First detection of *Theileria equi* in free-roaming donkeys (*Equus africanus asinus*) in Sri Lanka

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Abstract

Equine piroplasmosis (EP) is a tick-borne disease caused by *Theileria equi* and *Babesia caballi* in equids, including horses, donkeys, zebras, and mules. It is globally endemic with significant economic impact on the equine industry. Infected animals may serve as carriers, and they may be a source of infection for ticks, thereby posing a great challenge for disease management. Sri Lanka is a tropical country, where infections by various tick-borne parasites are common among livestock animals. However, infections by *T. equi* and *B. caballi* remain unstudied in Sri Lanka. Therefore, in the present study, we conducted an epidemiological survey to investigate the presence of *T. equi* and *B. caballi* in apparently healthy free-roaming donkeys. Blood samples were randomly taken from 111 donkeys in Mannar (n = 100) and Kilinochchi (n = 11) districts in Sri Lanka. Thin blood smears were prepared from the blood samples and subjected to microscopic examination. Additionally, blood DNA samples were prepared and screened for *T. equi* and *B. caballi* infections using species-specific PCR assays. Our results showed that 64 (57.7%) and 95 (85.6%) of the donkeys were positive for *T. equi* by microscopy and PCR, respectively. However, all samples were negative for *B. caballi*. Phylogenetic analysis of the *T. equi* 18S rRNA sequences detected two distinct genotypes, namely C and D. To our knowledge, this is the first report of *T. equi* in Sri Lanka and of genotype C in donkeys. The present study highlights the importance of monitoring the shrinking donkey population in Sri Lanka owing to EP caused by *T. equi*.

Keywords: *Babesia caballi*, donkey, epidemiology, genetic diversity, Sri Lanka, *Theileria equi*

1. Introduction

Equine piroplasmosis (EP) is an infectious disease caused by the intra-erythrocytic protozoan parasites *Theileria equi* and *Babesia caballi* in equids, including horses, donkeys, mules, and zebras (Tamzali, 2013; Tirosh-Levy et al., 2020). These parasite species have also been reported in other animals, such as camels (Qablan et al., 2012) and dogs (Fritz, 2010; de Sousa et al., 2018), raising concerns about their host specificity. However, the epidemiological significance of these non-equine hosts is unclear. In addition to morbidity and mortality, restricted international animal trade due to EP often results in severe economic impacts on the equine industry (Wise et al., 2014; Tirosh-Levy et al., 2020).

EP has a global distribution and is endemic in tropical, sub-tropical, and some temperate regions where competent tick vectors are present (De Waal 1990; Scoles and Ueti, 2015). Both *T. equi* and *B. caballi* are transmitted primarily by ixodid tick species belonging to the genera *Amblyomma*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Scoles and Ueti, 2015); iatrogenic and transplacental transmissions have also been reported (Allsopp et al., 2007; Chhabra et al., 2012; Wise et al., 2014). Although a large proportion of the infected animals remains asymptomatic, clinical disease characterized by fever, hemolytic anemia, icterus, hemoglobinuria, anorexia, weakness, weight loss, poor exercise tolerance, and sometimes death, is common among naïve horses in endemic countries (Tamzali, 2013; Wise et al., 2013). The animals recovered from the clinical disease and asymptomatically infected ones may remain as carriers for a long period (De Waal 1990; Rothschild, 2013; Wise et al., 2013). In the absence of reinfection, *B. caballi*-infected animals usually clear the parasites in four years, while *T. equi* persists for life (Rothschild, 2013; Tamzali, 2013; Wise et al., 2014). These carrier animals play a key role in the disease epidemiology because they may be a source of infection for the tick vectors (Wise et al., 2014). Therefore, the detection of carrier animals is vital for assessing the risk of EP and designing effective control measures.

In common with other protozoan parasites, *T. equi* and *B. caballi* also consist of genetically diverse populations (Tirosh-Levy et al., 2020). Based on 18S rRNA sequences, five *T. equi* genotypes (A, B, C, D, and E), and three *B. caballi* genotypes (A, B1, and B2) have been identified so far (Bhoora et al., 2009; Qablan et al., 2013; Liu et al., 2016; Bishop et al., 2020; Tirosh-Levy et al., 2020a). The performance of diagnostic tests is often compromised by these genetic diversities. For instance, competitive enzyme-linked immunosorbent assays (cELISAs) for *T. equi* and *B. caballi* infections were developed based on the equine merozoite antigen 1 (EMA-1) (Bhoora et al., 2010) and rhoptry-associated protein 1 (RAP-1) (Bhoora et al., 2010a), respectively. However, the *T. equi*-cELISA lacks the ability to detect all the *T. equi* genotypes (Bhoora et al., 2010), because EMA-1 is not found in genotype C (Knowles et al., 2018). Similarly, false negative results are common with the *B. caballi*-cELISA, because of the high genetic diversity of *rap-1* (Bhoora et al., 2010a; Rapoport et al., 2014). The genotypic diversity might also be associated with differences in virulence, because previous studies found that clinical cases involving *T. equi* were more commonly associated with genotype A than the other genotypes (Manna et al., 2018; Tirosh-Levy et al., 2021). Moreover, drug treatment outcomes might also depend on the genotypic diversity, because previous studies have shown that repeated treatment with imidocarb dipropionate cleared the Texas strain that belongs to genotype A but not *Theileria haneyi*, which is a newly described species that belongs to genotype C (Ueti et al., 2012; Sears et al., 2020). Therefore, identification of *T. equi* and *B. caballi* genotypes is very important because of their diagnostic, clinical, and therapeutic significances.

Sri Lanka, an island country in the Indian Ocean, has limited populations of horses and donkeys. The donkeys in Sri Lanka were thought to have been brought from northeastern Africa by Dutch and Arabian traders several centuries ago and were used mainly for the transportation of goods (Santiapillai et al., 1999; Liyanage, 2014). During the decades-long civil unrest in Sri Lanka (Ganguly, 2018), the donkeys in Mannar district were abandoned, and they have roamed freely around the district ever since (Liyanage, 2014). In common with other tropical countries, tick infestation and infection with tick-borne pathogens are very common among Sri Lankan

livestock populations (Sivakumar et al., 2012; Zhyldyz et al., 2019). Therefore, the feral donkeys are also at high risk for infection by tick-borne pathogens including *T. equi* and *B. caballi*, but such infections have not yet been investigated. In the present study, we investigated the presence and genetic diversity of *T. equi* and *B. caballi* in the free-roaming donkeys in Mannar and Kilinochchi districts of Sri Lanka.

2. Materials and Methods

2.1. *Blood sampling, smear preparation, and DNA extraction*

Blood samples were randomly taken in 2019 from 111 apparently healthy free-roaming donkeys (*Equus africanus asinu*) from two locations, namely Mannar (n = 100) and Kilinochchi (n = 11) districts in the northern province of Sri Lanka. Approximately 2 ml of blood were collected from the jugular vein of each animal into a sterile EDTA vacutainer tube. Thin blood smears were prepared from a drop of the blood and then air-dried (Houwen, 2002). Additionally, a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from the collected blood samples and then stored at -30 °C until use.

The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan, approved all the animal procedures (approval number 21-4).

2.2. *Microscopic examination for* T. equi *and* B. caballi *infections*

Thin blood smears were fixed with absolute methanol and then stained with 10% Giemsa solution (Barcia, 2007). The stained smears were observed under a light microscope, CX33 (Olympus, Tokyo, Japan) for the presence of *B. caballi* and *T. equi*.

2.3. *PCR screening for* T. equi *and* B. caballi *infections*

Previously described PCR assays based on 18S rRNA sequences were used to screen the donkey DNA samples to detect *T. equi* and *B. caballi* (Bashiruddin et al., 1999; Alhassan et al., 2005). Briefly, 1 µl of DNA sample was added to 9 µl of reaction mixture that contained 1× PCR buffer (Applied Biosystems, Branchburg, USA), 200 µM of each dNTP (Applied Biosystems, Vilnius, Lithuania), 0.5 µM of each forward and reverse primer (Table 1), 0.1 µl of 5U/µl Taq DNA polymerase (Applied Biosystems, Vilnius, Lithuania), and 5.9 µl of distilled water. The reaction mixture was then subjected to pre-denaturation at 95 ºC for 5 min, followed by 40 cycles that included a denaturation step at 95 ºC for 30 sec, an annealing step at 51 ºC (*T. equi*) or 55ºC (*B. caballi*) for 30 sec, and an extension step at 72 ºC for 1 min. After the final

elongation at 72 ºC for 7 min, the PCR products were resolved in a 1.5% agarose gel, stained with ethidium bromide, and then visualized under UV illumination. DNA samples that had been extracted from *in vitro* cultures of *T. equi* and *B. caballi* were used as positive controls, while reaction mixture without any template DNA was used as a negative control. Samples that produced bands of expected sizes (Table 1) were considered to be positive.

2.4. *Cloning, sequencing, and phylogenetic analysis of* T. equi *18S rRNA sequences*

A long fragment of 18S rRNA (≈1,600 bp) was amplified from *T. equi*-positive donkey DNA samples, using previously reported forward (Nbab_1F) and reverse (TB-rev) primers (Matjila et al., 2008; Oosthuizen et al., 2008) (Table 1). The PCR reaction mixture and cycling conditions were essentially as described previously (Sivakumar et al., 2019). The PCR amplicons were gel-extracted using a QIAamp DNA Blood Mini Kit (Qiagen) and then cloned into a PCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA). For each amplicon, two colonies were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan). The resulting sequences were analyzed using BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their origins, as well as to determine the identity scores that they shared with the corresponding sequences registered in GenBank. The identity scores shared among the newly generated sequences were determined using the MatGAT software (Campanella et al., 2003).

The Sri Lankan sequences, together with *T. equi* 18S rRNA sequences representing the five genotypes (A–E) derived from Sudan, Israel, Turkey, South Africa, Brazil, USA, Cuba, China, India, Spain, Chile, Iran, and Switzerland, were aligned using an online version of MAFFT (https://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2002). The resulting alignment was analyzed using MEGA X (Kumar et al., 2018) to predict the best-fitting substitution model. Subsequently, a maximum likelihood phylogeny was constructed based on the General Time Reversible substitution using Invariant plus Gamma-distributed sites (GTR+I+G) model (Felsenstein, 1981) with 1000 bootstrap replicates to estimate reliability.

3. Results and Discussion

In the present study, the presence and genetic diversity of *T. equi* and *B. caballi* were investigated in free-roaming donkeys in Sri Lanka. Microscopic examination revealed the presence of *T. equi* parasites on blood smears from 64 (57.7%) of 111 surveyed animals. Various morphological forms of *T. equi*, including Maltese-cross form, ring form, and irregular pyriforms, were observed (Fig. 1). However, morphological forms suggestive of *B. caballi*, such as large paired pyriforms, were not observed on any of the 111 blood smears examined.

Although microscopy techniques is commonly used for parasite detection, it lacks sensitivity (Zobba et al., 2008; Tirosh-Levy et al., 2020) and is therefore unsuitable for detecting carrier animals with very low parasitemia. Conversely, PCR assays have now superseded microscopy in epidemiological surveys, because of their high specificity and sensitivity (Tirosh-Levy et al., 2020). Therefore, conventional PCR assays based on 18S rRNA sequences were subsequently used for detecting *T. equi* and *B. caballi* DNA in the samples (Bashiruddin et al., 1999; Alhassan et al., 2005). These PCR assays have been widely used in epidemiological surveys to detect carrier animals. Our findings showed that 95 (85.6%) samples were PCR-positive for *T. equi*, whereas none of the samples were positive for *B. caballi*. Compared with *T. equi*, *B. caballi* has a limited geographical distribution and the prevalence of *T. equi* was reported to be higher than *B. caballi* globally. The absence of *B. caballi* in the surveyed donkeys, may suggest that this parasite species is probably not endemic to Sri Lanka. However, large-scale epidemiological surveys are essential to confirm this assumption due to the small sample size used in the present study.

The detection of *T. equi* in the surveyed donkeys was not surprising, because this parasite species have been reported in several equids other than horses, such as both domestic and wild donkeys and zebras. Similar to our findings, high infection rates of *T. equi* have been observed in donkeys (72.0%) in Kenya and Israel (89.0%) and zebras in Israel (62.0%), Kenya (100%), and South Africa (80.3%) (Bhoora et al., 2010b; Hawkins et al., 2015; Tarav et al., 2017; Tirosh-Levy et al., 2020b). The pronounced persistence of *T. equi* in the infected animals,

probably for the remainder of their lives, could be the reason for the high rate of positivity (Rothschild, 2013).

Although the infection rate of *T. equi* in the surveyed donkeys was high, severe clinical disease during the acute phase of infection is uncommon in this animal species (Laus et al., 2015). However, a previous study found that *T. equi*-infected donkeys had significantly lower values for RBC counts, hematocrit, and hemoglobin concentration compared with those of uninfected donkeys (Laus et al., 2015). Therefore, EP might be of health significance in donkeys, particularly as the donkey population in Sri Lanka is shrinking and in danger of extinction probably due to either poor management practices or infectious diseases (Santiapillai et al., 1999). Preservation efforts should also focus on management of various infectious diseases, including EP.

A previous study found several tick species belonging to the genera *Amblyomma, Dermacentor, Hyalomma,* and *Rhipicephalus* infest livestock and other animals including cattle, goats, pigs, dogs, and buffalo in Sri Lanka. However, tick species infesting in equines and prevalent in the study area are unknown. (Liyanaarachchi et al., 2015). Therefore, studies to identify tick species infesting donkeys in the study area are a priority. The *T. equi*-infected donkeys can act as carriers from which the parasite can be tick-transmitted to horses, where the infection may result in severe disease (Kumar et al., 2009). However, this possibility is unlikely in Sri Lanka because only a small number of horses are maintained in geographically distant areas. The horses are mainly found in the central region of the country with no close contact with the donkeys maintained in the northern part.

To analyze the genetic diversity of *T. equi* infecting the donkeys in Sri Lanka, a long fragment of 18S rRNA was amplified from 33 randomly selected PCR-positive DNA samples, and two clones per sample were sequenced. Sequences of both clones from 24 samples were identical to each other, while two sequences were obtained from the remaining nine samples. Therefore, a total of 42 18S rRNA sequences were registered with GenBank (Accession No. LC649067 – LC649098 and LC670594 – LC670603) and used for sequencing and phylogenetic

analysis. The Sri Lankan sequences shared 97.8–100% identity scores among them. These sequences also shared 95.0 – 100% identity scores with other sequences registered in GenBank. Based on the phylogenetic position, two genotypes, namely C and D, were detected (Fig. 2). Genotype D was the most prevalent; 36 sequences from 28 donkeys occurred in the relevant clade, while the remaining six sequences from five donkeys occurred in a clade representing genotype C.

Genotype C has a wide distribution, but had not been detected previously in donkeys (Tirosh-Levy et al., 2020). Therefore, the present study is the first to report genotype C in donkeys. Recently, a strain belonging to genotype C was characterized as a new *Theileria* species known as *T. haneyi*, which lacks EMA-1 (Knowles et al., 2018). Therefore, EMA-1-based diagnostic assays, such as ELISA and cELISA (Knowles et al., 2018) might not be suitable for serological survey of *T. equi* infection in Sri Lanka. Moreover, the presence of genotype C is worrisome, because drug-induced clearance of *T. haneyi* is extremely difficult (Sears et al., 2020). Genotype D was found in Africa, the Mediterranean region, and the Middle East (Tirosh-Levy et al., 2020). This genotype has been commonly detected in several African countries including Sudan (Salim et al., 2009), South Africa (Bhoora et al., 2020), Nigeria (Mshelia et al., 2020), and Gambia (Coultous et al., 2020). These observations, together with the origin of Sri Lankan donkeys, and the prevalence of genotype D may suggest that *T. equi* could have been introduced from Africa into Sri Lanka.

International movement of horses is highly restricted based on the epidemiological status of EP in the countries involved (OIE, 2021). In Sri Lanka, the endemicity of EP was however unknown, leading to several challenges such as inability of the horse management agencies to import into or export from Sri Lanka. The detection of *T. equi* in Sri Lanka is therefore important in terms of global epidemiology of EP and international of horse trade.

In conclusion, the present study reports the presence of *T. equi* and two of its genotypes in donkeys in Sri Lanka for the first time. Further studies to investigate the clinical significance of *T. equi* infection in donkeys are now a priority in this country.

Conflict of Interests

All authors declare no conflict of interests associated with this study.

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Figure legends:

Fig. 1. Morphology of *Theileria equi* detected in free-roaming donkeys in Sri Lanka. **A**. Maltese-cross form, **B**. ring form, and **C**. irregular pyriform.

Fig. 2. Phylogenetic analysis of *T. equi* 18S rRNA sequences. The sequences isolated from the donkeys in the present study, together with those retrieved from GenBank, were used to construct a maximum likelihood phylogeny. The sequences determined in the present study are indicated in bold font. The Sri Lankan sequences occurred in clades C and D.

Table 1. List of PCR primers used in the present study.

Target Gene	Primers	Sequence (5`- 3`)	Product (bp)	Reference
T. equi 18S rRNA	Bec-UF2	TCGAAGACGATCAGATACCGTCG	435	Alhassan et al., 2005
	Equi-R	TGCCTTAAACTTCCTTGCGAT		
B. caballi 18S rRNA	BCAF	TTCGCTTCGCTTTTTGTTTTTACT	659	Bashiruddin et al., 1999
	BCAR	GTCCCTCTAAGAAGCAAACCCAA		
T. equi 18S rRNA (Sequencing)	Nbab_1F	AAGCCATGCATGTCTAAGTATAAGCTTTT	≈ 1,600	Oosthuizen et al., 2008
	TB Rev	GAATAATTCACCGGATCACTCG		Matjila et al., 2008

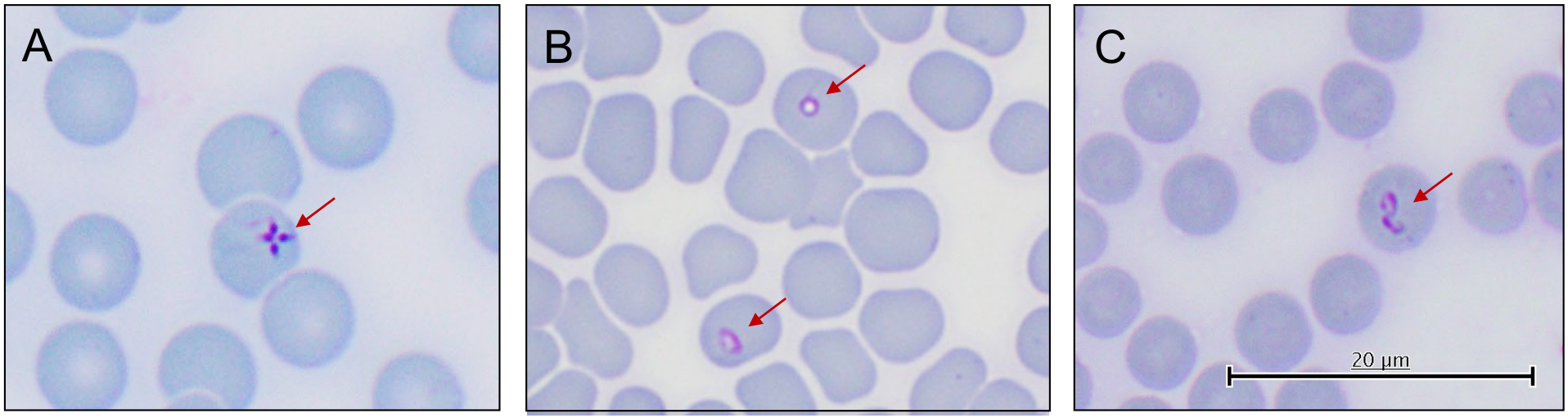
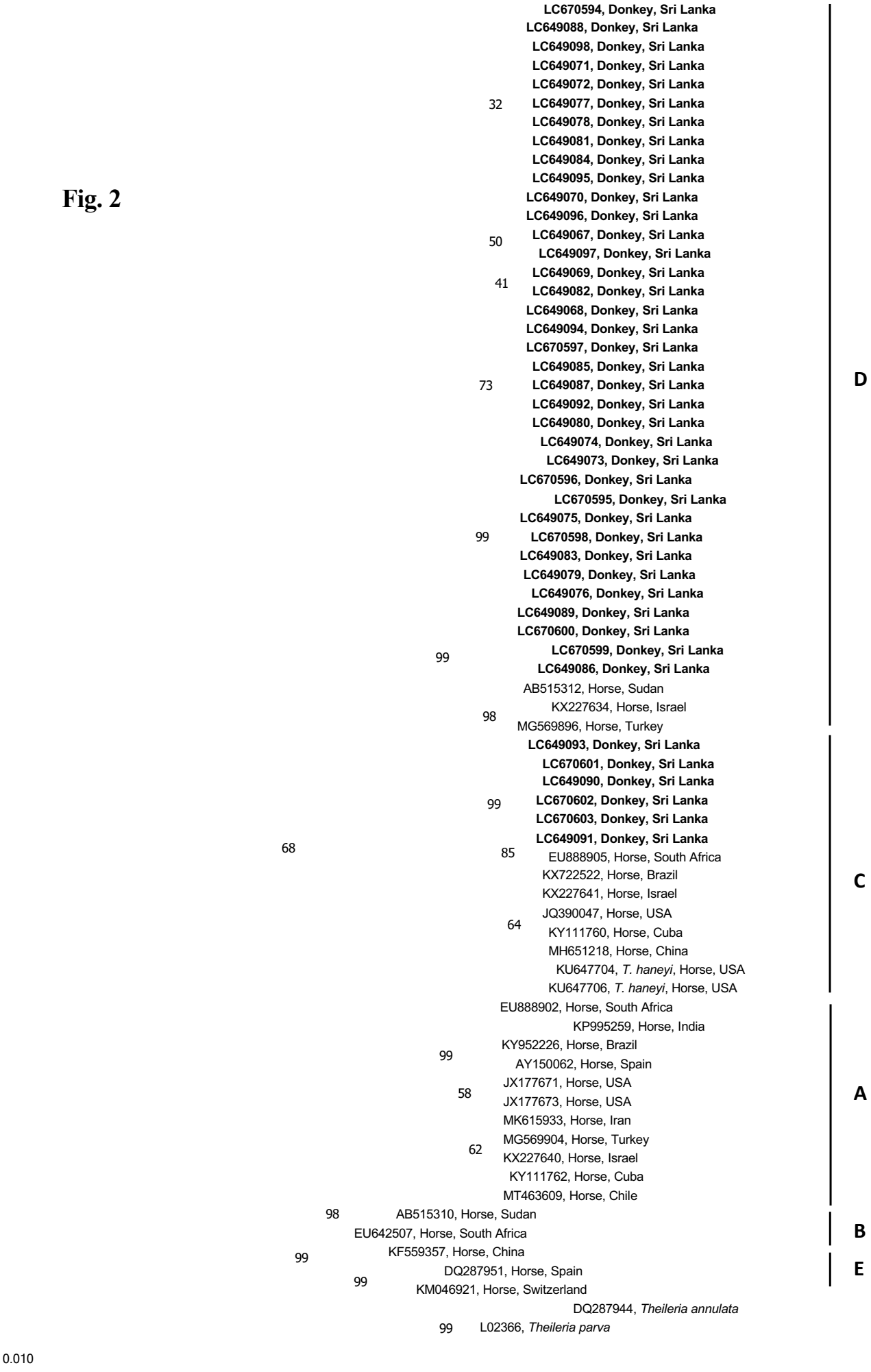


Fig. 1

Fig. 2


LC670594, Donkey, Sri Lanka
LC649088, Donkey, Sri Lanka
LC649098, Donkey, Sri Lanka
LC649071, Donkey, Sri Lanka
LC649072, Donkey, Sri Lanka
32
LC649077, Donkey, Sri Lanka
LC649078, Donkey, Sri Lanka
LC649081, Donkey, Sri Lanka
LC649084, Donkey, Sri Lanka
LC649095, Donkey, Sri Lanka
LC649070, Donkey, Sri Lanka
LC649096, Donkey, Sri Lanka
50
LC649067, Donkey, Sri Lanka
LC649097, Donkey, Sri Lanka
41
LC649069, Donkey, Sri Lanka
LC649082, Donkey, Sri Lanka
LC649068, Donkey, Sri Lanka
LC649094, Donkey, Sri Lanka
LC670597, Donkey, Sri Lanka
LC649085, Donkey, Sri Lanka
73
LC649087, Donkey, Sri Lanka
LC649092, Donkey, Sri Lanka
LC649080, Donkey, Sri Lanka
LC649074, Donkey, Sri Lanka
LC649073, Donkey, Sri Lanka
LC670596, Donkey, Sri Lanka
LC670595, Donkey, Sri Lanka
LC649075, Donkey, Sri Lanka
99
LC670598, Donkey, Sri Lanka
LC649083, Donkey, Sri Lanka
LC649079, Donkey, Sri Lanka
LC649076, Donkey, Sri Lanka
LC649089, Donkey, Sri Lanka
LC670600, Donkey, Sri Lanka
99
LC670599, Donkey, Sri Lanka
LC649086, Donkey, Sri Lanka
AB515312, Horse, Sudan
KX227634, Horse, Israel
98
MG569896, Horse, Turkey
D
LC649093, Donkey, Sri Lanka
LC670601, Donkey, Sri Lanka
LC649090, Donkey, Sri Lanka
99
LC670602, Donkey, Sri Lanka
LC670603, Donkey, Sri Lanka
LC649091, Donkey, Sri Lanka
68
85
EU888905, Horse, South Africa
KX722522, Horse, Brazil
KX227641, Horse, Israel
64
JQ390047, Horse, USA
KY111760, Horse, Cuba
MH651218, Horse, China
KU647704, T. haneyi, Horse, USA
KU647706, T. haneyi, Horse, USA
C
EU888902, Horse, South Africa
KP995259, Horse, India
99
KY952226, Horse, Brazil
AY150062, Horse, Spain
58
JX177671, Horse, USA
JX177673, Horse, USA
MK615933, Horse, Iran
62
MG569904, Horse, Turkey
KX227640, Horse, Israel
KY111762, Horse, Cuba
MT463609, Horse, Chile
A
98
AB515310, Horse, Sudan
EU642507, Horse, South Africa
B
99
KF559357, Horse, China
DQ287951, Horse, Spain
99
KM046921, Horse, Switzerland
E
DQ287944, Theileria annulata
99
L02366, Theileria parva
0.010