Theileria parva ribosomal internal transcribed spacer sequences exhibit extensive polymorphism and mosaic evolution: application to the characterization of parasites from cattle and buffalo

N. E. COLLINS* *and* B. A. ALLSOPP

Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort 0110, Pretoria, South Africa

(*Received 9 September 1998; revised 4 January 1999; accepted 4 January 1999*)

SUMMARY

We sequenced the rRNA genes and internal transcribed spacers (ITS) of several *Theileria parva* isolates in an attempt to distinguish between the causative agents of East coast fever and Corridor disease. The small subunit (SSU) and large subunit (LSU) rRNA genes from a cloned *T. p. lawrencei* parasite were sequenced; the former was identical to that of *T. p. parva* Muguga, and there were minor heterogeneities in the latter. The 5·8S gene sequences of 11 *T. parva* isolates were identical, but major differences were found in the ITS. Six characterization oligonucleotides were designed to hybridize within the variable ITS1 region; 93·5% of *T. p. parva* isolates examined were detected by probe TPP1 and 81·8% of *T. p. lawrencei* isolates were detected by TPL2 and/or TPL3a. There was no absolute distinction between *T. p. parva* and *T. p. lawrencei* and the former hybridized with fewer of the probes than did the latter. It therefore seems that a relatively homogenous subpopulation of *T. parva* has been selected in cattle from a more diverse gene pool in buffalo. The ITSs of both *T. p. parva* and *T. p. lawrencei* contained different combinations of identifiable sequence segments, resulting in a mosaic of segments in any one isolate, suggesting that the two populations undergo genetic recombination and that their gene pools are not completely separate.

Key words: *Theileria parva*, ribosomal RNA gene, internal transcribed spacer, oligonucleotide probes.

INTRODUCTION

Theileria parva is a tick-borne protozoan parasite of ruminants in sub-Saharan Africa where the major reservoir host is the African buffalo (*Syncerus caffer*). Three different subspecies of the parasite were thought to cause 3 clinically distinct diseases of cattle (Uilenberg, 1976; Lawrence, 1979), with *T. p. parva* causing East Coast fever, *T. p. lawrencei* causing Corridor disease and *T. p. bovis* causing January disease. More recently it has been suggested that there are no biological grounds for this distinction and that *T. parva* parasites should be classified, according to their host of origin, as cattle-derived or buffalo-derived (Norval, Perry & Young, 1992). We have preferred to use the trinomial system in this paper when the clinical symptoms induced in cattle were known, and where we had no such information we have indicated the host of origin. We have not used the cattle-derived/buffalo-derived terminology because of its misleading implication of exclusivity to one host.

East Coast fever was introduced into South Africa at the turn of the century and was only eradicated, at great cost, by 1954 (Anon., 1981). Vector ticks of the genus *Rhipicephalus* are still widespread, and *T. parva* parasites are still present in buffalo in parts of South Africa, where they occasionally cause outbreaks of Corridor disease. The danger of reintroducing East Coast fever into the cattle population makes it important to be able to distinguish between those forms of the parasite which cause the two diseases, and the practical objective of this work was to find DNA probes for this purpose.

While probes specific for variable regions in the small subunit (SSU) rRNA gene can distinguish between 6 *Theileria* species (Allsopp *et al*. 1993) no SSU rRNA probe will distinguish between the 3 subspecies, suggesting that their SSU rRNA genes are identical. This was confirmed during the present study. There is greater variation, both within and between species, in the large subunit (LSU) rRNA genes, and in the internal transcribed spacer (ITS) which separates the small and large subunit rRNA genes (Gerbi, 1986). We have previously demonstrated significant sequence differences between the 2 ITSs derived from a *T. p. lawrencei* clone (Kibe *et al*. 1994) so we decided in this study to examine the LSU rRNA gene and the ITS of a larger number of *T. p. parva* and *T. p. lawrencei* isolates.

* Corresponding author. Tel: +27 12 5299388. Fax: +27 12 5299 431. E-mail: nicola@ovisun.ovi.ac.za

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

MATERIALS AND METHODS

Parasites and preparation of DNA

DNA was extracted from schizont-infected lymphoblastoid cell cultures of 11 *T. parva* isolates (Table 1) as described by Allsopp & Allsopp (1988). DNA from the Zimbabwean *T. parva* isolates was provided by R. Bishop (personal communication). The South African *T. parva* DNA samples used in characterization studies were extracted either from schizont-infected lymphoblastoid cell cultures or from whole blood. Isolates from East Africa, Malawi and Zambia were obtained from the International Livestock Research Institute (ILRI), Nairobi, Kenya.

Amplification of T. parva *rRNA genes and ITS regions*

T. parva rRNA genes and ITS regions were amplified by the polymerase chain reaction (PCR) (Saiki *et al*. 1988). The positions of primers used in amplification reactions are shown in Fig. 1. Primers A and B, designed to amplify and clone eukaryotic 16S-like rRNA genes (Medlin *et al*. 1988; Sogin, 1990) were used to amplify the complete *T. p. lawrencei* 7344 SSU rRNA gene. Details of PCR primers used to amplify the ITS and LSU rRNA gene are shown in Table 2. Two *Theileria*-specific primer pairs, IL0 465 and IL0 467, and NC1 and NC2, were designed for amplification of the LSU rRNA gene from *T. p. lawrencei* 7344. IL0 466 and IL0 464 (Kibe *et al*. 1994), corresponding to conserved sequences at the 3′ end of the small subunit and at the 5′ end of the large subunit, were used to amplify *T. parva* ITS regions. The primers were modified (Table 2) such that they could be digested with a selection of restriction enzymes to facilitate directional cloning of PCR products.

Reactions were performed as described by Kibe *et al*. (1994) on a Hybaid HB-TR1 programmable heating block. Cycling conditions were: 95 °C for 1 min, 60 °C (SSU and ITS primer pairs) or 65 °C (LSU primer pairs) for 1 min and 72 °C for 1·5 min, for 30 cycles.

Cloning and sequencing of SSU rRNA gene amplification products

The *T. p. lawrencei* 7344 SSU rRNA products from 3 reactions were pooled to eliminate *Taq* polymerase copying errors which may have occurred early in any one reaction. Amplicons were cloned and sequenced as described by Allsopp *et al*. (1993).

Cloning and sequencing of LSU rRNA and ITS amplification products

The complete ITS of *T. p. lawrencei* Hluhluwe 3 was amplified as a single fragment, while the LSU rRNA gene was amplified from *T. p. lawrencei* 7344 in 2 sections. In each case, PCR products from 3 amplification reactions were pooled before cloning directly into the TA cloning vector, pGEM-T (Promega). For each fragment, 6 clones were pooled and shotgun cloning (Bankier, Weston & Barrell, 1987) was used to produce subclones in M13mp18. The Multiprime DNA labelling kit (Amersham) was used to label LSU rRNA or ITS amplification products, which were used to screen plaque lifts to select only those clones containing the correct insert. Recombinant phage plaques were picked and single-stranded templates were prepared for sequencing by the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977).

Another strategy was employed to gather ITS sequence information from the other isolates more rapidly. Restriction sites were added to the ITS amplification primers, IL0 466 and IL0 464, to facilitate directional cloning, internal sequencing primers were designed to avoid the need for shotgun sequencing, and a decision was taken to sequence only 1 strand of each amplicon. ITS regions were amplified from the remaining isolates (Table 1) using modified primers IL0 466 and IL0 464. For each isolate, amplification products from 3 PCR reactions were pooled, digested with *Eco*RI and *Hin*dIII, gel-purified and ligated into the sequencing vector M13mp19. Six clones from each isolate were sequenced separately using the M13 (–20) forward primer and the internal sequencing primers (Table 2) complementary to conserved regions within the *T. parva* ITS.

Experimental probe design and testing

The ITS sequences were aligned using CLUSTALW (Thompson, Higgins & Gibson, 1994) and the Genetics Data Environment (GDE) alignment editor (Smith, 1992) and 6 negative strand characterization probes were designed (Table 2).

Slot blot hybridization of PCR products (Saiki *et al*. 1986) was used to screen ITS amplification products with radio-isotope labelled oligonucleotide probes. PCR amplification of the ITS region of a number of *T. parva* isolates was performed as described previously, using unmodified primers IL0 466 and IL0 464. A 10 μl aliquot of each PCR product was denatured and slot-blotted onto replicate Hybond N+ filters (Amersham); 1 filter was prepared for each probe to be used. The filters were pre-hybridized in 0·5 M sodium phosphate buffer (pH 7·5) and 7% SDS (modified from Church & Gilbert, 1984) for at least 1 h at 65 °C in a Hybaid hybridization oven. Oligonucleotide probes were 3′ end-labelled with [α-^{32}P]dATP (3000 Ci/mmole, 10 mCi/ml) using terminal transferase (Promega) according to the manufacturer's instructions and

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

Table 1. *Theileria* stocks used as sources of DNA from which ITS regions were sequenced

Species origin	Stock/isolate code	Remarks	Reference
T. parva isolated from cattle	Muguga	Field isolate from Kiambu District, Kenya. East Coast fever reference stock.	Brocklesby, Barnett & Scott (1961)
	Muguga LN/MI/1	Schizont-infected lymph node material fom a bovine infected at ILRAD, Nairobi, with *T. parva* Muguga.	T. T. Dolan (personal communication)
	Muguga SM	Schizont-infected lymph node material from a bovine infected at the CTVM, Edinburgh, with *T. parva* Muguga.	C. G. D. Brown (personal communication)
	Schoonspruit	Isolated in 1937 from natural bovine ECF infection in Transvaal, South Africa.	Neitz (1948)
	T.p./CHI/16	Isolated from naturally infected bovine on Chikeya farm, Zimbabwe. Typed as 'cattle-derived' using MAb profile and *Tpr* & rRNA probes.	Bishop *et al.* (1994)
	T.p./AYR/12	Isolated from naturally infected bovine on Ayrshire farm, Zimbabwe. Typed as 'cattle-derived' using MAb profile and *Tpr* & rRNA probes.	Bishop *et al.* (1994)
T. parva isolated from buffalo	7344/G5/F5/E8	Clone generated from an isolate made in Laikipia District, Kenya, from naturally infected buffalo 7014. Classical Corridor disease in cattle.	Kariuki *et al.* (1990), Morzaria *et al.* (1995)
	Hluhluwe 3	Isolated from naturally infected buffalo in Natal, South Africa. Extensive passage through cattle, stable Corridor disease clinical picture.	Potgieter *et al.* (1988)
	KNP 2	Isolated from naturally infected buffalo in the Kruger National Park, eastern Transvaal, South Africa. Classical Corridor disease in cattle.	H. Stoltsz (personal communication)
	T.p./BAL/25	Animal experimentally infected by ticks collected from buffalo grazed paddock, Bally Vaughn Game Park, Zimbabwe. Typed as 'buffalo-derived' using MAb profile and *Tpr* probe.	Bishop *et al.* (1994)
	T.p./BAL/93	Produced severe reaction in cattle, typed as 'buffalo-derived' using MAb profile.	G. K. Kanhai and P. Spooner (personal communication)
	T.p./MAS/38	Natural infection in bovine kept on buffalo grazed paddock, Masuri farm, Zimbabwe. Typed as 'buffalo-derived' using MAb profile and *Tpr* & rRNA probes.	Bishop *et al.* (1994)
	T.p./MAS/40	Natural infection in bovine kept on buffalo grazed paddock, Masuri farm, Zimbabwe. Typed as 'buffalo-derived' using MAb profile and *Tpr* & rRNA probes.	Bishop *et al.* (1994)

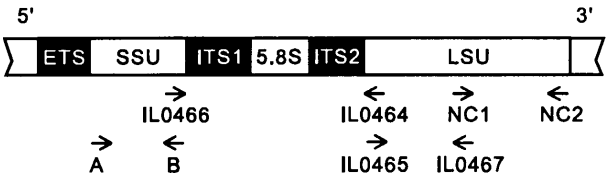

Fig. 1. Schematic diagram showing positions of primers used to amplify *Theileria parva* ITS and rRNA sequences. Coding regions are indicated by open bars, while transcribed spacers are indicated by solid bars. Not to scale. ETS: external transcribed spacer. ITS: internal transcribed spacer. SSU: small subunit. LSU: large subunit.

were added directly to the hybridization solution. Hybridization was carried out at $T_m - 20$ °C overnight. The filters were washed in 4 × SSC, 3 times at room temperature for 10 min, and once at $T_m - 5$ °C for 1 min. Filters were autoradiographed with Cronex 4 X-ray film (Du Pont) at −80 °C with an intensifying screen for 2·5 h or overnight.

RESULTS

Sequence of the small and large subunit rRNA genes of T. p. lawrencei *7344*

The *T. p. lawrencei* 7344 SSU rRNA gene sequence, the first to be obtained from this subspecies, was

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

Table 2. Oligonucleotides used for amplification, sequencing and probing of the *Theileria parva* ITS

Identity	Strand	T_m °C*	Sequence 5′–3′[b]	Remarks
Amplification oligonucleotides				
IL0 465	+	70·8	GAA AAT CCG TTG AAT TTG CCA ACG G	For use with IL0 467 to amplify 5′ half of LSU rRNA gene. Reverse complement of IL0 464
IL0 467	–	72·4	TGC GTC GAG AAG TTC ACT TGT GTT G	For use with IL0 465 to amplify 5′ half of LSU rRNA gene
NC1	+	75·3	CGC CAC GTT AAC TGC GTG TGA GG	For use with NC2 to amplify 3′ half of LSU rRNA gene
NC2	–	77·3	GAG CAC CTC GGG TAG AAT CTC AGC G	For use with NC1 to amplify 3′ half of LSU rRNA gene
mIL0 466	–	82·6	CGG AAT TCG AGC TCG GGA ACG TCT AGG GAA GTT TTG	Modified from Kibe *et al.* (1994), includes *Eco*RI and *Sac*I sites to facilitate cloning
mIL0 464	+	80·3	GCT CTA GAA GCT TCC GTT GGC AAA TTC AAC GGA TTT TC	Modified from Kibe *et al.* (1994), includes *Xba*I and *Hin*dIII sites to facilitate cloning
Sequencing oligonucleotides				
TPTS1	+	59·3	TAG TTT TAG TGG GAA GAT G	Sequencing primer for *T. parva* ITS
TPTS2	+	63·1	GAG TAC CCG GGT AAG C	Sequencing primer for *T. parva* ITS
TPTS3	+	64·1	GGT GGA TGT CTT GGC TC	Sequencing primer for *T. parva* ITS
TPTS4	+	59·3	TTG ATG AGT GAA ACT AAG C	Sequencing primer for *T. parva* ITS
Experimental probes				
TPP1	–	55·0	AGG TTA ATA AAT TAA CCC A	Specific for *T. p. parva* isolates
TPP2	–	50·7	TGT TAT TAA AAT TAA CCA A	Specific for Zimbabwean *T. p. parva* isolates
TPL1	–	59·3	CTA AAC CGT AAA ACC CAA T	Specific for group 1 *T. p. lawrencei* isolates
TPL2	–	Lowest 59·3	TAR[b] GTC CGA AGA CY[c]C W[d]AA A	Specific for group 2 *T. p. lawrencei* isolates
TPL3a	–	60·3	AAC CCW[d] AAW[d] GGG TTW[d] CTA AA	Specific for group 3 *T. p. lawrencei* isolates
TPL3b	–	57·1	CCT AAA AAG GGT TTC TAA A	Specific for group 3 *T. p. lawrencei* isolates

* T_m: the temperature at which 50% of the probe/target DNA duplexes are dissociated.
[b] R: G or A; [c]Y: T or C; [d]W: A or T.

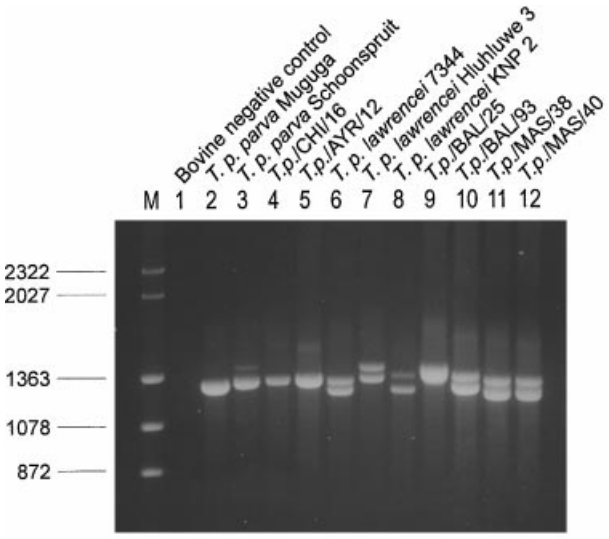

Fig. 2. *Theileria parva* ITS amplification products generated with primers IL0 464 and IL0 466. Lane M: λ *Hin*dIII and φX174 *Hae*III molecular weight markers. Lane 1: bovine DNA (negative control). Lanes 2–12: ITS amplicons from the *T. parva* isolates designated.

submitted to GenBank™ (accession number AF013418). It was 1741 bp in length and was identical to the previously known *T. p. parva* Muguga sequence (Genbank™ accession number L02366).

The LSU rRNA amplicon produced with primers IL0 465 and IL0 467 was 1564 bp in length; that produced with primers NC1 and NC2 was 1761 bp. The full sequence from primer IL0 465 to primer NC2 was submitted to Genbank™ with accession number AF013419. There were only minor sequence differences between the *T. p. lawrencei* 7344 and *T. p. parva* Muguga LSU rRNA gene sequences. Two regions in the *T. p. lawrencei* 7344 LSU rRNA gene sequence had deletions of 3 and 6 bp when compared to that of *T. p. parva* Muguga, and there were a number of point mutations.

Amplification of T. parva *ITS regions*

The ITS amplification products from *T. parva* isolates from cattle appeared to be homogeneous, while ITS amplicons of different sizes were obtained

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

from the *T. p. lawrencei* isolates (Fig. 2). Sequence information, however, indicated that the Zimbabwean isolates from cattle, T.p./AYR/12 and T.p./CHI/16, each also contained 2 ITS sequences which differed in size by only 11 bp.

Cloning and sequencing of T. parva *ITS amplification products*

The 2 ITS amplification products generated from *T. p. lawrencei* Hluhluwe 3 (Fig. 2, lane 7), were cloned and sequenced separately. The sequences were submitted to the Genbank™ database with accession numbers AF086733 and AF086734. ITS sequences on one strand only were obtained from *T. p. parva* Schoonspruit, T.p./CHI/16, T.p./AYR/12, Tpl KNP2, T.p./BAL/25, T.p./BAL/93, T.p./MAS/38 and T.p./MAS/40 using the rapid strategy. All of the sequences obtained were aligned with previously available *T. parva* ITS sequences (Kibe *et al*. 1994) using CLUSTALW (Thompson *et al*. 1994).

Variation in T. parva *ITS sequences*

The full length of the *T. parva* ITS sequences from the 3′ end of the SSU rRNA gene to the putative 5′ end of the LSU rRNA gene varied between 1058 and 1210 bp. The ITS1 varied between 500 and 600 bp in length and contained 3 variable regions (V1, V2 and V3). The ITS2 was approximately 300 bp long and could be divided into 2 variable regions (V4 and V5) separated by a conserved sequence.

The *T. parva* 5·8S gene was located by comparison with the 5·8S sequences of *Prorocentrum micans* (Maroteaux, Herzog & Soyer-Gobillard, 1985) and *Giardia ardeae* (van Keulen *et al*. 1991). The gene was approximately 170 bp in length (columns 720–890 in Fig. 3) starting 17 nucleotides upstream of the highly conserved GGAT motif (Nazar, 1984). The sequence was completely conserved between all of the *T. parva* isolates studied.

The sequences of all 6 cloned ITS amplicons from *T. p. parva* Schoonspruit, a South African isolate which causes East Coast fever (Neitz, 1948), were identical. This indicates that the 2 copies of the rDNA transcription unit in this isolate are probably identical, as has previously been shown for *T. p. parva* Muguga (Kibe *et al*. 1994).

The Zimbabwean isolates, T.p./AYR/12 and T.p./CHI/16 were obtained from cattle on two different farms, Ayrshire and Chikeya, where there were no buffalo, nor ticks which may have fed on buffalo (Bishop *et al*. 1994). These isolates were typed as 'cattle-derived' (or *T. p. bovis* under the trinomial classification system) by RFLP analysis and probing with *T. parva Tpr* repetitive and ribosomal DNA probes and screening with a panel of anti-schizont monoclonal antibodies (Bishop *et al*. 1994). Both isolates contained two ITS sequences, a longer one (ITS-L) and a shorter one (ITS-S), which differed in length by only 11 bp. The ITS-L sequences from the two isolates shared 98·3% identity while the two ITS-S sequences were identical.

Two ITS sequences were identified in all of the isolates from buffalo. *T. p. lawrencei* Hluhluwe 3 ITS-L contained a unique insert, approximately 70 bp in length (see Fig. 3, positions 183–264), located at the end of the first variable region (V1) in the ITS1. This insert accounted, in part, for the large size of *T. p. lawrencei* Hluhluwe 3 ITS-L in comparison to ITS amplification products from other isolates (Fig. 2). This insert was also found in *T. p. lawrencei* KNP 2 ITS-L.

ITS sequences were obtained from 4 Zimbabwean isolates whose monoclonal antibody profiles, rRNA gene patterns and *Tpr* RFLP genotypes were characteristic of isolates from buffalo (Bishop *et al*. 1994). Two of these isolates, T.p./MAS/38 and T.p./MAS/40, obtained from cattle kept on a paddock that was also grazed by buffalo on Masuri farm, each contained 2 ITS sequences. The ITS-L sequences shared 99·3% identity while the ITS-S sequences were identical. Differences in the ITS-L sequences of these isolates occurred as a few point mutations. By contrast, the 2 isolates obtained by experimental infection of cattle by ticks collected from a buffalo grazed paddock on a second farm, Bally Vaughn, T.p./BAL/25 and T.p./BAL/93 (Bishop *et al*. 1994) each contained two ITS sequences; all 4 of these ITS sequences were distinct. The longer ITS sequence of both of these isolates contained the large insert of approximately 70 bp which was previously observed at the end of V1 in *T. p. lawrencei* Hluhluwe ITS-L and *T. p. lawrencei* KNP 2 ITS-L. The sequence of this insert was closely similar to these 4 isolates, differing by only 2 or 3 point mutations per sequence.

A sequence mosaic in the T. parva *ITS*

Few of the *T. parva* ITS sequences were identical to any other over their full length. However, each ITS had sections homologous to those of a number of other ITS sequences within the variable regions. Consider, for example, the *T. p. parva* Schoonspruit sequence in the alignment shown in Fig. 3. It was initially similar to *T. p. parva* Muguga and *T. p. lawrencei* Hluhluwe 3 ITS-S (1–86); it later changed to resemble first *T. p. lawrencei* 7344 ITS-L (87–113) and then *T. p. lawrencei* Hluhluwe 3 ITS-S (114–178); at the end of the V1 region (181–247) and in the V2 region (419–558) it was again identical to *T. p. parva* Muguga; in the V3 region it was similar to *T. p. lawrencei* 7344 ITS-L and *T. p. lawrencei* Hluhluwe 3 ITS-L and ITS-S (592–720). The ITSs of the other isolates contained different combinations of such identifiable segments of sequence in the

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

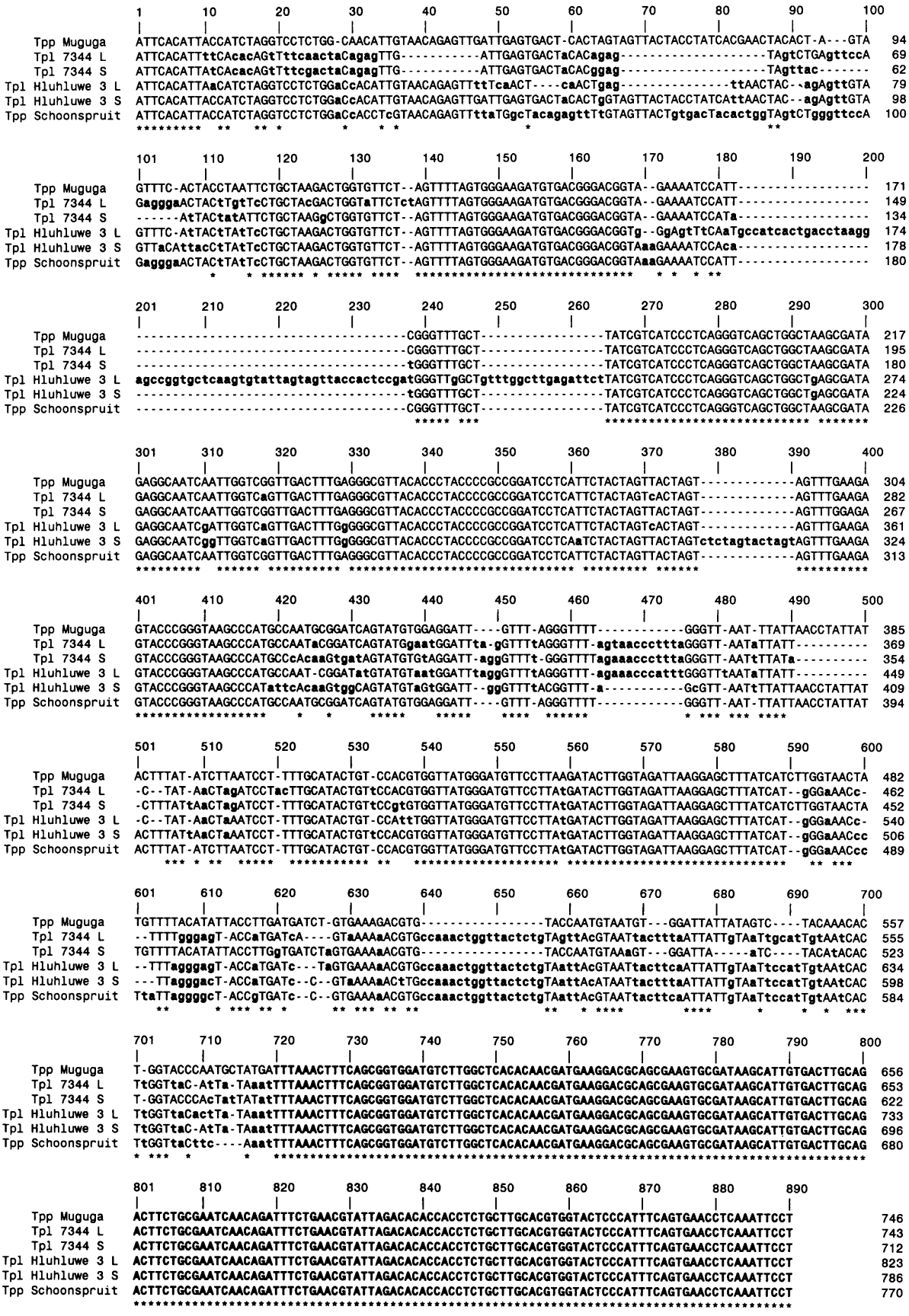

Fig. 3. CLUSTALW alignment of *Theileria parva* ribosomal ITS DNA sequences, from the beginning of the ITS 1 to the putative end of the 5·8S gene. This is indicated by bold capitals (720–890). Sequences shown are from *T. p. parva* Muguga, *T. p. lawrencei* 7014 (ITS-L and ITS-S), *T. p. lawrencei* Hluhluwe 3 (ITS-L and ITS-S) and *T. p. parva* Schoonspruit. The variable ITS1 regions are V1 (10–264), V2 (419–538) and V3 (590–719). Bases which differ

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

Type	No. of ITS sequences represented	Sequence
Cattle	3	GGATT----GTTT-AGGGTTTT----------GGGTT-AAT-TTATTAACCTATTATACTTTAT-ATC-TTAAT
	3	GGATT-AGGCTTT-AGGGTTTT----------GGGTT-AAT-TTATTAACCT-GCATACCATTTTA-CATAAAT
	1	GGATTTAGTGTTT-AGGGTTTT----------GGTT-AATTTTATTAAC---ACATACCATTTTA-CTTTAAT
Buffalo 1	1	GGATT--GGGTTTTACGGTTTA----------GCGTT-AATTTTATTAACCTATTATACTTTATTAAC-TAAAT
Buffalo 2	1	GGATT-AGGCTTT-AGGGTTTT----------GGGTCTT------CGGACCTATTTTGCTTAATTAACC--AAT
	1	GGATT-AGGCTTT-AGGGTTTT----------GGGTCTT------CGGACCTATTTTACTTAATTAACC--AAT
	1	GGATTCAGGGTTCTAGGGTTTA----------GAGTCTT------CGGACTTATTATACCATTTTATCTTTAAT
	1	GGATTTAGTGTTT-AGGGTTTT----------GGGTCTT------CGGACCTATTT-ACCATTTTATCTTTAAT
Buffalo 3	1	GGATTTA-GGTTTTAGGGTTT-AGTAACCC--TTTAGGGTT-AATATTATT----------C--TAT-AAC-TAGAT
	1	GGATT-AGGGTTTT-GGGTTTTAGAAACCC--TTTAGGGTT-AATTTTATA----------CTTTATTAAC-TAGAT
	1	GGATTTAGGGTTTTAGGGTTT-AGAAACCC--ATTTGGGTTTAATATTATT----------C--TAT-AAC-TAAAT
	1	GGATT----GTTT-AGGGTTTTAGAAACCC--ATTTGGGTTTAATATTATT----------CTATATTAAC-T-AAT
	1	GGATT-A--GTTTTAGGGTTT-AGAAACCC--TTTTGGGTTTAATATTATT----------C--TAT-AAC-TAAAT
	1	GGATT-AGGGTTT-AGCGTTTTAGAAACCC--TTTTGGGTTTAATATTATT----------C--TAT-AAC-TAAAT
	1	GGATCTAGGGTTTTAGGGTTTTAGAAACCC--TTTTGGGTTTAATATTATT----------C--TAT-AAC-TAAAT
	2	GGATT-AGGGTTT-AGGGTTTTAGAAACCCTTTTTAGGGTTTAATATTATT----------C--TATATTACCAAAT

Fig. 4. ITS1 region used for distinction between *Theileria p. parva* and *T. p. lawrencei* genotypes: represented are 7 sequences from 5 cattle isolates (1 group, 3 unique sequences) and 14 sequences from 7 buffalo isolates (3 groups, 13 unique sequences).

variable regions, resulting in a mosaic of ITS sequence segments.

Screening of T. parva *isolates with ITS experimental probes*

One variable region in the ITS1 appeared to be a likely target for distinguishing between *T. p. parva* and *T. p. lawrencei* isolates (Fig. 4). The *T. p. lawrencei* isolates could be divided into 3 groups on the basis of sequence differences in this region. Six experimental characterization oligonucleotides were designed (Table 2). The results of screening *T. parva* ITS amplification products with the 6 ITS probes are shown in Table 3. Of the *T. parva* isolates from cattle, 29 out of 31 (93·5%) were detected by TPP1; 19 out of 23 (81·8%) *T. parva* isolates from buffalo were detected by TPL2 and/or TPL3a.

DISCUSSION

The use of rRNA sequences to distinguish between T. parva *isolates*

Amplification of the *T. parva* ITS appeared to give two products from buffalo isolates and a single product from cattle isolates. However, the apparent single band obtained from Zimbabwean cattle isolates T.p./AYR/12 and T.p./CHI/16 actually contained two amplicons which differed in size by only 11 bp. Two ITS amplification products could indicate mixed populations of *T. parva* parasites, but this is not necessarily so since polymorphism between the two ITS sequences within a cloned *T. parva* parasite has been demonstrated (Kibe *et al*. 1994). The two non-stoichiometric bands obtained from *T. p. lawrencei* KNP 2 are more suggestive of the presence of two parasites in differing proportions.

The *T. parva* rRNA coding regions appear to be highly conserved. *T. p. parva* Muguga and *T. p. lawrencei* clone 7344 have identical SSU, and closely similar LSU, gene sequences, and all 11 *T. parva* 5·8S genes were identical. In contrast there was much intra-individual and intraspecific variation in *T. parva* ITS sequences, and many isolates gave two different sequences. Such extreme variation in ITS sequences has also been found in *Plasmodium falciparum* (Rogers, McConkey & McCutchan, 1995). The ITSs of both *T. p. parva* and *T. p. lawrencei* parasites contain different combinations of identifiable segments of sequence in the variable regions, resulting in a mosaic of ITS sequence segments. Such a mosaic might be expected if the different *T. parva* genotypes were the products of genetic recombination, and independent evidence has shown that sexual recombination does indeed occur during tick passage (Morzaria *et al*. 1992).

For the development of characterization probes we designed 6 oligonucleotides which hybridized to regions in the ITS1. The *T. p. parva* isolates hybridized with a limited subset of these probes, while the *T. p. lawrencei* isolates appeared to be more variable and hybridized with many more of them. The greater diversity of *T. p. lawrencei* isolates has also been demonstrated antigenically (Conrad *et al*. 1987) and all these findings support the suggestion that there has been a selection in cattle of a relatively homogenous subpopulation of *T. parva* from a much

from the *T. p. parva* Muguga sequence are shown in lower case bold; bases which are identical in all sequences are indicated by an asterisk below.

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

Table 3. Probing of *Theileria parva* ITS amplification products with experimental characterization oligonucleotides

T. parva isolates		Probes					
		T. p. parva		*T. p. lawrencei*			
Origin	Name/Isolate code	TPP1	TPP2	TPL1	TPL2	TPL3a	TPL3b
Cattle							
Kenya	Muguga LN/MI/1	+	−	−	−	−	−
	Muguga SM	+	−	−	−	−	−
	Muguga BJ2433	+	−	−	−	−	−
	Muguga BJ400	+	−	−	−	−	−
	Mariakani G28/E6/F8/E7	−	−	−	−	−	+
	Marikebuni G371	+	−	−	−	−	+
	Marikebuni F171	+	−	−	−	−	+
Uganda	Uganda BH3312	+	−	−	−	−	+
	Uganda BH257	+	−	−	−	−	+
Burundi	Burundi	+	−	−	−	−	+
Malawi	Malawi	+	−	+	−	−	−
Zambia	Zambia V1	+	−	+	−	−	−
	Zambia S105	+	−	+	−	−	−
	Zambia L1	+	−	+	−	−	−
South Africa	Schoonspruit	+	−	+	−	−	−
Zimbabwe	Boleni D770	+	−	+	−	−	−
(vaccine strain)	Boleni BH2 3369	+	−	+	−	−	−
Zimbabwe	T. p./CHI/16	+	+	−	−	−	−
	T. p./CHI/17	+	+	−	−	−	−
	T. p./CHI/18	+	+	−	−	−	−
	T. p./CHI/20	+	+	−	−	−	−
	T. p./CHI/21	+	+	−	−	−	−
	T. p./CHI/22	+	+	−	−	−	−
	T. p./CHI/23	−	−	+	+	+	+
	T. p./AYR/11	+	−	−	−	−	−
	T. p./AYR/12	+	−	−	−	−	−
	T. p./AYR/13	+	+	−	−	−	−
	T. p./AYR/14	+	−	−	−	−	−
	T. p./GAL/30	+	+	−	−	−	−
	T. p./AVE/9	+	+	−	−	−	−
	T. p./HUN/96	+	−	−	−	−	−
Buffalo							
Kenya	7344/G5/F5/E8	−	−	−	−	+	−
South Africa	Hluhluwe 3	−	+	+	−	+	−
	Hluhluwe #3	−	+	+	−	+	−
	Hluhluwe #5	−	+	+	−	+	−
	Hluhluwe #6	−	+	+	−	+	−
	Hluhluwe #7	−	+	+	−	+	−
	KNP 2	−	−	−	−	+	+
	LNP/94_1	−	−	−	+	−	−
	KNP/94_2	−	−	−	+	−	−
	KNP/94_3	−	−	−	+	−	−
	KNP/94_4	−	−	−	+	−	−
	Warmbaths 1	−	−	−	−	−	−
	Warmbaths 2	−	−	−	+	−	−
	Warmbaths 3	−	−	−	−	−	−
	9890 (1993)	−	−	−	−	−	+
Zimbabwe	T. p./BAL/25	−	−	−	+	+	+
	T. p./BAL/27	+	−	−	−	−	−
	T. p./BAL/93	−	−	−	+	−	−
	T. p./DOM/28	−	−	+	+	+	+
	T. p./MAS/37	+	−	−	−	+	+
	T. p./MAS/38	−	−	−	+	−	+
	T. p./MAS/39	−	−	+	+	+	+
	T. p./MAS/40	−	−	−	+	−	+

https://doi.org/10.1017/S0031182099004321 Published online by Cambridge University Press

larger and more diverse gene pool in buffalo (Koch *et al*. 1990; Bishop *et al*. 1994).

The probes gave no absolute distinction between all *T. p. parva* and *T. p. lawrencei* isolates, a predictable result in view of the sexual recombination which appears to take place between genotypes. We can, however, use the probes to assign a probability to the origin of a *T. parva* parasite. We see that 29 out of 31 *T. parva* isolates from cattle were detected by TPP1 while 19 out of 23 isolates from buffalo were detected by TPL2 and/or TPL3a. Extrapolation from these data suggests that a signal obtained with probe TPP1 indicates *T. p. parva* with a $> 90\%$ probability, while detection by probes TPL2 and/or TPL3a indicates *T. p. lawrencei* with a $> 80\%$ probability. There will be small numbers of isolates that react with both types of probes and it would not be possible to assign an origin to isolates such as these.

All of the isolates obtained from cattle in Zimbabwe were detected by probe TPP1, except for T.p./CHI/23. This anomaly is supported by other work, in which the same isolate has been shown, using anti-schizont monoclonal antibodies, to be similar to isolates from buffalo from elsewhere in Zimbabwe (Bishop *et al*. 1994). We obtained 3 ITS amplification products from T.p./CHI/23, which indicates that this is a mixed infection, and there is a strong suggestion that at least one component is a *T. p. lawrencei* genotype.

All of the South African *T. p. lawrencei* isolates from buffalo were detected by one or more of the *T. p. lawrencei* probes, and none of them by TPP1, which suggests that *T. p. parva* genotypes may not be carried by South African buffalo. This accords with the historic observation that East Coast fever was absent from South Africa until it was introduced from East Africa (Theiler, 1903). Obviously, however, a much larger number of samples needs examination in order to confirm this conclusion.

The trinomial system of classification of T. parva *parasites*

SSU rRNA sequence data are widely used for phylogenetic analysis (Maidak *et al*. 1997) but it is accepted that 'the resolving power of this method dissipates somewhere near the species level' (Wilson, 1995). Hence, the identity of the SSU rRNA genes of *T. p. parva* Muguga and the cloned *T. p. lawrencei* 7344 does not allow us to conclude whether or not they are different subspecies. The close similarity between the LSU rRNA genes of *T. p. parva* Muguga and *T. p. lawrencei* 7344 does, however, indicate that the two organisms are very closely related.

Subspecies of protozoa are commonly described on the basis of differences in host range, geographical distribution, pathological effects and epidemiological features. For example, *Trypanosoma brucei* is divided into three subspecies, namely *T. b. brucei*, a parasite solely of non-human animals in tropical Africa, *T. b. gambiense* which causes trypanosomiasis in humans in West and central Africa and *T. b. rhodesiense* which causes trypanosomiasis in humans in East Africa. Most *Trypanosoma brucei* isolates can be grouped into one of these subspecies by using isoenzyme analysis (Godfrey *et al*. 1990) although a small number cannot be grouped by these means. The continuum of types is therefore spread across different geographical areas, as well as across different sympatric hosts.

The situation in *T. parva* appears to be very similar, with the continuum of types spread across cattle and buffalo hosts but not geographically. *T. p. parva* appears to be a limited subpopulation of *T. parva* which is adapting to cattle as host, and in the process is diverging from the *T. p. lawrencei* population in buffalo. However, the divergence is currently limited by cattle and buffalo sympatry and sexual recombination of *T. parva* genotypes in the tick host which they share. Because the 2 populations are not separate gene pools absolute discrimination is not possible, but there are distinguishing features between most *T. p. parva* and most *T. p. lawrencei* parasites. In addition to the ITS sequence data described in this paper there are recognizable clinical differences between the diseases caused by the two parasites, and they react differently with certain monoclonal antibodies (Conrad *et al*. 1987, 1989). We believe, therefore, that *T. p. lawrencei* and *T. p. parva* are valid subspecies labels.

While most *T. p. lawrencei* isolates could be distinguished from *T. p. parva* and *T. p. bovis* isolates in this study, there was no pattern which distinguished between the latter two types. *T. p. parva* and *T. p. bovis* appear to represent subpopulations of *T. parva* which are both adapting to cattle as host and the differences between them may not be sufficient to distinguish them as separate subspecies. In distinction, the terms *T. p. parva* and *T. p. lawrencei* give a useful description of the majority of *T. parva* parasites and we recommend a general reversion to the use of these terms.

We thank Dr H. Stoltsz and Ms M. Dunsterville, Onderstepoort Veterinary Institute, Pretoria, South Africa, for the supply of *Theileria* cell culture material and blood samples. We are grateful to the International Livestock Research Institute, Nairobi, Kenya and Dr R. Bishop for the supply of *T. parva* DNA samples. This research was sponsored by the Agricultural Research Council of South Africa.

REFERENCES

ALLSOPP, B. A. & ALLSOPP, M. T. E. P. (1988). *Theileria parva*: genomic DNA studies reveal intraspecific sequence diversity. *Molecular and Biochemical Parasitology* **28**, 77–84.

ALLSOPP, B. A., BAYLIS, H. A., ALLSOPP, M. T. E. P., CAVALIER-SMITH, T., BISHOP, R. P., CARRINGTON, D. M., SOHANPAL, B. & SPOONER, P. (1993). Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. *Parasitology* **107**, 157–165.

ANON. (1981). The eradication of East Coast fever in South Africa. *Journal of the South African Veterinary Association* **52**, 71–73.

BANKIER, A. T., WESTON, K. M. & BARRELL, B. G. (1987). Random cloning and sequencing by the M13/dideoxynucleotide chain termination method. *Methods in Enzymology* **155**, 51–93.

BISHOP, R. P., SPOONER, P. R., KANHAI, G. K., KIARIE, J., LATIF, A. A., HOVE, T., MASAKA, S. & DOLAN, T. T. (1994). Molecular characterization of *Theileria* parasites: application to the epidemiology of theileriosis in Zimbabwe. *Parasitology* **109**, 573–581.

BROCKLESBY, D. W., BARNETT, S. F. & SCOTT, G. R. (1961). Morbidity and mortality rates in East Coast fever (*Theileria parva* infection) and their application to drug screening procedures. *British Veterinary Journal* **117**, 529–531.

CHURCH, G. M. & GILBERT, W. (1984). Genomic sequencing. *Proceedings of the National Academy of Sciences, USA* **81**, 1991–1995.

CONRAD, P. A., OLE-MOI YOI, O. K., BALDWIN, C. L., DOLAN, T. T., O'CALLAGHAN, C. J., NJAMUNGGEH, R. E. G., GROOTENHUIS, J. G., STAGG, D. A., LEITCH, B. L. & YOUNG, A. S. (1989). Characterization of buffalo-derived theilerial parasites with monoclonal antibodies and DNA probes. *Parasitology* **98**, 179–188.

CONRAD, P. A., STAGG, D. A., GROOTENHUIS, J. G., IRVIN, A. D., NEWSON, J., NJAMUNGGEH, R. E. G., ROSSITER, P. B. & YOUNG, A. S. (1987). Isolation of *Theileria* parasites from African buffalo (*Syncerus caffer*) and characterization with anti-schizont monoclonal antibodies. *Parasitology* **94**, 413–423.

GERBI, S. A. (1986). The evolution of eukaryotic ribosomal DNA. *BioSystems* **19**, 247–258.

GODFREY, D. G., BAKER, R. D., RICKMAN, L. R. & MEHLITZ, D. (1990). The distribution, relationships and identification of enzymic variants within the subgenus *Trypanozoon*. *Advances in Parasitology* **29**, 1–43.

KARIUKI, T. M., GROOTENHUIS, J. G., DOLAN, T. T., BISHOP, R. P. & BALDWIN, C. L. (1990). Immunization with *Theileria parva* parasites from buffaloes results in generation of cytotoxic T cells which recognize antigens common among cells infected with stocks of *T. parva parva*, *T. parva bovis* and *T. parva lawrencei*. *Infection and Immunity* **58**, 3574–3581.

KIBE, M. K., OLE-MOI YOI, O. K., NENE, V., KHAN, B., ALLSOPP, B. A., COLLINS, N. E., MORZARIA, S. P., GOBRIGHT, E. I. & BISHOP, R. P. (1994). Evidence for two single copy units in *Theileria parva* ribosomal RNA genes. *Molecular and Biochemical Parasitology* **66**, 249–259.

KOCH, H. T., KAMBEVA, L., OCAMA, J. G. R., MUNATSWA, F. C., FRANSSEN, F. F. J., UILENBERG, G., DOLAN, T. T. & NORVAL, R. A. I. (1990). Immunization of cattle against *Theileria parva bovis* and their exposure to natural challenge. *Veterinary Parasitology* **37**, 185–196.

LAWRENCE, J. A. (1979). The differential diagnosis of the bovine theilerias of southern Africa. *Journal of the South African Veterinary Association* **50**, 311–313.

MAIDAK, B. L., OLSEN, G. J., LARSEN, N., OVERBEEK, R., MCCAUGHEY, M. J. & WOESE, C. R. (1997). The RDP (ribosomal database project). *Nucleic Acids Research* **25**, 109–111.

MAROTEAUX, L., HERZOG, M. & SOYER-GOBILLARD, M.-O. (1985). Molecular organisation of dinoflagellate ribosomal DNA: evolutionary implications of the deduced 5·8S rRNA secondary structure. *BioSystems* **18**, 307–319.

MEDLIN, L., ELWOOD, H. J., STICKEL, S. & SOGIN, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene* **71**, 491–499.

MORZARIA, S. P., YOUNG, J. R., SPOONER, P. R., DOLAN, T. T., YOUNG, A. S. & BISHOP, R. P. (1992). Evidence of a sexual cycle in *Theileria parva* and characterisation of the recombinants. In *First International Conference on Tick-Borne Pathogens – An Agenda for Research* (ed. Munderloch, U. G. & Kurtti, T. J.), pp. 71–74. Saint Paul, Minnesota, USA.

MORZARIA, S. P., DOLAN, T. T., NORVAL, R. A. I., BISHOP, R. P. & SPOONER, P. R. (1995). Generation and characterization of cloned *Theileria parva* parasites. *Parasitology* **111**, 39–49.

NAZAR, R. N. (1984). The ribosomal 5·8S RNA: eukaryotic adaptation or processing variant? *Canadian Journal of Biochemistry and Cell Biology* **62**, 311–320.

NEITZ, W. O. (1948). Studies on East Coast fever. *South African Science* **1**, 133–135.

NORVAL, R. A. I., PERRY, B. D. & YOUNG, A. S. (1992). *The Epidemiology of Theileriosis in Africa*. Academic Press, London.

ROGERS, M. J., MCCONKEY, G. A., LI, J. & MCCUTCHAN, T. F. (1995). The ribosomal DNA loci in *Plasmodium falciparum* accumulate mutations independently. *Journal of Molecular Biology* **254**, 881–891.

SAIKI, R. K., BUGAWAN, T. L., HORN, G. T., MULLIS, K. B. & ERLICH, H. A. (1986). Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. *Nature, London* **324**, 163–166.

SAIKI, R. K., GELFAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B. & EHRLICH, H. A. (1988). Primer-directed amplification of DNA with a thermostable polymerase. *Science* **239**, 487–491.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.

SMITH, S. (1992). *Genetic Data Environment, version 2.0 : Documentation*. Harvard University and University of Illinois.

SOGIN, M. L. (1990). Amplification of ribosomal RNA genes for molecular evolution studies. In *PCR Protocols : A Guide to Methods and Applications* (ed. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J.), pp. 307–314. Academic Press, San Diego.

THEILER, A. (1903). The Rhodesian tick fever. *Transvaal Agricultural Journal* **1**, 93–110.

THOMPSON, J. D., HIGGINS, D. G. & GIBSON, T. J. (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through

sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

UILENBERG, G. (1976). Tick-borne livestock diseases and their vectors. 2. Epizootiology of tick-borne diseases. *World Animal Review* **17**, 8–15.

VAN KEULEN, H., HORVAT, S., ERLANDSEN, S. L. & JARROLL, E. L. (1991). Nucleotide sequence of the 5·8S and large subunit rRNA genes and the internal transcribed spacer and part of the external spacer from *Giardia ardeae*. *Nucleic Acids Research* **19**, 6050.

WILSON, K. H. (1995). Molecular biology as a tool for taxonomy. *Clinical Infectious Diseases* **20** (Suppl.), S117–S121.