Analysis of Trypanosoma sequences from Haemaphysalis flava (Acari: Ixodidae) and Tabanus rufuldens (Diptera: Tabanidae) collected in Ishikawa, Japan

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Abstract: Trypanosoma are known to be a diverse group of parasites that infect animals belonging to all classes in the subphylum Vertebrata and are important pathogens that affect human and animal health. Although many trypanosomatids have been found in mammals and birds in Japan, information regarding their invertebrate host is currently lacking. During our virome analyses of ticks and horse flies, several trypanosoma-like sequences were found. Further sequence characterization and PCR-based screening revealed trypanosomatids termed Trypanosoma sp. 17ISK-T2 and 17ISK-T22 in the nymphs of Haemaphysalis flava, and T. theileri-like sequences in Tabanus rufuldens. These results indicate that virome analysis by next-generation sequencing (NGS) can also be used as a tool for protozoan detection from arthropods. Further investigations will assist in understanding the diversity and transmission dynamics of these parasites in Japan.

Key words: Trypanosoma, Trypanosomatids, Trypanosomiasis, Trypanosoma theileri, tick, horse fly

INTRODUCTION

Trypanosomatids in the genus Trypanosoma are a diverse group of parasites that infect animals belonging to all classes in the subphylum Vertebrata. All Trypanosoma, except T. equiperdum Doflein, require invertebrate hosts for transmission between vertebrate hosts (Kaufer et al., 2017). Trypanosoma includes several important pathogens of humans and animals. For instance, African sleeping sickness caused by T. brucei gambiense Dutton and T. b. rhodesiense Stephens and Fantham is endemic in several African countries (Büscher et al., 2017), while Chagas disease caused by T. cruzi Chagas is a public health concern in Latin America (Pérez-Molina and Molina, 2018). Furthermore, atypical human infections of animal trypanosomes such as T. b. brucei Plimmer and Brandford, T. evansi Steel, or T. lewisi (Kent) have been reported and recent molecular diagnosis technique advances have allowed more frequent detection of these atypical infections (Truc et al., 2013).

While several trypanosomatids have been found from mammals and birds in Japan, their invertebrate hosts have not yet been elucidated (Table 1). In Japan, several cases of T. theileri (Laveran) infection have been reported in cattle (Sasaki, 1958; Iwata et al., 1959; Ishida et al., 2002; Matsumoto et al., 2011). Although T. theileri in general shows non-pathogenicity in cattle, the potential for exacerbating pathogenicity by concomitant infection with piroplasma or bovine leukemia virus has been observed (Iwata et al., 1959; Matsumoto et al., 2011). The prevalence of T. theileri in cattle is not well understood due to its non-pathogenicity in cattle occurring in a single infection. Furthermore, the vector species of T. theileri have remained obscure in Japan thus far. On the other hand, multiple Trypanosoma parasites have been observed in Japanese birds (Table 1). However, these parasites have not been classified into species, and their sequence information is not available (Table 1). This might be due to their low or unknown pathogenicity in the host (Kano, 1950; Hirayama et al., 2014). Moreover, human pathogenic T. lewisi was reported in Japan more than a century ago (reviewed by Irikura, 1906), and the recent distribution and endemic situation of the parasite remains unclear. Conversely, several Trypanosoma parasites have been found in ticks; however, their vertebrate host has not been identified (Table 1). Thus, information on domestic Trypanosoma parasites is limited.
### Table 1. Records of trypanosoma parasites in mammals, birds, and arthropods in Japan.

| Species | Vertebrate host | Invertebrate host | Reference |
|---------|----------------|-------------------|-----------|
| *Trypanosoma dionisii* | Mammalia (Eastern bent-wing bat) | Hemipteran insects | Maefie et al., 2018 |
| *Trypanosoma lewisi* | Brown rat and black rat | Flea | reviewed by Irikura, 1906; Sasaki, 1938; Iwata et al., 1959; Ishida et al., 2002; Matsumoto et al., 2011, etc. |
| *Trypanosoma theileri* | Cattle | Tabanidae | Hatama et al., 2007; Nakamoto et al., 2014 |
| *Trypanosoma sp. TSD1* | Hokkaido sika deer | unknown | It and Itagaki, 2003; Fujita and Watanabe, 2007 |
| *Trypanosoma sp.* | Black-browed reed warbler | unknown | Nagata, 2006 |
| *Trypanosoma sp.* | Black-faced bunting | unknown | Nagata, 2006 |
| *Trypanosoma sp.* | Brambling | unknown | Nagata, 2006 |
| *Trypanosoma sp.* | Brown-eared bulbul | unknown | Kano and Kimura, 1950 |
| *Trypanosoma sp.* | Bull-headed shrike | unknown | Hayashi et al., 1998 |
| *Trypanosoma sp.* | Cassin's kinglet | unknown | Nagata, 2006 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | reviewed by Kano and Kimura, 1950 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | Nagata, 2006 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | Kano and Kimura, 1950 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | reviewed by Kano and Kimura, 1950 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | Hayashi et al., 1998 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | reviewed by Kano and Kimura, 1950 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | Nakamoto et al., 2014 |
| *Trypanosoma sp.* | Chestnut-sided warbler | unknown | reviewed by Kano and Kimura, 1950 |

* Only morphological observation.
** Invertebrate host have been identified in overseas but not yet in Japan.
During our virome analyses of the ticks and horse flies, several trypanosoma-like sequences were found. Therefore, this study characterized the sequences and investigated their infection status among ticks and horse flies collected in Japan.

Materials and Methods

Tick and horse fly collection

Host questing ticks were collected from vegetation fields in several sites in the Ishikawa and Toyama Prefectures, Japan, in October 2017 by dragging as described previously (Kobayashi et al., 2020). The information regarding tick collection sites was listed in a previous report (Kobayashi et al., 2020). Furthermore, from April to June 2018, additional ticks were collected at the same sites.

Female tabanids were collected by sweeping in Ishikawa Prefecture, Japan, in August 2018. The collection sites were as follows: Point Saruyama, Yoshiura, Monzen-machi, Wajima City (37°19’26.1”N, 136°43’31.4”E); Fukami, Monzen-machi, Wajima City (37°17’58.4”N, 136°44’16.6”E); and Awazu, Misaki-machi, Suzu City (37°29’05.0”N, 137°19’52.6”E). The collected ticks and tabanids were divided into species by morphology and stored at −80°C until analyses. Incidentally, no blood-fed ticks and tabanids were contained in the specimens.

Next-generation sequencing

Trypanosomatid sequences were detected during the process of RNA virome analyses for questing and characterization of viruses of the ticks and tabanids. The next-generation sequencing (NGS) method for the ticks was described previously (Kobayashi et al., 2020). The fundamental technique of NGS for tabanids was the same as described previously (Kobayashi et al., 2020). In brief, tabanid samples were crushed in a medium by tissue lyser II (Qiagen) and filtered with a 0.45 µm filter. Then, nuclease cocktail [14 units of TURBO DNase (Invitrogen), 11.5 units of Baseline-zero DNase (epicentre), and 15 µg of RNase A (Nippon gene)] was added to the 380 µL filtrate, which was mixed into the tabanid pool in equal amounts (42.2–190 µL/pool). Preparation of the library for NGS was performed with NEB Next RNA first-strand and second-strand synthesis modules (New England Biolabs), NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs), and NEBNext Ultra II Ligation Module (New England Biolabs) according to the manufacturer’s protocol. Following purification of the libraries by Agencourt AMPure XP (Beckman Coulter), quantification was performed, and the libraries were amplified as required by NEBNext Ultra II Q5 Master Mix (New England Biolabs). The purified libraries were analyzed using a MiniSeq system (Illumina) with a MiniSeq Mid Output kit (300 cycles) (Illumina). The obtained reads were subjected to trimming and de novo assembly on a CLC Genomics Workbench version 12 (Qiagen). The resultant contigs were identified by BLASTN search. The sequence analyses were carried out by Genetyx software version 13 (Genetyx).

Screening and sanger-sequencing of trypanosomatids

In order to identify a trypanosomatid-positive pool, a polymerase chain reaction (PCR)-based screening was performed. 200–300 µL of phosphate-buffered saline (Sigma-Aldrich) was added to the homogenate and crushed again. DNA was extracted from 200 µL of the supernatant of the homogenate using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer’s protocol. The partial 18S ribosomal RNA (rRNA) gene was amplified by Prime Star Max DNA polymerase (Takara) with the primer set SSU-1 (5’-ATC TGC GCA TGG CTC ATT AC-3’) and SSU-2 (5’-CAC ACT TTG GTT CTT GAT TGA-3’) designated by Barratt et al. (2017) (Fig. 1). PCR was conducted under the following condition; 35 cycles at 98°C for 10 seconds, 55°C for 5 seconds, and 72°C for 7 seconds. The amplified products were confirmed by agarose gel electrophoresis. For additional PCR-based screening, a total of 114 pools composed of at least eight tick species [Amblyomma

![Fig. 1. A schematic illustration of the genetic structure of ribosomal RNA (rRNA) of trypanosomatid and the distribution of trypanosoma-like contigs. The black triangles on the illustration of the rRNA gene represent the position of the primer set used for the PCR-based screening. The lower two dotted boxes showed the distribution of contigs obtained from ticks (above) and tabanids (below).]
Phylogenetic analysis

(Kobayashi et al., 2016).

sequenced by the same method described previously.

analyses

Detection of trypanosoma-like sequences by NGS

used in this analysis are shown in Appendix 1.

6 (Tamura et al., 2013). The trypanosomatid sequences

G model by MEGA

+ or K2

method with a GTR

G

+ +

was constructed by the maximum likelihood (ML)

(Tamura et al., 2013). The phylogenetic dendrogram

suitable nucleotide substitution models by MEGA 6

MUSCLE (Madeira et al., 2019). Selections of the

ru/f_idens

trigonus

sapporoensis

Shiraki, 5

Tabanus iyoensis

pools (composed of 6

Ixodes

spp. (2 nymphs)
]

and 36 tabanid

female), and

23 females),

Neumann (6 nymphs, 10 males,

and 5 females),

Ixodes ovatus

spp. (105 larvae),

Saito (3 nymphs,

Haemaphysalis

(72 nymphs, 1 male,

and 5 females),

H. megaspinosa

(3 nymphs and 4 females),

Supino

Neumann (1 male),

H. formosensis

H. hystricis

Neumann (252 nymphs, 81 males, and 90 females),

282 Med. Entomol. Zool.

nT4_c22 705 45 9.41

Trypanosoma sp. KG1 gene for 18S ribosomal RNA,

partial sequence

Trypanosoma theileri isolate Cow 2095 clone 4 18S

ribosomal RNA gene, partial sequence; and internal

transcribed spacer 1, 5.8S ribosomal RNA gene, and

internal transcribed spacer 2, complete sequence

Trypanosoma minasense genes for 18S rRNA, ITS1,

5.8S rRNA, ITS2, 28S rRNA, partial and complete

sequence

Trypanosoma minasense genes for 18S rRNA, ITS1,

5.8S rRNA, ITS2, 28S rRNA, partial and complete

sequence

Trypanosoma theileri gene for 18S rRNA, 5.8S rRNA,

28S rRNA, partial and complete sequence

Trypanosoma minasense genes for 18S rRNA, ITS1,

5.8S rRNA, ITS2, 28S rRNA, partial and complete

sequence

Source Contig name Length (nt) Total read count Average coverage

H. flava nT4_c22 705 45 9.41

T. rufidens YA3_c42 678 40 8.48

YA3_c43 574 45 10.58

YA3_c45 807 57 9.99

YA3_c49 1057 65 8.59

YA3_c56 574 51 11.98

Result of blastn search

Accession no. e-value identity (%)

AB281091 3e-106 92.09, 99.10*

JX85185 0.0 100.00

AB362411 0.0 93.69

AB362411 0.0 96.42

AB007814 0.0 100.00

AB362411 0.0 95.30

* Two unknown sequence regions are contained inside one contig. The range of number shows the value in each frame.

testudinarium Koch (1 nymph), Haemaphysalis flava

Neumann (252 nymphs, 81 males, and 90 females),

H. formosensis Neumann (1 male), H. hystrix Supino

(3 nymphs and 4 females), H. longicornis Neumann

(72 nymphs, 1 male, and 5 females), H. megaspinosa

Saito (3 nymphs), Haemaphysalis spp. (105 larvae),

Ixodes ovasus Neumann (6 nymphs, 10 males, and

23 females), I. turdus Nakatsudai (3 nymphs and 1

female), and Ixodes spp. (2 nymphs)] and 36 tabanid

pools (composed of 6 Tabanus iyoensis Shiraki, 5 T.

sapporoensis Shiraki, 5 T. mandarinus Schiner, 2 T.

trigonus Coquillet, 2 T. chrysulus Loew, and 25 T.

rufidens Bigot) were used. The amplicon was sanger-

sequenced by the same method described previously

(Kobayashi et al., 2016).

Phylogenetic analysis

Multiple sequence alignment was performed by

MUSCLE (Madeira et al., 2019). Selections of the

suitable nucleotide substitution models by MEGA 6

(Tamura et al., 2013). The phylogenetic dendrogram

was constructed by the maximum likelihood (ML)

method with a GTR+G+I or K2+G model by MEGA 6

(Tamura et al., 2013). The trypanosomatid sequences

used in this analysis are shown in Appendix 1.

RESULTS

Detection of trypanosoma-like sequences by NGS

analyses

During the RNA virome analyses of ticks and

tabanids, trypanosoma-like contigs were detected

(Table 2) in the tick sample nT4 (combination of 7 pools

composed of larva, nymph, and adult H. flava; adult

H. formosensis; and larval Haemaphysalis spp., further

details can be found in Kobayashi et al., 2020), a total of

1,149,100 reads were obtained (Kobayashi et al., 2020).

Within this sample, one contig (nT4_c22) related to

the sequence of trypanosomatids was identified via a

BLASTN search (Table 1). The sequence showed high

similarity to the 18S rRNA gene of Trypanosoma sp.

KG-1, isolated from H. hystrix collected in Kagoshima

Prefecture, Japan (Thekisoe et al., 2007). Furthermore,

trypanosoma-like sequences were contained in the

resultant contigs produced from a total of 759,868 reads

in the tabanids sample, which was made up of eight T.

rufidens females. Four contigs similar to the sequences

of trypanosomatids were identified by the BLASTN

search, and two contigs (named YA3_c42 and YA3_

c49) were 100% identical to that of T. theileri (Table

1). Other contigs (YA3_c43, YA3_c45, and YA3_c56)

from the tabanids showed a 93–96% sequence identity

to the genes of T. minasense Chagas (Table 1). Multiple

sequence alignment revealed that two contigs (YA3_c42

and YA3_c49) and others (YA3_c43, YA3_c45, and

YA3_c56) corresponded to the 18S and 28S rRNA genes

of the Trypanosoma, respectively (Fig. 1).

PCR-based screening of trypanosomatids from the
ticks and tabanids

PCR-based screening was performed to identify

the trypanosomatid-positive pool, with the primer

set (SSU-1 and SSU-2), which targeted a highly

conserved region on the 18S rRNA gene among the

trypanosomatids (Fig. 1). The pool no. 17ISK-T22

(resultant contigs produced from a total of 759,868 reads

in the tabanids sample, which was made up of eight T.

rufidens females. Four contigs similar to the sequences

of trypanosomatids were identified by the BLASTN

search, and two contigs (named YA3_c42 and YA3_

c49) were 100% identical to that of T. theileri (Table

1). Other contigs (YA3_c43, YA3_c45, and YA3_c56)

from the tabanids showed a 93–96% sequence identity

to the genes of T. minasense Chagas (Table 1). Multiple

sequence alignment revealed that two contigs (YA3_c42

and YA3_c49) and others (YA3_c43, YA3_c45, and

YA3_c56) corresponded to the 18S and 28S rRNA genes

of the Trypanosoma, respectively (Fig. 1).

PC-
A trypanosomatid sequence was identified in only the pool 17ISK-T2 (made up of 6 nymphs of *H. flava*) (Table 3). The sequence exhibited 100% identity to that of 17ISK-T22 (data not shown), which suggested that trypanosomatids detected in the two different *H. flava* pools were the same species.

On the other hand, the trypanosomatid-positive pool was identified as the pool no. 18HF29, which consisted of one female *T. rufidens*, from the pooled NGS sample (Table 3). PCR-based screening was performed in the same manner to detect trypanosomatids in the other tabanid pools. However, no positive pool was detected from the other 36 tabanid pools.

### Phylogenetic relationships among trypanosomatids

Phylogenetic analysis was conducted based on about 1,000 nt sequences of the partial 18S rRNA gene amplified by SSU-1 and SSU-2. The phylogenetic dendrogram was constructed by the maximum likelihood (ML) method with a GTR+G+I model (Fig 2A). The trypanosomatids from the *H. flava* ticks (named *Trypanosoma* sp. 17ISK-T2 and T22, respectively, Table 3) formed a cluster with *Trypanosoma* parasites from *H. hystrics* (*Trypanosoma* sp. KG-1), badger (*P. pensani* Bettencourt and Franca LEM 110), and wombat (*T. copemani* Auster, Jefferies, Friend, Ryan, Adams and Reid H26 and wombat AAP). However, the 17ISK-T2 and 17ISK-T22 are located apart from these trypanosomatids. Moreover, the trypanomatid detected from *T. rufidens* was located in the *T. theileri* clade (data not shown).

Recent studies revealed that several *Trypanosoma* parasites, which were related to the 17ISK-T2 and 17ISK-T22, were found in animals and ticks (Madeira et al., 2009; Marotta et al., 2018a, b). However, there were short overlap sequences among the 17ISK-T2, 17ISK-T22, and related species. Therefore, further phylogenetic analysis was performed using the available sequences of each related species. The dendrogram constructed by the ML method with a K2+G model using aligned about 300 nt of 18S rRNA genes was shown in Fig. 2B. In the dendrogram, the 17ISK-T2 and 17ISK-T22 were related to *T. caninum* Madeira, Almeida, Barros, Olireira, Sousa, Alres, Miranda, Schubach and Marzochi, a recently described *Trypanosoma* species found in a domestic dog (*Canis lupus familiaris* Linnaeus) in Brazil (Madeira et al., 2009). Furthermore, the 17ISK-T2, 17ISK-T22, and trypanosomatids from ticks formed a clade in the dendrogram (Fig. 2B).

To confirm the phylogenetic relationships between *Trypanosoma* sp. 18HF29 and trypanosomatids in *T. theileri* clade, ML tree was constructed based on partial 18S rRNA gene (548 nt) by K2+G model (Fig. 3). Although several *T. theileri* patasites have been isolated in Japan, *Trypanosoma* sp. 18HF29 formed a clade with *T. theileri* strain KM (GenBank accession no. AB007814) in the dendrogram (Fig. 3).

### Discussion

Several trypanosomatid sequences had been detected in data from the RNA virome of ticks and tabanids. The system for RNA virome analysis was established by a previous study (Kobayashi et al., 2020) and the host microorganisms such as protozoans or bacteria were assumed to be excluded during the small-pore filtration step, at least in principle. However, several bacteria or trypanosomatids were detected from the resultant contigs, which were almost all derived from their rRNA genes. Furthermore, many sequence reads derived from host rRNA genes were also detected in spite of the nuclease treatment of samples. Ribosomes were composed of rRNAs forming higher-ordered structures and ribosomal proteins (Alberts et al., 2014). Therefore, it seems that the rRNA genes were easy to retain because RNase A used in the nuclease treatment step in this study digests single-stranded RNA only. In addition, a typical eukaryotic cell contains millions of ribosomes in the cytoplasm (Alberts et al., 2014). Furthermore, previous studies indicated that a rich rRNA was contained in the extracellular vesicles derived from *Trypanosoma* parasite (Bayer-Santos et al., 2014; Garcia-Silva et al., 2014). These extracellular vesicles probably play a role in protection of inside RNAs from the nuclease.

A previous study reported a novel *Trypanosoma* parasite named *Trypanosoma* sp. KG-1 isolated from pooled adult *H. hystrics* collected in Kagoshima Prefecture in Japan (Thekiso et al., 2007). Another report showed that three trypanosomatid isolates were
obtained from the nymphs of *H. flava* collected from Kagoshima, Tokushima, and Fukushima Prefectures in Japan (Fujita and Watanabe, 2007). The latter study suggested that the trypanosomatids from *H. flava* and the KG-1 were two different species because they were discriminable by morphology (Fujita and Watanabe, 2007). However, the sequences of these trypanosomatids from *H. flava* are not available. In this study, two trypanosomatid sequences were detected from two pools of *H. flava* nymphs, and their sequences were different from that of the *Trypanosoma* sp. KG-1. *Trypanosoma* sp. 17ISK-T2 and 17ISK-T22 were detected in the same tick species and developmental stages indicated that they are probably the same species.

![Phylogenetic characterization of trypanosomatids detected from ticks in this study. (A) The phylogenetic dendrogram was constructed based on the nucleotide sequences (about 1,000 nt) by the ML method with the use of the GTR+G+I model. The bootstrap values of more than 50 required by bootstrap test (1000 replicates) (Felsenstein, 1985) are shown in the next to the branches. The origin of each trypanosomatids is indicated in parenthesis. *Bodo caudatus* and *Trypanoplasma borreli* were used as an outgroup. The accession numbers of trypanosomatids used in this analysis are shown in Appendix 1. (B) The phylogenetic dendrogram was constructed based on the nucleotide sequences (about 300 nt) by the ML method with the use of the K2+G model. The bootstrap values of more than 50 required by bootstrap test (1000 replicates) (Felsenstein, 1985) are shown in the next to the branches. Trypanosomatids isolated from ticks are represented using illustrations. Trypanosomatids which are identified in this study are indicated by a black circle and bold-faced. The origin of each trypanosomatids is indicated in parenthesis. The accession numbers of the trypanosomatids used in this analysis are shown in Appendix 1.](image-url)
as that reported by Fujita and Watanabe (2007). In this study, the trypanosomatids were not detected from other tick species, suggesting that this *Trypanosoma* might be an *H. flava*-specific species.

*Trypanosoma* sp. 17ISK-T2 and 17ISK-T22 formed a clade with trypanosomatids from ticks and a dog in the phylogenetic dendrogram in this study. All trypanosomatids from ticks are yet to be detected in their vertebrate hosts (Thekisoe et al., 2007; Marotta et al., 2018a, b). However, *T. caninum*, which was isolated from dogs in Brazil, was located in the sister clade of 17ISK-T2 and 17ISK-T22. *Trypanosoma pestanai*, which is a related species of 17ISK-T2 and 17ISK-T22, was isolated not only from a badger (isolate LEM 110) but also from a dog (German isolate; Dyachenko et al., 2017). Therefore, the vertebrate host of *Trypanosoma* sp. 17ISK-T2 and 17ISK-T22 might be dogs or a related species in the Canidae family such as a raccoon dog or fox in Japan. *Haemaphysalis flava* is known as a tick species parasitizing various animals including large and medium-sized mammals and birds (Takada et al., 2019), and the species has been collected in 36 species in five order of Aves so far (Yamauchi, 2001). A variety of trypanosomatids was found in Japanese birds, but their invertebrate hosts have not been identified. Therefore, it cannot deny the possibility that *Trypanosoma* sp. 17ISK-T2 and 17ISK-T22 are an avian *Trypanosoma* species, although they are phylogenetically distant from previously known avian trypanosomatids such as *T. corvi* Stephens and Christophers, mend, Baker, *T. culicavium* Votypka, Szabová, Rádrová, Zídková and Svobodová, *T. thomasafrica* Slapeta, Morin-Adeline, Thompson, McDonnel, Sheils, Gilchrist, Votypka and Vogelnest, and *T. avium* Votypka, Szabová, Rádrová, Zídková and Svobodová. Interestingly, *T. amblyommi* Marotta, Dos Santos, Cordeiro, Barros, Bell-Sakyi and Fonseca and *T. rhipicephalis* Marotta, Dos Santos, Cordeiro, Matos, Barros, Madeira, Bell-Sakyi and Fonseca were isolated from ticks collected from white-lipped peccary (*Tayassu pecari*) and cattle, respectively (Marotta et al., 2018a, b). Thus, these infested animals appear to be vertebrate hosts of these trypanosomatids. Therefore, to identify the vertebrate host of these tick-associated trypanosomatids, further investigations are required.

In this study, two contigs shared 100% identity to a sequence of *T. theileri* and were detected from a tabanid sample. However, the other three contigs from the same tabanid sample showed 93–96% identity to that of *T. minasense* as the highest score sequence by BLASTN search. It appeared that all of the contigs were derived from a single pool (pool no. 18HF29) because only one pool was positive by PCR-based screening using a universal primer set targeting the 18S rRNA gene of trypanosomatids. In fact, only partial sequences of the 28S rRNA gene of *T. theileri* were available on the International Nucleotide Sequence Database (DDBJ/EMBL/NCBI). Therefore, no corresponding sequence of *T. theileri* was found for the three contigs (YA3_c43, YA3_c 45, and YA3_c 56). Even though several contigs shared 100% identities to the sequence of *T. theileri*, we could not conclude that the detected sequences were from a *T. theileri* as morphological data was not available.

The phylogenetic analysis between *Trypanosoma* sp. 18HF29 and trypanosomatids in *T. theileri* clade.
indicated that *Trypanosoma* sp. 18HF29 formed a clade with Japanese *T. theileri* strain KM (GenBank accession no. AB007814). However, the information of this strain (e.g., host species and location) was not available. Interestingly, other Japanese strains of *T. theileri* (Esashi 9, Esashi 12, and Obihiro) formed a different cluster, and it was located far from *Trypanosoma* sp. 18HF29 and *T. theileri* strain KM. The strain Esashi 9, Esashi 12, and Obihiro were isolated from cattle in Hokkaido, Japan, suggesting that *T. theileri* may be maintained in local transmission cycle. However, the dendrogram was constructed based on short 18S rRNA sequence. To understand the host specificity and natural history of *T. theileri* and related species, further investigations are needed.

The vector species of *T. theileri* have remained obscure in Japan thus far. Therefore, this study is the first detection report of *T. theileri*-like parasite from field-caught vector insect (*T. rufidens*). In fact, *T. rufidens* is known as a tabanid species sucking the blood from large mammals (e.g., cattle and horse) and humans (Nagasawa, 1967; Kano and Shinonaga, 2003). A previous study showed that the tabanids belonging to the genus *Haematopota*, *Hybomitra*, and *Tabanus* were regarded as a vector species of *T. theileri* in Germany (Böse et al., 1987). Furthermore, *T. theileri*-like trypanosomatids were detected from the tabanidae genera *Chrysops*, *Haematopota*, *Hybomitra*, and *Tabanus* in Poland and Russia (Ganyukova et al., 2018; Werszko et al., 2020). Therefore, various types of tabanid species appeared to play a role as vectors of *T. theileri* and related species. *Trypanosoma* sp. TSD1, which is a related species of *T. theileri*, was isolated from sika deer in Hokkaido (Hatama et al., 2007). Although the vector species of this parasite has not been identified, tabanids seem to contribute to their transmission in nature as *T. cervi* Kingston and Morton, which is a related *Trypanosoma* species in deer, is also transmitted by tabanids (Fisher et al., 2013). Thus, *T. theileri* and related *Trypanosoma* might be transmitted between cattle and deer by the tabanids. Additional investigations will assist with understanding the transmission dynamics of these parasites in nature.

In conclusion, two different species of trypanosomatid sequences were detected during the RNA virome analyses of ticks and tabanids. Further sequence characterizations and PCR-based screening revealed that trypanosomatids called *Trypanosoma* sp. 17ISK-T2 and 17ISK-T22 were detected from the nymphs of *H. flava*, and *T. theileri*-like sequences were found from *T. rufidens*. These results indicate that virome analysis by NGS can also be used as a tool for protozoan detection from arthropods. Further investigations will assist with understanding the diversity and transmission dynamics of these parasites, and this fundamental information will likely contribute to the identification of the potential risk of zoonotic infections in humans.

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| Clade                      | Species name                        | Common name                        | Scientific name                        | Origin                  | Accession no.          |
|---------------------------|-------------------------------------|------------------------------------|----------------------------------------|-------------------------|------------------------|
| Aquatic clade             | Trypanosoma binneyi                 | Platypoeculus obesus              | Ornithorynchus anatinus               | Australia               | AJ13235                  |
|                           | Trypanosoma chattoni                | Northern leopard frog             | Rana pipiens                          | USA                     | AF119807                |
|                           | Trypanosoma cobriti                 | Stone loach                       | Noemacheilus barbatulus              | England                 | AJ809143                |
|                           | Trypanosoma epimeneophilis          | Barramundi                        | Lates calcarifer                      | China                   | MG589625                |
|                           | Trypanosoma fallii                  | American toad                     | Anaxayus americus                     | Canada                  | AJ1F8806                |
|                           | Trypanosoma granulatum              | European ed                       | Anguilla anguila                      | United Kingdom          | KJ620551                |
|                           | Trypanosoma maga                    | African common toad               | Metaxyphias regularis                | Africa                  | AS091557                |
|                           | Trypanosoma neureilhitei           | Edible frog                       | Rana esculenta                        | Yugoslavia              | AK18809                 |
|                           | Trypanosoma ranarum                 | Bull frog                         | Rana catesbienae                     | Canada                  | AK18810                 |
|                           | Trypanosoma rotatorius              | Bullfrog                          | Rana catesbienae                     | Canada                  | AK18810                 |
| Trypanosoma brucei clade  | Trypanosoma brucei brucei          | Tsetse flies                      | Glossina pallidipes                  | Kenya                   | KR002998632             |
|                           | Trypanosoma brucei gambia           | Human                             | Homo sapiens                          | Nigeria                 | A009141                 |
|                           | Trypanosoma brucei rhodesiens       | Tsetse flies                      | Glossina pallidipes                  | Central African Republic | KP307026                |
|                           | Trypanosoma congolense              | Tsetse flies                      | Glossina affinis                     | Central African Republic |                           |
|                           | Trypanosoma equiperdum              | Horse                             | Equus caballus                        | China                   | A009153                 |
|                           | Trypanosoma evansi                  | Water buffalo                     | Bulbasula arnee                      | Thailand                | AY04050                  |
|                           | Trypanosoma godfreyi                | Tsetse fly                         | Glossina ruminata                    | The Gambia              | A009151                 |
| Trypanosoma simiae        | Trypanosoma simiae                 | Zeua castoro                      | Glossina pallidipes                  | Kenya                   | A040608                 |
| Trypanosoma vivax         | Trypanosoma vivax                   | Rat                               | Rattus ratti                          | Brazil                  | A012441                 |
| Trypanosoma conorhini     | Trypanosoma conorhini               | Hybomitra tarandina               | Hybomitra tarandina                  | Austria                 |                           |
| Trypanosoma brucei clade  | Trypanosoma brucei brucei          | Tsetse flies                      | Glossina pallidipes                  | Kenya                   | A009153                 |
|                           | Trypanosoma brucei gambia           | Human                             | Homo sapiens                          | Nigeria                 | A009141                 |
| Trypanosoma brucei clade  | Trypanosoma brucei rhodesiens       | Tsetse flies                      | Glossina affinis                     | Central African Republic | KP307026                |
| Trypanosoma congolense    | Trypanosoma congolense              | Tsetse flies                      | Glossina affinis                     | Central African Republic |                           |
| Trypanosoma equiperdum    | Trypanosoma equiperdum              | Horse                             | Equus caballus                        | China                   | A009153                 |
| Trypanosoma evansi        | Trypanosoma evansi                  | Water buffalo                     | Bulbasula arnee                      | Thailand                | AY04050                  |
| Trypanosoma godfreyi      | Trypanosoma godfreyi                | Tsetse fly                         | Glossina ruminata                    | The Gambia              | A009151                 |
| Trypanosoma simiae        | Trypanosoma simiae                 | Zeua castoro                      | Glossina pallidipes                  | Kenya                   | A040608                 |
| Trypanosoma vivax         | Trypanosoma vivax                   | Rat                               | Rattus ratti                          | Brazil                  | A012441                 |
| Trypanosoma conorhini     | Trypanosoma conorhini               | Hybomitra tarandina               | Hybomitra tarandina                  | Austria                 |                           |
| Trypanosoma brucei clade  | Trypanosoma brucei brucei          | Tsetse flies                      | Glossina pallidipes                  | Kenya                   | A009153                 |
|                           | Trypanosoma brucei gambia           | Human                             | Homo sapiens                          | Nigeria                 | A009141                 |
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| Trypanosoma congolense    | Trypanosoma congolense              | Tsetse flies                      | Glossina affinis                     | Central African Republic |                           |
| Trypanosoma equiperdum    | Trypanosoma equiperdum              | Horse                             | Equus caballus                        | China                   | A009153                 |
| Trypanosoma evansi        | Trypanosoma evansi                  | Water buffalo                     | Bulbasula arnee                      | Thailand                | AY04050                  |
| Trypanosoma godfreyi      | Trypanosoma godfreyi                | Tsetse fly                         | Glossina ruminata                    | The Gambia              | A009151                 |
| Trypanosoma simiae        | Trypanosoma simiae                 | Zeua castoro                      | Glossina pallidipes                  | Kenya                   | A040608                 |
| Trypanosoma vivax         | Trypanosoma vivax                   | Rat                               | Rattus ratti                          | Brazil                  | A012441                 |
| Trypanosoma conorhini     | Trypanosoma conorhini               | Hybomitra tarandina               | Hybomitra tarandina                  | Austria                 |                           |
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|                           | Trypanosoma brucei gambia           | Human                             | Homo sapiens                          | Nigeria                 | A009141                 |
| Trypanosoma brucei clade  | Trypanosoma brucei rhodesiens       | Tsetse flies                      | Glossina affinis                     | Central African Republic | KP307026                |
| Trypanosoma congolense    | Trypanosoma congolense              | Tsetse flies                      | Glossina affinis                     | Central African Republic |                           |

*Free-living species.*