of the nucleus and the anterior end (NA), length of the free flagellum (FF) and sizes of the kinetoplast and nucleus. Nuclear index (NI) and kinetoplast index (KI) were calculated as follows: NI=(PK+KN)/NA, and KI=(PK+KN)/KN. Blood films on glass slides examined in this study were deposited in The National Science Museum, Tokyo, Japan under the registration numbers NSMT-Pr 194-196 (*T. kuseli* sp. n. from three Siberian flying squirrels), NSMT-Pr 197 (*T. otospermophili* from a Richardson's ground squirrel) and NSMT-Pr 198 (*T. otospermophili* from a Columbian ground squirrel).

### Polymerase chain reaction and nucleotide sequencing

The methods for parasite DNA extraction, polymerase chain reaction (PCR) amplification of fragments of the SSU and LSU rDNA and nucleotide sequencing and alignment using the Clustal W multiple alignment programme (Thompson et al. 1994) are described in our previous study (Sato et al. 2005). The nucleotide sequences reported in this paper are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB175625, AB175626 and AB190228.

### Infection and monitoring of parasitaemia

Mongolian jirds were bred in the Institute for Animal Experiments, Hirosaki University School of Medicine. Inbred Bagg albino/c (BALB/c) mice were purchased from a commercial breeder. All animal experiments were performed according to Hirosaki University's Guidelines on Animal Experimentation.

*Trypanosoma otospermophili* from a Columbian ground squirrel was expanded by in vitro culture as described

previously (Sato et al. 2003). Cultured trypanosomes were resuspended in supplemented RPMI 1640 medium in the absence of fetal calf serum. In the first experiment (experiment I), three 4-week-old jirds of either sex were injected subcutaneously with 10 mg of prednisolone tertiary-butylacetate (suspension of Codelcortone®-TBA, Merck, Rahway, NJ, USA), then intraperitoneally (i.p.) with $2\times10^5$ cultured trypanosomes. After injection of these parasites in jirds, the course of parasitaemia was monitored by counting the number of trypanosomes in the peripheral blood using a hemocytometer. Briefly, the blood sample was collected from the orbital venous plexus on days 3, 5, 7, 9 and 13 post-infection (p.i.) and diluted in 0.83% $NH_4Cl$–Tris buffer with heparin. In the second experiment (experiment II), four young (6-week-old) and six old (10-month-old) jirds of either sex, both groups treated with prednisolone, and 8-week-old male mice treated with/without prednisolone (six mice/group) were infected i.p. with $2\times10^5$ trypanosomes.

### Histological examination

Animals were killed by over-inhalation of ethanol, and thin smears of blood and pleural effusion, if available, were made. In addition, organ stamps of the peritoneum, pleura, liver, spleen and kidney were prepared. These specimens were stained in Giemsa's solution. Organs, such as the lungs, heart, liver, spleen, kidneys, intestines and brain, were then fixed in 10% neutral-buffered formalin. Trimmed organ blocks were dehydrated in a series of alcohol, cleared in xylene and embedded in paraffin. Thin sections (5-μm thick) were stained with hematoxylin–eosin according to a standard technique. Some representative stained specimens of blood and pleural effusion films and organ stamps on glass slides prepared from infected jirds with *T. otospermophili* were deposited in The National Science Museum, Tokyo, Japan under the registration numbers NSMT-Pr 199-221.

## Results

Trypanosomes were detected in blood samples from 1 of 10 Richardson's ground squirrels necropsied on 14 June 2003 (1 male squirrel), from 3 of 16 Siberian flying squirrels necropsied on either 14 June 2003 (1 male and 1 female squirrel) or 13 July 2003 (1 male squirrel) and 1 of 10 Columbian ground squirrels necropsied on 26 June 2004 (1 female squirrel). All other sciurid species examined were negative for trypanosomes as well as other blood-dwelling parasites. Trypanosomes from the ground squirrels showed a typical "*lewisi*-like" morphology on the stained blood films, whereas those from the flying squirrels appeared stumpy partially due to a shorter free flagellum (Fig. 1; see Table 1 for measurements). Another conspicuous morphological character of the latter trypanosome species was an anteriorly positioned kinetoplast, located at the midpoint between the nucleus and the posterior end.

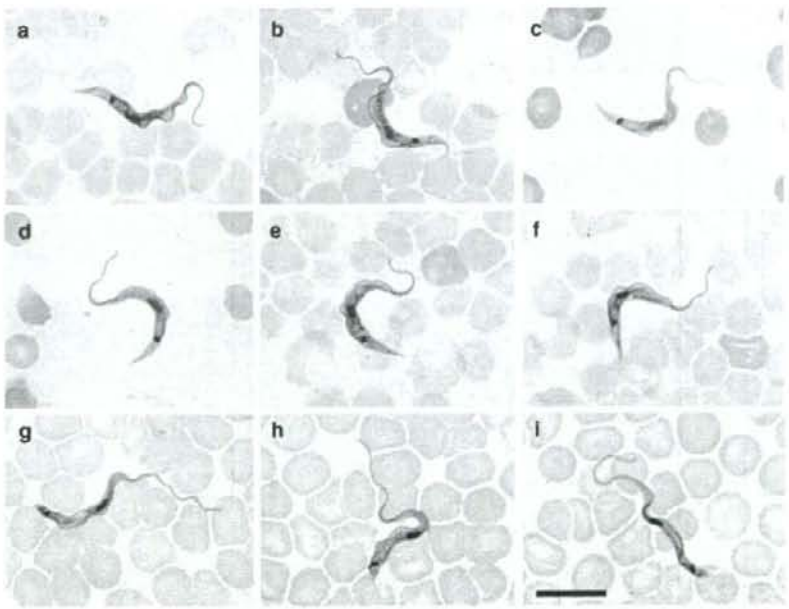


**Fig. 1** Developed trypomastigotes of *Herpetosoma* trypanosomes in the peripheral blood of sciurid hosts. **a**–**f** *T. kuseli* sp. n. in Siberian flying squirrels; **g**–**i** *T. otospermophili* in a Richardson's ground squirrel (Giemsa's stain, scale=10 μm)

**Table 1** Morphometric comparison of *Trypanosoma* (*Herpetosoma*) *kuseli* sp. n. with seven other recorded sciurid *Trypanosoma* spp. belonging to the subgenus *Herpetosoma*[a]

| Parasite | Host genus | Number of samples | Total length | Width | PK | KN | NA | FF | NI | KI | Kinetoplast | Nucleus | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. kuseli* sp. n. | *Pteromys* | 13 | 32.1±0.8 (30.8–33.6) | 2.1±0.2 (1.7–2.4) | 7.0±0.5 (6.0–7.6) | 5.3±0.7 (4.2–6.4) | 11.9±1.0 (9.9–13.3) | 7.0±0.8 (5.6–8.4) | 1.0±0.1 | 2.3±0.2 | 1.0×0.6 | 2.2×0.9 | The present study |
| *Trypanosoma* sp. | *Tamiasciurus* | 100 | 32.4±2.3 (25.2–38.2) | 1.7 | 3.9 | 9.9 | 10.2 | 8.4 | 1.4 | 1.4 | 1.1×0.8 | 2.7×1.4 | Dorney (1967) |
| *T. indicum* | *Funambulus* | 100 | 28.2 (19.7–33.8) | 2.6 (1.4–4.7) | 3.4 (1.9–4.7) | 7.2 (3.8–9.5) | 12.3 (11.6–13.0) | 5.9 (3.8–8.5) | 0.9 | 1.5 | 1 (0.5–1.4) | 2.1×1.2 (1.4–3.1×0.9–2.4) | Todd (1963) |
| *T. xeri* | *Xerus* | 40 | 30 (26–32) | 2.2 (1.8–2.5) | 3.9 (2.5–5.5) | 14 (12–18) | | 8 (6–10) | | 1.3 | | 2.2 (1.8–2.5) | Ranque (1977) |
| | | 100 | 28.2±1.9 (24.0–33.6) | 2.6±0.3 (1.6–3.6) | 5.4±0.5 (4.4–7.1) | | 4.3±0.3 (3.5–5.1) | 12.8±1.1 (10.0–16.0) | 3.5±0.1 (3.3–3.9) | 1.8±0.3 (1.6–2.1) | | 2.7±0.1 (2.4–3.0) | Marinkelle and Abdalla (1978) |
| *T. parkeri* | *Marmota* | 10 | 29.7 (27.3–32.9) | 1.5 (1.4–2.1) | 2.1 (1.4–2.8) | 5.2 (1.7–9.1) | 10 | 11.5 (9.1–20.3) | 0.7 | 1.4 | | | Dias 1938; Dorney (1967) |
| *T. obospermophili* | Spermophilus | 13 | 35.9±1.0 (34.9–37.9) | 1.5±0.2 (1.1–1.7) | 2.3±0.3 (1.9–2.7) | 9.2±1.0 (7.2–10.2) | 8.8±1.5 (6.4–11.0) | 15.5±1.6 (13.3–17.8) | 1.4±0.3 | 1.3±0.1 | 0.9×0.6 | 2.4×0.8 | The present study |
| | | 37 | 47.6±2.7 (39.0–55.0) | 2.6±0.5 (1.5–4.0) | 4.5±1.0 (3.0–7.5) | 16.6±1.3 (13.0–19.5) | 12.7±2.4 (6.0–21.0) | 13.8±2.8 (6.0–24.0) | 1.7 | 1.3 | | 3.8±0.8 (3.0–6.0) | Hilton and Mahrt (1972) |
| *T. tamiasi* | *Tamias* | 100 | 34.5±2.1 (27.9–38.6) | 1.7 | 4 | 12 | 10.8 | 7.7 | 1.5 | 1.3 | 1.1×0.9 | 2.9×1.2 | Dorney (1967) |
| | | 75 | 34.8±1.7 (28.6–38.6) | 1.1±0.3 (0.7–3.0) | 3.9±0.6 (2.1–5.1) | 10.8±0.9 (6.6–12.5) | 11.1±1.3 (8.1–13.9) | 9.0±1.3 (6.1–13.1) | 1.3 | 1.4 | | | Woo et al. (1980) |
| *T. sciuri* | *Funisciurus* | | 41.0 | | 4.0 | | | 7.0 | | | | | Schwetz (1933) |

[a] For abbreviations, see the "Materials and methods".

*Trypanosoma* (*Herpetosoma*) *kuseli* sp. n.

The body of the trypomastigotes in the peripheral blood ($n$=13), which measured 25.1±1.3 by 2.1±0.2 μm, had a curved anterior end and a spindle-shaped posterior end, with the free flagellum being relatively short at 7.0±0.8 μm in length. The oval nucleus, which measured 2.2×0.9 μm, was situated at the midpoint between both ends. The oval kinetoplast, which measured 1.0×0.6 μm, was situated at the midpoint between the nucleus and posterior end (Fig. 1a–f). It was noted that the undulating membrane was not wide. Additional measurements of this new species are given in Table 1 as well as the measurements for known squirrel trypanosomes of the same subgenus. The SSU and LSU rDNA nucleotide sequences of this new species are deposited and available in the DDBJ/EMBL/GenBank databases under the accession numbers AB175626. Information regarding the site and mode of parasite proliferation is not yet available.

Taxonomic summary

Type host: *Pteromys volans* (Linnaeus 1758).

Site of infection: Trypomastigotes in the peripheral blood.

Prevalence: 19% (3 of 16 hosts autopsied on either 14 June or 13 July 2003)

Locality: Unknown. The observed materials were collected from squirrels imported from China to Japan as personal pets.

Specimens deposited: The National Science Museum, Tokyo, Japan: syntype (NSMT-Pr 194), paratypes (NSMT-Pr 195 and NSMT-Pr 196).

Etymology: The species is named in honour of Prof. John R. Kusel, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK, who has encouraged our experimental studies of *T.* (*H.*) *grosi* and stimulated our interests to a wide range of trypanosomes.

Remarks

The described species should be classified in the subgenus *Herpetosoma* rather than the subgenus *Megatrypanum*. Trypanosomes of the latter subgenus have a wide undulating membrane and an anteriorly situated kinetoplast near the nucleus (Hoare 1972). A relatively short free flagellum and a more anteriorly positioned kinetoplast, compared with *T. lewisi* and most "lewisi-like" species of the subgenus *Herpetosoma*, have been described in other *Herpetosoma* species, such as *Trypanosoma zapi* from jumping mice (*Zapus* spp.; Davis 1952) and *Trypanosoma aunawa* from an insectivorous bat (*Miniopterus tristris*; Ewers 1974). Furthermore, the SSU and LSU rDNA nucleotide sequences indicate that this species is closely related to *T.* (*H.*) *otospermophili*, and to a lesser extent other *Herpetosoma* trypanosomes of the Muridae family, rather than *Trypanosoma talpae* classified in the subgenus *Megatrypanum* (Hamilton et al. 2005; Sato et al. 2005). As shown in Tables 1 and 2, no squirrel *Herpetosoma* species recorded to date show an identical morphology with this new species, and there are no collection records of any trypanosomes from the genus *Pteromys* and other relative genera of flying squirrels.

The infection course of squirrel *Herpetosoma* trypanosomes in jirds

*Trypanosoma otospermophili* from a Columbian ground squirrel was expanded successfully in culture. Live *T. otospermophili* from a Richardson's ground squirrel and *T. kuseli* sp. n. from Siberian flying squirrels were not available; therefore, in vitro culture was not attempted. To

**Table 2** Classification of squirrels (the family Sciuridae) and recorded *Herpetosoma* trypanosome species[a]

| Subfamily | Tribe | No. of genera | Representative genus | Trypanosome species (hosts)[b] |
|---|---|---|---|---|
| Ratufinae | | 1 | *Ratufa* | – |
| Sciurillinae | | 1 | *Sciurillus* | – |
| Sciurinae | Pteromyini | 15 | *Pteromys, Petaurista, Glaucomys* | *T. kuseli* sp. n. (*Pteromys volans*) |
| | Sciurini | 5 | *Sciurus, Tamiasciurus* | *Trypanosoma* sp. (*Tamiasciurus* spp.) |
| Callosciurinae | Callosciurini | 12 | *Callosciurus, Dremomys, Exilsciurus* | – |
| | Funambulini | 1 | *Funambulus* | *T. indicum* (*Funambulus* spp.) |
| Xerinae | Xerini | 3 | *Xerus, Spermophilopsis, Atlantoxerus* | *T. xeri* (*Xerus* spp.) |
| | Marmotini | 6 | *Marmota, Spermophilus, Tamias* | *T. parkeri* (*Marmota* spp.)<br>*T. otospermophili* (*Spermophilus* spp.)<br>*T. tamiasi* (*Tamias* spp.) |
| | Protoxerini | 6 | *Funisciurus, Heliosciurus, Protoxerus* | *T. sciuri* (*Funisciurus* spp.) |

[a] Classification of squirrels follows a newly proposed system by Steppan et al. (2004).
[b] Bar means that no trypanosomes have been recorded.

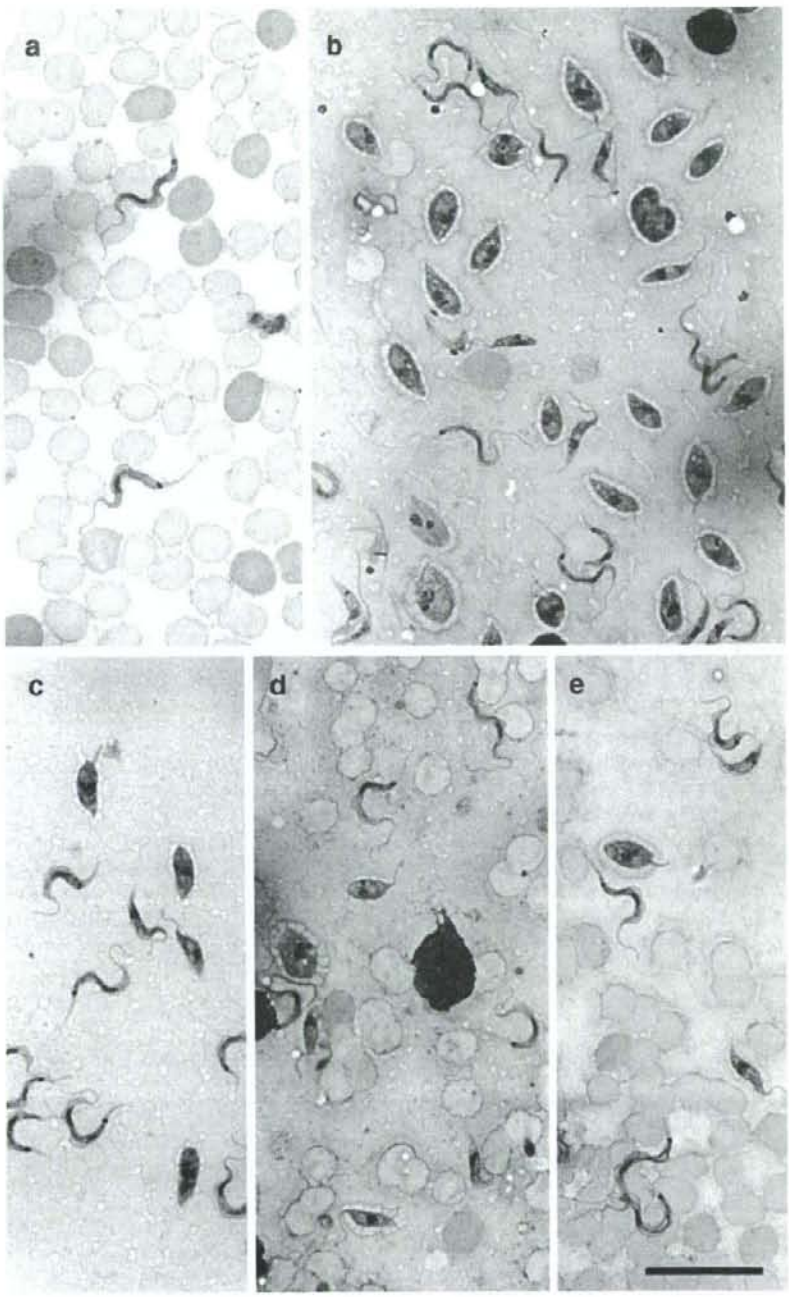


**Fig. 2** Dividing and developed *T.* (*H.*) *otospermophili* in various organs (**a**, **c**, smear; **b**, **d**, **e**, impression smear) of a prednisolone-treated jird at day 7 p.i. **a** Peripheral blood; **b** peritoneal cavity; **c** pleural effusion; **d** spleen; **e** kidney (Giemsa's stain, scale=20 μm)

assess the usability of jirds as experimental hosts for sciurid *Herpetosoma* trypanosome species, *T. otospermophili* was injected i.p. to prednisolone-treated jirds.

In experiment I, two of three jirds died abruptly on day 7 p.i. with marked hydrothorax, although no parasitaemia was recorded on day 5 p.i. Pleural effusion was 0.5 ml in each dead jird and contained $2.4\times10^8$ proliferating and developed trypanosomes/ml in each case (Fig. 2). In these two jirds, proliferating and developed trypanosomes were also seen in the peripheral blood and organ impression smears, including the peritoneum, spleen and kidney. One live jird exhibited parasitaemia, with $3.0\times10^7$ developed trypanosomes/ml on day 7 p.i., but no parasitaemia was recorded on days 9 and 13 p.i. This jird was killed on day 17 p.i., and no gross lesions were found in any organs. However, proliferating and developed trypanosomes were abundant in a pleural impression smear.

In experiment II, parasitaemia was detected only in jirds (Table 3), and again, one old jird died due to marked hydrothorax on day 9 p.i. Counting of trypanosomes in pleural effusion was not performed due to autolysis, but it was observed to contain abundant trypanosomes. Remaining animals were killed on day 13 p.i., and one old jird was noted to have fibrinous pleurisy. No parasites were detected in any blood or organ impression smears. Histological examination revealed some lesions associated with pleurisy, but no foci of parasite proliferation were detected in any organs.

## Discussion

Table 2 summarises the relationship among eight known squirrel *Herpetosoma* trypanosome species following a newly proposed classification of the Sciuridae by a molecular phylogenic approach (Steppan et al. 2004), although there exists great controversy between paleontologists and molecular phylogenists (Mercer and Roth 2003). Recent molecular phylogenic analyses (Mercer and Roth 2003; Steppan et al. 2004) indicate that flying squirrels (Tribe Pteromyini) and tree squirrels (Tribe Sciurini) are phylogenetically close, comprising the subfamily Sciurinae (see Table 2). Of particular note, a novel species, *T. kuseli* sp. n., in Siberian flying squirrels has a morphology distinct from that of seven other squirrel *Herpetosoma* species that exhibit a typical "lewisi-like" morphology (Dorney 1967; Hoare 1972; Riedel 1975).

Dorney (1967) detected undescribed *Herpetosoma* trypanosomes from eastern and western American chipmunks (*Tamias striatus* and *Eutamias minimus*) and the American red squirrel (*Tamiasciurus hudsonicus*) in Wisconsin. He did not specify a name for them as he was of the opinion that a new species designation required not only an occurrence in a new host genus (a possible criteria proposed by Levine 1965) but also biological information on all aspects of their life history, which characterised the species from existing species (Dorney 1967). Trypanosomes from chipmunks (the genera *Tamias* and *Eutamias*) were later designated as *T.* (*H.*) *tamiasi* by Riedel (1975), who conducted laborious transmission experiments and intensive microscopic observations, whereas those from red squirrels (the genus *Tamiasciurus*) remained to be characterised and designated. It is usually difficult to maintain sufficient parasite resources and naive rodents as natural hosts for the parasite. Due to this reason, PCR-based genetic characterisation of an isolated trypanosome species, such as rDNA, has a great advantage. This technology enables feasible comparison with recorded species, promoting reliable designation of trypanosomes collected in the field and understanding of the phylogeographical relationships of *Herpetosoma* trypanosome species, which may only be partially known. Considering that rodents comprise the largest mammalian order with 29 families, 443 genera and approximately 2,015 species [see Dr. J. Decher's homepage, University of Vermont (URL: http://www.uvm.edu/~jdecher/Lecture19.html)], and *Herpetosoma* trypanosomes have adapted most successfully in rodent hosts with an assumption that one host genus might have one unique species (Levine 1965), designated *Herpetosoma* species are certainly too small in number to date.

**Table 3** Parasitaemia in laboratory rodents after intraperitoneal injection of $2\times10^5$ *T. otospermophili*

| Animal | Parasitaemia[a] | | | | |
|---|---|---|---|---|---|
| | 3 days | 5 days | 7 days | 9 days | 13 days |
| Young jirds | 0/4 | 0/4 | 2/4 ($2.5/5.0\times10^4$/ml) | 2/4 ($5.0/10.0\times10^4$/ml) | 0/4 |
| Old jirds | 0/6 | 1/6 ($2.5\times10^4$/ml) | 2/6 ($2.5/20.0\times10^4$/ml) | 2/5 ($2.5/2.5\times10^4$/ml) | 0/5 |
| Prednisolone-treated mice | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Naive mice | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |

[a] Number of positive animals/number of examined animals. The level of parasitaemia is indicated in parentheses.

Similarly, even for the Sciuridae family, which contains 50 genera and 273 species (Hoffman et al. 1993), records of only eight *Herpetosoma* trypanosome species at present might be too small in number, and more species wait to be described using molecular taxonomic approaches.

The SSU rDNA nucleotide sequence of *T. kuseli* sp. n. was compared with that of several *Herpetosoma* species of ground squirrels (*T. otospermophili*) and murid hosts (*T. grosi*, *T. lewisi*, *T. musculi* and *T. micoroti*) reported in our previous study (Sato et al. 2005). The sequences of the two squirrel *Herpetosoma* species were distinctly separate from those of the murid species, and there were base changes at 19 positions between the 2,218-bp SSU rDNA sequence of *T. kuseli* sp. n. and the 2,215-bp SSU rDNA sequence of *T. otospermophili*. Between *T. lewisi* and *T. musculi*, there was only a single base change at position 1,321 of their 2,219-bp SSU rDNA sequences, despite their distinct biological characters, such as host specificity and manner of parasite reproduction (Hoare 1972; Haag et al. 1998). In contrast, three isolates of *T. grosi* from different *Apodemus* spp. had base changes at six positions of their 2,219-bp SSU rDNA sequences (Sato et al. 2005). Although the use of isoenzyme patterns (Mohamed et al. 1987) or lectin-binding patterns (Maraghi et al. 1989) also helps to differentiate *Herpetosoma* species, PCR-based molecular taxonomic approaches using a minimum volume of sample are superior to these methods, particularly when a new species is recorded. The most appropriate target for PCR amplification and sequencing for taxonomic purposes should be determined in the future from the increasing amount of data on recorded species with well-defined morphological and biological characters, host specificity and geographical distribution.

A laboratory reproduction of sciurid infection with *Herpetosoma* trypanosomes, *T. otospermophili* from a Columbian ground squirrel, using prednisolone-treated jirds was partially successful. We could detect only low levels of parasitaemia (up to $2.0\times10^5$/ml of blood) by *T. otospermophili* in jirds, compared with those resulting from long-lasting *T. grosi* and short-lasting *T. lewisi* infection in prednisolone-treated jirds (Sato et al. 2003–2005). Hydrothorax killed some infected jirds with *T. otospermophili*, and the pleural effusion contained $2.4\times10^8$ developed as well as dividing trypanosomes/ml (Fig. 2). It is uncertain whether hydrothorax was caused specifically by the trypanosomes or not. Furthermore, dividing trypanosomes were also seen in the peritoneal cavity, and to a lesser degree in the peripheral blood, spleen and kidney. Based on these observations, it is possible that *T. otospermophili*, at least in laboratory jirds, proliferates in the body cavity. It is unclear whether *T. kuseli* sp. n. can proliferate in laboratory jirds like *T. otospermophili*. However, this should be investigated, when live trypanosomes are available, as it is essential to develop an experimental host for trypanosomes that is easier to handle or manipulate in the laboratory than the natural hosts.

Although rodent trypanosomes of the subgenus *Herpetosoma* lack essentially medical and economic importance, the study of their evolutionary process or adaptation to diverse members of Rodentia through molecular phylogenetic approaches is important in order for parasitologists to understand the host adaptation process of an ancient parasite. In addition, mammalogists could use the information from these studies to aid elucidation of the interactions between host animals of the past and present (Noyes et al. 2002; Hamilton et al. 2005; Sato et al. 2005).

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