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▣ ORIGINAL ARTICLE

Phylogenetic Analysis of Ruminant *Theileria* spp. from China Based on 28S Ribosomal RNA Gene

Huitian Gou, Guiquan Guan, Miling Ma, Aihong Liu, Zhijie Liu, Zongke Xu, Qiaoyun Ren, Youquan Li, Jifei Yang, Ze Chen, Hong Yin* and Jianxun Luo*

State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Key Laboratory of Grazing Animal Diseases MOA, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science, Xujiaping 1, Lanzhou, Gansu 730046, P. R. China

Abstract: Species identification using DNA sequences is the basis for DNA taxonomy. In this study, we sequenced the ribosomal large-subunit RNA gene sequences (3,037-3,061 bp) in length of 13 Chinese *Theileria* stocks that were infective to cattle and sheep. The complete 28S rRNA gene is relatively difficult to amplify and its conserved region is not important for phylogenetic study. Therefore, we selected the D2-D3 region from the complete 28S rRNA sequences for phylogenetic analysis. Our analyses of 28S rRNA gene sequences showed that the 28S rRNA was useful as a phylogenetic marker for analyzing the relationships among *Theileria* spp. in ruminants. In addition, the D2-D3 region was a short segment that could be used instead of the whole 28S rRNA sequence during the phylogenetic analysis of *Theileria*, and it may be an ideal DNA barcode.

Key words: *Theileria* sp., 28S rRNA, phylogeny, cattle, sheep, China

INTRODUCTION

Members of the genus *Theileria* are hemoprotozoan parasites, which have global economic and veterinary importance in ruminants. The clinical signs range from a life-threatening disease to mild or subclinical infections, depending on the infectious agent. These parasites are transmitted by hard ticks and they have complex life cycles in vertebrate and invertebrate hosts. Unlike *Babesia* species, which infect only erythrocytes, *Theileria* species can infect leukocytes and erythrocytes [1]. At least 9 species of *Theileria* have been reported in China. The causative agents of bovine theileriosis in China are *T. annulata, T. orientalis, T. mutans,* and *T. sinensis* [2,3]. Ovine theileriosis is caused mainly by *T. lestoquardi, T. luwenshuni, T. uilenbergi, T. ovis,* and *T. seperate* [4]. Of the ovine *Theileria* present in China, *T. luwenshuni* and *T. uilenbergi* are considered to be the most pathogenic forms [5,6]. Of the bovine *Theileria* spp., *T. annulata* is the most virulent species and it affects large numbers of cattle [3,7]. The nomenclature of the members of the benign bovine *Theileria* spp. group is controversial [8-11]. No consensus has been reached but we used the nomenclature *T. orientalis* instead of *T. sergenti/buffeli/orientalis* in this paper [12].

In general, the classification of members of the genus *Theileria* is based on the parasite morphology, host, disease pathology, vector ticks, and geographic origin. Recently, molecular markers, such as the major piroplasma surface protein (MPSP), small subunit ribosomal RNA gene (18S), and rRNA internal transcribed spacer region (ITS), have been used in the phylogenetic analysis of *Theileria* spp. [13,14]. The 28S rRNA forms part of the rRNA transcriptional unit, which occurs in tandem repeats arranged in ribosomal clusters throughout the genome [15]. Recently, several molecular phylogeny studies have focused on 28S rRNA [16-18]. However, the phylogenetic analysis of *Theileria* using 28S rRNA has not been reported previously.

The 28S rRNA gene comprises a mixture of conserved and divergent regions. The divergent regions of the 28S rRNA gene are known as "D"-regions and are numbered from the 5′ to 3′ direction in the rRNA [15,19] (Fig. 1). The D2 and D3 segments of the 28S rRNA are usually selected in phylogenetic studies because it is easy to design primers for them and there are many informative sites [20]. Therefore, an increasing number of studies have moved from 18S rRNA to the D2 and D3

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* Corresponding authors (vectorparasit@126.com; yinhong@caas.net.cn)

segments of 28S rRNA to analyze phylogenetic relationships [21-24].

In this study, we obtained 28S rRNA sequences and D2-D3 fragments from 13 *Theileria* spp. isolates collected in China. The phylogenetic analysis was performed using the maximum parsimony (MP) and Bayesian inference (BI) methods to determine the relationships among *Theileria* spp.

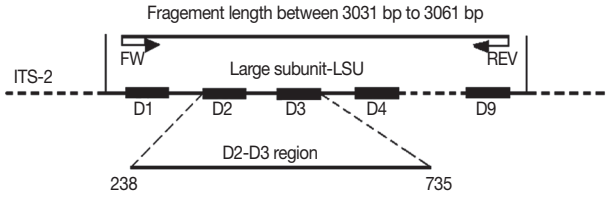

Fig. 1. Primers used for amplification and sequencing of 28S rDNA and approximate lengths of fragments. The sketch below indicates the positions of the D2-D3 region. The numbers indicate base pair positions of the alignment results for 15 isolates of *Theileria* spp. used in this study.

MATERIALS AND METHODS

Parasites and animals

Thirteen Chinese isolates of *Theileria* were used in this study, which comprised 9 isolates infective to cattle, i.e., *T. orientalis* Ningxian, *T. orientalis* Wenchuan, *T. orientalis* Lintan, *T. sinensis* Lintan, *T. sinensis* Weiyuan, *T. annulata* Ningxia, *T. annulata* Sanmenxia, *T. annulata* Inner Mongolia, and *T. annulata* Xinjiang, and 4 isolates infective to sheep, i.e., *T. ovis* Xinjiang, *T. uilenbergi* Longde, *T. luwenshuni* Weiyuan, and *T. luwenshuni* Ninxian. Detailed information on the 13 isolates is shown in Table 1.

Experimental animals (cattle and sheep) aged 6-12 months were purchased from an area where theileriosis had not been reported. One month before the experiments, all animals were

Table 1. Host and origin of *Theileria* spp., and GenBank accession number of 28S rDNA and 18S rDNA sequences used in phylogenetic analysis

Species	Origin	Host	Type	Length (bp)	Accession no.
Theileria annulata	Sanmenxia (China) Yining (China)	Cattle	28S 18S	3,038 1,739	JN696675 EU073963
T heileria annulata	Inner Mongolia (China)	Cattle	28S 18S	3,037 1,740	JN696678 EU083801
Theileria annulata	Xinjiang (China)	Cattle	28S 18S	3,040 1,742	JN696671 EU083799
Theileria annulata	Ningxia (China)	Cattle	28S 18S	3,050 1,740	JN696676 EU083800
Theileria orientalis	Ningxian (China)	Cattle	28S 18S	3,060 1,738	JN696679 EU083803
Theileria orientalis	Wenchuan (China)	Cattle	28S 18S	3,061 1,876	JN696677 EU083804
Theileria orientalis	Lushi (China) Liaoyang (China)	Cattle	28S 18S	3,061 1,738	JN696670 EU083802
Theileria sinensis	Lintan (China)	Cattle	28S 18S	3,058 1,741	JN696681 EU274472
Theileria sinensis	Weiyuan (China)	Cattle	28S 18S	3,057 1,738	JN696673 EU277003
Theileria ovis	Xinjiang (China)	Sheep	28S 18S	3,056 1,744	JN696672 FJ603460
Theileria luwenshuni	Weiyuan (China) Lintan (China)	Sheep	28S 18S	3,058 1,743	JN696680 AY262115
Theileria luwenshuni	Ningxian (China)	Sheep	28S 18S	3,058 1,744	JN696669 AY262118
Theileria uilenbergi	Longde (China)	Sheep	28S 18S	3,051 1,742	JN696674 AY262120
Theileria parva	Kenya1	Buffalo	28S 18S	3,268 1,740	AF218825 L02366
Theileria parva	Kenya2	Buffalo	28S 18S	3,294 1,739	AF013419 AF013418
Babesia bovis	Shanxian (China)	Buffalo	28S 18S	2,878 1,653	JN391431 AY603398

splenectomized. Ten days before the experiment, blood films were taken from the ears of the animals, which were stained with Giemsa and examined to confirm the absence of hemoparasites [25]. The animal experiments were approved and performed according to the guidelines of Institutional Animal Care and Use Committee, and the number of IACUC is SYXK2010-0003.

Extraction and sequencing of DNA

Nine cattle and 4 sheep were inoculated with 10 ml of cryopreserved infectious blood containing different *Theileria* isolates. When the parasitemia level reached >5%, blood was drawn from the jugular vein and collected in tubes using heparin as an anticoagulant. Erythrocytes were isolated and the parasite DNA was obtained using a DNA MiniKit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The amount of DNA isolated was assessed photometrically. Negative control DNA was obtained from the experimental animals prior to inoculation [26].

A pair of 28S rRNA sequence piroplasma universal primers were used based on the sequences of *T. parva* (GenBank no. AF218825 and no. AF013419) and a previous study [25]. The primers were as follows: forward, 5′(1011)-CTAGTAACG-GCGAGCGAAGA-3′(1030); reverse, 5′(4056)-AGGCGTTCAGTCATTATCCAA-3′(4036). The numbers in parentheses indicate the nucleotide positions of the consensus sequence. The PCR amplification conditions and the generation of sequences used the same method as a previous study [25]. The 18S rRNA sequence of each isolate was downloaded from the GenBank (Table 1).

Sequence alignment

Contigs were assembled using the Lasergene SeqMan, and the sequences produced were checked against GenBank to verify that the sequences were from the appropriate *Theileria* species. The sequences were initially compared with public sequences using the BLAST program at NCBI, USA (http://www.ncbi.nlm.nih.gov/). The distance matrices for the aligned sequences, where all gaps were ignored, were calculated using the Kimura's 2-parameter method in MEGA4. The D2-D3 segments with junction sequences from base pair positions 238-735 were selected based on the alignment results of 15 isolates.

Phylogenetic tree reconstruction

Phylogenetic trees of the complete 28S rRNA, D2-D3 region, and 18S rRNA were constructed using the maximum parsimony (MP) and Bayesian inference (BI). The MP trees were constructed using the PAUP4.0 [27]. Gaps were treated as missing data, with equal weightings for transitions and transversions, and a heuristic search was made using the TBR branch-swapping algorithm [28]. BI analyses of each dataset were conducted separately using the MrBayes 3.1.2 [29]. The Bayesian analyses were initiated using a GTR+I+Γ model with no initial values assigned to these parameters and were performed with 4 chains of 1.0×10^6 generations for the 28S rRNA dataset, 2.0×10^5 generations for the D2-D3 dataset, and 1.0×10^6 generations for the 18S rRNA dataset. The Markov chains were sampled at intervals of 100 generations. After discarding the burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The posterior probabilities (PP) were obtained for appropriate clades. Clades with PP ≥ 95% were considered to be highly supported. One *Babesia bovis* isolate was used as the outgroup. Finally, the phylogenetic trees constructed using the 2 methods were viewed using the TreeView program (version 32).

RESULTS

rRNA gene sequences

The lengths of the PCR products of the 28S rRNA gene ranged from 3,037 bp (*T. annulata* Inner Mongolia) to 3,061 bp (*T. orientalis* Wenchuan and *T. orientalis* Lushi). In the aligned sequences of the 28S rRNA gene, 526 sites were variable and 399 sites were parsimony-informative according to the criterion of the maximum parsimony. The nucleotide frequencies were 0.253 (A), 0.262 (T), 0.207 (C), and 0.278 (G). The transition/transversion rate ratios were 2.609 (purines) and 1.417 (pyrimidines). The percentage sequence divergence ranged from 0.1% to 5.8% at the intraspecies level, and from 1.2% to 11% at the interspecies level. The lengths of the selected D2-D3 segments varied from 467 bp (*T. annulata* Inner Mongolia) to 485 bp (*T. orientalis* Wenchuan). The percentage of sequence divergence ranged from 0.2% to 4.7% at the intraspecies level, and from 4.4% to 28% at the interspecies level. During this region, 192 sites were variable, and 141 sites were parsimony-informative. The nucleotide frequencies were 0.263 (A), 0.286 (T), 0.194 (C), and 0.256 (G). The transition/transversion rate ratios were 1.526 (purines) and 3.438 (pyrimidines). For 18S rRNA, the variation of sequences ranged from 0.1% to 1.2% at the intraspecies level, and from 1.6% to 4.0% at the interspecies level.

Phylogenetic relationships of *Theileria* spp.

To compare the differentiation of different species of *Theileria*, phylogenetic trees were constructed for the 28S rRNA gene, D2-D3 region, and 18S rRNA gene (Fig. 2A-C). The Bayesian analysis produced essentially the same topology as the maximum parsimony analyses, so the 3 trees generated by PAUP are shown here with the bootstrap proportions (BP) and the posterior probabilities (PP) produced by the Bayesian analyses. Fig. 2A is based on the 28S rRNA gene and it shows that the 15 *Theileria* isolates were divided into 3 clades; 1 clade for *T. annulata*, another clade for *T. parva*, while the remaining *Theileria* spp. constituted the remaining clade. The phylogenetic tree generated for the D2-D3 region (Fig. 2B) showed that *T. annulata* and *T. parva* constituted 1 clade. However, the biggest difference in the phylogenetic tree based on the 18S rRNA gene was that *T. uilenbergi* represented 1 clade and the remaining *Theileria* spp. constituted another clade (Fig. 2C).

DISCUSSION

In this study, we used 1 pair of piroplasma universal primers for 28S rRNA, which has been shown to amplify the DNA of *Babesia* spp. and *Theileria* spp. successfully [25]. The amplification efficiency was the same for the 2 piroplasma genera but the lengths of the amplified fragment were different for *Babesia* (2,878-3,017 bp) and *Theileria* (3,037-3,061 bp) [25]. This shows that different indel events have occurred in each species of piroplasma. However, its high amplification efficiency means that this pair of primers could potentially be used to develop a method for diagnosing piroplasmosis and for use in epidemiological investigations.

The 18S rRNA gene is used most commonly in studies of *Theileria* but it has a high degree of conservation and does not always discriminate among closely related species [30,31]. The 28S rRNA gene comprises tandem conserved and diverse regions. Given these structural characteristics, several recent molecular phylogeny studies have focused on 28S rRNA [32,33]. Some studies have proved the "D"-regions (especially the D2-D3 segment) are suitable markers for applications in DNA-based species identification and phylogenetic analysis in nematodes and fish [21,24,34]. This is the first study to use the complete sequence of 28S rRNA and the D2-D3 segment as phylogenetic markers to analyze the relationships among *Theileria* species. Our results showed that the complete and partial 28S rRNA gene can exhibit better intraspecific diversity and closer interspecific relationships among *Theileria* spp. than 18S rRNA. However, from intra-/interspecific distance and phylogenetic relationships, D2-D3 segment has also been proved as a potential marker in *Theileria* species.

A comparison of the results of the current study and previous phylogenies obtained using other markers (ITS genetic regions and MPSP) [11,14,35] showed that the biggest difference was the phylogenetic relationships among 3 ovine *Theileria* (*T. luwenshuni*, *T. uilenbergi*, and *T. ovis*) isolated from the northwest region of China. *T. luwenshuni* and *T. uilenbergi* are considered highly pathogenic, whereas *T. ovis* is considered to be benign because it causes subclinical infections in small ruminants [4,36]. *T. luwenshuni* and *T. uilenbergi* shared the same shape, host, vector tick, pathogenicity, and distribution, but their phylogenetic relationships were distant in the 28S rRNA and D2-D3 segment trees. These results also provided evidence that *T. luwenshuni* and *T. uilenbergi* are 2 different and valid species [30,31].

T. sinensis is a new species that was first isolated from cattle in the Gansu province of China and *Haemaphysalis qinghaiensis* was shown to be its vector [37]. However, it is still uncertain whether this *Theileria* isolate should be treated as a new *Theileria* species or if it is an existing species. Recent studies compared different genes of *T. sinensis* with other *Theileria* spp. and found that *T. sinensis* was different from known *Theileria* species [14,38]. In this study, the 3 phylogenetic trees based on 28S rRNA, D2-D3 segment, and 18S rRNA showed that *T. sinensis* always formed an obvious clade, which was distant from other *Theileria* species. This supports the conclusion that *T. sinensis* should be classified as an independent and valid *Theileria* species, which agrees with previous phylogenetic studies based on MPSP and ITS [39,40].

The 2 most important species in cattle and water buffalo are *T. parva*, as the causative agent of the Corridor disease in cattle in South Africa, and East Coast fever (ECF) in eastern and central Africa, and *T. annulata*, which causes tropical theileriosis. In the present study, 2 species formed 1 clade in the phylogenetic trees based on 28S rRNA and 18S rRNA. However, this result was also the same as previous studies which used other molecular markers, such as ITS and MPSP [39,40].

In conclusion, our study demonstrated that 28S rRNA was useful for determining the relationships among *Theileria* species. The D2-D3 segment of the 28S rRNA appears to be a potentially useful target for phylogenetic studies of *Theileria* spp. The regions investigated in this study will enrich our knowl-

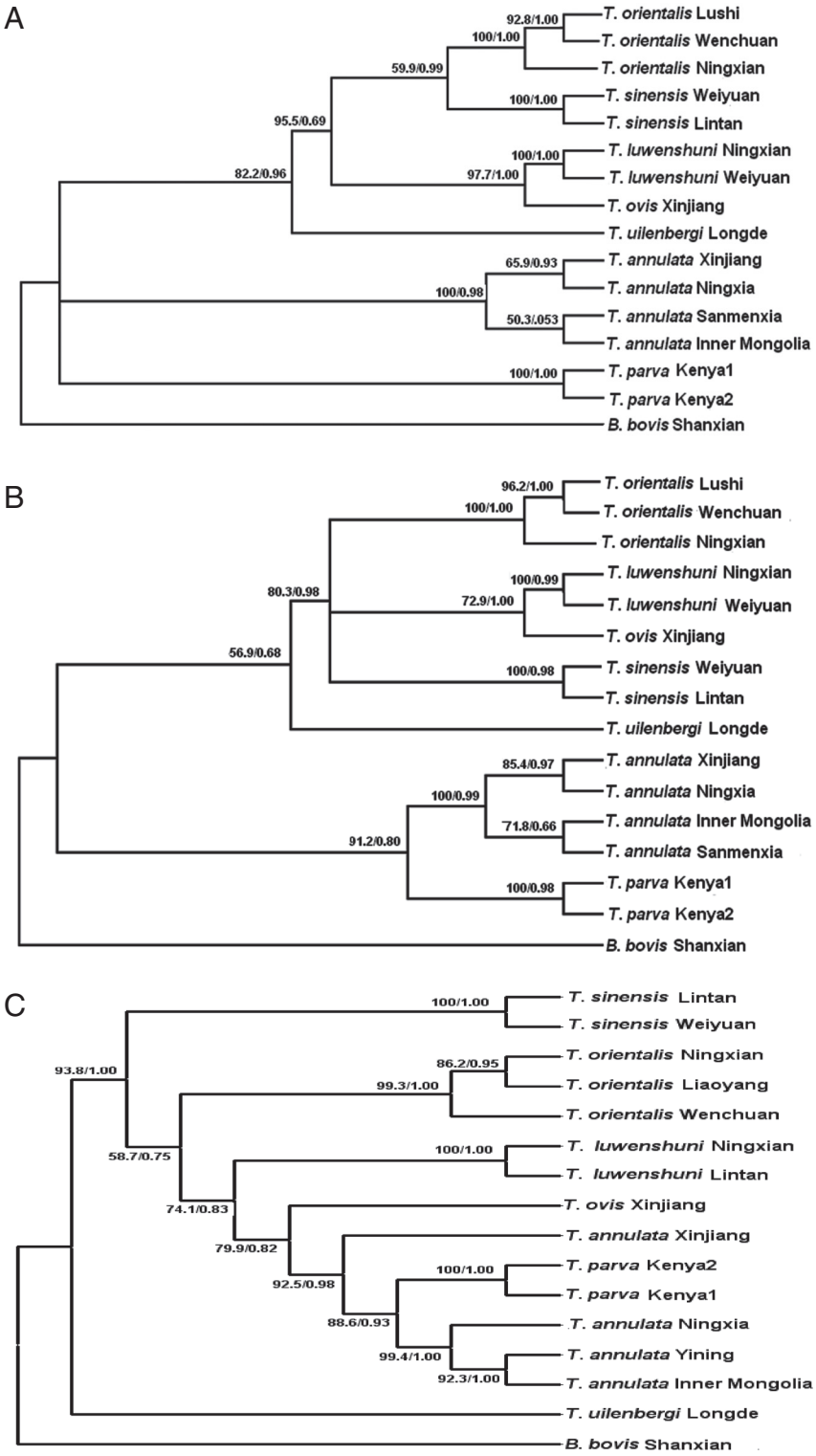

Fig. 2. Phylogenetic trees based on the complete 28S rDNA gene (A), D2-D3 region (B), and 18S rDNA gene (C) from *Theileria* spp., which were computed using the maximum parsimony (MP) and Bayesian inference (BI) algorithms. Clades with bootstrap (BP) support and posterior probabilities (PP) are marked at the nodes. One isolate of *Babesia bovis* was used as the outgroup.

edge of the phylogenetic relationships among *Theileria* species.

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Conflict of interest

We have no conflict of interest related with this study.

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