Characterization of SSU and LSU rRNA genes of three *Trypanosoma* (*Herpetosoma*) *grosi* isolates maintained in Mongolian jirds

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(*Received 28 April 2004; revised 19 July 2004; accepted 22 July 2004*)

SUMMARY

Trypanosoma (*Herpetosoma*) *grosi*, which naturally parasitizes *Apodemus* spp., can experimentally infect Mongolian jirds (*Meriones unguiculatus*). Three isolates from *A. agrarius*, *A. peninsulae*, and *A. speciosus* (named SESUJI, HANTO, and AKHA isolates, respectively) of different geographical origin (AKHA from Japan, and the others from Vladivostok), exhibited different durations of parasitaemia in laboratory jirds (2 weeks for HANTO, and 3 weeks for the others). To assess the genetic background of these *T. grosi* isolates, their small (SSU) and large subunit (LSU) ribosomal RNA genes (rDNA) were sequenced along with those of 2 other *Herpetosoma* species from squirrels. The SSU rDNA sequences of these 3 species along with available sequences of 3 other *Herpetosoma* trypanosomes (*T. lewisi*, *T. musculi* and *T. microti*) seemed to reflect well the phylogenetic relationship of their hosts. Three isolates of *T. grosi* exhibited base changes at 2–6 positions of 2019-base 18S rDNA, at 5–29 positions of 1817/1818-base 28Sα rDNA, or 1–5 positions of 1557–1559-base 28Sβ rDNA, and none was separated from the other 2 isolates by rDNA nucleotide sequences. Since base changes of *Herpetosoma* trypanosomes at the level of inter- and intra-species might occur frequently in specified rDNA regions, the molecular analysis on these regions of rodent trypanosomes could help species/strain differentiation and systematic revision of *Herpetosoma* trypanosome species, which must be more abundant than presently known.

Key words: *Herpetosoma* trypanosomes, *Trypanosoma grosi*, SSU rDNA, LSU rDNA, *Meriones unguiculatus*, *Apodemus* spp.

INTRODUCTION

Rodent trypanosomes of the subgenus *Herpetosoma* show rigid specificity of their vertebrate hosts (Hoare, 1972), even though the vectors such as fleas are shared among multiple hosts (Molyneux, 1969*a*, *b*; Maraghi & Molyneux, 1989). Due to their narrow host range, only 2 species of *Herpetosoma* trypanosomes have been studied in detail using common laboratory rodents; i.e., *Trypansoma* (*H.*) *lewisi* and *Trypanosoma* (*H.*) *musculi*, which naturally infect rodents of the genera *Rattus* and *Mus*, respectively (reviewed by Dusanic, 1975; Viens, 1985; Albright & Albright, 1991; Monroy & Dusanic, 2000). Experimental observations on other *Herpetosoma* trypanosomes might be possible only when natural rodent hosts had been prepared with special individual efforts (Molyneux, 1969*a*, *b*, 1970). As reported previously (Sato *et al*. 2003, 2004), *Trypanosoma* (*H.*) *grosi* naturally infecting *Apodemus* spp. causes persistent infection in laboratory Mongolian jirds (*Meriones unguiculatus*) as well. In our previous studies (Sato *et al*. 2003, 2004), we noticed different infection courses of *T. grosi* isolated originally from *Apodemus agrarius* (SESUJI isolate) and *Apodemus peninsulae* (HANTO isolate) in Vladivostok, Russia; the parasitaemia due to these two isolates in jirds terminated at 2 or 3 weeks post-infection (p.i.), respectively.

Morphologically, trypanosomes of the subgenus *Herpetosoma* resemble closely each other, which is generally called '*lewisi*-like' after the type species of *T. lewisi* (Davis, 1952; Hilton & Mahrt, 1972; Hoare, 1972). The rigid 'host specificity' of *Herpetosoma* trypanosomes might provide a clue for differentiation of trypanosome species found in the blood; i.e., it is impossible to infect hosts from different genera or

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Parasitology (2005), **130**, 157–167.
DOI: 10.1017/S0031182004006493 Printed in the United Kingdom

higher taxonomic categories with a singlle *Herpetosoma* species. However, exceptions to this rigid host specificity are sometimes found in experimental transmission (Culbertson, 1941; Mühlpfordt, 1969; Sato *et al*. 2003). In this regard, Noyes *et al*. (2002) detected natural infection with *Herpetosoma* trypanosomes of a novel genotype distinct from *T. grosi* from 2 individuals of *Apodemus sylvaticus* caught in Cheshire, England. In addition, as mentioned above and shown by Chiejina *et al*. (1993), we know the presence of trypanosome populations of a single species that differ phenotypically e.g. different courses of parasitaemia in the same laboratory host. At present, there are no assays to assess the significance of *Herpetosoma* trypanosome isolates originating from different host species of the same genus, or showing different biological characters, or to differentiate the observed species with certainty. The present study was designed to assess the genetic background of *T. grosi* isolates with different origins and/or different biological characters by investigating the small (SSU) and large subunit (LSU) ribosomal RNA genes (rDNA). In addition, 2 squirrel-parasitic species of *Herpetosoma* trypanosomes including *Trypanosoma* (*H.*) *otospermophili* were similarly characterized. Our results would provide the framework for future studies on abundant *Herpetosoma* species and/or strains collected from different rodent genera or species in the same or different geographical regions.

MATERIALS AND METHODS

Parasites

Two isolates of *T. grosi* (SESUJI and HANTO isolates) originated from a striped field mouse, *A. agrarius*, and a Korean field mouse, *A. peninsulae*, which were trapped in Vladivostok, Russia, as described previously (Sato *et al*. 2003). Another isolate of *T. grosi* (named AKHA isolate after the Japanese name of the host 'akha-nezumi') originated from a large Japanese field mouse, *A. speciosus speciosus*, trapped on 9 June 2003 in Takko, Aomori, Japan. Each isolate was expanded by *in vitro* cultures on monolayers of jird's renal cells in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0·3% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphotericin B, and 10% foetal calf serum (Sato *et al*. 2003). *Trypanosoma lewisi* was isolated from a wild brown rat, *Rattus norvegicus*, caught in Shihezi, Xinjiang, China on 13 September 2001 as follows. A renal tissue piece was cut aseptically from this rat, immersed in supplemented RPMI 1640 medium, kept at 4 °C for 9 days, and brought to the laboratory. The material was kept for 5 days under sterile conditions and 5% CO_2 at 37 °C. Unknown numbers of trypanosomes swimming in the culture medium were inoculated intraperitoneally (i.p.) into a 6-week-old female SD rat injected subcutaneously (s.c.) with 20 mg methylprednisolone acetate (Depo-Medrol® sterile aqueous suspension; Pharmacia & Upjohn Co., Tokyo) simultaneously. Prednisolone treatment of experimental hosts is known to increase the level of parasitaemia (Dusanic, 1975; Viens, 1985; Albright & Albright, 1991; Sato *et al*. 2003). Nine weeks later, 4×10^6 trypanosomes taken from the peripheral blood of this rat were injected into another 15-week-old female SD rat treated similarly with prednisolone. *Trypanosoma* (*H.*) *otospermophili* and another *Herpetosoma* trypanosome species were collected from an imported Richardson's ground squirrel, *Spermophilus richardsonii*, and Siberian flying squirrel, *Pteromys volans*, sacrificed on 14 June 2003 for a survey of zoonotic agents in imported pet-rodents. Details of these 2 species will be reported separately. Parasite materials for DNA extraction were preserved at −80 °C until use.

Infection and monitoring of parasitaemia

Mongolian jirds 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.

Trypanosoma lewisi preparations for infection were collected from the peripheral blood of prednisolone-treated rats at days 15 to 77 p.i., and re-suspended in supplemented RPMI 1640 medium containing heparin but free of foetal calf serum. Groups of jirds were inoculated i.p. with *T. lewisi*, and injected s.c. with 10 mg methylprednisolone acetate at infection, or injected i.p. with 0·4 ml of SD rat serum every 1·5 days for 2 weeks of infection. *Trypanosoma grosi* preparations for infection were described previously (Sato *et al*. 2003, 2004). After injection of these parasite suspensions in jirds, the course of parasitaemia was monitored by counting the number of trypanosomes in the peripheral blood on a haemocytometer. The blood sample was collected from the orbital venous plexus and diluted in 0·83% NH_4Cl-Tris buffer with heparin.

Polymerase chain reaction (PCR) and nucleotide sequencing

Parasite DNA was prepared using GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the instructions provided by the manufacturer.

Amplification was performed in 50 μl of a solution containing 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, each dNTP at 0·2 mM, each primer at 0·5 μM (see Fig. 4, Table 1), parasite DNA,

https://doi.org/10.1017/S0031182004006493 Published online by Cambridge University Press

Table 1. Primers used to amplify and sequence a special segment of rDNA of *Herpetosoma* trypanosomes

Segment no.*	Primer name†		Sequence	Annealing temp. (°C)‡	Position of 5′-end§
	For amplifying	For sequencing			
1	F: ITS1		5′-TCCGTAGGTGAACCTGCGG-3′	62	Unknown (IGS)
	F: ITS2		5′-GCTGCGTTCTTCATCGATGC-3′	62	Unknown (IGS)
	R: ssu/148R		5′-TGTATTAGCTTGGCGTTTCGCC-3′		177 (18S)
		R: ssu/157R	5′-CGCCAAGTTATCCACTGTTG-3′		159 (18S)
2	F: TRY/ssu-35F		5′-TGTCTGTGTGTGCCACGTAA-3′	63	-35 (IGS)
	R: ssu/352R		5′-GGCTGATAGGGCAGTTGTTC-3′		369 (18S)
3	F: ssu/1F		5′-TGATTCTGCCAGTAGTCATA-3′	62 (59–65)	9 (18S)
	R: ssu/997R		5′-.AGTGTCACCGAAAAGGAGAGC-3′		1025 (18S)
		F: NSF573/19	5′-CGCGGTAATTCCAGCTCCA-3′		631 (18S)
		F: TRY927F	(see below)		776 (18S)
4	F: TRY927F		5′-GAAACAAGAAACACGGGAG-3′	55	776 (18S)
	R: TRY927R		5′-CTACTGGGCAGCTTGGA-3′		1716 (18S)
		F: ssu561F	5′-TGGGATAACAAAGGAGCA-3′		907 (18S)
		R: ssu561R	5′-CTGAGACTGTAACCTCAAAGC-3′		1475 (18S)
5	F: ssu/1447F		5′-GCTTTGAGGTTACAGTCTCA-3′	62 (59–65)	1455 (18S)
	R: ssu/2168R		5′-CTACAGCTACCTTGTTACGA-3′		2195 (18S)
		F: NSF1179/18	5′-AATTTGACTCAACACGGG-3′		1551 (18S)
		R: TRY927R	(see above)		1716 (18S)
6	F: ssu/1447F		(see above)	65 (59–65)	1455 (18S)
	R: TRY5.8SR		5′-AAATATGGCATGCACGGGGATG-3′		159 (5.8S)
7	F: ITS5		5′-GGAAGTAAAAGTCGTAACAAGG-3′		2165 (18S)
	R: TRY5.8SR		(see above)	60–62	159 (5.8S)
	R′: 28S/475R		5′-TCTATGTCACTCTCTTTCCA-3′	60	447 (28Sα)
		F: TRY5.8SF	(see below)		39 (5.8S)
8	F: SSU/2088F		5′-CGATGATGGTGCAATACAGG-3′	63	2087 (18S)
	R:TRY5.8SR2		5′-CAAAAGAGCTTCTCCCATGC-3′		134 (5.8S)
9	F: TRY5.8SF		5′-GAAGAACGCAGCAAAGTGCGAT-3′		39 (5.8S)
	R: TRY28S/207R		5′-GTATTCAGCAACGGGTTTGC-3′	66	226 (28Sα)
	R′: ITS4		5′-TCCTCCGCTTATTGATATGC-3′	62	58 (28Sa)
10	F: TRY5.8SF		(see above)	60–63	39 (5.8S)
	R: 28S/475R		(see above)	(59–63)	447 (28Sα)
11	F: TRY5.8SF2		5′-GATGGATGACTTGGCTTCCT-3′	66	11 (5.8S)
	F: TRY28S/3F		5′-GACCTGAGTGTGGCAGGACT-3′	63–66	4 (28Sα)
	R: .TRY28S/814R		5′-GACTCCTTGGTCCGTGTTTC-3′		707 (28Sα)
		F: TRY28S/211F	(see below)		211 (28Sα)
		R: TRY28S/207R	5′-GTATTCAGCAACGGGTTTGC-3′		226 (28Sa)
12	F: TRY28S/211F		5′-ACCCGTTGCTGAATACAACC-3′	66	211 (28Sα)
	R: TRY28S/814R		(see above)		707 (28Sα)
		F: 28S/532F	(see below)		530 (28Sα)
13	F: 28S/258F		5′-CCAAAGAAGGTGTCAGCCCAT-3′	65–66	249 (28Sα)
	F: 28S/532F		5′-CCTTGTTGTGTACCCGTTGA-3′	65	530 (28Sα)
	R: NLR1432/23		5′-GTTGTTACACACTCCTTAGCGGA-3′		1374 (28Sα)
		F: TRY28S/695F	5′-GAAACACGGACCAAGGAGTC-3′		688 (28Sα)
		F: NLF1105/22	(see below)		1010 (28Sα)
14	F: TRY28S/695F		5′-GAAACACGGACCAAGGAGTC-3′	65	688 (28Sα)
	R: NLR1432/23		5′-GTTGTTACACACTCCTTAGCGGA-3′		1374 (28Sα)
15	F: NLF1105/22		5′-CCGAAGTTTCCCTCAGGATAGC-3′		1010 (28Sα)
	R: NLR2362/20		5′-ACATTCAGAGCACTGGGCAG-3′	65–66	460 (28Sβ)
	R′: NLR2781/19		5′-CCGCCCCAGYCAAACTCCC-3′	65–66	1085 (28Sβ)
		F: 28S/1405F	5′-TTATGCAAGGTCGGAAGGAC-3′		1170 (28Sα)
		F: 28S/1911F	(see below)		1664 (28Sα)
		F: 28S/2055F	5′-AAAGGGGCAACAGAGAACCT-3′		1776 (28Sα)
		R: 28Sbeta/66R	(see below)		85 (28Sβ)
		R: 28S/2556R	5′-TACACCACTTGGATGCATCAC-3′		424 (28Sβ)
16	F: 28S/1405F		(see above)		1170 (28Sα)
	R: NLR2362/20		(see above)	65	460 (28Sβ)
	R′: NLR2781/19		(see above)	63–65	1085 (28Sβ)
		28Sbeta/66R	(see above)		85 (28Sβ)
17	F: 28S/1911F		5′-TTCGGATACTGAGCACAACG-3′		1664 (28Sα)
	R: 28Sbeta/66R		5′-CTTCCCTGAGTGCACCTTTC-3′	62 (62–65)	85 (28Sβ)
	R′: NLR2781/19		(see above)	63	1085 (28Sβ)
		F: 28S/2055F	(see above)		1776 (28Sα)
		F: 28Sbeta/246F	(see below)		283 (28Sβ)
18	F: 28Sbeta/246F		5′-TCTCACTTCTGCATGGCTGT-3′	63	283 (28Sβ)
	R: TRY28S/endR		5′-TCTTGTGATCATGGAGATACTTGGCGC-3′		78 (28Sζ)
		F: NLF2551/21	(see below)		643 (28Sβ)

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Table 1. (*Cont.*)

Segment no.*	Primer name†		Sequence	Annealing temp. (°C)‡	Position of 5′-end§
	For amplifying	For sequencing			
19	F: NLF1999/19		5′-CCGCAKCAGGTCTCCAAG-3′	60	25 (28S*β*)
	R: NLR2781/19		(see above)		1085 (28S*β*)
		F: 28S/2809F	5′-GTGATGCATCCAAGTGGTGT-3′		404 (28S*β*)
20	F: NLF2551/21		5′-GGGAAAGAAGACCCTGTTGAG-3′	60–65	643 (28S*β*)
	R: NLR3284/21		5′-TTCTGACTTAGAGGCGTTCAG-3′		158 (28S*δ*)
		F: 28S/3398F	5′-AACCGTGCTGACAAACTGAA-3′		992 (28S*β*)
21	F: NLF2551/21		(see above)	63	643 (28S*β*)
	R: TRY28S/endR		5′-TCTTGTGATCATGGAGATACTTGGCGC-3′		78 (28S*ζ*)
		R: NLR2781/19	(see above)		1085 (28S*β*)
22	F: NLF3090/24		5′-AGGGAACGTGAGCTGGGTT	65	1411 (28S*β*)
	R: TRY28S/endR		5′-TCTTGTGATCATGGAGATACTTGGCGC-3′		78 (28S*ζ*)
	R′: TRY28S/endR1		5′-GGCAGGCAGAGCCTACCAGA-3′	62	43 (28S*ζ*)
		F: 28S/4027F	(see below)		30 (28S*δ*)
		F: TRY/ITS6F1	5′-TATGCGCTTGCTTCTGAAAA-3′		113 (ITS6)
		F: TRY/ITS6F2	5′-TTGTGTTGTGTGTGGTGGTG-3′		196 (ITS6)
		F: TRY/ITS7F1	5′-CCACCTTCTCCTCCTTC-3′		323 (ITS6)
23	F: 28S/4027F		5′-CGTGAGGGAAGTATGGGGTA-3′	65	30 (28S*δ*)
	R: TRY28S/endR		5′-TCTTGTGATCATGGAGATACTTGGCGC-3′		78 (28S*ζ*)
		F: TRY/ITS7F2	5′-TTGTGCAACATGCAGACAGA-3′		546 (ITS6)
24	F: 28S/4027F		(see above)	62	30 (28S*δ*)
	R: TRY5S/R2		5′-CCGTACTAACGAGGCCTGTG-3′		Unknown (ITS7?)
		F: TRY/ITS7F1	(see above)		323 (ITS6)
		R: TRY/IGSR1	(see below)		173 (ITS7)
25	F: 28S/4027F		(see above)	62–65	30 (28S*δ*)
	R: TRY/IGSR1		5′-GCGGGTGTGCAGTATCTTTT-3′		173 (ITS7)
		F: TRY/ITS7F1	(see above)		323 (ITS6)
		F: TRY/ITS7F2	(see above)		546 (ITS6)

* Number corresponds to the number attached to bars (amplified segments) shown in Fig. 4.
† Primers used for amplifying and sequencing a special segment are shown as 'for amplifying', and those only for sequencing as 'for sequencing'. Parts of used primers are similar to those employed previously by other groups (Van de Peer *et al*. 2000; [oberon.fvms.ugent.be:8080/rRNA/primers/] Noyes *et al*. 2002; and the homepage of R. Vilgalys laboratories, Department of Biology, Duke University [www.biology.duke.edu/fungi/mycolab/primers.htm]), and the primer names used here follow the original. The rest were designed by us referring to known sequences of *trypanosomatids such as T. lewisi* (DDBJ/EMBL/GeneBank Accession no. AJ223566), *T. congolense* (U22315), *T. cruzi* (L22334), and *L. major* (AC005806). F: forward, and R: reverse.
‡ Suitable annealing temperature for PCR amplification with the range in parenthesis where applicable.
§ The position of 5′-end of primers on rDNA regions of *T. grosi* (DDBJ/EMBL/GeneBank Accession no. AB175622) instead of showing the length of each amplicon.

and 1·25 U of AmpliTaq Gold® polymerase (PE Applied Biosystems, Inc., Foster City, CA, USA). Each cycle of PCR consisted of denaturation for 45 sec at 94 °C, hybridization for 60 sec at 55–66 °C (Table 1), and extension for 90 sec at 72 °C. This cycle was repeated 30–35 times. Amplified PCR products were analysed by electrophoresis using 1·3% agarose gel and ultraviolet visualization after ethidium bromide staining. Amplicons of interest were semi-purified with QIAquick® PCR purification kit (Qiagen Sciences, Inc., Germantown, MD, USA), and sequenced directly using aforementioned primers (Table 1) on ABI PRISM™ 377 (PE Applied Biosystems, Inc.). DNA sequences were aligned using the CLUSTAL W multiple alignment program (Thompson *et al*. 1994) availabe on the internet [hypernig.nig.ac.jp/homology/clustalw-e.shtml]. To clarify the relationship of nucleotide sequences of a certain rDNA region, unrooted neighbour-joining (N-J) trees with bootstrap analysis using 1000 steps were prepared on the same web site. The nucleotide sequences reported here are available in the DDBJ/EMBL/GenBank databases under Accession numbers AB175622–AB175626.

Statistical analysis

Differences between two groups were examined for significance using the Student's *t*-test. A *P* value less than 0·05 denoted statistical significance.

RESULTS

Course of parasitaemia by T. grosi (*AKHA isolate*) *and* T. lewisi *in jirds*

Parasitaemia due to AKHA isolate of *T. grosi* was observed for more than 2 weeks but not beyond 3 weeks p.i. (Fig. 1). This indicates that the course of parasitaemia by AKHA isolate resembles that of SESUJI isolate rather than that of HANTO isolate (see Sato *et al*. 2003). Persistent infection of AKHA isolate in the *vasa recta* of the kidney was observed as in other isolates of *T. grosi*, and the infection induced

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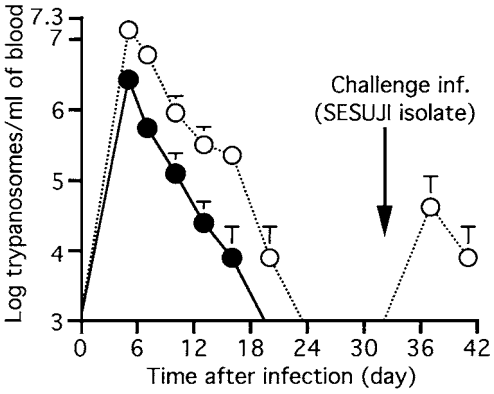

Fig. 1. Time-course of parasitaemia by AKHA isolate of *Trypanosoma grosi* in jirds. Naive (closed circles; $n=3$) and prednisolone-treated (open circles; $n=3$), 8-week-old female jirds were inoculated i.p. with 2×10^5 *T. grosi* (AKHA isolate), and challenged i.p. with 1×10^6 *T. grosi* (SESUJI isolate) at day 32 p.i. Blood was examined at days 5, 7, 10, 13, 16, 20, 24, 32, 37 and 41 p.i. No symbol means no detectable trypanosomes in the blood. In this experiment, parasitaemia after the challenge infection was noted in 1 of 3 prednisolone-treated jirds. The same course of primary infection was seen in 6-week-old male jirds inoculated with 4×10^4 *T. grosi* (AKHA isolate), albeit the level of parasitaemia was lower. Data are mean + S.D.

a high resistance to the challenge infection even with a different isolate (SESUJI) of *T. grosi*.

Parasitaemia by *T. lewisi* in jirds was observed up to day 9 p.i. regardless of treatment of infected jirds with prednisolone (Fig. 2) or rat serum (Fig. 3). In a preliminary experiment in which 2 each of naive 6-week-old male jirds were inoculated i.p. with 1×10^7, 1×10^6, 1×10^5, 1×10^4, and 1×10^3 *T. lewisi*, jirds inoculated with 1×10^7 or 1×10^6 trypanosomes showed peaks of parasitaemia at day 6 p.i., whereas in jirds inoculated with 1×10^5 or 1×10^4 trypanosomes at day 9 p.i., and in jirds with 1×10^3 trypanosomes, no parasitaemia was detected. Accordingly, the course of parasitaemia by *T. lewisi* in jirds was somewhat different in different host-parasite systems, but shorter than that of *T. grosi*. Without prednisolone treatment, *T. lewisi* infection in jirds elicited complete resistance to homologous reinfection (Fig. 2), but partial resistance to heterologous challenge infection by *T. grosi* (Fig. 3).

rDNA sequences of Herpetosoma *trypanosomes*

Based on the known sequences of SSU and LSU rDNAs of several trypanosomatid species (see footnote for Table 1), primers were selected or designed to amplify and sequence rDNA segments dispersed from the 3′-end region of the intergenic spacer region (IGS) through 18S and 5·8S to the 3′-end region of LSU of 3 *T. grosi* isolates and 2 *Herpetosoma* species of squirrels (Fig. 4, Table 2).

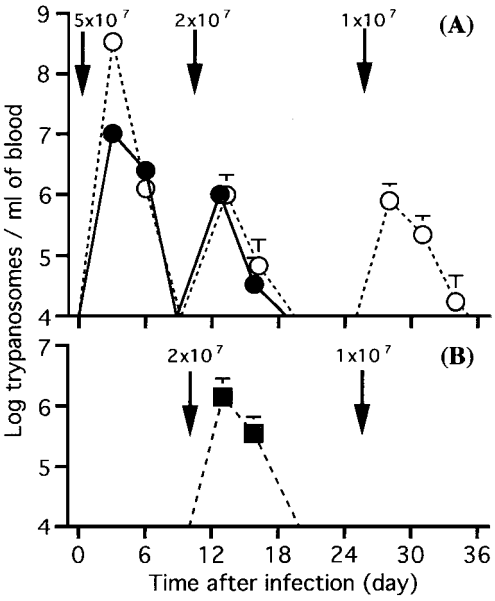

Fig. 2. Time-course of parasitaemia by *Trypanosoma lewisi* in jirds with/without prednisolone treatment. (A) Naive (closed circles; $n=3$) and prednisolone-treated (open circles; $n=3$), 10-week-old female jirds were inoculated i.p. with 5×10^7 *T. lewisi* from an infected rat. At day 10 p.i., all these jirds were treated with prednisolone and challenged with 2×10^7 *T. lewisi*. At day 25 p.i., jirds were re-challenged with 1×10^7 *T. lewisi* without prednisolone treatment. (B) Naive 6-week-old male jirds ($n=3$) were inoculated i.p. with 2×10^7 *T. lewisi*, and challenged with 1×10^7 *T. lewisi* at day 15 p.i. These two experiments were simultaneously conducted on a time-schedule as illustrated using the same inoculum. Blood was examined at days 3, 6, 9, 13, 16, 20, 25, 28, 31, 34 and 37 p.i. No symbols means no detectable trypanosomes in the blood. Data are mean + S.D.

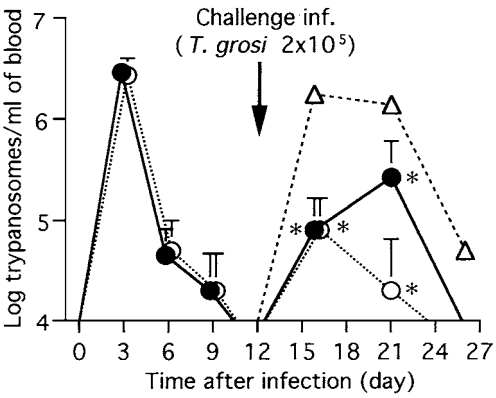

Fig. 3. Cross-immunity of *Trypanosoma lewisi* infection against *Trypanosoma grosi* infection in jirds. Naive 7-week-old male jirds were divided into 3 groups; Group A (closed circles, $n=5$) was treated at an interval of 1·5 days with rat serum. Groups B (open circles, $n=5$) and C (open triangles, $n=3$) were untreated. Groups A and B were inoculated i.p. with 1×10^7 *T. lewisi*, and all groups were challenged i.p. with 2×10^5 *T. grosi* (SESUJI isolate) at day 12 p.i. Blood was examined at days 3, 6, 9, 12, 16, 21 and 26 p.i. Data are mean + S.D. * $P<0·05$ compared with Group C.

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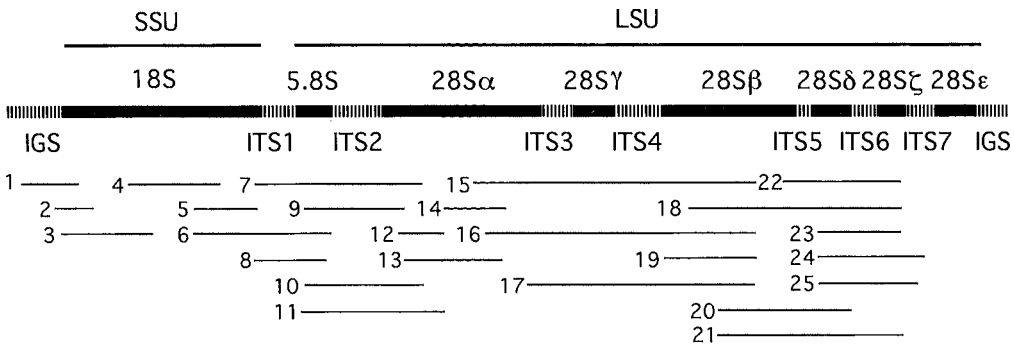

Fig. 4. Schematic organization of rDNA of *Herpetosoma* trypanosomes with segments amplified in this study. The approximate regions of amplified segments are shown by bars, and an attached number indicates the combination of primers shown in Table 1. Any length shown here is not proportional to the substantial length of each rDNA region.

To check the reliability of our sequencing and to visualize the highly variable region of 18S rDNA for differentiation of *Herpetosoma* trypanosomes, the SSU rDNA sequence of *T. grosi* with some additional information was plotted as a secondary structure (Fig. 5), based on the provided configuration of *Trypanosoma cruzi* SSU rDNA secondary structure on the internet (www.rna.icmb.utexas.edu/) (Cannone *et al*. 2002). Most of the base changes (substitution, deletion or insertion) to *T. cruzi* sequence by *Herpetosoma* trypanosomes occurred in the anterior and middle 1/3 parts of the whole SSU sequence, particularly in the region where strands were not coupled. When the change occurred in the coupled region i.e. loops, it often increased the affinity between coupled bases. Similarly, inter- and intra-species changes of *Herpetosoma* trypanosomes occurred in the anterior 2/3 region; 24 positions in the anterior, 19 positions in the middle, and 2 positions in the posterior 1/3 of SSU rDNA. Subsequent to SSU rDNA, 5·8S and 5 parts of 28S rDNA (α, γ, β, δ and ζ) with internal transcribed spacer (ITS) regions (ITS 1–7) of 3 isolates of *T. grosi* and 2 *Herpetosoma* species from squirrels were sequenced and compared (Table 2). More than 70% of all changes within each unit in different species were situated at positions 531–1065 (corresponding to 30% of the whole length) of 28Sα, at positions 817–974 (similarly, 10%) of 28Sβ, and at positions 8–18 (similarly, 6%) of 28Sδ of *T. grosi* (SESUJI), whereas sequences of 5·8S and 28Sγ of all species were identical. Fig. 6 illustrates the unrooted N-J tree of hitherto known 18S rDNA sequences of *Herpetosoma* trypanosomes with *Trypanosoma rangeli*, *T. cruzi* and *Leishmania major*, indicating a close association of *T. grosi* with *T. lewisi* and *T. musculi*, but not with *T. microti* or squirrel *Herpetosoma* trypanosomes, *T. otospermophili* and *Trypanosoma* sp. from a Siberian flying squirrel. The latter 2 species were closely associated with each other. Similarly prepared, 4 N-J trees based on sequences of ITS1 – ITS2, 28Sα, 28Sβ, or all segments of 28S including 4 ITS regions showed an identical schema, although the relationship among the 3 isolates of *T. grosi* was variably positioned.

DISCUSSION

Although approximately 40 *Herpetosoma* trypanosomes that parasitize rodents and insectivores have been named (Hoare, 1972), the number must be too small when considering the huge number and the wide variety of rodents in the world (*ca*. 2015 species, 443 genera, and 29 families) (see Dr J. Decher's website, University of Vermont [www.uvm.edu/~jdecher/Lecture19.html]; Wilson & Reeder, 1993). As have been actively accomplished in other trypanosomatid species (Maslov *et al*. 1996; Haag, O'Huigin & Overath, 1998; Stevens & Gibson, 1999; Stevens *et al*. 1999; Stevens *et al*. 1999), molecular biological approaches should allow critical differentiation of the species/strains and clarify the relationship between members of the subgenus *Herpetosoma*. Using such an approach, Stevens *et al*. (1999) removed *T. rangeli* that had been classified for a long time in the subgenus *Herpetosoma* despite of its exceptionally wide host range (Hoare, 1972), from this subgenus. To date, complete 18S rDNA sequences have been reported for only 3 species of *Herpetosoma* trypanosomes, *T. lewisi*, *T. musculi* and *T. microti* (Haag *et al*. 1998; Stevens *et al*. 1999), along with partial 18S rDNA sequences from *T. microti*, *T. evotomys*, *T. grosi* and an unidentified trypanosome species from *A. sylvaticus* (Noyes *et al*. 2002). Therefore, differential diagnosis of the *Herpetosoma* trypanosomes remains essentially in the same state as feared more than 30–50 years ago by Davis (1952) and Hoare (1972). Indeed, nobody could differentiate the *Herpetosoma* trypanosomes with certainty based on morphological and biological criteria alone.

Hilton & Mahrt (1972) conducted intensive cross-transmission experiments of '*T. otospermophili*' using 5 *Spermophilus* squirrels in North America, and observed the course of parasitaemia and the morphology of collected parasites. They found

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Table 2. Comparison of SSU and LSU rDNA organization of trypanosomatids including 6 *Herpetosoma* trypanosomes*

Species name	SSU			LSU												DDBJ/EMBL/ GeneBank Accession no.
	IGS	18S	ITS1	5.8S	ITS2	28Sα	ITS3	28Sγ	ITS4	28Sβ	ITS5	28Sδ	ITS6	28Sζ	ITS7	28Sε	
T. (*H.*) *grost*																
SESUJI isolate	(286)	2219	340	172	491	1818	30	214	43	1559	23	183	631	80	(147)	—	AB175622
HANTO isolate	(374)	2219	351	172	479	1818	30	214	37	1558	23	183	628	80	(317)	—	AB175623
AKHA isolate	(412)	2219	348	172	505	1817	31	215	40	1557	25	183	641	79	(173)	—	AB175624
T. (*H.*) *otospermophili*	(27)	2215	420	172	490	1803	56	214	45	1528	43	184	561	(10)	—	—	AB175625
Trypanosoma (*H.*) sp. from a flying squirrel, *Pteromys volans*	(27)	2218	420	172	496	1812	67	214	46	1541	54	184	558	(43)	—	—	AB175626
T. (*H.*) *lewisi*	—	2219	—	—	—	—	—	—	—	—	—	—	—	—	—	—	AJ223566
T. (*H.*) *musculi*	—	2219	—	—	—	—	—	—	—	—	—	—	—	—	—	—	AJ223568
T. (*H.*) *microti*	—	2217	—	—	—	—	—	—	—	—	—	—	—	—	—	—	AJ009158
T. (*Schizotrypanum*) *cruzi*	—	2315	526	172	509	1934	53	212	40	1619	71	182	584	78	381	—	AF245382 and L22334
Leishmania major	4017	2204	256	172	667	1780	90	213	330	1525	33	183	322	73	253	129	AC005806
Crithidia fasciculata	—	2206	367	171	416	1781	125	212	32	1523	79	183	263	73	248	133	Y00055
Homology among *Herpetosoma* sp.†	—	98–99	68–99	100	61–94	96–99	38–91	100	71–88	97–98	52–86	95–98	82–90	—	—	—	
Homology among *T. grosi* isolates†	97–100	99	92–98	100	96–98	98–99	96–100	100	94–100	99	69–100	98–100	95–99	98–100	—	—	

* The number of bases in each rDNA region is compared. The base number in partially sequenced regions is shown in parenthesis. Dash means unknown.
† Homology of nucleotide sequences.

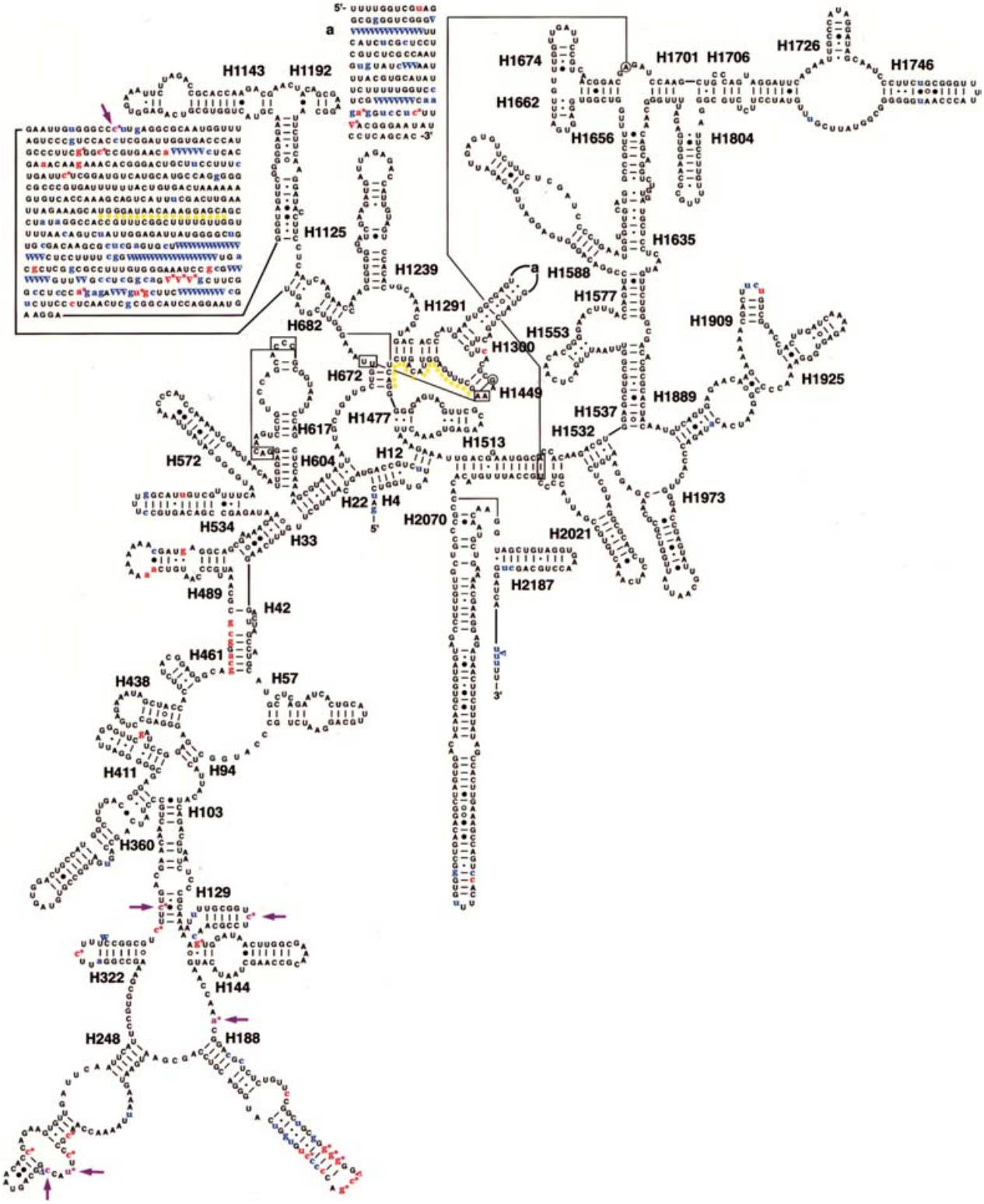

Fig. 5. Position of base changes in 18S rDNA among 3 *Trypanosoma grosi* isolates or among 6 species of *Herpetosoma* trypanosomes, plotted on the secondary structure of *T. grosi* (SESUJI isolate). *Herpetosoma* spp. compared here include *T. lewisi* (DDBJ/EMBL/Gen Bank Accession no. AJ009156), *T. musculi* (Accession no. AJ223568), *T. microti* (Accession no. AJ009158), *T. grosi*, *T. otospermophili*, and *Trypanosoma* sp. from *Pteromys volans*. Symbols in large letter are conserved bases among *Trypanosoma cruzi* (Accession no. AF245382) and all *Herpetosoma* spp. examined here. Symbols in small letter are bases changeable among these *Trypanosoma* spp.: blue-coloured letter, changed from *T. cruzi*; red-coloured letter, changeable among different *Herpetosoma* spp.; violet-coloured letter, changeable bases among *T. grosi* xenodemes; letter with asterisk, not restricted at the level indicated by colour but also higher level(s); and △ with letter, base insertion by the level indicated by colour. Bases accompanied with yellow marks are the positions of primers used by Noyes *et al.* (2002).

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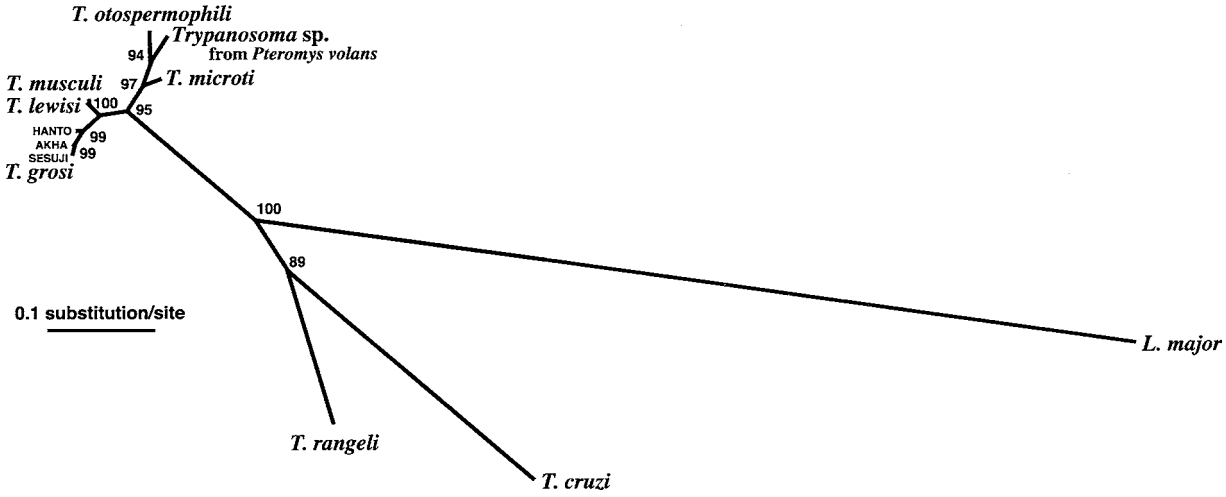

Fig. 6. Unrooted N-J tree of 18S rDNA sequences of 6 *Herpetosoma* species and *T. cruzi* mentioned in the legend for Fig. 5, with *T. rangeli* (DDBJ/EMBL/GenBank Accession no. AJ009160) and *L. major* (Accession no. AC005806). Note that *Herpetosoma* trypanosomes from Murinae, Arvicolinae, and Sciuridae are divided into 2 branches, and located far distantly from other trypanosomatids including *T. rangeli*. Bootstrap scores expressed as percentages of 1000 replicates are given.

different intensities and durations of parasitaemia after injection of the same inoculum in different host species and significantly different mensural values of trypanosomes collected from different host species used in their experiments. In their experimental cases, we could ascribe these differences to host-induced variations. However, finding these differences in a natural infection makes species differentiation difficult. The presence of *Herpetosoma* trypanosome isolates showing different durations of parasitaemia, however, could be seen in *T. grosi* (SESUJI and AKHA *vs.* HANTO isolates) as shown here and in our previous study (Sato *et al.* 2003). The course of parasitaemia by all *T. grosi* isolates in jirds is distinct from the shorter course of parasitaemia by *T. lewisi* in the same host (Mühlpfordt, 1969; the present study). Furthermore, Chiejina *et al.* (1993) reported different intensities and durations of parasitaemia by Partinico II and Portuguese isolates of *T. musculi* that originated from *Mus musculus* in Sicily, Italy and *Mus spretus* in Lisbon, Portugal, respectively. Species differentiation of these *Herpetosoma* trypanosomes originating from different host species of the same genus but showing different biological characters is faced by special difficulties.

Therefore, the main aims of the present study were the following. (1) To clarify the reliability of genotypes on different *T. grosi* isolates showing different biological characters, that have been seen in experimental infection, or the molecular biological relationships of *T. grosi* isolates of different origins. (2) To identify the highly variable rDNA region of *Herpetosoma* trypanosomes where inter- or intra-species differentiations are possible. (3) To demostrate the feasibility of molecular biological approaches, particularly using rDNA sequences, for systemic arrangement of *Herpetosoma* species or their taxonomic differentiation. Analyses of 2 additional *Herpetosoma* trypanosomes that originated from ground and flying squirrels (Sciuridae: the subfamilies Sciurinae and Petauristinae, respectively) provided an opportunity to compare the sequences of trypanosomes from phyogenetically distant hosts from *Rattus* (Muridae: the subfamily Murinae) for *T. lewisi*, *Mus* (similarly, Murinae) for *T. musculi*, *Apodemus* (similarly, Murinae) for *T. grosi*, and *Microtus* (similarly, the subfamily Arvicolinae) for *T. microti*.

The basic framework of rDNA of *Herpetosoma* trypanosomes was inferred based on previous studies using other trypanosomatid species (Hasan, Turner & Cordingley, 1984; White, Rudenko & Borst, 1986; Spencer *et al.* 1987). Prior to this study, complete 18S rDNA sequences are available for only three species of *Herpetosoma* trypanosomes, while no sequences are available for other parts of *Herpetosoma* rDNA. The possible positions of the base change in 18S rDNA are plotted on a diagram of the secondary structure (Fig. 5), using these 3 known sequences and newly added 5 sequences of *T. grosi* and 2 squirrel *Herpetosoma* species. Most of the changes occurred in uncoupled regions of the strand, particularly the anterior and middle 1/3 of the whole 18S rDNA sequences. Noyes *et al.* (2002) successfully amplified and sequenced part of this region (a segment between positions 907 and 1475 of 18S rDNA of *T. grosi*) of several *Herpetosoma* spp. such as *T. lewisi*, *T. musculi*, *T. grosi*, *T. microti* and *T. evotomys* for their characterization. They found a higher homology of the sequences between *T. microti* and *T. evotomys*, both of which are parasitic to rodents of the subfamily Arvicolinae (*Microtus* spp. and *Clethrionomys* spp., respectively), compared with 3 other species parasitic to rodents of the subfamily Murinae. At the same

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time, they found occasionally trypanosomes distinct from *T. grosi* from *A. sylvaticus* based on sequencing of the aforementioned amplicon, and indicated that the trypanosome closely resembled *T. evotomys* and *T. microti*, though it might be different. They emphasized that host range is not a sufficient taxonomic character to erect a new species or to differentiate the species of *Herpetosoma* trypanosomes.

Based on a molecular biological approach used by Noyes *et al.* (2002), we recently confirmed that trypanosome isolates (SESUJI and HANTO isolates) from *A. agrarius* and *A. peninsulae* in Vladivostok, Russia were *T. grosi* (Sato *et al.* 2004). These two isolates showed distinct courses of parasitaemia in experimentally-infected jirds (Sato *et al.* 2003). With the addition of another isolate of *T. grosi* from *A. speciosus* in Japan (AKHA isolate), we compared rDNA sequences of these three isolates in this study. The results showed that these 3 exhibit base changes at 6 positions throughout the whole length of 18S rDNA (2219 bases) as well as several changes in other regions of rDNA including 28S rDNA. This suggests that isolates of the same *Herpetosoma* species collected in the same geographical region could express minor variation, not inevitably identical, since SESUJI and HANTO isolates collected at the same location express the same amount of differences in rDNA sequences as was found between each of them and a geographically-separated isolate (AKHA isolate).

The highly variable rDNA regions of *Herpetosoma* trypanosomes where inter- or intra-species differentiation might be possible are not arbitrarily dispersed as supposed. The 18S rDNA region amplified and sequenced by Noyes *et al.* (2002) is a good candidate for species or strain differentiation. In addition, the sequence of a region prior to this 18S rDNA segment is valuable for checking any base changes at inter- or intra-species levels, since this anterior 1/3 part of 18S rDNA contains all 6 positions where base changes were found among different *T. grosi* isolates, and almost an equivalent number of base changes among different *Herpetosoma* species as in the middle 1/3 part part of 18S rDNA targeted by Noyes *et al.* (2002). The remaining 1/3 of the 18S rDNA contains few base change positions, even when *Herpetosoma* trypanosomes and *T. cruzi* are compared. As stated previously, sequences of several specified regions of 28S rDNA segments and multiple ITS regions are similarly highly variable. N-J trees constructed by sequences of 18S rDNA or other regions showed identical configurations. *Herpetosoma* trypanosome species from hosts of Murinae (*T. lewisi*, *T. musculi* and *T. grosi*), those from Arvicolinae (*T. microti*), and those from Sciuridae (*T. otospermophili* and *Trypanosoma* sp. from *Pteromys volans*) are clearly separated. With regard to coevolution, accumulated molecular phylogenetic information on *Herpetosoma* trypanosomes may provide an insight on geographical dispersion or divergence of rodents and ecological interactions of different rodent species. Simultaneously, molecular biological approaches of *Herpetosoma* trypanosomes such as 18S rDNA sequencing may help the description of a new species beyond the fear of some researchers such as Davis (1952), Hilton & Marhr (1972), or Hoare (1972), since we could assess directly or retrospectively the newly-proposed species regardless of tentative phenotypical characters that might occur often in individual hosts.

This work was supported in part by Grants-in-Aid (Nos. 13460137, 13575039, 13670240, and 15390134) from the Japan Society for the Promotion of Science. In addition, we acknowledge the Japan Health Science Foundation for the scholarship to H. Sato, which allowed invaluable discussion with Professor John R. Kusel, IBLS, University of Glasgow, on our series of work on *Herpetosoma* trypanosomes.

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