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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Phylogenetic analysis of *Theileria equi* and *Babesia caballi* sequences from thoroughbred mares and foals in Trinidad

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Abstract

The agents of equine piroplasmosis, *Theileria equi* and *Babesia caballi*, are endemic in Trinidad, West Indies. While transmission is mainly by ixodid ticks, transplacental transmission of *T. equi* has also been reported. This disease has contributed to foetal losses as well as morbidity and mortality of neonatal foals and adult horses. Previous 18S rRNA–based phylogenetic studies indicated a noticeable degree of variation within and among *B. caballi* and *T. equi* isolates from different geographical regions. The objective of this study was to evaluate the diversity of *T. equi* and *B. caballi* obtained from horses in Trinidad by amplifying a region of the 18S rRNA gene. The phylogenetic trees for *T. equi* sequences obtained from horses in 2006 and 2011–2013 revealed that Trinidad sequences were of genotype A. Additionally, all of the *B. caballi* sequences from Trinidad were grouped together with other *B. caballi* sequences of genotype A. However, *T. equi* sequences from horses in Saint Kitts and Nevis clustered with sequences of genotype C. This study also identified two genotypes of *T. equi* in the equine population of Brazil. All of the *T. equi* and *B. caballi* sequences obtained from horses in Trinidad belong to genotype A and were similar to *T. equi* and *B. caballi* sequences of the same genotype that were submitted to GenBank™ databases. Countries in close proximity to Trinidad have *T. equi* sequences belonging to genotype C; therefore, movement of horses between these countries can introduce a new genotype of *T. equi* into the equid population of Trinidad.

Keywords *Babesia caballi* · Equine piroplasmosis · Horses · Phylogenetic analysis · *Theileria equi* · Trinidad

Introduction

Equine piroplasmosis is endemic in Trinidad, West Indies, with a seroprevalence of 76.1% for *Babesia caballi* and 30.6% for *Theileria equi* (Sant et al. 2016). This disease is mainly transmitted via the ixodid tick vectors of the genera *Rhipicephalus*, *Hyalomma*, *Amblyomma*, and *Dermacentor* (De Waal 1992; Ketter-Ratzon et al. 2017). The genera of ixodid ticks that have been reported in Trinidad and Tobago are *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus* (Basu and Charles 2017). Loftis et al. (2014) confirmed the presence of *B. caballi* in 31.3% of horses and 67.4% of donkeys in Saint Kitts and Nevis and *T. equi* in 23.2% of horses from St. Kitts. Ticks collected from the horses and donkeys in that study were identified as *Dermacentor nitens* (Loftis et al. 2014). Other than transmission by ticks, the use of contaminated needles, syringes, surgical instruments and blood transfusions including blood doping practices have contributed to mechanical transmission of this disease. In utero infections of foals which occur transplacentally can result in abortions, stillbirths, neonatal piroplasmosis, and the development of healthy carriers (Allsopp et al. 2007). This disease has contributed to foetal losses, has increased the morbidity and mortality of both neonatal foals and adult horses, and has thus limited the international movement of horses. Infected horses usually become asymptomatic carriers and for this reason, their movement across borders may be restricted.

Serological assays are important to epidemiological surveys; however, cross-reactivity of genetically closely related

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species has been reported (Mans et al. 2015; Gondim et al. 2017). Molecular-based assays have improved the sensitivity and specificity of detection (Mans et al. 2015). Additionally, phylogenomics has vastly improved the understanding of the relatedness between different organisms (Chan and Ragan 2013).

With the vast improvement in technology over the last decade, comparative genomics and phylogenomics have become important tools in veterinary medicine. This technological evolution has provided tools to identify new ways to study parasites through the linkage of molecular biology with ecology and epidemiology (Forrester and Hall 2014). The *equi merozoite antigen*, β-tubulin, and 18S rRNA genes have been used to diagnose *T. equi* and *B. caballi* infections using conventional PCR (cPCR) (Cacciò et al. 2000; Battsetseg et al. 2002; Alhassan et al. 2005). However, the 18S rRNA gene was considered to be superior compared to the other genes due to its low substitution rate, constrained and conserved function, and occurrence in multiple copies (Qablan et al. 2013). Phylogenetic analyses of *T. equi* based on the 18S rRNA gene have revealed the presence of five different genotypes, A, B, C, D, and E, based on the presence of distinct clades (Qablan et al. 2013; Liu et al. 2016; Ketter-Ratzon et al. 2017). There is less sequence variation in the 18S rRNA gene of *B. caballi* compared to *T. equi*, and as a result, three genotypes (A, B, and C) have been reported for *B. caballi* (Bhoora et al. 2009; Qablan et al. 2013).

Trinidad, the southernmost island of the Caribbean which is just north of the South American continent, is located at 10.69° N, 61.22° W. There has been no phylogenetic study of *T. equi* or *B. caballi* in thoroughbred horses in Trinidad, West Indies; thus, the aim of this study was to identify the genotypes of *T. equi* and *B. caballi* present in thoroughbred mares and their foals in Trinidad based on the 18S rRNA gene.

Materials and methods

Blood samples

The estimated sample size was 100 foals based on an a priori estimate of 100 foals being born annually in Trinidad. EDTA blood samples were collected from 111 pregnant mares in the fifth month of pregnancy during 2011–2013. The mares in this study were distributed over seven different breeding farms located throughout Trinidad. Five breeding farms were located in North Trinidad, one in South Trinidad, and one in Central Trinidad. In this study population, 89 of the 111 mares gave birth to live foals. There was an abortion rate of 19.8% in this study as 22 mares lost their fetuses between the second and eleventh month of pregnancy (median = fourth month of pregnancy). Samples from aborted fetuses were not collected. EDTA blood samples were also collected from each of their live foals (89 in total) within the first 36 h of birth to detect transplacental transmission of these piroplasms (Sant et al. 2016).

Genomic DNA extraction and conventional PCR for *T. equi* and *B. caballi*

Genomic DNA (gDNA) was extracted from 100 μL of whole blood from mares and foals using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, MD, USA) according to the manufacturer's instructions.

The 18S rRNA gene was amplified to detect *T. equi* using the forward and reverse primers BEC-UF2 (5′ TCGAAGAC GATCAGATACCGTCG) and Equi-R (5′ TGCCTAAA CTTCCTTCCTTGCGAT) (Sigma-Aldrich Inc. St Louis, MO, USA) respectively which yielded a product of approximately 400 base pairs (bp) (Steinman et al. 2012). The reaction conditions were performed as described by Steinmann et al. (2012) with the following modification: an annealing temperature of 54.2 °C was used instead of 60.5 °C based on the results obtained from a gradient PCR.

B. caballi gDNA was amplified using the primers BEC-UF2 (5′TCGAAGACGATCAGATACCGTCG) and Cab-R (5′CTCGTTCATGATTTAGAATTGCT) (Sigma-Aldrich Inc. St Louis, MO, USA) which also targeted the 18S rRNA gene. PCR was conducted as described by Alhassan et al. (2005); however, the annealing temperature was decreased to 51.3 °C based on results obtained from a gradient PCR to yield a product of approximately 540 bp. Positive control DNA was obtained from horses confirmed to be positive for *T. equi* or *B. caballi* by nucleotide sequencing. PCR grade water was used as a negative control. PCR products were visualized using gel electrophoresis under UV light.

To evaluate if there were any changes in the genotypes of *T. equi* present in the equine population, four sequences obtained from amplifying a portion of the V4 hypervariable region of the 18S rRNA gene from equine thoroughbred horses collected in 2006 were included in this study.

Sequencing

A total of 21 positive *T. equi* samples and four positive *B. caballi* samples by cPCR were sequenced using the ABI3730XL DNA sequencer (Macrogen Inc., South Korea). Table 1 contains the *T. equi* and *B. caballi* sequences and their accession numbers obtained from this study that were deposited on GenBank EMBL and DDBJ databases.

Construction of data sets for *Theileria equi*

Due to differences in the positions of the amplified PCR products along the 18S ribosome, two separate data sets were constructed for *T. equi*. For each data set, at least two *T. equi*

Table 1 List of accession numbers for the *T. equi* and *B. caballi* sequences obtained from horses in Trinidad that are available in the GenBank™ EMBL, and DDBJ databases

Accession number	Name of piroplasm	Year obtained	Genotype
KT285377	*Theileria equi*	2011	Unknown
KU289089	*Theileria equi*	2011	Unknown
KU289090	*Theileria equi*	2011	Unknown
KU289091	*Theileria equi*	2011	Unknown
KU289092	*Theileria equi*	2011	Unknown
KU289093	*Theileria equi*	2011	Unknown
KU289094	*Theileria equi*	2011	Unknown
KU289095	*Theileria equi*	2011	Unknown
KX896429	*Theileria equi*	2013	Unknown
KX896430	*Theileria equi*	2013	Unknown
KX896431	*Theileria equi*	2012	Unknown
KX896432	*Theileria equi*	2011	Unknown
KX896433	*Theileria equi*	2011	Unknown
KX896434	*Theileria equi*	2011	Unknown
KX896435	*Theileria equi*	2011	Unknown
KX896436	*Theileria equi*	2011	Unknown
KY053282	*Theileria equi*	2006	Unknown
KY053283	*Theileria equi*	2006	Unknown
KY053284	*Theileria equi*	2006	Unknown
KY053285	*Theileria equi*	2006	Unknown
KU289099	*Babesia caballi*	2011	Unknown
KU289100	*Babesia caballi*	2011	Unknown
KU289101	*Babesia caballi*	2011	Unknown
KU289102	*Babesia caballi*	2011	Unknown

samples of each known genotype were included to determine the genotype of *T. equi* present in the equine population of Trinidad. Their accession numbers, countries, and their genotypes are displayed in Table 2.

The first data set contained four of the Trinidad *T. equi* sequences obtained from thoroughbred horses in 2006 (accession numbers KY053282 to KY053285). These sequences were aligned with 19 *T. equi* sequences from GenBank representing all of the known genotypes. All of the sequences were cut and aligned to 335 bp using the MUSCLE Alignment program incorporated into the MEGA 7.0 program (Kumar et al. 2016). The second data set generated for *T. equi* contained 17 of the *T. equi* sequences obtained from thoroughbred mares and foals sampled between 2011 and 2013. These sequences were aligned with 17 closely related *T. equi* sequences of known genotype from GenBank and cut to a length of 383 bp using the MUSCLE Alignment program.

Construction of data set for *Babesia caballi*

The four *B. caballi* sequences obtained from horses in Trinidad were aligned with 11 *B. caballi* sequences submitted to GenBank. Six of the 11 sequences were of known genotype. Their accession numbers, countries of origin, and genotypes are displayed in Table 2. The sequences were 509 bp following alignment using the MUSCLE Alignment incorporated into MEGA package version 7.0 program.

Phylogenetic analyses

For all data sets, phylogenetic analyses were carried out using the Jukes–Cantor model with a discrete Gamma distribution (J+G model) as recommended by jMODELTEST v2.1.10 (Fungiflora and Gascuel 2003; Darriba et al. 2012). A Bayesian approach implemented in the Markov chain Monte Carlo (MCMC) inference framework of the BEAST v1.10.3 software package (Drummond and Rambaut 2007; Lemey et al. 2009; Suchard et al. 2018) was used to estimate phylogenies. For the performance of best tree and clock priors, we estimated the marginal likelihoods using the path sampling (PS) and stepping stone sampling methods (Baele et al. 2012). To ensure convergence of effective sampling size parameters (ESS > 200), analyses were run for 30–50 million generations, with a 10% burn-in period that was more than adequate to achieve stationarity. The program Tracer v1.7.1 (Rambaut et al. 2018) was used to ensure that stationarity was achieved. TreeAnnotator v1.10.0 (part of the BEAST package) was used to summarize the posterior tree distribution and FigTree v1.4.3 to visualize the annotated maximum clade credibility (MCC) phylogenetic trees.

Results

Twenty-seven (24.3%) mares and four (4.5%) foals were positive for *T. equi* by cPCR. Four (3.6%) of the mares were positive for *B. caballi* by cPCR.

The sequences that were used to construct the phylogenetic trees from Trinidad were 98–100% homologous with sequences of genotype A from South Africa (EU642508, Z15105, and EU642507), Israel (KX227629), Sudan (AB515310), Spain (AY150062), and the USA (JX177672) for *T. equi*. The *B. caballi* sequences from Trinidad were 98–100% homologous with *B. caballi* sequences from South Africa (Z15104, EU642514, and EU642512), Spain (AY534883), and Jordan (JN596980) in the NCBI database using BLASTn program (Altschul et al. 1990).

Phylogenetics for *T. equi* sequences from Trinidad

The primers that were used to detect *T. equi* in the thoroughbred horses in 2006 were the RLB F2 and RLB R2 primers which amplified nucleotides between 2,592,374 and 2,592,772 of the complete sequence of *Babesia equi* strain WA chromosome 1 (CP001669). However, the BEC-UF2

Table 2 List of the accession numbers and genotypes of *T. equi* and *B. caballi* from different countries worldwide

Ascension number	Name of piroplasm	Country of origin	Genotype
KX227629	*Theileria equi*	Israel	A
EU642508	*Theileria equi*	South Africa	A
Z15105	*Theileria equi*	South Africa	A
JX177672	*Theileria equi*	USA	A
AY150062	*Theileria equi*	Spain	A
EU642512	*Babesia caballi*	South Africa	A
JN596980	*Babesia caballi*	Jordan	A
AY534883	*Babesia caballi*	Spain	A
EU642507	*Theileria equi*	South Africa	B
JN596980	*Babesia caballi*	Jordan	A
AY534883	*Babesia caballi*	Spain	A
EU642507	*Theileria equi*	South Africa	B
AB515310	*Theileria equi*	Sudan	B
Z15104	*Babesia caballi*	South Africa	B
JQ390047	*Theileria equi*	Mexico	C
KJ573372	*Theileria equi*	Brazil	C
KX227641	*Theileria equi*	Israel	C
EU888903	*Theileria equi*	South Africa	C
EU642514	*Babesia caballi*	South Africa	C
JF827602	*Babesia caballi*	Jordan	C
AB515308	*Theileria equi*	Sudan	D
AB515311	*Theileria equi*	Sudan	D
AB515315	*Theileria equi*	Sudan	D
KF559357	*Theileria equi*	China	E
HM229407	*Theileria equi*	South Korea	E
KM046918	*Theileria equi*	Switzerland	E
KJ573373	*Theileria equi*	Brazil	Unknown
JX049129	*Theileria equi*	Saint Kitts and Nevis	Unknown
KJ908940	*Babesia caballi*	Romania	Unknown
KP792452	*Babesia caballi*	Turkey	Unknown
KM882893	*Babesia caballi*	Iran	Unknown
JQ288736	*Babesia caballi*	Mongolia	Unknown
KU879021	*Babesia caballi*	Malaysia	Unknown

and Equi-R primers used in this study amplified nucleotides along 2,592,911–2,593,264 of the complete sequence of *Babesia equi* strain WA chromosome 1 (CP001669). As different portions of the 18S ribosome were amplified, two separate MCC phylogenies were constructed.

The phylogeny that was constructed for the *T. equi* sequences obtained from the thoroughbred horses in 2006 is shown in Fig. 1a which also contains sequences belonging to the five known genotypes (A, B, C, D, and E). The four *T. equi* sequences obtained from the horses in Trinidad (KY053282, KY053283, KY053284, and KY053285) were clustered with *T. equi* sequences of genotype A with high statistical support (posterior probability of 1). It is important to note that two of the *T. equi* sequences obtained from the regions (St Kitts and Nevis and Brazil) clustered with those of genotype C. Brazil also had a sequence clustered in genotype A.

Figure 1b shows the phylogeny with the *T. equi* nucleotide sequences obtained from thoroughbred horses in Trinidad from 2011 to 2013. The 17 *T. equi* sequences from Trinidad were closely related to *T. equi* sequences of genotype A (KX227629, EU642508, Z15105, JX177672, and AY150062). Three of the 17 *T. equi* samples from Trinidad (KX896429, KX896430, and KX896431) were obtained from foals with clinical signs of equine piroplasmosis. These foals were 4 h, 3 days, and 3 weeks of age respectively. The foals were born naturally with no complications, and they did not have any previous contact with ticks or infected blood.

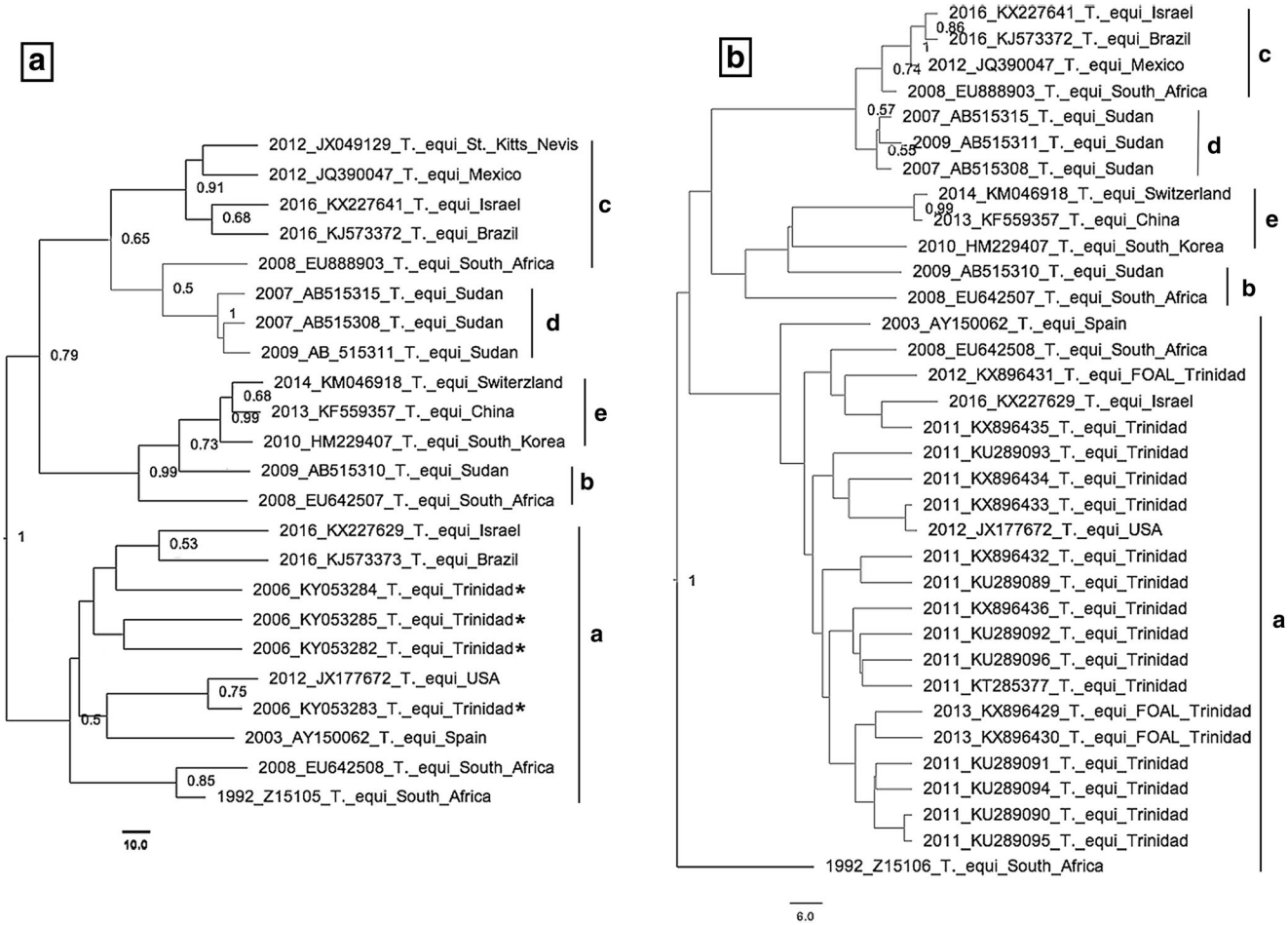

Fig. 1 Maximum clade credibility phylogenies of *Theileria equi* 18S rRNA sequence genotypes A, B, C, D, and E. A tree including 24 nucleotide sequences of a length of 335 bp (**a**) and a tree including 35 nucleotide sequences of a length of 383 bp (**b**) are shown. Only posterior probability estimates of > 50% are indicated. An unrooted tree is shown

Phylogenetics for *B. caballi* sequences from Trinidad

The phylogeny in Fig. 2 shows that the *B. caballi* sequences obtained from thoroughbred horses in Trinidad were closely related to other *B. caballi* sequences in GenBank. All of the *B. caballi* sequences obtained from thoroughbred horses in Trinidad were grouped into a single clade with other *B. caballi* sequences of genotype A with a posterior probability of 0.91 (EU642512, JN596980, and AY534883) (Table 2). Other *B. caballi* sequences of unknown genotype included in this clade were from Romania (KJ908940), Turkey (KP792452), and Iran (KM882893). The *B. caballi* sequences from Trinidad were closely related to the sequences of genotype A as they showed 98 to 100% homology.

Discussion

The positive *T. equi* result of three of the foals in this study further supports the fact that transplacental transmission of *T. equi* can occur as the foals were born without any complications and did not have any contact with ticks or infected blood.

This study demonstrates that the *T. equi* and *B. caballi* sequences obtained from the horses in Trinidad were closely related to other *T. equi* and *B. caballi* sequences submitted to GenBank from many different countries of the world.

Theileria equi samples from both data sets (2006 and 2011–2013) all belonged to genotype A with a high posterior probability. Similar findings were reported in the USA by Hall et al. (2013); therefore, this may indicate that these genes are highly conserved and that the genotypic diversity observed within the 18S rRNA gene for *T. equi* is not due to multiple gene copies within their genomes (Eickbush and Eickbush 2007; Mans et al. 2011; Hall et al. 2013). Mans et al. (2011) also suggested that genotypic diversity observed in the 18S rRNA gene is due to conventional divergent evolution, linked with mutations due to insertions or deletion within the genome. Even though the samples in this study were from different time periods (2006 and 2011–2013), they all clustered with genotype A sequences. This similarity may be explained

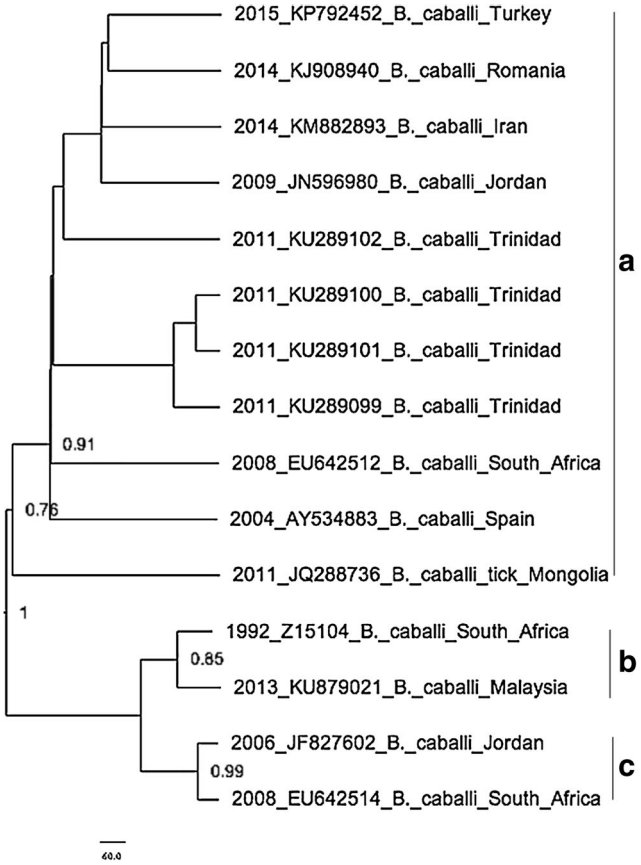

Fig. 2 Maximum clade credibility phylogeny of *Babesia caballi* 18S rRNA sequence genotypes A, B, and C. The tree analysis involved 16 sequences of a length of 509 bp. Only posterior probability estimates of > 50% are indicated. An unrooted tree is shown

by a single common source of introduction of *T. equi* into Trinidad's equine population.

It is interesting to note that *T. equi* sequences of genotype C have been previously reported in Brazil (Ketter-Ratzon et al. 2017). One of the other *T. equi* sequences from Brazil that was used to construct the phylogeny clustered with *T. equi* sequences of genotype A, indicating that there are at least two genotypes of *T. equi* in Brazil. *T. equi* sequence obtained from St. Kitts and Nevis clustered with sequences of genotype C. This finding is important as there appears to be diversity in *T. equi* sequences from islands in close proximity to Trinidad. The presence of a different genotype in St. Kitts and Nevis may be related to the origin of the agent and the observation that *Dermacentor nitens* was the major tick vector associated with equine piroplasmosis in St. Kitts and Nevis, while *Rhipicephalus microplus* was found to be the main tick species on horses in Trinidad (Loftis et al. 2014; Sant et al. 2016). Phylogenetic analyses of *Babesia rossi* erythrocyte membrane antigen 1 (Br*EMA1*) gene sequences enabled the identification of 13 genotypes. Four of these genotypes could be linked to mild, moderate, or severe clinical disease (Matjila et al. 2009; Bhoora et al. 2010). It is not known whether the different *T. equi* 18S rRNA genotypes can be associated with clinical differences in equine piroplasmosis cases. Therefore, the challenge remains in discovering the pathogenicity of the different genotypes of *T. equi*. This study shows the importance of safe guarding borders, proper biosecurity measures, and testing of animals before movement in order to prevent the introduction of a new genotype. Further work through full genome sequencing can provide some of the answers to these questions to improve knowledge on the diversity of this piroplasm.

As with observations for *T. equi*, *B. caballi* sequences from Trinidad all appeared to cluster within genotype A suggesting limited diversity of the *B. caballi* hemoprotozoan parasite in Trinidad. This can be a result of a single introduction of a source of *B. caballi* into the equine population of Trinidad.

Conclusion

T. equi and *B. caballi* sequences obtained from thoroughbreds in Trinidad appear to predominantly belong to a genotype classified as A respectively.

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Compliance with ethical standards

All procedures performed in this study involving animals were in accordance with the ethical standards of The University of the West Indies, St. Augustine at which the studies were conducted.

Conflict of interest The authors declare that there are no conflicts of interest.

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