Full Paper

Widespread distribution of *Trypanosoma* (*Herpetosoma*) *grosi* in Japanese field mice (*Apodemus speciosus*)

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Abstract

The species variations and geographical distributions of trypanosomes in Japan are still largely unknown. To evaluate the endemic status of the *Trypanosoma* spp. in the wildlife of Japan, we collected 20 *Trypanosoma*-positive blood samples from *Apodemus speciosus* captured at selected 11 sites on the four main islands (Hokkaido, Honshu, Shikoku, and Kyushu) and on Awaji Island. The representative *Trypanosoma*-positive rates were 25.0% (1/4) at Aomori (a northern part of Honshu), 83.3% (5/6) at Noto peninsula and 37.5% (3/8) at Mt. Hakusan (Ishikawa prefecture, a central area of Honshu). Morphometric analysis of the trypomastigotes detected on the Giemsa-stained blood smears showed that all isolates were within the range of parameter indexes for *Trypanosoma* (*Herpetosoma*) *grosi*. Molecular analysis based on a partial sequence (522 bp) of the 18S small subunit of ribosomal RNA locus confirmed the identification. Since atypical human cases of animal-specific *Trypanosoma* spp. infections are becoming increasingly recognized, the widely distributed and highly endemic *T. grosi* presence in Japanese field mice should be monitored as a potential source of human trypanosomiasis.

Key words: *Trypanosoma grosi*, *Herpetosoma*, *Apodemus speciosus*, trypanosomiasis, 18S rRNA

Introduction

Trypanosomes are flagellated protozoan parasites that infect a wide range of organisms, including host animals and vector insects, as digenetic parasites. In humans, only two species of *Trypanosoma* are known to be causative agents of human trypanosomiasis. These species include *T. cluzi*, which causes American trypanosomiasis (Chagas disease), and *T. brucei* (*T.b.*) *gambiense* and *T.b. rhodesiense,* which cause African trypanosomiasis (African sleeping sickness). While native human trypanosomiasis does not exist outside the American and African continents, atypical types of human trypanosomiasis caused by the animal-specific trypanosomes are increasingly recognized [reviewed in 9, 22]. Considering the origin of human trypanosomiasis, which appears to be the result of evolutionary re-adaptation of related animal species [20], and considering the phenomenon of host switching that has been observed in *T. lewisi* [10], human cases of atypical trypanosomiasis and the *Trypanosoma* spp. in animals near human habitats should be carefully studied, since those cases may serve as a precursory indication of novel trypanosomiasis in humans.

Little is known about the distribution of *Trypanosoma* spp. in domestic and wild animals of Japan. In primates, early tests for the presence of trypanosomes in *Macaca fuscata* from 6 different areas were all negative [24]. Since then, no cases have been reported, except for the imported *Trypanosoma (Megatrypanum) minasense* infection that was detected in quarantined neotropical primates [19]. The presence of *T. theileri* has been previously

62 reported in Japanese deer [3] and cattle; the sequence data (accession number AB007814) was
63 directly submitted to the DNA Data Bank of Japan (DDBJ), and was used in a trial phylogeny
64 analysis for *T. theileri* [13]. While, from wild rodents, unidentified *Trypanosoma* spp. isolated
65 from *Apodemus speciosus* in the Iwate prefecture [6] and an isolate of *T.* (*Herpetosoma*) *grosi*
66 from *A. speciosus* captured at the Aomori prefecture, have been reported [17].

67 To evaluate the geographical and species distribution of *Trypanosoma* in Japanese wild
68 life, we analyzed *Trypanosoma* spp. detected from captured wild rodents at various sites in
69 Japan.

70

71 **Materials and Methods**

72

73 **Rodent blood sample collection**

74 *Trypanosoma* spp. was collected from infected wild rodents at various sites in Japan between
75 2001 and 2007 (Fig. 1, Table 1). Among these, the field mice from the Sirakami Mountains
76 (Aomori prefecture, northern Honshu region; 36°56’53.37N, 136°49’49.15”E), Noto
77 peninsula (coastal region of Ishikawa prefecture, central Honshu; 36°5’48.86”N,
78 136°41’45.09”E), and Mt. Hakusan (a mountain range in the Ishikawa prefecture;
79 40°34’25.56”N, 140°16’43.61E), were specifically collected to evaluate the prevalence of
80 *Trypanosoma* spp. Although the remaining samples were collected for the purpose of other

81 research [14, 15], the accidental discovery of trypanosomes by microscopic examination led
82 to these samples being used in our study. All rodents were captured using Sherman traps (live
83 traps). The cardiac puncture blood was smeared on a slide grass immediately after the
84 sacrifice. In addition, 200-300 µl blood from the rodents was also stored in a 1.5 ml tube, at
85 4°C in the field and -20°C in the laboratory, for the further DNA analyses.

86

87 **Microscopic examination and morphological identification of *Trypanosoma* sp.**

88 The Giemsa stained blood smear samples were microscopically examined under high
89 magnification (1000×), and the *Trypanosoma*-positive blood samples were subjected to
90 further molecular analyses. Measurement of morphometric parameters and calculations of
91 indexes (Table 2) performed as previously reported [5, 7].

92

93 **DNA extraction and molecular identification**

94 DNA was extracted from whole blood samples using the QuickGene DNA Tissue Kit S
95 (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. The extracted genomic
96 DNA was concentrated to a 10 ng/µl aqueous solution by ethanol precipitation, and stored at -
97 20°C for future experimentation.

98 A partial DNA fragment belonging to the 18S small subunit ribosomal RNA (18S
99 rRNA) gene locus (561 bp) of the *Trypanosoma* species was amplified by nested polymerase

chain reaction (PCR) [11]. The reaction was carried out in 0.2 ml thin-walled PCR tubes and run on a MyCycler (BioRad Laboratories, CA, USA) using the following primers: outer primers, TRY927F 5′-GAAACAAGAAACACGGGAG-3′ and TRY927R 5′-CTACTGGGCAGCTTGGA-3′; inner primers, SSU561F 5′-TGGGATAACAAAGGAGCA-3′ and SSU561R 5′-CTGAGACTGTAACCTCAAAGC-3′. The optimized PCR conditions were as follows. Approximately 10 ng of DNA template was amplified in a 20 µl reaction mixture containing 0.4 µM of each primer, 0.8 mM of each deoxynucleotide triphosphate (dNTP), and 1 U of PrimeSTAR HS DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and 1× PrimeStar reaction buffer. The cycle parameters for the first round of PCR were as follows: initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 55°C for 15 s, and 72°C for 60 s, and a final extension at 72°C for 2 min. For the second round of PCR, 1 µl of the PCR mixture from the initial PCR round was used as template with a similar reaction mixture. The cycle conditions used for the second round were also the same except for the extension time, which was decreased to 30 s. The PCR products were electrophoresed on 2.0% L03 agarose (TaKaRa Bio Inc.) with ethidium bromide, and visualized on an ultraviolet transilluminator, the Gel Doc™ EZ Imager (BioRad Laboratories). The target bands were excised from the gel and purified using the Quantum Prep™ Freeze ‘N Squeeze DNA Gel Extraction Spin Columns (BioRad Laboratories) according to the manufacturer's instructions.

These PCR amplicons were cloned into the EcoRV site of the pBluescript II SK(+) plasmid (Stratagene, CA, USA), using the Mighty Cloning Reagent Set (blunt-end) (TaKaRa

120 Bio Inc.). The cloning was performed according to the manufacturer's instructions. The
121 recombinant plasmids were transformed into *Escherichia coli* DH5α (Stratagene) and
122 screened on Luria Broth (LB) agar plates supplemented with 100 mg/l of ampicillin. The
123 clones were selected as individual *E. coli* DH5α colonies and cultured overnight in 2 ml LB
124 broth supplemented with 100 mg/l of ampicillin. The culture was collected and pelleted, and
125 plasmid DNA extracted using the QIAGEN® Plasmid Mini Kit (QIAGEN K.K., Tokyo, Japan)
126 according to the manufacturer's instructions.

127 Full-length sequences were confirmed using the T3 and T7 primers with the ABI Prism
128 BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan) on an
129 Applied Biosystems 3130 Genetic Analyzer (Life Technologies). All nucleotide sequences
130 were confirmed using both forward and reverse data reads.

131 The partial sequences (522 bp) of the 18S rRNA gene locus of the *Trypanosoma* species
132 confirmed in this study were subjected to a homology search using the nucleotide basic local
133 alignment search tool (BLASTn) algorithm on the DNA databank of Japan (DDBJ). The
134 reference *T. grosi* sequences which provided a 99- to 100%-match, which were; the AKHA
135 strain (AB175624), 110 strain (AY043355), HANTO strain (AB175623), SESUJI strain
136 (AB175622), and Cha1 strain (FJ694763), were aligned on MEGA5 [21].

137

138 **Results and discussion**

139

140 Morphologically, all captured rodents were identified as large Japanese field mice
141 (*Apodemus speciosus*), which are known to be distributed all over Japan [4]. The positive
142 rates of *Trypanosoma* species (Table 1) confirmed by microscopic examination were 25.0%
143 (4/16) at Mts. Sirakami, Aomori, and 83.3% (5/6) and 37.5% (3/8) at Noto peninsula and Mt.
144 Hakusan, respectively, which are both in the Ishikawa prefecture. In addition to the
145 prevalence data, we confirmed the presence of the *Trypanosoma* sp. in *A. speciosus* obtained
146 from all four main islands (Hokkaido, Honshu, Shikoku, and Kyushu) and the Awaji Island in
147 Japan (Fig. 1). To date, there are no other data about the endemic status of *Trypanosoma* spp.
148 in Japanese wild rodents; however, these preliminary data suggest that *Trypanosoma* sp.
149 infection in *A. speciosus* might be common.
150 All the measured dimensions and calculated indexes of observed trypomastigotes (Fig. 2,
151 Table 2) were within the range found in previous *T. grosi* data [7, 8]. Although polymorphic
152 forms of *T. grosi* trypomastigote in the host blood stream were previously found to occur as a
153 mixture of the slender and stout shapes in Poland isolates [8], in this study, all
154 trypomastigotes were of the slender-shaped type: body lengths and widths ranged from 20.5
155 to 21.2 μm and from 1.5 to 1.8 μm, respectively. These sizes are not within the length and
156 width ranges of 14.7 ± 2.3 and 1.8 ± 0.3, respectively, previously reported for the stout-
157 shaped type [8]. Rather, our data followed the morphological data of *T. grosi* isolated in
158 Eastern Slovakia [7]. Although further experimental data are required to explain the
159 morphological variation observed in *T. grosi*, it is noteworthy that the blood smear

preparations in this study were conducted at the sites using the live traps. As discussed in previous publications, the morphological development of *Trypanosoma* into the insect form might be partly dependent on temperature condition [1]. In the case of delayed smear preparation, the temperature of the blood appeared to drop to the room temperature level, we also observed the staut-shaped variants (data not shown).

All of the sequenced clones, which included 522 bp of the 18S rRNA gene locus, were completely identical and showed 100% similarity to the reference data of *Trypanosoma grosi,* AKHA (Japan isolate), HANTO (Russia isolate), SESUJI (Russia isolate) [18], and 110 (England isolate) [12]. We also observed a single nucleotide substitution (A to G) at the 1027 bp position in the confirmed sequence (based on the AY043355) from Cha1 strain (China isolate) [2]. Although the 522 bp sequence appeared to be too short for the evaluation of intra-species diversity, the nested PCR system was useful and convenient as a molecular taxonomic tool of *Trypanosoma* spp. [11] for performing species-level identification.

In this study, we have confirmed the wide distribution and highly endemic status of *T. grosi* in Japanese field mice. Considering the increasing number of reported cases of atypical human *T. lewisi*-like infection [16, 23], as a same rodent-infecting Stercoraria (formerly known as the *lewisi* group) [5], the presence of *T. grosi* near human habitats must be closely monitored as a potential source of human trypanosomiasis.

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266

267

268 **Figure legend**

269

270 **Fig. 1 Sample collection sites**

271

272 **Fig. 2 Representative micrograph of a trypomastigote detected in this study.** The
273 trypomastigote displayed a slender form with a sharp posterior end, a distinct kinetoplast
274 located at a distance from the posterior tip, a rod-shaped nucleus between the anterior and
275 middle region of the body, a weakly developed undulating membrane with the attached
276 marginal flagellum extended along the complete length of the body, and a well-developed free
277 flagellum. All of these morphological features matched the morphology of Stercoraria (the *T.*
278 *lewisi* group [5]). The measured morphological parameters were within the range of the *T.*
279 (*Herpetosoma*) *grosi,* trypomastigote form (Table 2).

Table.1 Background information of detected *Trypanosoma grosi* in this study.

(a)

Collection site (latitude, longitude)	Date	Positive rate
Shoubudani, Noto, Ishikawa (36°56'53.37"N, 136°49'49.15"E)	May.12-25.2007	5/6
Mt. Hakusan, Ishikawa (36°5'48.86"N, 136°41'45.09"E)	Jun.14-15.2006	3/8
Mts. Shirakami, Aomori (40°34'25.56"N, 140°16'43.61"E)	Jun.23.2006	1/4

(b) Other positive samples

Collection site	Date	Sample no.
Sapporo, Hokkaido	Sep.2003-Jun.2004	4
Mt. Yunomaru, Nagano	Sep.2003	1
Mt. Happu, Nagano	Sep.2003	1
Yachiho village, Nagano	Fe.2004	1
Mt. Tsurugi, Tokushima	Apr.2003	1
Mt. Yae, Kagoshima	Jan.2003	1
Uchinoura, Kagoshima	Feb.2004	1
Awaji Island, Hyogo	May.2001	1

Table 2. Observed dimensions (in µm) and calculated indexes of *Trypanosoma grosi* compared to reference data

Collection site (Identification)	Host	BL[a)]	BW[b)]	FF[c)]	NL[d)]	NI[e)]	KI[f)]	Reference
Shoubudani,Noto, Ishikawa (*T. grosi*)	*A. speciosus*	20.5 ± 1.1[g)]	1.5 ± 0.4	7.0 ± 0.59	2.9 ± 0.24	1.4 ± 0.11	1.4 ± 0.05	This study
Hakusan, Ishikawa (*T. grosi*)	*A. speciosus*	20.9 ± 1.7	1.5 ± 0.3	7.1 ± 0.90	3.1 ± 0.31	1.2 ± 0.17	1.3 ± 0.09	This study
Shirakami Mt. range, Aomori (*T. grosi*)	*A. speciosus*	21.2 ± 1.4	1.8 ± 0.3	7.1 ± 0.68	2.6 ± 0.31	1.4 ± 0.19	1.4 ± 0.06	This study
Eastern Slovakia (*T. grosi*)	*A. agrarius*	21.1 ± 3.1	1.5 ± 0.3	8.9 ± 1.8	2.7 ± 0.6	1.4 ± 0.4	1.5 ± 0.2	[7]
Poland (*T. grosi* kosewiense subsp.: slender form)	*A. flavicollis*	23.0 ± 2.3	1.4 ± 0.3	7.6 ± 1.4	2.7 ± 0.4	1.2 ± 0.3	1.5 ± 0.1	[8]
Poland (*T. grosi* kosewiense subsp.:stout form)	*A. flavicollis*	14.7 ± 2.3	1.8 ± 0.3	8.6 ± 1.5	2.4 ± 0.3	1.8 ± 0.4	1.3 ± 0.1	[8]

[a)]BL, body length; [b)]BW, body width at the nucleus level, excluding the undulating membrane; [c)]FF, free flagellum length; [d)]NL, nucleus length; [e)]NI, nuclear index = PN / NA; [f)]KI, kinetoplastic index= PN / KN, KN indicates distance from kinetoplast to nucleus center; Data of posterior end to nucleus center (PN) and nucleus center to anterior end (NA) were not shown and represented as the calculated indexes NI and KI; [g)]Mean ± standard deviation is shown (n = 10).

Fig. 1

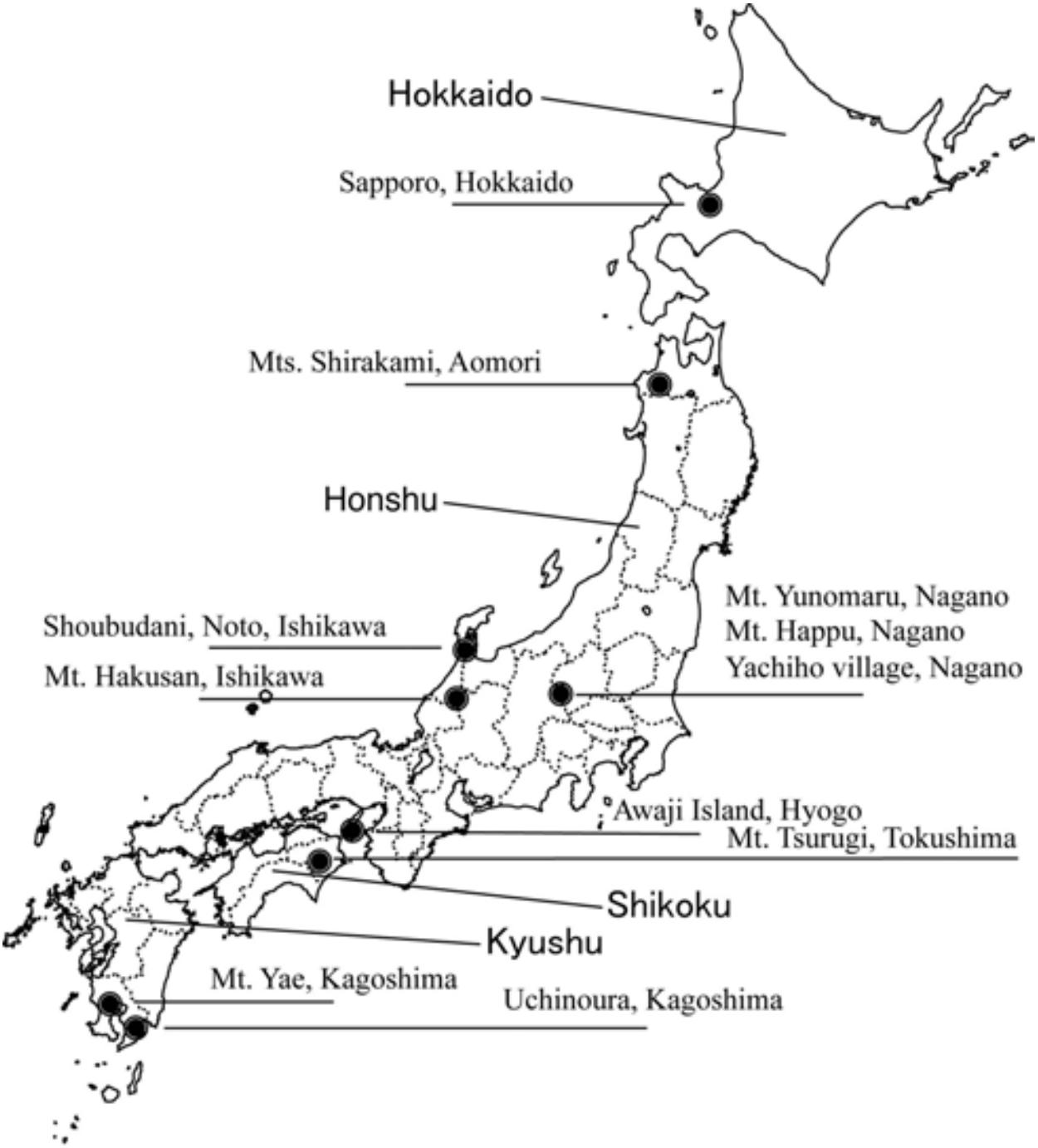
Hokkaido
Sapporo, Hokkaido
Mts. Shirakami, Aomori
Honshu
Mt. Yunomaru, Nagano
Mt. Happu, Nagano
Yachiho village, Nagano
Shoubudani, Noto, Ishikawa
Mt. Hakusan, Ishikawa
Awaji Island, Hyogo
Mt. Tsurugi, Tokushima
Shikoku
Kyushu
Mt. Yae, Kagoshima
Uchinoura, Kagoshima

Fig. 2