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Veterinary Parasitology 147 (2007) 221–230
veterinary parasitology
www.elsevier.com/locate/vetpar

Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among *Theileria* species

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Received 19 January 2006; received in revised form 6 April 2007; accepted 9 April 2007

Abstract

The genomic region spanning the two ribosomal RNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene was cloned and sequenced from sixteen *Theileria* isolates. Each *Theileria* species possessed ITS1 and ITS2 of unique size(s) and species specific nucleotide sequences. Varying degrees of ITS1 and ITS2 intra- and inter-species sequence polymorphism were found among ruminant *Theileria* species. The spacers were most polymorphic in the agent of tropical theileriosis, *Theileria annulata*, and were more conserved in two benign species, *Theileria buffeli* and *Theileria sergenti* Chitose. Phylogenetic analysis of the rDNA ITS1-5.8S rRNA gene-ITS2 region clearly separated each taxon, placing them in three clusters. One held *T. annulata, Theileria parva*, and *Theileria mutans*, with the latter two most closely related. The second held *T. sergenti* Ikeda, *T. sergenti* Chitose, and *T. buffeli*, with the latter two most closely related. The third cluster held the *Theileria ovis* isolates.
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Keywords: Ribosomal RNA intergenic spacer; Internal transcribed spacer; 5.8S rRNA gene; *Theileria* species; Phylogenetic tree; Polymorphism

1. Introduction

Theileria are tick-transmitted hemoprotozoan parasites of worldwide economic and veterinary importance in ruminants. Clinical signs range from life-threatening disease to mild or subclinical infections depending on the infectious agent (Barnett, 1977; Levine, 1985). Classification of members of the Theileriidae Family is based on parasite morphology, vertebrate host, disease pathology, vector tick, geographic origin, and, more recently, molecular markers such as the major piroplasm surface protein or the small subunit ribosomal RNA (SSU rRNA) gene (Levine, 1985; Chae et al., 1999; Gubbels et al., 2000).

The SSU rRNA gene is widely used as a taxonomic marker for these organisms, but it is highly conserved and may not always reliably discriminate between closely related species. The rRNA internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), which are separated by the 5.8S gene and flanked by the SSU and large subunit rRNA genes in most eukaryotes, are not subject to the same functional constraints as the

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doi:10.1016/j.vetpar.2007.04.007

rRNA genes. As a result, the spacer regions are subject to higher evolutionary rates leading to greater variability in both nucleotide sequence and length (Hillis and Dixon, 1991). The ITS regions are therefore valuable in more discrete phylogenetic separation of closely related species, recognition of new species, determination of conspecificity between isolates, discrimination within a species, and differentiation between piroplasm species and subspecies (Fazaeli et al., 2000; Zahler et al., 1998a,b; Collins and Allsopp, 1999; Dubey et al., 2001).

In the Family Theileriidae, only the agent of East Coast Fever, *Theileria parva*, has been studied at this locus to date (Collins and Allsopp, 1999). In this study, cloned sequences of the genomic region spanning the rRNA ITS1, 5.8S gene, and ITS2 of *Theileria* isolates from different hosts, of different pathogenicity, and from different geographic regions were analyzed to gain an understanding of the primary structure and genetic polymorphism of this locus within this genus.

2. Materials and methods

2.1. Theileria isolates

Theileria spp. infected blood samples were collected into ethylenediamine tetraacetic acid-K_3 (EDTA) via jugular venipuncture from cattle originating from Elazig (samples E1–3) and Malatya (samples M5–7), and sheep from Erzincan (samples Er8, 9, and 11), Turkey. Parasitemias were detectable on Giemsa-stained blood films from all animals, but none showed clinical signs of theileriosis at the time of blood collection. Three USA bovine *Theileria* isolates were from two clinical cases in Texas and Arkansas, and from a clinically normal steer in Oklahoma (the latter two samples were provided by A.A. Kocan, Department of Entomology and Plant Pathology, College of Agricultural Sciences and Natural Resources, Oklahoma State University, Stillwater, OK). The *Theileria buffeli* isolate from a clinical case in Michigan, USA was previously reported (Cossio-Bayugar et al., 2002). *Theileria sergenti* Chitose, *T. sergenti* Ikeda, and *Theileria mutans* Intona have been previously described (Kim et al., 1998; Chae et al., 1999; Morzaria et al., 1990).

Small subunit ribosomal RNA gene sequence analysis was used to confirm the species designations for the *Theileria* isolates used in this study, except for *T. mutans* Intona (GenBank accession no. AF078815) and the *T. buffeli* from Michigan, which were previously reported (Chae et al., 1999; Cossio-Bayugar et al., 2002).

2.2. DNA preparation

Genomic DNA (gDNA) was extracted from blood samples from cattle and sheep in Turkey by a modified previously described method (D'Oliveira et al., 1995). Blood (50 μl) was combined with 500 μl of saponin lysis mixture (0.22% NaCl, 0.015% saponin, 1 mM EDTA) and centrifuged at 11,600 × *g* for 1 min. The resulting pellets were washed three times by centrifugation with 500 μl of saponin lysis mixture. The final pellets were incubated at 56 °C for 1 h in 100 μl buffer (40 mM KCl, 10 mM Tris–HCl [pH 8], 0.5% Tween 20) containing 100 μg/ml proteinase K, then incubated at 95 °C for 10 min and then stored at 4 °C until use. *T. mutans* Intona and the Michigan *T. buffeli* gDNA samples were available from earlier studies (Chae et al., 1999; Cossio-Bayugar et al., 2002). Genomic DNA was purified from the remaining samples using a standard phenol-chloroform extraction method (Sambrook et al., 1989).

2.3. Small subunit ribosomal RNA gene amplification and analysis

SSU rRNA genes were amplified from the Turkey *Theileria* isolates using Proofpro DNA polymerase (Continental Lab Products) and from the other isolates using the Advantage 2 PCR Enzyme System (BD Biosciences) according to a previously reported protocol (Holman et al., 2003). Single band amplicons viewed on ethidium bromide stained agarose gels were directly ligated into plasmid vector pCR TOPO 2.1 and TOP10 *Escherichia coli* cells were transformed (Invitrogen). The resulting colonies were screened by colony PCR and plasmid DNA was purified from overnight cultures of selected clones containing the correct size insert using a modified alkaline lysis mini preparation (Qiagen QIAprep).

PCR with nested primers was performed using 1 μl primary PCR product as template with primers AN and BN (5′-GCT TGT CTT AAA GAT TAA GCC ATG C-3′ and 5′-CGA CTT CTC CTT CCT TTA AGT GAT AAG-3′) (Schoelkopf et al., 2005) or 989A and 990A (5′-GGT AGG GTA TTG GCC TAC CGT-3′ and 5′-AAA GTC CCT CTA AGA AGC-3′) if multiple or no visible products resulted from primary PCR. The amplicons were evaluated and cloned as above, except for E2 and E4, which were directly sequenced.

The amplicons were sequenced in both directions as previously described (Holman et al., 2003; Schoelkopf et al., 2005). The resulting sequences were aligned and analyzed using Sequencher 3.0 (Gene Codes Corporation

Inc.). Consensus SSU rRNA gene sequences were determined from alignments of three clones for each isolate and subjected to BLAST similarity searches (Altschul et al., 1990) (GenBank database; National Center for Biotechnology Information, National Institutes of Health). GenBank accession numbers were assigned to the obtained sequences.

2.4. Ribosomal DNA intergenic spacer region amplification and analysis

The rDNA spanning the ITS1-5.8S gene-ITS2 region was amplified from *Theileria* sp. gDNA using the forward strand SSU rRNA gene primer 1055F (5′-GGT GGT GCA TGG CCG-3′) and the reverse strand large subunit rRNA gene primer LSUR300 (5′-T(A/T)G CGC TTC AAT CCC-3′) as described (Holman et al., 2003). Thermal cycling included initial denaturation at 96 °C for 3 min followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min and hold at 4 °C.

Nested PCR was performed as previously described (Holman et al., 2003) with forward primer ITSF (5′-GAG AAG TCG TAA CAA GGT TTC CG-3′) and reverse primer LSUR50 (5′-GCT TCA CTC GCC GTT ACT AGG-3′). The thermal cycling protocol was as detailed above, except for initial denaturation at 96 °C for 2 min and annealing at 55 °C. The nested PCR products were evaluated and cloned.

The amplicons were sequenced in both directions, aligned, and analyzed as above. At least three ITS1-5.8S-ITS2 region clones were analyzed for each isolate and pair wise sequence comparisons were performed using Genestream 2 (Institut de Génétique Humaine, Montpelier, France; http://xylian.igh.cnrs.fr/) (Pearson et al., 1997). GenBank accession numbers were assigned to the obtained sequences.

The *Theileria* sp. rDNA ITS1-5.8S-ITS2 sequences obtained in this study were aligned with corresponding sequences from *T. parva* (GenBank accession nos. U03602, AF086733 and AF086734) and *Plasmodium vivax* (GenBank accession no. AF316893), for a total of 64 sequences, using CLUSTAL W (http://www.ebi.ac.uk/clustalw) (Thompson et al., 1994). The alignment was manually adjusted to maximize similarity among related sequences (alignment available upon request). The consensus 5.8S rRNA gene sequences for all of the above taxa, excluding *P. vivax*, were also separately aligned.

A phylogenetic tree was generated from the aligned rDNA ITS1-5.8S-ITS2 sequences using the neighbor-joining algorithm in PAUP* version 4.0b10 (Swofford, 1998). Molecular distances were estimated by the Kimura two parameter model (Kimura, 1980). The nodes were tested for robustness by 1000 bootstrap replications. *P. vivax* served as the out group.

3. Results

3.1. SSU rRNA gene analysis

The bovine hemoparasite from Turkey was confirmed to be *Theileria annulata* by SSU rRNA gene sequence analysis. E1, 2 and 3, and M7 were identical in sequence to the sequence for *T. annulata* in the GenBank database (GenBank accession no. M64243) except for a cytosine inserted at position 1119, yielding a sequence 1741 base pairs in length. Microheterogeneity ranging from 0.1 to 0.2% was seen among sequences from Malatya isolates 5 and 6 and *T. annulata* (M64243). Identity of 99.5% was found between the Elazig and Malatya consensus SSU rDNA sequence and a 995 bp SSU rRNA gene fragment from *T. annulata* from France reported in the GenBank database (accession no. AY150056).*Theileria ovis* was identified in the sheep samples from Erzincan, Turkey by SSU rRNA gene sequence analysis. A full length gene sequence of 1747 bp was obtained from Erzincan 8 and 1675 bp and 1000 bp gene fragments from Erzincan 9 and Erzincan 11, respectively. The consensus gene sequence differed in two base positions from that of *T. ovis* reported from the Sudan and Turkey (GenBank accession nos. AY260171 and AY260172, respectively) and in five base positions from *T. ovis* from Spain (GenBank accession no. AY533144). The Erzincan *T. ovis* sequence shared less identity, 97.2%, with that of *Theileria lestoquardi* (GenBank accession no. AF081135).

T. buffeli was identified in all of the bovine samples from the USA by SSU rRNA gene analysis, with a gene length of 1740 bp. The sequences were designated *T. buffeli* Tx, *T. buffeli* Ark, and *T. buffeli* Ok, and the common sequence as USA *T. buffeli*. Each isolate possessed a consensus sequence identical to the others and also to the *T. sergenti* Chitose SSU rRNA gene sequence obtained in this study, as well as those of the Michigan *T. buffeli* (*T. buffeli* Mi) and *Theileria* type A in the GenBank database (U97047).

The *T. sergenti* Ikeda SSU rRNA gene was 1748 bp in length and shared 99.9% identity with *Theileria* sp. type B SSU rRNA gene (GenBank accession no. U97048) with a single base substitution of thymidine for cytosine at position 783.

The SSU rRNA gene sequences obtained in this study were assigned GenBank accession numbers AY508462

and AY524666 (*T. annulata* E1), AY508473 (*T. annulata* E2), AY508463 (*T. annulata* E3), AY508465–AY508466 and AY508469 (*T. annulata* M5), AY508468–AY508470 (*T. annulata* M6), AY508471–AY508472 (*T. annulata* M7), AY661515 (*T. sergenti* Ikeda), AY661514 (*T. sergenti* Chitose), AY661511, AY508453–AY508455 (*T. ovis* Er8), AY508456 (*T. ovis* Er9), AY508459–AY508461 (*T. ovis* Er11), and AY661511, AY661512, and AY661513 (*T. buffeli* isolates from Arkansas, Texas, and Oklahoma, respectively).

3.2. rRNA ITS1-5.8S gene-ITS2 genomic region analysis

ITS1 varied in length from 482 to 1634 bp and was longer than the ITS2 region (268–525 bp) in all *Theileria* isolates. Taxon-specific sizes were found for each region, as shown in Table 1. Intraspecies variation in length was greatest in *T. annulata*, with 15 different lengths in both the ITS1 and the ITS2 among 24 clones (Table 1; Fig. 1). In the other taxa, the ITS1 length varied more than the ITS2. *Theileria sergenti* Chitose clones were of two ITS1 lengths and one ITS2 length, the lowest amount of intraspecies size variation found among the taxa. The *T. buffeli* clones were of three different ITS1 lengths and one ITS2 length (Table 1).

T. mutans Intona ITS1-5.8S-ITS2 clones were the most conserved and of identical length with only 0.2–0.5% intraspecies sequence polymorphism (Table 1). This minor polymorphism may be explained by TAQ error as it results from single base substitutions in the ITS1 of two clones, a single base substitution in the 5.8S gene of one clone, and a single base substitution in the ITS2 of a single clone. Thirteen *T. buffeli* clones produced ITS1-5.8S-ITS2 of three different lengths with 0.1–2.4% sequence polymorphism. Five *T. sergenti* Chitose ITS1-5.8S-ITS2 clones were of two lengths with polymorphism of 0.6–2.1%. The eleven *T. ovis* cloned sequences were of four different lengths ranging in variation from 0.2–1.8%. Each of the four *T. sergenti* Ikeda clones were of different lengths with 2.0–3.4% sequence polymorphism.

In contrast, among 28 *T. annulata* clones there were 19 different ITS1-5.8S-ITS2 lengths with sequence variation exceeding 17% (Table 1). Much of the polymorphism was attributable to insertions/deletions of blocks of conserved sequence, as shown the examples for ITS2 in Fig. 1. Single base polymorphisms also occurred throughout the ITS1-5.8S-ITS2 region. Some of these were in variable regions, some were clearly substitutions between two bases with fair representation of each in all clones sequenced, and some were limited in occurrence (Fig. 1). For example, in Fig. 1, two single base polymorphisms in different positions were found in only one cloned ITS2 sequence each, which suggests that they resulted from Taq polymerase error. Despite the expected contribution of Taq error to ITS1-5.8S-ITS2 sequence variation based on the reported Taq error rate of 2.2×10^{-5} errors per nucleotide per cycle (Lundberg et al., 1991), overall there were very few instances of single base substitutions found in only one

Table 1
Intra-isolate variation in the *Theileria* rRNA internal transcribed spacers 1 and 2 (ITS1 and ITS2) determined from cloned sequences

Isolate	*N*	Length in base pairs		Percent variation	
		ITS1	ITS 2	ITS1	ITS 2
T. buffeli Ark	3	640, 641	275	0–<1	<1
T. buffeli Mi	4	640, 642	275	<1–3	0–<1
T. buffeli Ok	3	642	275	<1	0–<1
T. buffeli Tx	3	642	275	<1	<1–2
T. sergenti Chitose	5	628, 631	277	0–2	<1–2
T. sergenti Ikeda	4	1628–34	524–525	1–4	<1–2
T. mutans Intona	3	598	347	<1	0–<1
T. annulata Elagzig 1	4	492–512	268–276	0–12	7–20
T. annulata Elagzig 2	6	482–509	277–354	6–14	7–26
T. annulata Elagzig 3	4	493–511	264–326	0–11	0–30
T. annulata Malatya 5	4	488–506	274–339	0–12	<1–31
T. annulata Malatya 6	3	486–498	270–278	7–12	10–26
T. annulata Malatya 7	3	490–504	279–345	10–11	16–33
T. ovis Erzincan 8	3	763,764	434	<1–12	<1–11
T. ovis Erzincan 9	5	751–764	434	<1–3	<1
T. ovis Erzincan 11	3	764	434	<1	<1

The isolate, total number of clones sequenced from each, sequence lengths in base pairs, and the range of percent variation among clones for each isolate are shown.

```
T.annM6-18  TTAATTCTATCCAAAACAACCTAATGGTGATCCCCAAAGGGTT----------ACCCA------AGGCA---------------------
T.annE2-12  TTAATTCTATCCAAAACAACCTAATGGTTGTCCCCAACGGGTTAGCCTAGTGAACCCATCTGGGTAGCTGGTGATCCCCAAAGGACCCTA
T.annM5-13  TTAATTCTATCCAAAACAACCTAATGGTGATCCCCAACGGGTTAGCCTAATGAACCCACCCGGGTAGCTGGTGATCCCTAAAGGACCCTA
T.annM5-8   TTAATTCTATCCAAAACAACCTAATGGTGATCCCTAACGGGAT----------ACCCT------AAGCCG--------------------
T.annE1-14  TCAATTCTATCCAAAACAACCTAATGGTGATCCCTAACGGGTT----------ACCCA------AGGCCC--------------------
T.annE1-13  TTAATTCTATCCAAAACAACCTAATGGTGATCCCCAAAGGGTTAGCCAA----------------AGCCG--------------------
            * **************************  **** ** *** *                       **
             1                          2

T.annM6-18  -------------------------TTGGATGTGAGTGACTGCCCC---------------TAGGTGGGTGGTTGATTTTAGGCTCTGGT
T.annE2-12  GGTACCTGGGACTTAAAGCCCTAGTTTGGATGTGAGTGACTACCAAATAACTCCGTTTGGATGTTTGGGTGGTTGATTTTAGGCTCTGGT
T.annM5-13  GGTACCTGGGACTTAAAGCCCTAGTTTGGATGTGAGTGACTGCCCC---------------TGTTTGGGTGGTTGATTTTAGGCTCTGGT
T.annM5-8   ------------------------TTTGGATGTGAGTGACTGCCTAATAACTCCGTTTGG--AGGTGGGTGGTTGATTTTAGGCTCTGGT
T.annE1-14  ------------------------TTTGGATGTGAGTGACTGCCC------------------TTTGGGTGGTTGATTTTAGGCTCTGGT
T.annE1-13  ------------------------TTTGGATGTGAGTGACTGCCC------------------CTTGGGTGGTTGATTTTAGGCTCTGGT
                                     **************** **                     *************************

T.annM6-18  CACTAAAGTCGTTGCTATGGTATGAAACTATGCTGTATCTAATACTCTATGTTTTATACTTCAATGTCCAGTAGACTCAGAG-TCCCGGG
T.annE2-12  CACTAATGTCGTTGCTATGGTATGAAACTATGCTGTATCTAATACTCTATGTTTTATACTTCAATGTCCAGTAGACTCAGAGTCCCGGGG
T.annM5-13  CACTAAAGTCGTTGCTATGGTATGAAACTATGCTGTATCTAATACTCTATGTTTTATACTTCAATGTCCAGTAGACTCAAGAGTC-GGTT
T.annM5-8   CACTAATGTCGTTGCTGTGGTATGAAACTATGCTGTATCTAATACTCTATGTTTTATACTTCAATGTCCAGTAGACTTAGAG-TCCCGGG
T.annE1-14  CACTAAAGTCGTTGCTATGGTATGAAACTATGCTGTATCTAATACTCTATGTTTTATACTTCAATGACCAGTAGACTCAAAG-TC-GGTT
T.annE1-13  CACTAAAGTCATTGCTATGGTATGAAACTATGCTGTATCTAATACTCTATGTTTTATACTTCAATGTCCAGTAGACTCAGAG-TCCCGGG
            ****** *** ***** ************************************************* ********** *     *  *
                      3     4                                                 5

T.annM6-18  ACTTCCA--TTCCC------ACCTATACTAGACCATCTAGTATTCGTGTTGAAATTTTGTTCCTGAGATTGGGTGAGATGATCCAC 270
T.annE2-12  ACTTCCA--TTCCCACCATCACCTATACTAGTCCATCTAGTATTCGTGTTACAATTTTGTTCCTGAGATTGGGTGAGATGATCCAC 354
T.annM5-13  AGCTCAAACCTCCCACCATCACCTATACTAGTCCATCTAGTATTCGTGTTACAATTT-GTTCCTGAGATTGGGTGAGATGATCCAC 339
T.annM5-8   ACTTCCA--TTCCCACTATCACCTATACTAGTCCATCTAGTATTCGTGTTACAATTTTGTTCCTGAGATTGGGTGAGATGATCCAC 291
T.annE1-14  AGCTCTA-CCTCCCACCATCACCTATACTAGTCCATCTAGTATTCGTGTTACAATTTTGTTCCTGAGATTGGGTGAGATGATCCAC 275
T.annE1-13  ACTTCCA--CTCCCAAC---------ACT--CCCACGTG------GAGTATCAATTTTGTTCCTGAGATTGGGTGAGATGATCCAC 258
            *  ** *   *****               * ***  *       * **  *****  ****************************
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Fig. 1. CLUSTAL W sequence alignment of the ITS2 region from clones of four *T. annulata* isolates: Malatya 6 clone 18 (T.annM6–18), Elazig 2 clone 12 (T.ann E2–12), Malatya 5 clones 8 and 13 (T.annM5–8 and T.annM5–13), and Elazig 1 clones 13 and 14 (T.annE1–13 and T.annE1–14). Nucleotides conserved in all sequences are indicated by asterisks under the sequence. Underlined fragments show that sequence conservation across isolates occurred even in variable regions. Sequence lengths are indicated at the end of each sequence. Note that sequence length variation is due largely to deletions of sequence blocks in conserved regions. Variable single base positions in conserved regions are noted below the sequence (K = G or T; M = A or C; R = A or G; W = A or T; Y = C or T). Five single base polymorphisms in conserved regions limited to one clone in the sequences shown are in bold type and numbered below the sequence. Of these, 1 and 2 are found in other sequenced clones not shown and 3, 4 and 5 only occur in the clones shown.

cloned full ITS1-5.8S-ITS2 sequence (data not shown), which would be clearly indicative of Taq error.

ITS1-5.8S-ITS2 interspecies variation (Table 2) was greater than intraspecies variation as evidenced by the phylogenetic relationships inferred by the neighbor-joining algorithm. The tree topology separates the full ITS1-5.8S-ITS2 nucleotide sequences from the *Theileria* species into three well-supported branches with a common ancestor (Fig. 2). The first branch holds two nodes dividing *T. annulata* sequences from the *T. parva* and *T. mutans* sequences, but with well-supported bootstrap values for separation among all three species. The second branch also holds two nodes separating the *T. buffeli* and the *T. sergenti* Chitose sequences from the *T. sergenti* Ikeda sequences, again with well-supported separation among all three species. Branch three contains a single node holding the *T. ovis* sequences.

The *T. buffeli* clade is further separated into two groups, one composed primarily of the *T. buffeli* Tx and *T. buffeli* Ok clones and one composed of the *T. buffeli* Mi and *T. buffeli* Ark clones. This distribution is not absolute as one *T. buffeli* Mi clone is in the *T. buffeli* Tx/Ok group. None of the other isolates showed clear separation of sequences into groups. In particular, no

Table 2
Comparative *Theileria* intra- and inter-species pair wise percent variation (range) in ITS1-5.8S-ITS2 sequences

	T. b	T. s Ch	T. s Ik	T. m In	T. a E	T. a M	T. o E
T. buffeli	<1–2	6–7	63–64	45–46	45–46	45–56	48–49
T. sergenti Chitose		<1–2	63–64	45–46	45–46	44–47	48–50
T. sergenti Ikeda			2–3	64–65	67–69	68–69	59–61
T. mutans Intona				<1	40–41	39–42	47–48
T. annulata Elagzig					0–16	6–17	49–51
T. annulata Malatya						<1–17	49–52
T. ovis Erzincan							<1–2

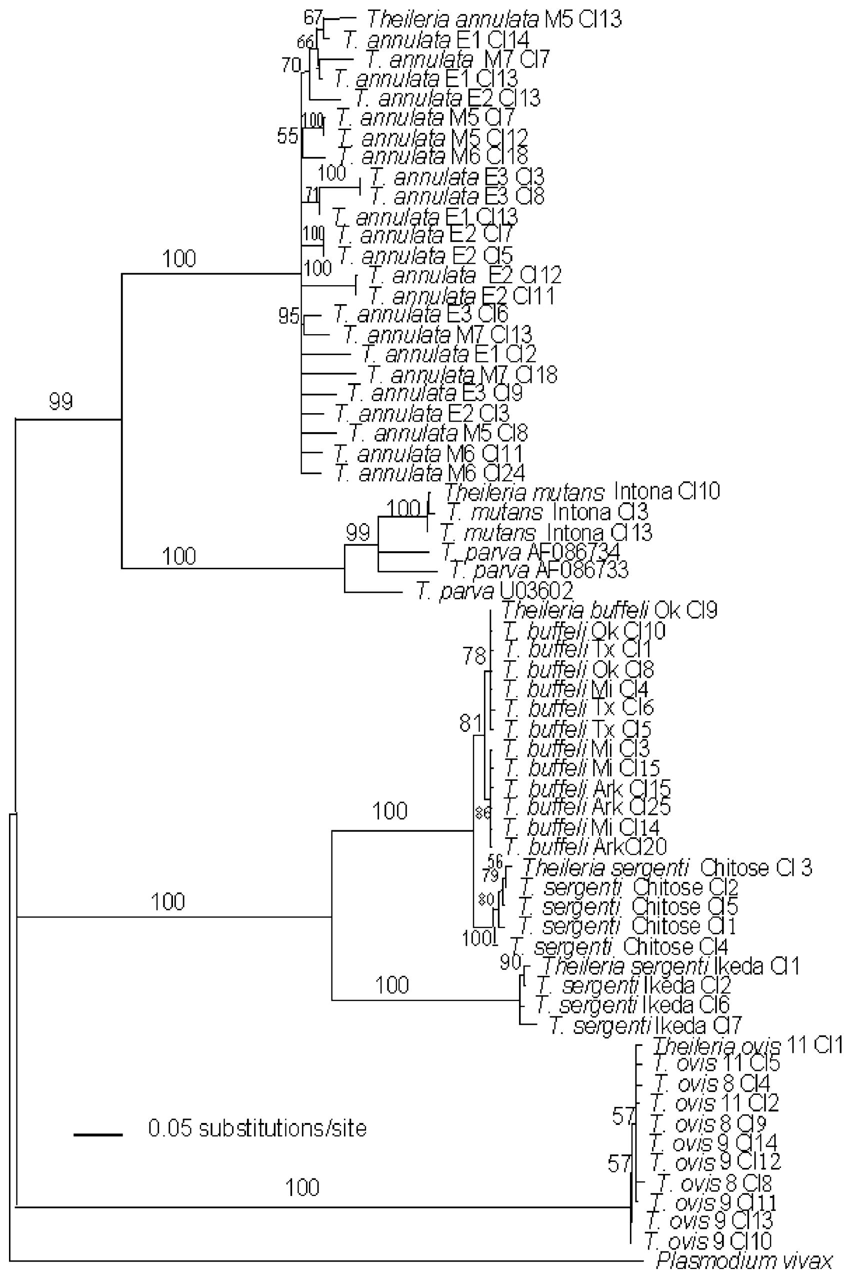

Fig. 2. Phylogenetic relationships among the ruminant *Theileria* spp. based on the rRNA intergenic spacer region sequences. The tree was constructed using the neighbor-joining algorithm with molecular distances estimated by the Kimura-2 parameter model. *Theileria mutans* Intona, *Theileria annulata* Malatya, *T. annulata* Elazig, *Theileria buffeli* Arkansas, *T. buffeli* Michigan, *T. buffeli* Oklahoma, *T. buffeli* Texas, *Theileria sergenti* Chitose, *T. sergenti* Ikeda, and *Theileria ovis* Erzincan, and three corresponding sequences from *Theileria parva* (AF086734, AF086734, and U03602) were included. *Plasmodium vivax* (AF316893) served as the outgroup. Bootstrap values are shown as percentages at each node based on 1000 replicates. Branch lengths correlate to the number of substitutions inferred according to the scale shown.

pattern is evident in the distribution of the *T. annulata* clones based on either geographic origin or animal isolate (Fig. 2). Clones from isolates E1 and M7 show the most intra-isolate disparity, being separated in the tree, whereas E2, E3, and M5 each show closely grouped clones.

The ITS1-5.8s rDNA-ITS2 cloned sequences were assigned GenBank accession numbers AY661516–AY661519 for *T. sergenti* Ikeda, AY661520–AY661522 and AY661530–AY661531 for *T. sergenti* Chitose, AY661523–AY661529 and AY661532–AY661537 for *T. buffeli* USA, AY663653–AY663655

T.parva	AAACTTTCAGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.mutans Intona	AAACTTTCAGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.annulata M	AAACTTTCAGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.annulata E	AAACTTTCAGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.buffeli US	A**C**ACTTT**T**AGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.sergenti Ch	A**C**ACTTT**T**AGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.sergenti Ik	A**C**ACTTT**T**AGCGGTGGATGTCTTGGCTCACACAACGATGAAGGAC
T.ovis	AAACTT**AT**AGCGGTGGATGTCT**C**GGCTCA**T**ACAACGATGAAGGAC

T.parva	GCAGCGAAGTGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.mutans Intona	GCAGCGAAGTGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.annulata M	GCAGCGAAGTGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.annulata E	GCAGCGAAGTGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.buffeli US	GCAGCGAA**T**TGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.sergenti C	GCAGCGAA**T**TGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.sergenti I	GCAGCGAA**T**TGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT
T.ovis	GCAGCGAA**T**TGCGATAAGCATTGTGACTTGCAGACTTCTGCGAAT

T.parva	CAACAGATTTCTGAACGTATTAGACACACCACCTCTGCTTGCACG
T.mutans Intona	CAACAGATTTCTGAACGTATTAGACACACCACCTCTGCTTGCACG
T.annulata M	CAACAGATTTCTGAACGTATTAGACACACCACCTCTGCTTGCA**T**G
T.annulata E	CAACAGATTTCTGAACGTATTAGACACACCACCTCTGCTTGCA**T**G
T.buffeli US	CAACAGATTTCTGAACGTATTAGACACACCACCTCTGCTTGCACG
T.sergenti Ch	CAACAGATTTCTGAACGTATTAGACACACCACCTCTGCTTGCACG
T.sergenti Ik	CAACAGATTTCTGAACGTATTAGACACACCAC-TCT--TT---**T**G
T.ovis	CAACAGA**C**TTCTGAACGTAT**A**AGACACACCACCTCT**C**CTTG**G**A**A**G

T.parva	GTGGTACTCCCATTTCAGTGAACCT
T.mutans Intona	GTGGTACTCCCATTTCAGTGAACCT
T.annulata M	GTGGTACTCCCATTTCAGTGAACCT
T.annulata E	GTGGTACTCCCATTTCAGTGAACCT
T.buffeli US	GTGGTACTCCCATTTCAGTGAACCT
T.sergenti Ch	GTGGTACTCCCATTTCAGTGAACCT
T.sergenti Ik	GTGGTACTCCCATTTCAGTGAACCT
T.ovis	-TGG**CT**CTCCCA**A**TTCAGTGAACCT

Fig. 3. Consensus 5.8S rRNA gene sequences from *Theileria mutans* Intona, *Theileria annulata* Malatya, *T. annulata* Elazig, *Theileria buffeli* (identical sequences for Ark, Mi, Ok, and Tx are shown as U.S.), *Theileria sergenti* Chitose, *T. sergenti* Ikeda, and *Theileria ovis*, aligned with *Theileria parva* (AF086734 and U03602). Nucleotide differences from the *T. parva* sequence are shown in bold type. Gaps inserted to maximize the alignment are shown as dashes.

for *T. mutans* Intona, AY672746–AY672756 for *T. ovis* Erinzcan, AY684822–AY684835 for *T. annulata* Elazig, and AY684835–AY684845 for *T. annulata* Malatya.

3.3. 5.8S rRNA gene analysis

The 5.8S genes from all of the *Theileria* spp. were each 159 bp in length, except for that of *T. sergenti* Ikeda, which was 153 bp in length. Consensus 5.8S gene sequences determined for each isolate are shown aligned in Fig. 3. The Mi, Ark, Ok, and Tx *T. buffeli* isolates all possessed identical 5.8S gene sequences (shown as USA *T. buffeli*), which were also identical to that of *T. sergenti* Chitose (Fig. 3). The *T. sergenti* Ikeda 5.8s rRNA gene sequence was also identical to that of *T. sergenti* Chitose and USA *T. buffeli* except for deletions at positions 132, 136, 142, and 145–147, which resulted in the shorter gene length. *T. annulata* Elazig and Malatya shared identical sequences. *T. parva* and *T. mutans* also shared identical 5.8S sequences, which differ from the *T. annulata* sequence in a single base position. *T. ovis* possesses 10 unique substitutions and one deletion not found in the 5.8S genes of the other *Theileria* spp. Microheterogeneity likely attributable to sequencing and/or Taq error was seen (not shown).

4. Discussion

Two rRNA transcriptional units located on different chromosomes are described in *T. parva* (Kibe et al., 1994; Gardner et al., 2005). In our study, only the USA. *T. buffeli* ITS1-5.8S-ITS2 sequences separated into two distinct groups in phylogenetic analysis (Fig. 2). Unlike *T. parva*, however, the separation found in *T. buffeli* was among isolates rather than within them. The separation places mainly the Oklahoma and Texas *T. buffeli* sequences in one group, and those of Arkansas and Michigan in another group. The geographic separation is not absolute; one of the four Michigan sequences lies

within the Oklahoma and Texas cluster. These results might be explained by preferential PCR skewing the proportion of the various amplification products or chance selection of clones for analysis. Although there was no clear evidence for two discrete rRNA transcriptional units within any of the *Theileria* isolates, the different ITS1-5.8S-ITS2 sequences obtained suggests the likelihood of multiple rRNA transcriptional units in these species.

Of the *Theileria* species included in the current study, the greatest polymorphism in the ITS was in the pathogenic agent, *T. annulata*, the causative agent of tropical theileriosis. Collins and Allsopp (1999) suggest that different isolates of *T. parva*, also a pathogenic species, show intermixed identifiable sequence segments in the ITS regions that might result from genetic recombination if the gene pools are not completely separate. In *T. annulata*, the high degree of polymorphism both within an isolate and between isolates may also be due to the presence of mixed parasite populations within a given isolate as suggested for *T. parva* (Collins and Allsopp, 1999). Although *T. annulata* ITS were not composed of intermixed sequence segments as reported in *T. parva*, the deletions and/or insertions of blocks of sequence might be explained similarly. Higher levels of genetic variation among pathogenic versus benign organisms may be related to higher parasitemias during acute disease leading to greater numbers of organisms being ingested by the vector tick. This would then provide a more diverse pool during gametogenesis in the tick gut, resulting in greater opportunity for recombination to occur (Collins and Allsopp, 1999). It is also possible that a higher rate of parasite proliferation in the pathogenic species increases the likelihood of random mutation. *Theileria sergenti* Ikeda, which is considered moderately pathogenic for cattle, shows greater polymorphism in ITS regions than the benign *T. sergenti* Chitose, but less than the pathogenic *T. annulata*.

The contribution of PCR to sequence variation due to Taq error is well-documented and likely accounts for some of the ITS sequence polymorphism in the *Theileria* spp. (Williams et al., 1999). However, a similar degree of variability should be evident in all clones if it were due to PCR induced experimental error since all PCR assays were conducted in the same manner. In fact, *T. annulata* showed the highest amount of sequence variation although it had the shortest ITS regions of the isolates examined, whereas *T. sergenti* Ikeda showed the least variation but had the longest ITS regions.

The assignment of the binomen *T. sergenti* to two parasites of cattle that are distinguishable by molecular analysis, disparate clinical presentation, and differential susceptibility to diminazene aceturate, but transmitted by the same vector ticks is debated (Uilenberg et al., 1985; Sugimoto et al., 1991; Fujisaki et al., 1994; Onuma et al., 1998; Kawazu et al., 1999; Chansiri et al., 1999; Sako et al., 1999; Baek et al., 2002). In our study, the more pathogenic *T. sergenti* Ikeda, isolated from ticks collected at Ikeda common pasture located at Nasu Highlands in Tochigi prefecture, and the benign *T. sergenti* Chitose, isolated from cattle in Chitose, Hokkaido, Japan correspond to these two agents (Matsuba et al., 1992; Fujisaki et al., 1985). The taxonomic separation of these two isolates is also supported by the results of this study. *Theileria sergenti* Ikeda possesses ITS1 and ITS2 regions twice the length of those of *T. sergenti* Chitose and also diverges from *T. sergenti* Chitose in the 5.8S rRNA gene sequence. Moreover, *T. sergenti* Ikeda and *T. sergenti* Chitose share only 36% identity in the ITS regions, the same identity found between *T. sergenti* Ikeda and the clearly divergent *T. mutans* Intona. Phylogenetic analysis based on the ITS1-5.8S-ITS2 genomic region further supports this separation with the tree topology indicating a common ancestor for *T. sergenti* Ikeda anad *T. sergenti* Chitose, but clear divergence of the two taxa with well-supported bootstrap values (Fig. 2).

Previously it was reported that benign *T. sergenti* isolates and *T. buffeli* in the USA share identical SSU rRNA gene sequences, which was confirmed in our study (Chae et al., 1998, 1999). Our study also shows identical 5.8S rRNA genes, nearly identical ITS2, and highly similar ITS1 sequences between the USA *T. buffeli* and *T. sergenti* Chitose isolates. The ITS1 of *T. sergenti* Chitose was shorter than those of the *T. buffeli* isolates and separation of *T. sergenti* Chitose and the USA *T. buffeli* isolates was strongly supported in phylogenetic analysis, but with recent divergence (Figs. 1 and 2). Other studies show that there are differences between *T. sergenti* and *T. buffeli* in major piroplasm protein sequences and transmissibility by *Haemaphysalis* ticks (Gubbels et al., 2000; Uilenberg et al., 1985). These combined results suggest a recent evolutionary divergence of these two organisms, perhaps related to their geographic separation. Studies of this locus from other *T. buffeli* geographic isolates, especially from Australia and Africa, might provide additional information for defining species, subspecies and/or strains of the benign bovine *Theileria* species complex.

In our study, the 5.8S rRNA gene of the generally benign *T. mutans* was identical to that of *T. parva* and considerable ITS sequence conservation was found

between *T. mutans* and *T. parva* as well (Fig. 1). These two species lie in the same group in the phylogenetic tree constructed from ITS1-5.8S-ITS2 sequences, sharing a common recent ancestor with recent divergence of *T. mutans* from *T. parva* (Fig. 2). Nevertheless, their separation is well-supported by bootstrap values. The topology of the phylogenetic tree based on this locus thus suggests a closer evolutionary relationship between less pathogenic *T. mutans* and pathogenic *T. parva* than between *T. parva* and pathogenic *T. annulata*. *Theileria parva* and *T. mutans* also share identical 5.8S rRNA gene sequences, which differ from that of *T. annulata* in one base position. These results contrast from previous phylogenetic analyses based on SSU rRNA gene sequences that place *T. parva* and *T. annulata* closer to each other than to *T. mutans*, which in each case has occupied a well separated branch (Chae et al., 1999; Gubbels et al., 2000; Stockham et al., 2000).

Distinctive taxon-specific rRNA ITS1-5.8S-ITS2 region characteristics were found between the *Theileria* spp. included in the present study, with each taxon possessing ITS1 and ITS2 regions of unique size(s), taxon-specific nucleotide sequences, and varying degrees of polymorphism. This study demonstrates that this locus is applicable in defining relationships between parasites that are highly similar, such as is seen between the USA *T. buffeli* and *T. sergenti* Chitose parasites. It also underscores the importance of evaluating multiple cloned sequences from multiple isolates when possible, in order to determine the variation within a species to provide a valid base of comparison to other organisms.

Acknowledgments

We thank Ertugrul Ozdemir, Bulent Bakici and Cafer Yazici for their kind help during sample collection. This research was supported in part by the Texas Agricultural Experiment Station Project 89870.

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