Molecular characterization of a new *Trypanosoma (Megatrypanum) theileri* isolate supports the two main phylogenetic lineages of this species in Japanese cattle

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Abstract

Trypanosoma (Megatrypanum) theileri is a cosmopolitan, usually non-pathogenic, trypanosome of cattle transmitted by blood-sucking arthropods, mainly tabanid flies. Several *T. theileri* strains isolated from domestic and wild ruminants via co-culturing with mammalian feeder cells or blood cells have been characterized morphologically and genetically. Here, we cultured a new trypanosome isolate from a Holstein cow in Hokkaido, Japan and performed morphological and molecular characterization studies. The new isolate (Obihiro strain) was co-cultivated with Madin–Darby bovine kidney (MDBK) cells in GIT medium supplemented with 10% fetal bovine serum. Trypomastigotes and epimastigotes, but not intracellular parasites, were identified in the culture. Analysis of the V7-V8 region of *18S rRNA* sequences showed that the Obihiro strain is positioned within the subgenus *Megatrypanum.* A dendrogram based on whole internal transcribed spacer rDNA sequence showed that the Obihiro strain clustered in the lineage TthII together with the Japanese isolates of *T. theileri*, Esashi 9, and Esashi 12, and isolates from Zambia and USA. *Trypanosoma*

theileri of the KM strain and a *T. theileri*-like trypanosome isolated from deer (TSD1 strain) clustered in the lineage TthI, separate from the Obihiro strain. Based on a partial cathepsin L-like protein gene analysis, the Obihiro strain clustered with isolates of the TthIIF genotype, which includes *T. theileri* from Vietnam, Sri Lanka, and Brazil. Our analyses of the *T. theileri* Obihiro strain provide relevant insights into its genetic diversity in Japanese cattle and corroborate the host-specificity of cattle and deer trypanosomes of the subgenus *Megatrypanum*.

Keywords: genotyping; *In vitro* culture; morphology; *Megatrypanum;* Stercoraria; *Trypanosoma theileri*

Introduction

Trypanosoma (Megatrypanum) theileri is a non-pathogenic or weakly pathogenic parasite of domestic cattle, which was discovered in South Africa by Theiler in 1902 (Cross et al. 1971; Hoare 1972). This trypanosome has a global distribution, including in Japan (Sasaki 1958; Woo et al. 1970; Greco et al. 2000; Rodrigues et al. 2003; Lee et al. 2010). *T. theileri* belongs to the subgenus *Megatrypanum* of the section Stercoraria along with other non-pathogenic trypanosome species of ruminants such as *T. melophagium* (sheep), *T. theodori* (goats), *T. cervi* (deer), and other trypanosomes of wild ruminants (Rodrigues et al. 2003; Rodrigues et al. 2006; Hatama et al. 2007; Garcia et al. 2011; Martinkovic et al. 2012; Fisher et al. 2013).

T. theileri is cyclically transmitted by blood-sucking insects, mainly tabanid flies (Hoare 1972). Böse *et al.* (1987) suggested that *T. theileri* is transmitted to cattle by contamination of oral mucosa with metacyclic trypomastigotes from gut contents or feces of infected tabanids. The hard tick *Hyalomma anatolicum* has also been reported as a potential vector of *T. theileri*

(Morzaria et al. 1986).

The low pathogenicity of *T. theileri* in healthy ruminants has generally been attributed to low parasitemia. However, in animals that are immunocompromised, pregnant, or infected with bovine leukemia virus, *T. theileri* propagates in the blood, and high parasitemia may cause clinical disease (Matumoto et al. 2011; Sood et al. 2011).

T. theileri and *T. theileri*-like trypanosomes from the blood of cattle, buffalo, sheep, and deer have been cultivated *in vitro* (Rodrigues et al. 2003; Hatama et al. 2007; Nalbantoğlu et al. 2008). The co-cultivation of *T. theileri* with either blood cells or feeder cells are necessary for long-term cultivation (Verloo et al. 2000; Rodrigues et al. 2003; Van Hellemond et al. 2007; Lee et al. 2010). For example, *T. theileri* isolated from a Holstein cow in Taiwan (TWTth1 strain) was cultured with kidney cells of infant hamsters as feeder cells (Lee et al. 2010), and *T. theileri* isolated from cattle and buffalo in Brazil (Tthc1-3 and -4 and Tthb1-11 strains) were co-cultured with SF9 insect cells in Grace's medium or LLCMK2 mammalian cells in DMEM medium (Rodrigues et al. 2003).

97 *T. theileri* have been isolated from buffy coat and adapted to *in vitro*
98 cultures during an epidemiological survey of Japanese cattle (Ishida et al. 2002).
99 Moreover, a *T. theileri*-like trypanosome isolated from a Japanese sika deer
100 (*Cervus nippon yesoensis*) was cultivated using deer renal cells as feeder cells
101 (Hatama et al. 2007). To date, however, experimental cross-infections of cattle
102 with *T. theileri*-like trypanosome from deer (Kingston and Morton 1975) and
103 infections of deer with *T. theileri* from cattle (Bose et al. 1987) have been
104 unsuccessful.
105 Cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) have been found
106 to be infected with genotypes of different linages of *T. theileri* in Brazil
107 (Rodrigues et al. 2003; Rodrigues et al. 2006). However, the *T. theileri* isolates
108 from water buffalo and cattle in Sri Lanka and Vietnam have been found to
109 belong to the same lineage (Yokoyama et al. 2015; Weerasooriya et al. 2016),
110 suggesting that inter-host species cross-transmission of *T. theileri* and *T.*
111 *theileri*-like trypanosomes should not be ruled out. In this regard, Böhm et al.
112 (2007) found that wild deer constitute a source of numerous parasitic infections

of livestock. Therefore, transmission of *T. theileri*-like trypanosomes from wild deer to cattle might represent a potential risk for dairy cattle infection in Japan and possibly for the dairy industry.

In the present study, a new *T. theileri* strain from a dairy cattle blood sample was co-cultivated with Madin–Darby bovine kidney (MDBK) cells. To determine the lineage and genotype of the new isolate, we used three genetic markers to compare it with previously described *T. theileri* of cattle and *T. theileri*-like trypanosomes of deer from Japan and other countries.

Materials and methods

Isolation and in vitro *culture of* T. theileri

In 2012, active mobile *T. theileri* were detected in a blood sample collected from a female Holstein on the research farm of Obihiro University of Agriculture and Veterinary Medicine in Hokkaido Prefecture. After propagation of *T. theileri* by culturing of the blood sample, the specimens were transferred and co-cultured with MDBK cells (NBL-1 strain provided by Japanese Cancer Research Resources Bank) as feeder cells using GIT medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS). The *T. theileri* strain co-cultivated with MDBK cell was maintained via continuous sub-culturing twice per week with MDBK cells at 37ºC in a 5% CO_2 atmosphere. The *T. theileri* strain was also axenically cultivated without MDBK cell using GIT medium supplemented with 10% FBS or HMI-9 medium supplemented with 10% FBS (Hirumi and Hirumi 1991). The strain was then cryopreserved in FBS supplemented with 10% dimethyl sulfoxide at -80ºC, by using liquid nitrogen.

138

139 *Morphological analysis of isolated* T. theileri

140 *T. theileri* was co-cultured with MDBK cells in chamber glass slides
141 (Matsunami Glass Ind., Ltd., Tokyo, Japan). After 3 days, the culture supernatant
142 was discarded, and the MDBK cells and *T. theileri* were washed with PBS,
143 air-dried, and fixed with 100% methanol for 10 min at room temperature (~25°C).
144 For indirect fluorescence antibody tests (IFAT), the specimens were blocked with
145 5% skim milk in PBS for 1 h at room temperature and then incubated with a
146 primary antibody (anti-recombinant *T. congolense* α-tubulin serum) (Suganuma
147 et al. 2016). The slides were then incubated with a secondary antibody (Alexa
148 Fluor 488 goat anti-rabbit IgG [H + L]; Thermo Fisher Scientific K.K.) with
149 Hoechst 33342 (Dojindo, Co. Ltd., Kumamoto, Japan). Specimens were
150 observed using confocal laser scanning microscopy (Leica TCS SP5; Leica
151 Microsystems, Wetzlar, Germany).

152 The specimens were then stained with 10% Giemsa solution for 10 min.
153 The morphology of *T. theileri* was observed using a light microscope (Nikon

154 TCS5; NIKON CORPORATION, Tokyo, Japan), and we determined the total
155 length (TL) and width at the widest point of the cell body (MW), width at the
156 widest point of the undulating membrane (UM), distance between the posterior
157 end and central kinetoplast (PK), distance between the kinetoplast and center of
158 the nucleus (KN), distance between the center of the nucleus and anterior end
159 (NA), and length of the free flagellum (FF) from these observations. Nuclear (NI)
160 and kinetoplast (KI) indices were also calculated as follows: NI = (PK+KN)/NA
161 and KI = (PK+KN)/KN (Hoare 1972).
162
163 *Molecular characterization of* T. theileri
164 The total DNA of the Obihiro strain was extracted and purified using
165 TE-saturated phenol (Sigma-Aldrich Japan) and phenol-chloroform-isoamyl
166 alcohol solution (Sigma-Aldrich Japan) (Sambrook et al. 2006). Purified total
167 genomic DNA samples were stored at -30ºC until use.
168 The Tth625 fragment (Rodrigues et al. 2003), partial cathepsin L-like
169 protein (*CATL*) gene (Rodrigues et al. 2010), V7-V8 region of 18S ribosomal

RNA (*18S rRNA*) gene (Da Silva et al. 2004), and internal transcribed spacer 1 (ITS1) (Njiru et al. 2005) and whole ITS (ITS1 + 5.8S rRNA + ITS2) regions (Da Silva et al. 2004) of the *T. theileri* genome were amplified using the respective primer pairs (Supplementary Table S1). The amplicons were cloned into a pCR 2.1 cloning vector (Thermo Fisher Scientific K.K., Tokyo, Japan) and were sequenced using an ABI3100 genomic analyzer (Thermo Fisher Scientific K.K.). Contigs were constructed from each sequenced fragment by using the GeneStudio program (http://genestudio.com).

To clarify the taxonomical position of the new isolate among trypanosome species, the V7-V8 region of *18S rRNA* sequences of *T. theileri* Obihiro strain that was determined in the present study (Accession No.: LC385952) was subjected to dendrogram construction by neighbor-joining (NJ) method along with the following reference sequences retrieved from the NCBI database: *T. theileri* Esashi 12 (AB569250), Esashi 9 (AB569249), KM (AB007814); *Trypanosoma* sp. TDS1 (AB569248); *T. cruzi* Esmerald (AY785564 and AF362827); *T. rangeli* Macias (AJ012415); *T. rangeli* B450 (AY230240); *T.*

186 *brucei gambiense* DAL972 (FN554966); *T. evansi* Tansui (D89527); *T.*
187 *equiperdum* Botat1.1 (LC386039); *T. brucei* TREU927 (AC012647); and *T.*
188 *congolense* TS07210 (JN673389).

189 To clarify the intra-species relationship among *T. theileri* and *T.*
190 *theileri*-like trypanosome strains/isolates, whole ITS region (LC385951) and
191 partial *CATL* (LC385983) sequences of the Obihiro strain were determined. A
192 dendrogram based on whole ITS sequences was constructed by NJ method
193 using whole ITS sequence of the Obihiro strain and reference sequences
194 retrieved from the NCBI database (*T. theileri/T. theileri*-like trypanosome from
195 Japan [AB569248, AB569249, AB569250, and AB007814], Austria [KY412803],
196 Brazil [AY773698 and AY773699], Zambia [JN673395-JN673397], and USA
197 [AY773700, JX178172, JX178173, JX178164–JX178189, and JX853182–
198 JX853185]). The partial *CATL* sequence determined in this study and reference
199 sequences from the NCBI database (*T. theileri* from Brazil [GU299352,
200 GU299354, GU299367, GU299371, GU299375, GU299378, GU299401,
201 GU299404–GU299407, GU299413, and HQ664735], Sri Lanka [AB930146–

202 AB930168], the Philippines [JX860298 and JX860299], Vietnam [AB742558–
203 AB742662], Thailand [HQ543057, HQ543060–HQ543062, HQ543066,
204 HQ543068, and HQ543071–HQ543074], Cameroon [HQ664748 and
205 HQ664750], USA [GU299391 and GU299392], Germany [GU299415 and
206 GU299416]; and *T. vivax* [EU753811 and EU753807]) were also included in the
207 dendrogram construction by NJ method. These dendrogram analyses were
208 conducted using primer region-trimmed nucleotide sequences and the
209 neighbor-joining method implemented in the MEGA 7 software program.
210

Results

Culture of T. theileri *with MDBK cells*

The *T. theileri* Obihiro strain was co-cultivated with MDBK cells as feeder cells using GIT medium supplemented with 10% FBS. The strain was unable to propagate without the feeder cells or using HMI-9 (Hirumi and Hirumi 1991), which is a medium used to cultivate African trypanosomes (data not shown).

Morphological analysis of cultivated T. theileri

In addition to the typical epimastigote forms, trypomastigote and intermediate forms, in which the kinetoplast is located adjacent to the nucleus, were also observed in the *in vitro* culture (Fig. 1A, B). The body of the trypomastigotes of the Obihiro strain in culture (*n* = 150) had a polymorphic length of 37.7–84.1 µm (mean ± standard deviation: 60.0 ± 7.8 µm), a relatively narrow cell body width of 0.7–2.9 µm (1.7 ± 0.4 µm), and a long free flagellum of 3.8–33.4 (17.6 ± 5.6 µm). The nucleus was observed relatively near the anterior end and the kinetoplast was observed relatively near the nucleus. The mean

227 values of the nuclear and kinetoplast indices were calculated as 1.62 and 5.3,
228 respectively. Trypanosomes adhered to MDBK cells via the posterior end
229 (Supplementary movie S1); however, no intracellular forms (amastigotes) were
230 observed in Giemsa-stained smears, IFAT, or transmission electron microscopic
231 observations (data not shown).
232
233 *Molecular characterization of cultivated* T. theileri
234 The amplicons obtained from each PCR reaction were observed at the
235 expected sizes using agarose gel electrophoresis (Supplementary Fig. S1). The
236 homologies of partial *CATL*, V7-V8 region of 18S rRNA, whole ITS, and Tth625
237 fragment sequences with those of *T. theileri* isolates available in GenBank were
238 100%, 100%, 99%, and 86%, respectively (Supplementary Table S2).
239 A dendrogram analysis based on V7-V8 region of *18S rRNA* clearly
240 indicated that the Obihiro strain belonged to the subgenus *Megatrypanum* in
241 section Stercoraria (Fig. 2A). A dendrogram analysis by using whole ITS
242 sequence revealed that the Obihiro strain was nested in the lineage TthII

243 together with Esashi 9 and 12, which are *T. theileri* strains isolated from a cow in
244 another city in the same prefecture (Hokkaido) in Japan, and isolates from puku
245 antelope in Zambia and white-tailed deer in USA (Fig. 2B). Another Japanese
246 isolate of *T. theileri* (KM strain) clustered in the lineage TthI together with isolates
247 from Austria, Brazil, and USA (Fig. 2A, B). In a dendrogram analysis based on
248 partial *CATL* sequence, the Obihiro strain clustered with isolates from TthIIF
249 genotype, which includes *T. theileri* from Vietnam, Sri Lanka, and Brazil (Fig.
250 2C).
251

252 **Discussion**

253 *T. theileri* has been considered a non-pathogenic trypanosome in cattle;
254 however, some clinical cases caused by *T. theileri* infection were reported
255 (Matumoto et al. 2011; Sood et al. 2011). The prevalence of *T. theileri* in cattle in
256 Hokkaido Prefecture in Japan was reported to be 2.57% in 1958 based on
257 microscopic observations of blood smears (Sasaki 1958). Some *T. theileri* and *T.*
258 *theileri*-like isolates were identified in cattle and deer (Urakawa and Majiwa
259 2001; Hatama et al. 2007); however the phylogenic lineage and genotype of *T.*
260 *theileri* in Japan remained unclear. Therefore, we performed molecular analyses
261 to reveal the genotype of a new *T. theileri* strain isolated from a Holstein cow in
262 Hokkaido.

263 The isolated *T. theileri* Obihiro strain was co-cultivated with MDBK cells
264 as feeder cells using GIT medium supplemented with 10% FBS, following
265 previous studies (Verloo et al. 2000; Rodrigues et al. 2003; Hatama et al. 2007;
266 Lee et al. 2010). Van Hellemod *et al.* (2007) suggested that the metabolic
267 pathways of *T. theileri* are completely different from those of salivarian

268 trypanosomes. Our findings are in line with the above result as we confirmed
269 that the Obihiro strain could not be maintained at 37ºC *in vitro* without feeder
270 cells.

271 Lee et al. (2013) performed microscopic examination of Giemsa-stained
272 specimens, IFAT and transmission electron microscopic and reported that,
273 similar to *T. cruzi, T. theileri* could invade host cells and propagate in the
274 amastigote form in the cytosol. Moreover, Lee et al. (2013) showed that the
275 number of trypanosomes invading host cells differed from that invading feeder
276 cells; for example, the numbers of *T. theileri*-parasitized SVEC cells were
277 significantly fewer than the numbers that parasitized H9c2 and RAW 264.7 cells.
278 In the present study, although several *T. theileri* adhered to the MDBK cells via
279 their posterior end (Supplementary movie S1), intracellular trypanosomes were
280 not observed in the culture (data not shown). These results indicate that the
281 co-culture with MDBK cells as feeder cells might be suitable for maintaining the
282 extracellular stage of *T. theileri* but not the intracellular stages.

283 The dendrogram analysis based on the V7-V8 region of 18S rRNA
284 sequences clearly showed that the Obihiro strain belonged to the subgenus
285 *Megatrypanum*. Nucleotide sequence similarity analyses of partial *CATL*, *18S*
286 *rRNA*, and whole *ITS* region sequences based on BLASTn searches revealed
287 that the Obihiro strain has high sequence similarity (99%–100%) with previously
288 reported *T. theileri*. In contrast, the nucleotide sequence similarity of the Tth625
289 fragment of the Obihiro strain with that of other *T. theileri* strains was relatively
290 low (86%). Rodrigues *et al.* (2003) developed a Tth625-PCR protocol as a
291 species-specific PCR for *T. theileri*. Nucleotide polymorphism among Tth625
292 fragments has also been detected in *T. theileri* in Taiwan (TWTth1) and Brazil
293 (Accession Nos. AF537201 and AF537202) (Lee et al. 2010; Rodrigues et al.
294 2003), thus, indicating that Tth625 fragments can potentially be used as
295 taxonomic markers and for genotyping of *T. theileri*.
296 The population of sika deer on Hokkaido Island has increased markedly
297 over the past 30 years, and the economic damage to agriculture and forestry
298 caused by these deer is estimated at more than 1.5 billion Japanese Yen per

299 year (Kaji 1995; Uno et al. 2006). The high number of wild sika deer in Hokkaido
300 increases the possibility of their encroaching into areas where domestic animals
301 are reared. Wild deer have been reported to be a source of several infectious
302 cattle diseases (Böhm et al., 2007; Jilintai et al. 2008; Jilintai et al. 2009; Yokoi et
303 al. 2009; Masuzawa et al. 2011). *T. theileri*-like trypanosomes were isolated from
304 wild sika deer in Japan (Hatama et al., 2007), and inter-species transmission of
305 trypanosomes among cattle and wild deer was hypothesized. However, this was
306 not supported by genetic analyses, because *T. theileri* strains isolated in
307 Hokkaido prefecture (*T. theileri* Obihiro and *T. theileri* Esashi strains; TthII
308 lineage) were clustered in different lineages of the *Trypanosoma* TSD strain (TthI
309 lineage), also isolated from wild sika deer in same area. Although the present
310 study and previous analyses showed distinct clusters of isolates from cattle
311 within the lineage TthII (but not from deer and buffalo), some studies in Asian
312 countries have shown, based on dendrograms of partial *CATL* sequence, that
313 cross-transmission of *T. theileri* and *T. theileri*-like trypanosomes may occur
314 between different host species such as water buffalo and cattle. To date, only a

315 low number of *T. theileri* and *T. theileri*-like isolates from East Asian countries
316 have been genetically analyzed (Yokoyama et al. 2015; Weerasooriya et al.
317 2016). Further researches must isolate and molecularly characterize these
318 parasites to evaluate the possibility of inter-species transmissions of *T. theileri*
319 and *T. theileri*-like trypanosomes in Japan and other East Asian countries.

320 Although partial *CATL* sequence has recently been widely applied for
321 analysis of genetic diversity and host specificity of *T*. *theileri* strains, to the best
322 of our knowledge, the diversity of *T. theileri* isolates in Japan is yet to be
323 analyzed based on the *CATL* gene. Therefore, further studies should investigate
324 the genetic diversity of *T. theileri* in different host species based on Tth625
325 fragment, ITS, or *CATL* sequences. Such analyses might shed light on the
326 host-specificity of *T. theileri* in Japan, thus, indicating the likelihood of
327 transmission between cattle and deer.

328 In conclusion, the trypanosome species successfully isolated in this
329 study from cow blood in Obihiro City, Hokkaido, and cryopreserved has been
330 morphologically and genetically identified as *T. theileri*. The findings suggest that

331 the Obihiro strain is a new *T. theileri* strain belonging to TthII clade. The genetic

332 information obtained for the *T. theileri* Obihiro strain will provide a valuable

333 resource that can be used to reveal the genetic relationship of subgenus

334 *Megatrypanum* trypanosome species in Japan and in other countries.

335

Acknowledgements

We thank Ms. Yoko Matsushita for her excellent technical assistance. This study was partially supported by the “International Collaborative Research Program for Tackling the NTDs (Neglected Tropical Diseases) Challenges in African Countries” from the Japan Agency for Medical Research and Development, AMED. The study was financially supported by the Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number 16K18793 [Grants-in-Aid for Young Scientists (B)] and the AMED/JICA SATREPS (17jm0110006h0005).

Competing interests:

The authors declare no competing interests in association with this study.

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477

Figure legends

Fig. 1 Morphological analysis of *Trypanosoma theileri*

(A) Images of Giemsa-stained *T. theileri*. The trypomastigote form (upper panel) and epimastigote and intermediate forms (lower panel) were observed under a microscope. The scale bar represents 10 μm. The arrow and arrowhead indicate the nucleus and kinetoplast, respectively

(B) Images of indirect fluorescence antibody test of *T. theileri* trypomastigote and epimastigote forms. *T*. *theileri* was subjected to indirect immunofluorescence staining by using an anti-Tc α-tubulin antibody (green signal) and was observed under a confocal laser scanning microscopy. The nucleus and kinetoplast DNA were stained with Hoechst 33342, and the structures are shown in red. DIC: differential interference contrast image; Arrow: nucleus; arrowhead: kinetoplast

Fig. 2 Dendrograms of *Trypanosoma* species using V7-V8 region of *18S rRNA* (A), whole internal transcribed spacer (*ITS*) (B), and partial *CATL* (C) sequences

494 Bootstrap values (>60) are shown on the tree nodes. The Japanese
495 isolates are highlighted in blue in panel B. The major lineages TthII and TthI are
496 denoted in both panels B and C
497
498 Supplementary fig. S1. PCR products of *Trypanosoma theileri* Obihiro strain.
499 All of the PCR-amplified target loci had expected sizes. M: 100 bp
500 marker; Lane 1: V7-V8 region of *18S rRNA*; Lane 2: whole internal transcribed
501 spacer (*ITS*); Lane 3: Tth625 fragment; Lane 4: partial *CATL*; and Lane 5: *ITS1.*
502

Fig. 1

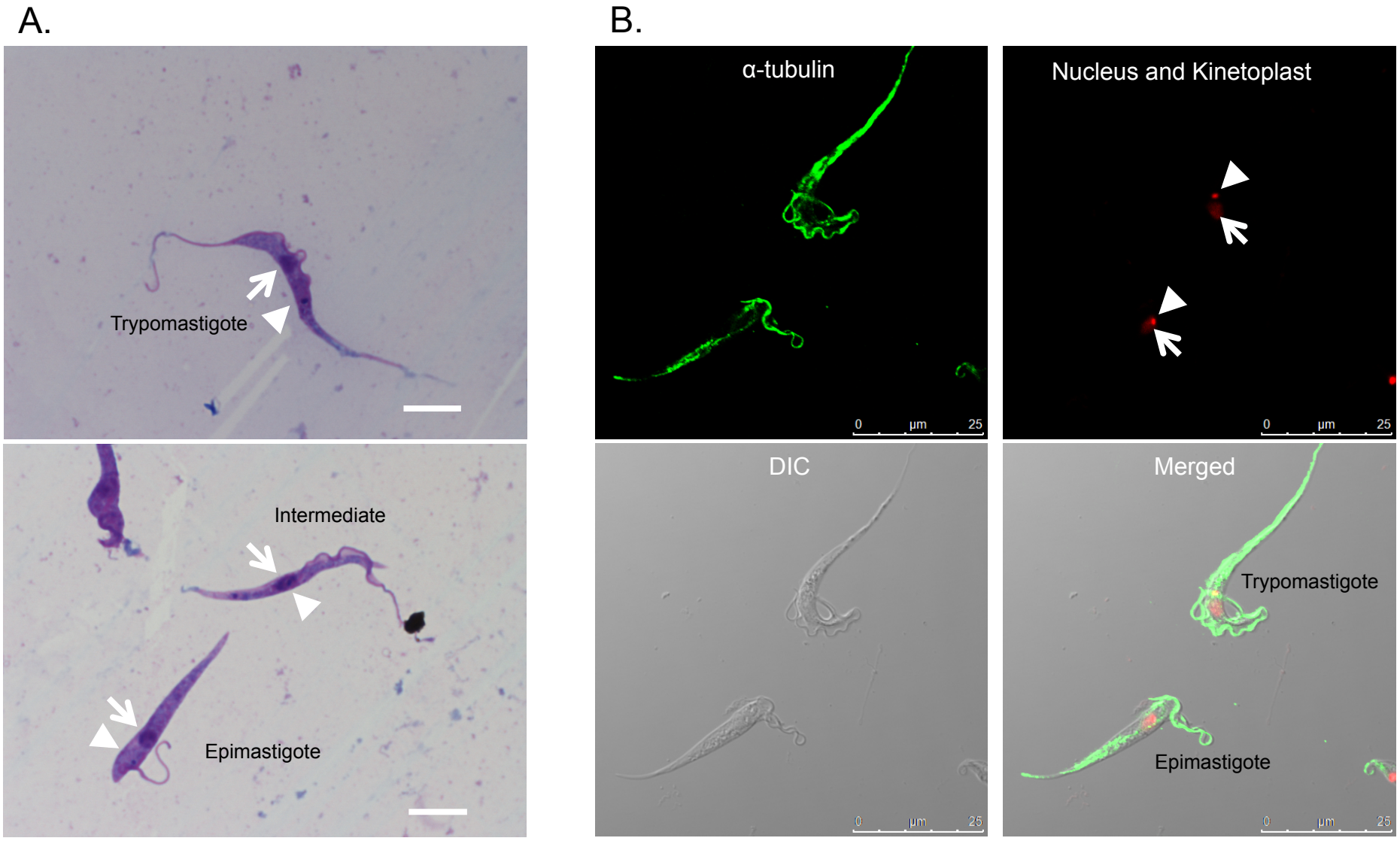

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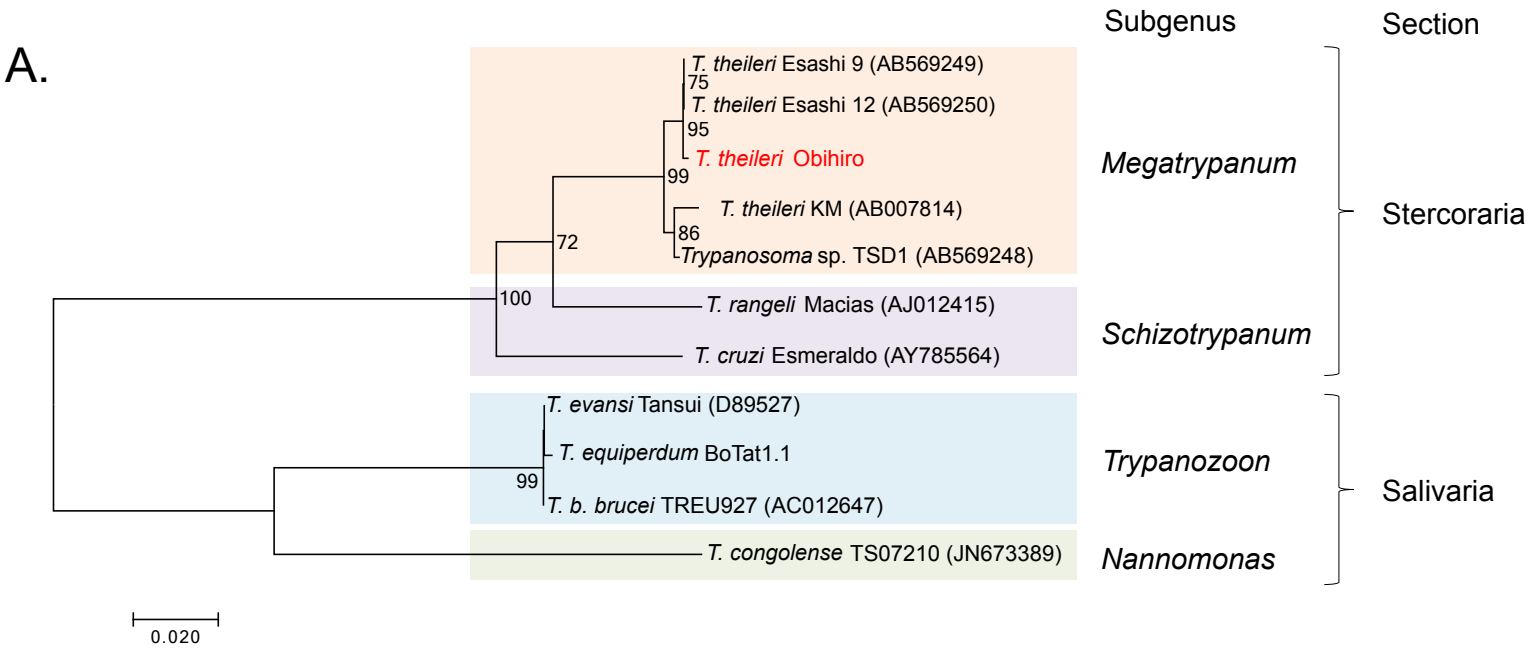
Fig. 2



Suganuma *et al.*

Fig. 2 B.

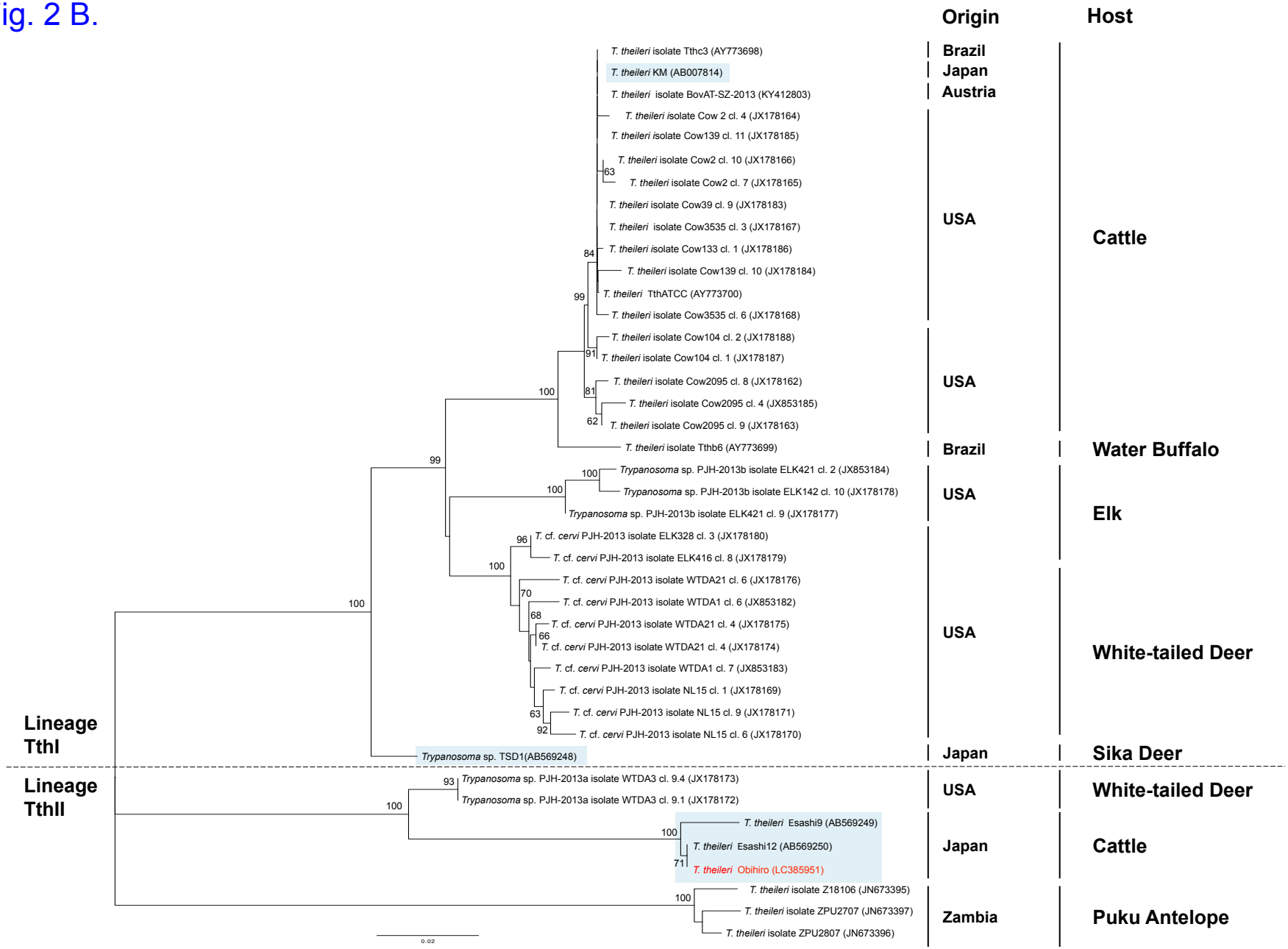


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Fig. 2

C.

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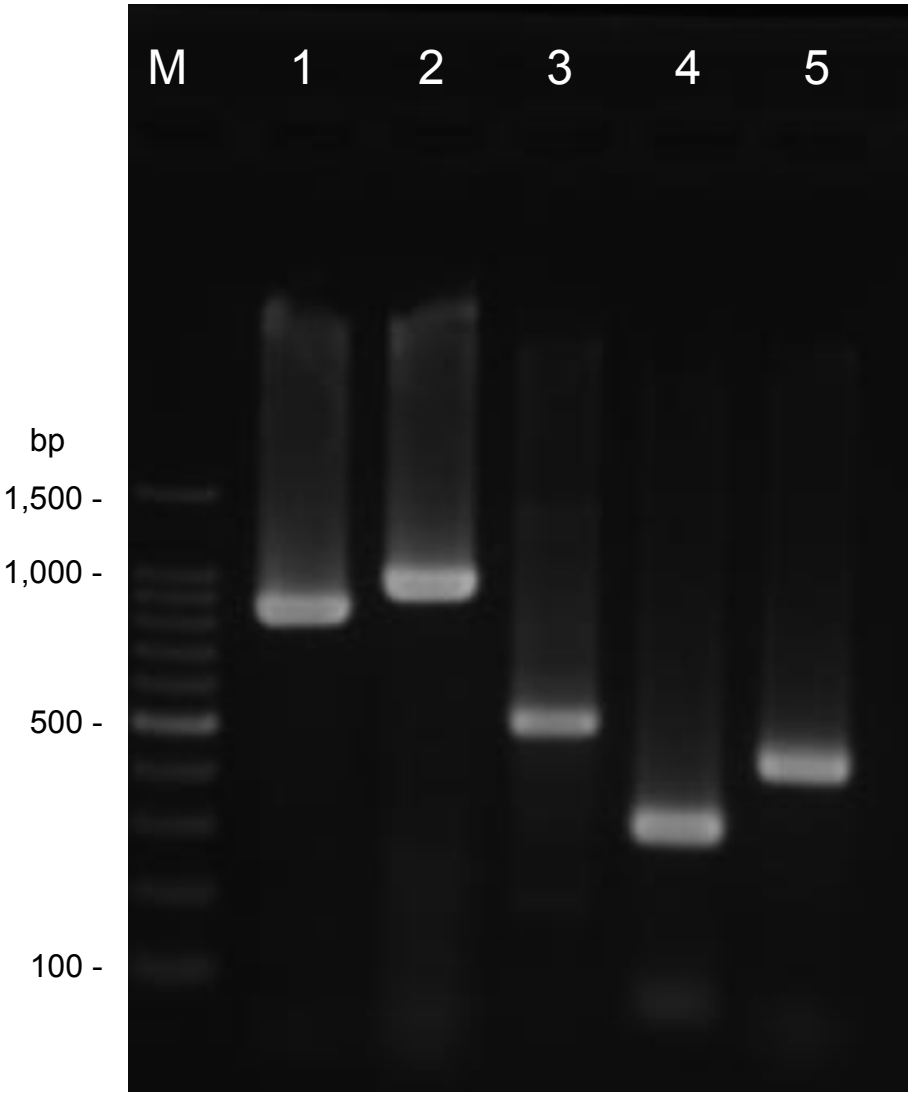
Supplementary fig. S1



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Supplementary Table 1. PCR conditions used in the present study

Target locus[a]		Sequence	Size	Reference
V7-V8 region of 18S rRNA	Forward (609F)	5′-CAC CCG CGG TAA TTC CAG C-3′	876 bp	Da Silva *et al.* 2004
	Reverse (706R)	5′-CTG AGA CTG TAA CCT CAA -3′		
ITS	Forward (IR1)	5′-GCT GTA GGT GAA CCT GCA GCA GCT GGA TCA TT-3′	997 bp	
	Reverse (IR2)	5′-GCG GGT AGT CCT GCC AAA CAC TCA GGT CTG-3′		
ITS1	Forward (ITS CF)	5′ -CCG GAA GTT CAC CGA TAT TG-3′	396 bp	Njiru *et al.* 2006
	Reverse (ITS BR)	5′-TTG CTG CGT TCT TCA ACG AA-3′		
Catepsin L-like	TthCATL1	5′-CGT CTC TGG CTC CGG TCA AAC-3′	289 bp	Rodrigues *et al*. 2010
	DTO155	5′-TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA-3′		
Tth625 fragment	Tth625a	5′-CCG CTG GAG CTA AGA ATA GA-3′	485 bp	Rodrigues *et al*. 2003
	Tth625b	5′-AAT TGC ATA AAC ACA GCT CCC-3′		

Supplementary Table 2. *Trypanosoma theileri* sequences used in this study

Target locus	GenBank Accession No.	NCBI BLAST highest identity			Reference
		Isolate	Accession No.	Identity %	
Partial *CATL*	LC385983	*T. theileri* isolate BU50	LC125455.1	100	Yokoyama *et al.* 2016
V7-V8 region of *18S rRNA*	LC385952	*T. theileri* isolate Cow 2073 clone 9	JX178182.1	100	Fisher *et al*. 2013
ITS	LC385951	*T. theileri* isolate Esashi12	AB569250.1	99	Hatama *et al.* 2007
Tth625 fragment	LC426018	*T. theileri* isolate Rodrigues A	AF537202.1	86	Rodorigues *et al.* 2003