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Short Communication

Genetic diversity in *Trypanosoma theileri* from Sri Lankan cattle and water buffaloes

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ABSTRACT

Trypanosoma theileri is a hemoprotozoan parasite that infects various ruminant species. We investigated the epidemiology of this parasite among cattle and water buffalo populations bred in Sri Lanka, using a diagnostic PCR assay based on the cathepsin L-like protein (CATL) gene. Blood DNA samples sourced from cattle ($n = 316$) and water buffaloes ($n = 320$) bred in different geographical areas of Sri Lanka were PCR screened for *T. theileri*. Parasite DNA was detected in cattle and water buffaloes alike in all the sampling locations. The overall *T. theileri*-positive rate was higher in water buffaloes (15.9%) than in cattle (7.6%). Subsequently, PCR amplicons were sequenced and the partial *CATL* sequences were phylogenetically analyzed. The identity values for the CATL gene were 89.6–99.7% among the cattle-derived sequences, compared with values of 90.7–100% for the buffalo-derived sequences. However, the cattle-derived sequences shared 88.2–100% identity values with those from buffaloes. In the phylogenetic tree, the Sri Lankan CATL gene sequences fell into two major clades (TthI and TthII), both of which contain *CATL* sequences from several other countries. Although most of the *CATL* sequences from Sri Lankan cattle and buffaloes clustered independently, two buffalo-derived sequences were observed to be closely related to those of the Sri Lankan cattle. Furthermore, a Sri Lankan buffalo sequence clustered with CATL gene sequences from Brazilian buffalo and Thai cattle. In addition to reporting the first PCR-based survey of *T. theileri* among Sri Lankan-bred cattle and water buffaloes, the present study found that some of the CATL gene fragments sourced from water buffaloes shared similarity with those determined from cattle in this country.

1. Introduction

Various species of *Trypanosoma* parasites are known to infect several wild and domestic animals worldwide. Species of mammalian trypanosomes were divided by Hoare (1964) into two major sections, Stercoraria and Salivaria. Trypanosomes of the Salivaria section, which include *Trypanosoma vivax*, *T. congelense*, *T. brucei*, and *T. evansi*, are transmitted to their vertebrate host during the blood meal of a parasite-infected insect vector, in which parasite development is terminated in the salivary glands (Haag et al., 1998; Van Den Abbeele et al., 2010; Vickerman et al., 1998). In contrast, for the trypanosomes of the Stercoraria section, which include *T. cruzi*, *T. melophagium*, and *T. theileri*, the final stages of parasite development take place in the lower

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digestive tract of the vectors and, therefore, these parasite species are transmitted by contamination with parasite-infected insect vector excreta (Schuster and Schaub, 2000). Among the *Trypanosoma* parasites, *T. vivax*, *T. congelense*, and *T. brucei* are considered to be the most pathogenic to susceptible animals (Steverding, 2008). However, *T. evansi* also causes a severe wasting disease in infected cattle (Brun et al., 1998; Desquesnes et al., 2013). On the other hand, *T. theileri* has also been implicated in sporadic clinical cases in domestic animals in various countries (Doherty et al., 1993; Sood et al., 2011; Villa et al., 2008). Therefore, the epidemiology of *T. theileri* should be studied. *Trypanosoma theileri* is a large *Trypanosoma* parasite vectored by tabanid flies (Böse et al., 1987) and ticks (Latif et al., 2004; Shastri and Deshpande, 1981).

Several genetic marker genes, such as the small subunit rRNA (ssrRNA), internal transcribed spacer 1 (ITS1), cytochrome b (Cyt b), spliced leader (SL), glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH), and cathepsin L-like protein (CATL) genes, have been used to analyze genetic variation in *T. theileri* parasites from several host animals, including cattle, buffaloes, and deer (Fisher et al., 2013; Garcia et al., 2011a,b; Rodrigues et al., 2006, 2010). In these phylogenetic analyses, the *T. theileri* gene sequences always clustered independently according to the host species (Fisher et al., 2013; Garcia et al., 2011a,b; Rodrigues et al., 2006, 2010). These findings led the researchers in these studies to conclude that *T. theileri* genotypes might be host-specific. However, genetic diversity in *T. theileri* has only been investigated in a small number of countries, including Brazil, Venezuela, Thailand, Vietnam, Philippines, and the United States (Fisher et al., 2013; Garcia et al., 2011a,b; Rodrigues et al., 2006, 2010; Sivakumar et al., 2013; Ybañez et al., 2013). Therefore, further analyses of *T. theileri* populations in other endemic countries are required to gain a more comprehensive understanding of the genetic diversity and host specificity of this parasite.

The presence of *T. theileri* in Sri Lankan water buffaloes, based on morphological and indirect immunofluorescence antibody tests, has previously been reported (Weilgama et al., 1989). However, molecular detection methods have not been used to detect *T. theileri* in Sri Lanka. Therefore, the aim of the present study was to determine the prevalence of *T. theileri* in cattle and water buffaloes bred in different geographical locations of Sri Lanka, using a diagnostic PCR assay based on the CATL gene, and to analyze genetic diversity in the parasites. Our findings indicated that *T. theileri* is a common parasite among the cattle and water buffaloes bred in Sri Lanka, and that the parasite populations are genetically diverse. Additionally, our results indicated that some of the water buffalo-derived *T. theileri CATL* nucleotide sequences determined in the present study shared high identity scores with those from cattle.

2. Materials and methods

2.1. Blood sampling and DNA extraction

The blood samples collected from cattle and water buffaloes in Sri Lanka have been described previously (Sivakumar et al., 2012b, 2014). Briefly, 316 cattle reared in four different districts (Nuwara Eliya, Polonnaruwa, Ampara, and Jaffna), and 320 buffaloes bred in three different districts (Polonnaruwa, Mannar, and Mullaitivu) were sampled in April and June 2011 and in February 2013, respectively. All the animals were over 1 year-old and apparently healthy during the sampling. DNA samples were extracted from 200 μl of whole blood using a commercial kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The DNA samples were stored at –20 °C until further use. These DNA samples were previously used for investigations on bovine *Babesia* and *Theileria* parasites (Sivakumar et al., 2012b, 2014).

2.2. Detection of T. theileri using a diagnostic PCR assay

A previously established *T. theileri*-specific PCR assay, based on the CATL gene, was employed to screen the DNA samples (Rodrigues et al., 2010). The PCR reaction mixtures and cycling conditions described by Rodrigues et al. (2010) were used with modifications. Briefly, PCR reactions contained 1 μl of 10× PCR reaction buffer, 200 μM dNTPs (Applied Biosystems, Branchburg, NJ), 0.5 μM of forward (5′-CGTCTCTGGCTCCGGTCAAAC-3′) and reverse (5′-TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA-3′) primers, 0.5 units of Taq polymerase (Applied Biosystems), and 5.9 μl of double distilled water. After the addition of 1 μl of each DNA sample, the mixture was subjected to an enzyme activation step at 95 °C for 5 min, followed by 45 cycles each including a denaturing step at 95 °C for 30 s, an annealing step at 55 °C for 1 min, and an extension step at 72 °C for 1 min. After final elongation at 72 °C for 10 min, the PCR products were analyzed by agarose gel electrophoresis. PCR amplicons close in size to the positive control were considered *T. theileri*-positive.

2.3. Cloning, sequencing, and phylogenetic analysis

PCR amplicons with high band-intensities, detected from both cattle and water buffaloes, were cloned into plasmid vectors and sequenced as described previously (Sivakumar et al., 2012a). For each cloned product, the both strands of a single clone were sequenced. Resultant sequences were then analyzed by basic local alignment tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identity values among the CATL gene sequences were calculated using the Emboss needle online program (http://emboss.bioinformatics.nl/cgi-bin/emboss/needle). Single nucleotide polymorphisms among the Sri Lankan *CATL* sequences were analyzed using DnaSP v5 software (Librado and Rozas, 2009). The gene sequences generated in the present study, together with those previously reported from different host species in several other countries, were used to construct a phylogenetic tree. After aligning the gene sequences using MAFFT (Katoh et al., 2002), a neighbor-joining tree (Saitou and Nei, 1987) was constructed using the Tamura 3-parameter model (Tamura, 1992) hosted by MEGA version 6.0 (Tamura et al., 2013).

Table 1
PCR detection of *T. theileri* in Sri Lankan cattle and water buffaloes.

Districts	Cattle			Water buffaloes		
	No. sample	No. positive	% (CI[b])	No. sample	No. positive	% (CI)
Nuwara Eliya	83	6	7.2 (3.4–14.9)	ND	–	–
Polonnaruwa	84	8	9.5 (4.9–17.7)	118	8	6.8 (3.5–12.8)
Ampara	88	7	8.0 (3.9–15.5)	ND	–	–
Jaffna	61	3	4.9 (1.7–13.5)	ND	–	–
Mannar	ND[a]	–	–	146	35	24.0 (17.8–31.5)
Mullaitivu	ND	–	–	56	8	14.3 (7.4–25.7)
Total	316	24	7.6 (5.2–11.1)	320	51	15.9 (12.3–20.4)

[a] ND, sampling was not done.
[b] 95% confidence interval.

2.4. Statistical analysis

The 95% confidence intervals for the positive rates were computed using the open epi online program (http://www.openepi.com/v37/Proportion/Proportion.htm), based on the method described by Wilson (1927).

3. Results and discussion

The diagnostic PCR assay revealed the presence of *T. theileri* in the cattle and water buffaloes bred in all the surveyed districts of Sri Lanka (Table 1). The overall *T. theileri*-positive rate was relatively higher in water buffaloes (15.9%) than in cattle (7.6%). However, the prevalences of *T. theileri* among water buffaloes bred in Polonnaruwa and Mullaitivu did not significantly differ from those determined for the cattle populations in the surveyed districts. On the other hand, *T. theileri*-positive rate among the water buffaloes in Mannar was significantly higher than those reported for the cattle and has contributed to the higher overall prevalence in water buffaloes in comparison to cattle. Therefore, it seems that *T. theileri*-positive rates in Sri Lanka might be influenced by the geographical location. The Mannar district is characterized by hot and humid climate, and further studies to investigate the possible effect of different climatic conditions on the distribution of tabanid flies might shed an additional light on the discrepancies between the prevalences of *T. theileri* in different geographical locations within Sri Lanka.

Although *T. theileri* is known to be relatively less virulent, the parasite sometimes becomes troublesome when present in co-infections with other pathogens (Mansfield, 1977; Wells, 1976). Therefore, we analyzed for possible co-infections based on the *Babesia* (*B. bovis* and *B. bigemina*) and *Theileria* (*T. annulata* and *T. orientalis*) infection data obtained from the same DNA samples in previous investigations (Sivakumar et al., 2012b, 2014). In the present study, 83.3% of the *T. theileri*-positive cattle and 86.3% of the *T. theileri*-positive water buffaloes were co-infected with *Babesia* and/or *Theileria* parasites (Table 2). Among the *T. theileri*-infected cattle, 10, 5, 4, and 1 were co-infected with one, two, three, and four *Babesia* and/or *Theileria* species, respectively. In contrast, out of 44 *T. theileri*-positive water buffaloes that had co-infections, 43 had dual infections with *T. orientalis*, while the remaining animal was co-infected with *B. bigemina* and *T. orientalis*. The discrepancies relating to the different co-infection profiles of the cattle and water buffaloes are caused by differences in the positive rates of *Babesia* and *Theileria* species between the cattle and water buffaloes in Sri Lanka (Sivakumar et al., 2012b, 2014). Nevertheless, the high *T. theileri* co-infection rates are of concern in Sri Lanka. Therefore, further investigation on the clinical status of co-infected animals is now a priority in this country.

Table 2
Co-infections of *T. theileri* with *Babesia* and *Theileria* parasites in Sri Lankan cattle and water buffaloes.

Combination[a]	No. positive	
	Cattle	Water buffaloes
5 parasites		
T. theileri + *B. bovis* + *B. bigemina* + *T. annulata* + *T. orientalis*	1	ND[b]
4 parasites		
T. theileri + *B. bovis* + *B. bigemina* + *T. orientalis*	2	ND
T. theileri + *B. bovis* + *T. annulata* + *T. orientalis*	2	ND
3 parasites		
T. theileri + *B. bovis* + *T. orientalis*	2	ND
T. theileri + *B. bovis* + *B. bigemina*	1	ND
T. theileri + *B. bigemina* + *T. orientalis*	1	1
T. theileri + *T. annulata* + *T. orientalis*	1	ND
2 parasites		
T. theileri + *T. orientalis*	6	43
T. theileri + *B. bigemina*	4	ND
Total (%[c])	20 (83.3)	44 (86.3)

[a] Co-infections were determined based on the *Babesia* (*B. bovis* and *B. bigemina*) and *Theileria* (*T. annulata* and *T. orientalis*) infection data obtained from the same DNA samples in previous investigations (Sivakumar et al., 2012b, 2014).
[b] ND, not detected.
[c] Expressed as a percentage of *T. theileri*-infected animals.

A total of 15 and 9 PCR amplicons from cattle and water buffaloes, respectively, were cloned and sequenced. All the CATL gene fragments amplified in the present study were 289-bp in length, but high genetic variation was found among the *CATL* sequences. Single nucleotide polymorphisms were frequently detected, as shown in Fig. 1. The identity values among the cattle-derived *CATL* sequences (AB930145–AB930159) were 89.6–99.7%, while the buffalo-derived sequences (AB930160–AB930168) exhibited 90.7–100% identity values. In contrast, the buffalo-derived *CATL* sequences had 88.2–100% sequence identity with those of cattle. Two cattle-derived *CATL* sequences (AB930146 and AB930147) shared 100 and

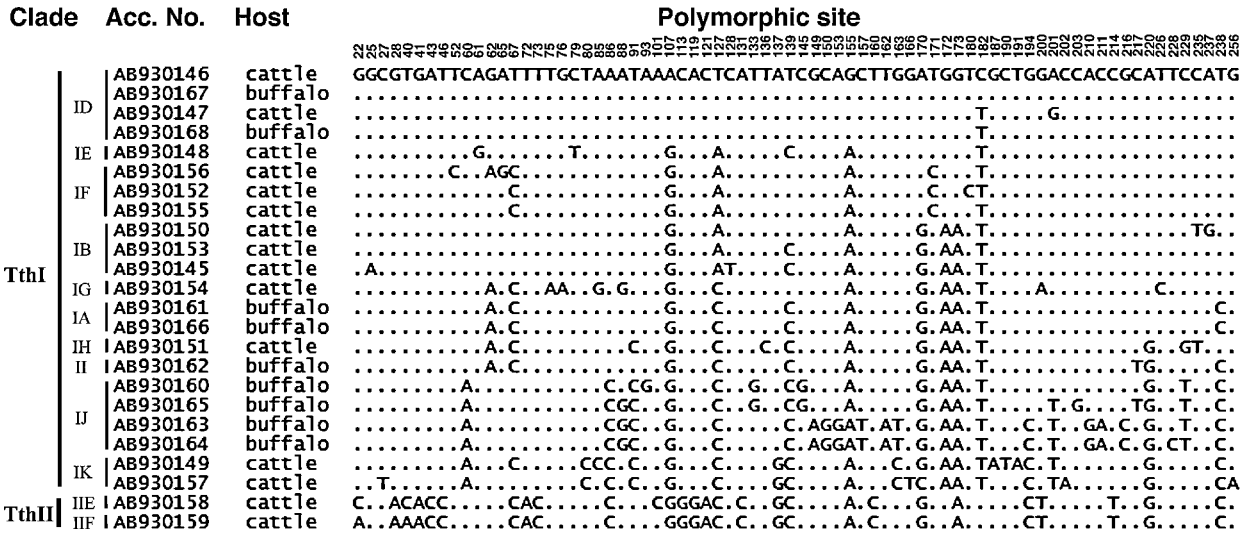

Fig. 1. Polymorphic sites within *T. theileri* CATL gene sequences isolated from cattle and water buffaloes in Sri Lanka. The CATL sequences generated in the present study were analyzed by DnaSP v5 software (Librado and Rozas, 2009) using a cattle-derived sequence (AB930146) as a reference for determining the polymorphic sites within the 289 nucleotides amplified by the diagnostic PCR assay. Clades refer to the clade names in Fig. 2. Dots denote nucleotides that are identical to the reference sequence. Note that there is high genetic diversity among the Sri Lankan *T. theileri CATL* sequences and that two buffalo-derived sequences (AB930167 and AB930168) share high sequence identity scores with two cattle-derived sequences (AB930146 and AB930147) in clade ID.

99.3% identity values with a buffalo-derived sequence (AB930167), and these cattle-derived sequences also shared 99.7% identity with another sequence (AB960168) derived from a Sri Lankan water buffalo. Similarly, a sequence from cattle (AB930151) shared a high identity score (97.9%) with buffalo-derived sequences (AB930161, AB930162, and AB930166). Genetic diversity levels among the *T. theileri* CATL gene sequences detected in Sri Lanka are in agreement with those of previous studies, which also found that *CATL* sequences are highly diverse among *T. theileri* isolates from different countries (Garcia et al., 2011a,b; Rodrigues et al., 2006, 2010). Several past studies have suggested that the *T. theileri* genotypes that infect cattle and water buffaloes might differ from each other (Fisher et al., 2013; Garcia et al., 2011a,b; Rodrigues et al., 2006, 2010). Based on ssrRNA and ITS nucleotide sequences, Rodrigues et al. (2006) also showed that the *T. theileri* genotypes from Brazilian cattle and water buffaloes differed from each other. In a recent study, which used several marker genes, the *T. theileri* genotypes obtained were described as being host-specific in Brazil and Venezuela (Garcia et al., 2011b). To the best of our knowledge, therefore, our study is the first to report high levels of sequence identity among directly PCR-amplified CATL DNA sequences of *T. theileri* from cattle and water buffaloes.

In the phylogenetic tree, the *CATL* sequences determined in the present study were found in multiple clades (Fig. 2). Although most of the cattle- and buffalo-derived sequences clustered independently, a few *CATL* sequences from water buffaloes (AB930167, AB930168, and AB930162) clustered together with the cattle sequences. These findings are consistent with the sequencing data analysis described above. We also found that a Brazilian sequence (HQ664735) from water buffalo shared 100 and 99.6% identity values with a buffalo-derived sequence (AB930162) from Sri Lanka and with two cattle-derived sequences (HQ543073 and HQ543074) from Thailand, respectively. Although these results question the host specificity of *T. theileri CATL* variants in cattle and buffaloes from Sri Lanka, the findings might be inconclusive as only the partial CATL gene fragments were analyzed. On the other hand, researchers have used several different marker genes to investigate genetic diversity in *T. theileri* in the recent past (Fisher et al., 2013; Garcia et al., 2011a,b; Rodrigues et al., 2006, 2010). In an extensive study using large DNA data set generated from cultured *T. theileri* isolates, Garcia et al. (2011b) demonstrated the high concordance between the genotypes of several genotypic makers, such as the ssrRNA, ITS1, Cyt b, SL, gGAPDH, and CATL genes, and found that the genotypes were host-specific. However, our present study examined only the DNA samples extracted from the host blood and analyzed the genetic variations based on partial *CATL* sequences. Therefore, the present findings may not allow us to reach a conclusion regarding the host-specificity of *T. theileri* genotypes in Sri Lanka.

A previous study demonstrated that the tabanid flies can harbor diverse genetic variants of *T. theileri* at a given time (Rodrigues et al., 2006). Therefore, the *T. theileri* genotypes in cattle could have been transmitted to the water buffaloes in Sri Lanka, as both the host animals are used to be in close contact with each other, especially during grazing, in the sampled locations. However, although different *T. theileri* genotypes are transmitted to the host animals, all the genotypes will not be selected for the establishment of infections, as the host specificity of the vector borne parasites is a complex issue that can be influenced by several factors, such as biology, ecology, physiology and behavioral features of vectors, vector-host interactions, and host immunity (Brooks and McLennan, 2002; Hellgren

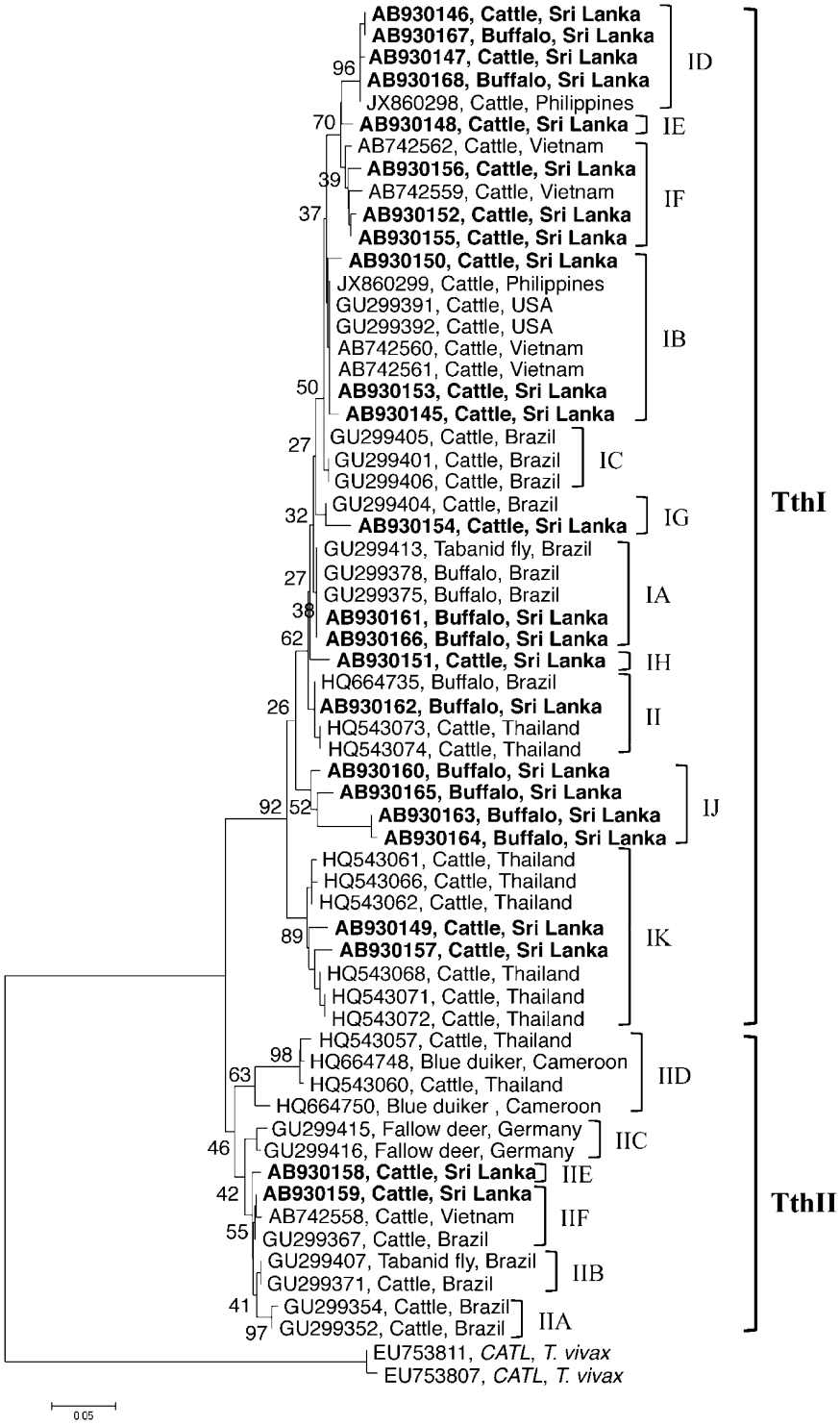

Fig. 2. Phylogenetic analysis of *T. theileri* CATL gene sequences. The *CATL* sequences determined in the present Sri Lankan study, together with those reported from several other countries, were used to construct a neighbor-joining phylogenetic tree (Saitou and Nei, 1987), based on the Tamura 3-parameter model (Tamura, 1992) using MEGA version 6.0 software (Tamura et al., 2013). The minor clades within the two major clades TthI and TthII are designated as IA to IK, and IIA to IIF, respectively, in the present study. Note that the two buffalo-derived sequences (AB930167 and AB930168) cluster together with two cattle-derived sequences (AB930146 and AB930147) from Sri Lanka in clade ID. Additionally, a single Sri Lankan buffalo-derived sequence (AB930162) was found in clade II with a Brazilian buffalo-derived sequence (HQ664735) and two Thai cattle-derived sequences (HQ543073 and HQ543074).

et al., 2008). Therefore, the present findings, which showed that some of the water buffalo-derived *CATL* sequences shared similarity to cattle-derived sequences, might be due to yet unestablished infections with different genotypes in these host animals. So that, the detection of *CATL* sequences that share similarity between cattle and water buffaloes in the present study does not necessarily mean that these *CATL* variants lack host-specificity. Therefore, future studies in Sri Lanka should analyze a large number of sequences belonging to several genotypic marker genes using cultured *T. theileri* isolates from different host animals. Nevertheless, the present findings are useful for furthering our understanding of the relationship between *T. theileri* genotypes and host-specificity. In conclusion, in addition to reporting the first PCR-based survey of *T. theileri*, the present study found evidence that some *T. theileri* CATL DNA sequences obtained from naturally infected cattle and water buffaloes in Sri Lanka may be shared among these hosts.

Conflict of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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