CHAPTER 5

Characterization of *Theileria parva* parasites occurring in buffalo (*Syncerus caffer)* in South Africa: In search of cattle-type p104 alleles

For the LORD giveth wisdom: out of his mouth cometh knowledge and understanding. ***Proverbs 2:6***

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"All truths are easy to understand once they are discovered; the point is to discover them." ***Galileo Galilei***
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5.1 Abstract

A recent study on characterization of South African *T. parva* field samples, based on the p67 gene, revealed the presence of an allele associated with parasites responsible for East Coast fever (ECF) in East Africa. This finding has increased concerns about the possibility of the recurrence of ECF in South Africa, and necessitated further characterization of *T. parva* parasites occurring in South African buffalo in search of cattle-type alleles. PCR-RFLP and sequencing of the variable region of the p104 gene was performed on samples obtained from South African *T. parva* parasites originating from cattle on farms with suspected theileriosis and from buffalo. RFLP profile analysis using BioNumerics revealed two major cluster groups correlating broadly with geographic origin; group A consisted primarily of samples from Hluhluwe-iMfolozi Game Park while group B consisted largely of samples from the Kruger National Park. Three subgroups were identified within each main group. In subgroup A2 within group A, PCR-RFLP profiles from cattle samples which came from a farm in Ladysmith grouped with *T. parva* Muguga, a cattle-derived stock responsible for ECF in East Africa. p104 nucleotide sequences and inferred amino acid sequences obtained from these samples were identical to that of Muguga in the region analyzed, confirming the cluster analysis results. While these results suggest the presence of a cattle-derived *T. parva* parasite, reports of cattle-to-cattle transmission could not be substantiated and ECF was not diagnosed on this farm. p104 sequences from other cattle samples grouped with samples obtained from buffalo suggesting that these parasite populations may have originated from buffalo. A p104 sequence similar to that of allele 1 (*T. parva* Muguga) was obtained from a buffalo sample from KNP. Although alleles 2 (from the cattle-derived *T. parva* Marikebuni) and 3 (from *T. parva* Boleni) grouped in sub-clade A2, none of the p104 sequences obtained in this study grouped closely with either of these alleles. Sequences obtained from three buffalo *T. parva* samples from KNP grouped closely with the p104 allele 4 sequence from the buffalo-derived isolate *T. parva* 7014. Since its eradication, ECF has not been reported in South Africa; the significance of the different p104 alleles in the epidemiology of cattle theileriosis in South Africa is not clear and therefore still needs to be established.

5.2 Introduction

Cattle theileriosis caused by *Theileria parva* is a disease of major economic importance in eastern, central and southern Africa (Young *et al*., 1988). Infections of cattle by *T. parva* parasites result in three recognized disease syndromes, East Coast fever (ECF), January disease and Corridor disease. The natural tick vectors of *T. parva* in southern Africa include *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* (Lawrence *et al*., 1983; Lessard *et al*., 1990). *Rhipicephalus appendiculatus* is widespread in South Africa, and, in the presence of *T. parva*-infected buffalo, the vector ticks can transmit the parasite to naïve cattle resulting in Corridor disease. Corridor disease remains an important form of theileriosis in South Africa where it is a controlled disease. No cases of ECF have been reported in South Africa since its eradication between 1946 and 1955 (Anonymous, 1981). It is not known whether ECF-causing *T. parva* parasites were transmitted to South African buffalo during the ECF epidemic or if there exists in buffalo an ancestral subpopulation of *T. parva* parasites that could become adapted to cattle.

In a recent study, size differentiation and sequence variation of the central region of the *T. parva* antigen gene, p67, were used to characterize South African *T. parva* field samples (Chapter 4; Sibeko *et al*., 2010). A p67 allele (allele 1) identical to that of Muguga, a *T. parva* stock from Kenya which causes ECF, was obtained from cattle samples from Ladysmith. This finding is of concern to the cattle industry in South Africa, since Potgieter *et al*. (1988) showed that buffalo-derived *T. parva* parasites causing Corridor disease can be maintained by passage between cattle and the tick vector. Presumably the persistence of *T. parva* carrier cattle in South Africa could eventually result in the selection of *T. parva* parasites adapted to cattle. Therefore there is continuous concern that ECF could re-emerge and a serious need to establish if there are cattle-type *T. parva* parasites in buffalo in South Africa.

A range of assays has been developed and used in several studies to differentiate between cattle- and buffalo-derived *T. parva* stocks (Minami *et al.*, 1983; Allsopp *et al*., 1989; Conrad *et al*., 1989; Bishop *et al.*, 1993; Collins and Allsopp, 1999). Size polymorphisms displayed by *T. parva* antigen genes, PIM, p104, p150 and p67, have been used to develop several molecular tools for characterization of *T. parva* stocks, exploiting the variable regions of these genes (Geysen *et al*., 1999; Bishop *et al*., 2001). PCR-RFLP analysis using these antigen genes demonstrated polymorphism in field stocks of *T. parva* in Kenya, although the majority of field stocks isolated from two regions of Zambia were relatively homogeneous (Geysen

et al., 1999). Recently, mini- and micro-satellite markers have been developed for characterizing *T. parva* stocks which enable detection of higher levels of polymorphism than PCR-RFLP methods (Oura *et al.,* 2003; 2005). However, the use of microsatellite markers is limited to parasite clones and cannot be directly used to characterize field samples, which usually contain complex mixtures of multiple *T. parva* strains.

The gene coding for the p104 antigen was selected for PCR-RFLP analysis since there is limited polymorphism in this gene, especially amongst cattle-type alleles, thus allowing distinction of buffalo-type from cattle-type alleles (Geysen *et al*., 1999). *Theileria parva* field samples obtained from buffalo often comprise multiple strains of *T. parva* parasites, which complicate characterization by PCR-RFLP. To overcome this challenge, p104 PCR products were cloned and sequenced.

5.3 Materials and methods

5.3.1 Sample collection

Blood samples were collected in EDTA vaccutainer tubes from buffalo from three game parks, and cattle from farms where cattle theileriosis was suspected, in South Africa. The *T. parva*-specific real-time PCR assay was used to screen for *T. parva*-positive samples using the reaction and cycling conditions described previously (Chapter 3; Sibeko *et al*., 2008). A total of 100 *T. parva*-positive samples, including 91 buffalo and nine cattle samples (Table 5.1), were investigated. In addition to these, two DNA samples from cattle-derived *T. parva* stocks, Muguga (Brocklesby *et al*., 1961) and Katete (Geysen, 2000), from Kenya and Zambia, respectively, were also analysed as reference samples.

5.3.2 DNA isolation

The High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) was used for extraction of DNA from 200 µl of EDTA blood samples. DNA extractions were performed according to the method described by the kit manufacturers, except that DNA was eluted in 100 µl elution buffer. DNA was stored at 4°C until further analysis.

5.3.3 Analysis of the p104 gene from *T. parva* samples using PCR-RFLP

The *Theileria* semi-nested p104 PCR-RFLP was performed as described by De Deken *et al.* (2007). Briefly, the variable region of the p104 gene was amplified using primers p104F2 (5'-CCA CCA TCT CCT AAA CCA CCG TT-3') and p104R (5'-TAA GAT GCC GAC TAT TAA TGA CAC CAC AA-3') for the primary PCR, and primers p104nF (5'-ACC ACC GTT TGA TCC ATC ATT CA-3') and p104R for the secondary PCR. Five microlitres of genomic DNA was used in a 25 µl amplification reaction for the primary PCR, and 0.5 µl of the primary PCR product was used as a template for the secondary PCR, using the reaction and cycling conditions previously described (De Deken *et al.*, 2007). Amplicons were digested overnight with the restriction enzyme *Alu*I and the digested products were separated on a 10% polyacrylamide gel before DNA detection by SYBR® green (SIGMA-ALDRICH, USA) for RFLP analysis.

Table 5.1 Geographic origin and source of blood samples (n=100) used for characterization of *T. parva* parasites

Geographical location	Province	Sample Name*	Source of blood sample	Year of collection/ Reference
Hluhluwe-iMfolozi Park (n=39)	KwaZulu-Natal	HIP 1, HIP 3, HIP 4, **HIP 5**, HIP 6, HIP 7, HIP 8, HIP 9, HIP 10, HIP 11, HIP 12, HIP 13, HIP 14, HIP 15, HIP 16, HIP 18, **HIP 19**, HIP 20, HIP 21, HIP 22, HIP 23, HIP 24, HIP 25, HIP 26, HIP 27, HIP 28, HIP 29, HIP 30, HIP 31, HIP 32, HIP 33, HIP 34, HIP 35, HIP 36, HIP 37, HIP 39, HIP 40, HIP 41, HIP 42	Buffalo	2004
Kruger National Park (n=46)	Mpumalanga	KNP 47, KNP 48, KNP 49, KNP 50, KNP 61, KNP 62, **KNP 63,** KNP 66, KNP 67, KNP 68, **KNP 102,** KNP AA5, **KNP AB47**, KNP AD3, KNP B2, **KNP B10**, KNP B22, KNP D11, KNP D24, KNP E7, KNP E18, KNP E20, KNP F9, KNP G2, KNP G11, KNP H8, KNP H15, KNP I23, **KNP L6**, KNP L27, KNP M2, KNP M12, KNP N1, KNP N8, KNP O1, KNP O11, **KNP P7,** KNP S17, KNP U3, KNP U20, KNP V5, **KNP W8**, KNP X4**, KNP Y4**, KNP Y19, KNP Z4	Buffalo	2003
Ladysmith (n=6)	KwaZulu-Natal	Lad 2, Lad 6, **Lad 10**, Lad 11, **Lad 15, Lad 17**	Bovines	2003
Mabalingwe Game Reserve (n=6)	Limpopo	**Mab A13**, **Mab BB43**, Mab B21**, Mab BB37**, Mab A22, **Mab BB38**	Buffalo	2004
Lydenburg (n=1)	Mpumalanga	Lyd N254	Bovine	2004
Bloemfontein (n=1)	Free-State	**Bloe B**	Bovine	2004
Schoonspruit (n=1)	Mpumalanga (former Transvaal)	**Schoonspruit**	Bovine	Neitz (1948), Collins (1997)

Samples shown in bold were used for sequencing

5.3.4 PCR-RFLP profile analysis

PCR-RFLP patterns were analysed using BioNumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium). Normalization of the RFLP profiles was done using the molecular weight marker 100 bp DNA Ladder (Fermentas Life Sciences, Germany), which was run in two lanes per gel of fifteen wells. DNA fragments of less than 100 bp were excluded from the analysis as their size could not be estimated accurately using the 100 bp DNA ladder; moreover, in some instances these fragment had run out of the gel. The software was used to calculate Dice coefficients of similarity, to cluster the RFLP profiles and to generate dendrograms by the unweighted-pair group method using average linkages (UPGMA). The most appropriate settings for optimization and tolerance, as determined by the software, were calculated. Samples with similar RFLP profiles obtained from different animals were defined as clusters.

5.3.5 Cloning and sequencing of p104 PCR products

PCR products from 19 *T. parva* samples, representative of each cluster group, were selected for cloning and sequencing (shown in bold in Table 5.1). PCR products were cloned into the pCR®2.1-TOPO® cloning vector (Invitrogen, Carlsbad, USA). The presence of inserts in recombinants was confirmed by colony PCR following the secondary PCR protocol used above. At least four clones were sequenced from each sample; sequencing was performed by the Genetic Service Facility of the University of Antwerp in Belgium.

5.3.6 Sequence analysis

Sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield *et al*., 1995; Staden, 1996; Staden *et al*., 2000). A multiple sequence alignment of p104 amino acid sequences was performed using MAFFT version 6 (Katoh *et al*., 2002) (http://align.bmr.kyushu-u.ac.jp/mafft/software), and maximum parsimony and Bayesian analyses were used to produce phylogenetic trees. The maximum parsimony analysis was performed in PAUP*4.0b10 (Swofford, 2003) with 1000 random addition sequence followed by bisection-reconnection (TBR) branch swapping and branch support was assessed with 100 bootstrap replicates. The Bayesian analysis was performed using MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003). A Markov chain Monte Carlo run of five million generations consisting of four parallel MCMC chains was performed.

5.4 Results

5.4.1 p104 PCR-RFLP profile analysis

A p104 PCR product of ~800 bp in size was obtained from all samples analyzed in this study (results not shown). From visual analysis, cattle samples, Lad 02, Lad 06, Lad 10 (from Ladysmith) and *T. parva* Schoonspruit had an identical PCR-RFLP profile to that of *T. parva* Muguga, a stock from Kenya responsible for ECF (Figure 5.1).

The cluster analysis using BioNumerics revealed two major groups, A and B, and the clustering correlated broadly with geographic origin of the samples (Figures 5.2 and 5.3). Group A consisted mainly of samples obtained from Hluhluwe-iMfolozi Game Park (39/49, 79.6%) and group B consisted largely of samples from Kruger National Park (KNP) (42/51, 82.4%), although KNP samples were also present in group A. Three subgroups were identified in each of the two major groups. Subgroup A1 consisted mainly of samples from Hluhluwe-iMfolozi while samples from Ladysmith and KNP appeared in subgroups A2 and A3. The majority of samples (42/50, 84%) in subgroups B1, B2 and B3 came from KNP. Samples from Hluhluwe-iMfolozi produced a relatively homogeneous fingerprint, with most (32/39, 82%) appearing in subgroup A1, while those from KNP buffalo samples were heterogeneous and appeared in all six fingerprint groups (Figures 5.2 and 5.3). The p104 RFLP profiles from four cattle samples, Lad 02, Lad 06, Lad 10 and *T. parva* Schoonspruit, with profiles similar to that of *T. parva* Muguga from visual inspection, grouped with *T. parva* Muguga on the dendrogram (Figures 5.2 and 5.3). These cattle samples clustered in group A amongst p104 profiles from buffalo samples from Hluhluwe-iMfolozi (Figures 5.2 and 5.3). Other cattle samples, Lad 11, Lad 15, Lad 17, Bloe B and Lyd N254 grouped with samples in group B which contained mainly buffalo samples from KNP. The *T. parva* Muguga-like cattle-type p104 fingerprints were not obtained from any of the buffalo samples analyzed in this study.

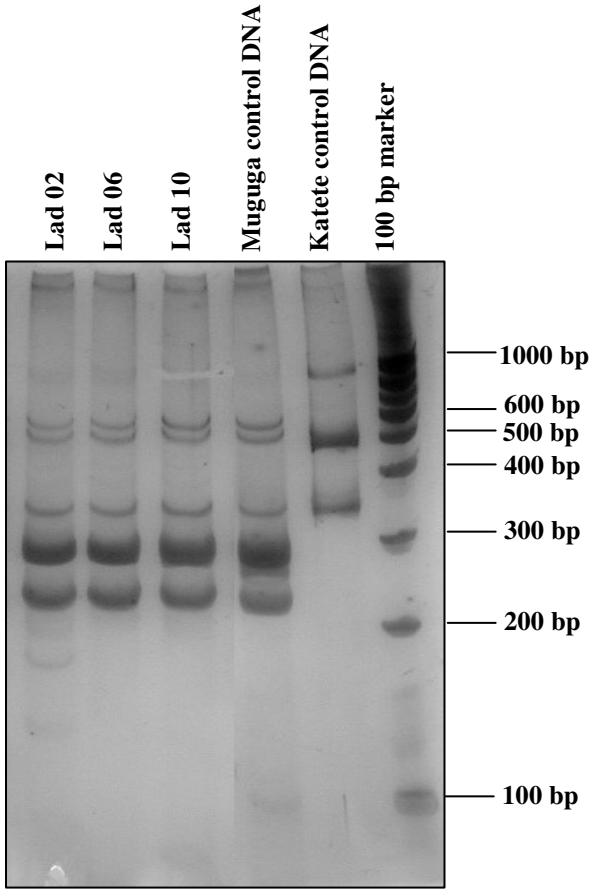


Figure 5.1 p104 gene *Alu*I RFLP profiles showing the *T. parva* Muguga RFLP profile and identical profiles obtained from cattle samples from a farm near Ladysmith.

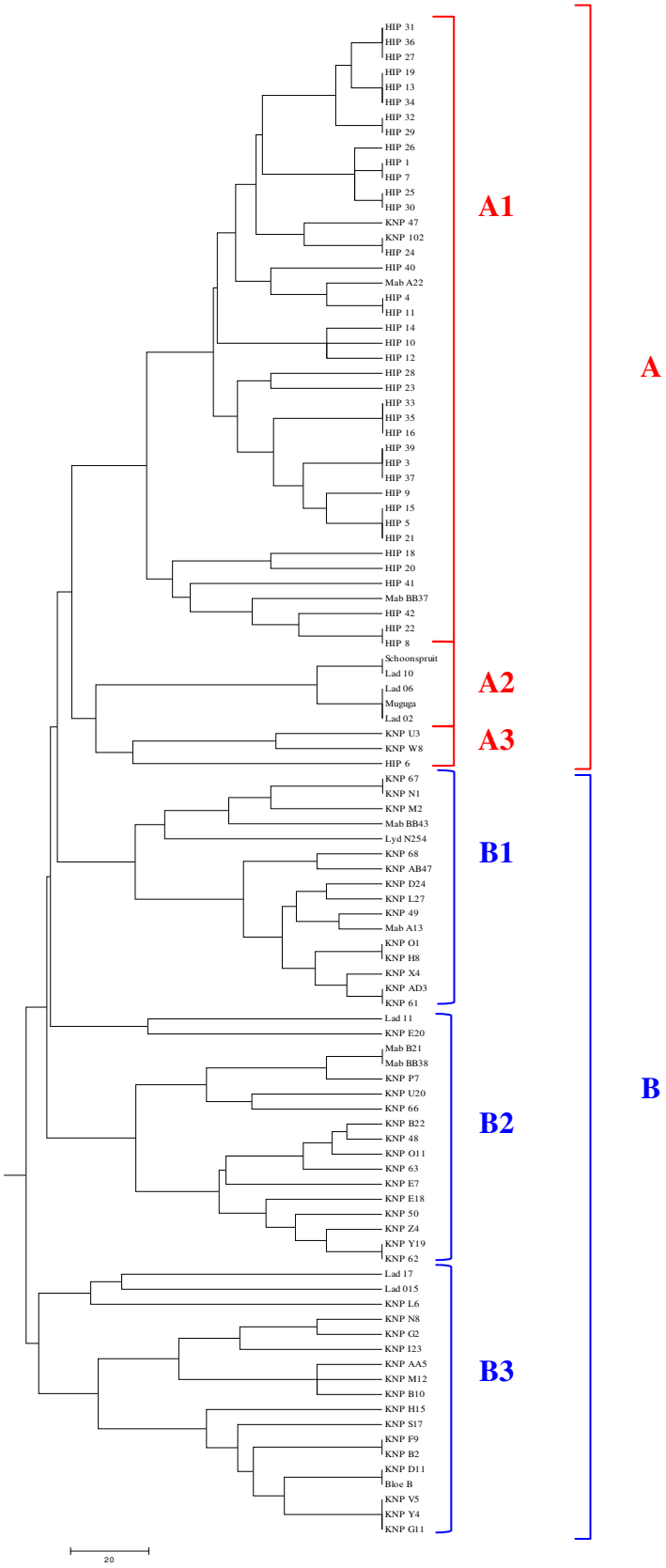


Figure 5.2 A simplified similarity dendrogram for p104 PCR-RFLP profiles cluster analysis based on the Dice Coefficient analysis produced using BioNumerics v5.1.

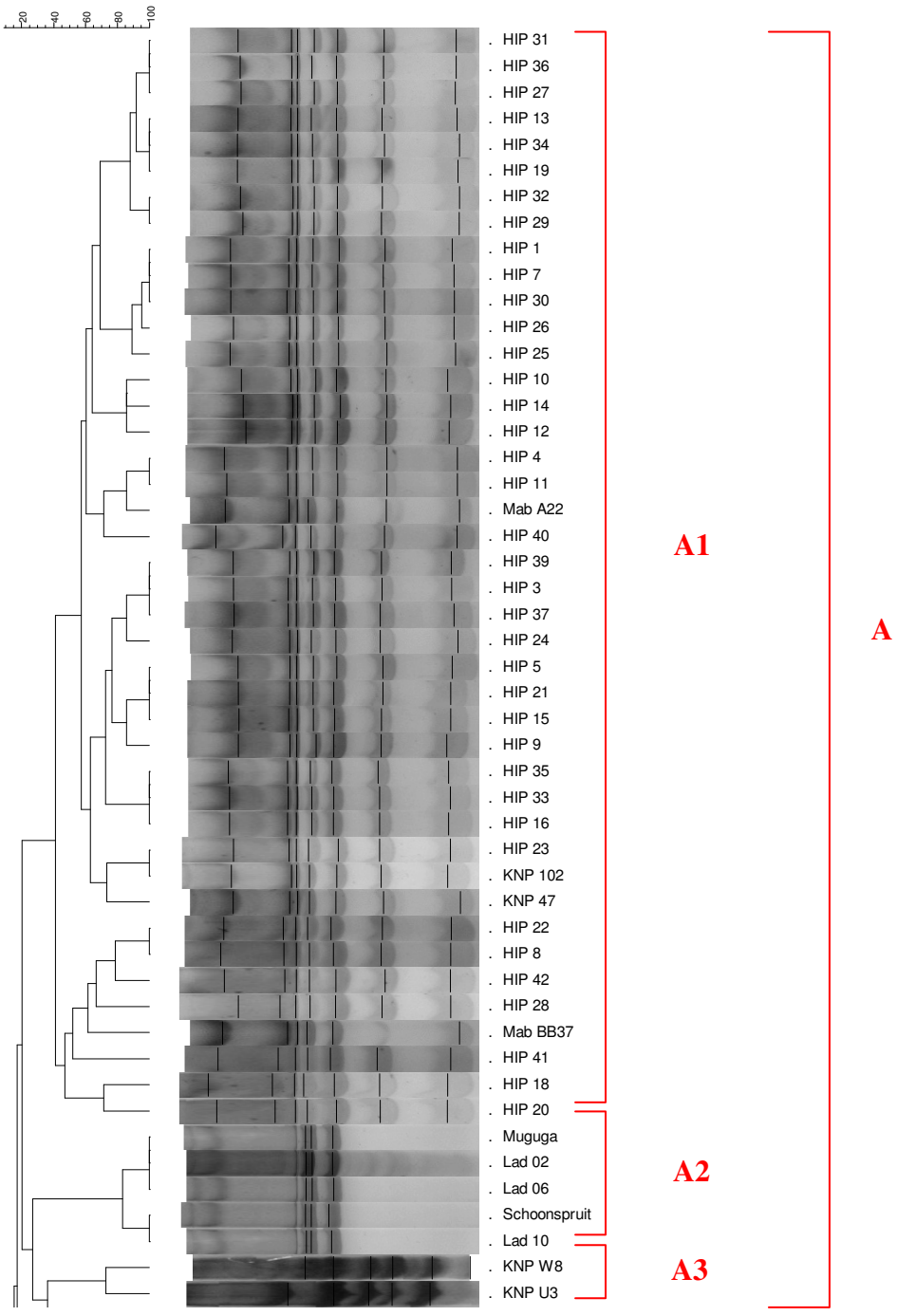
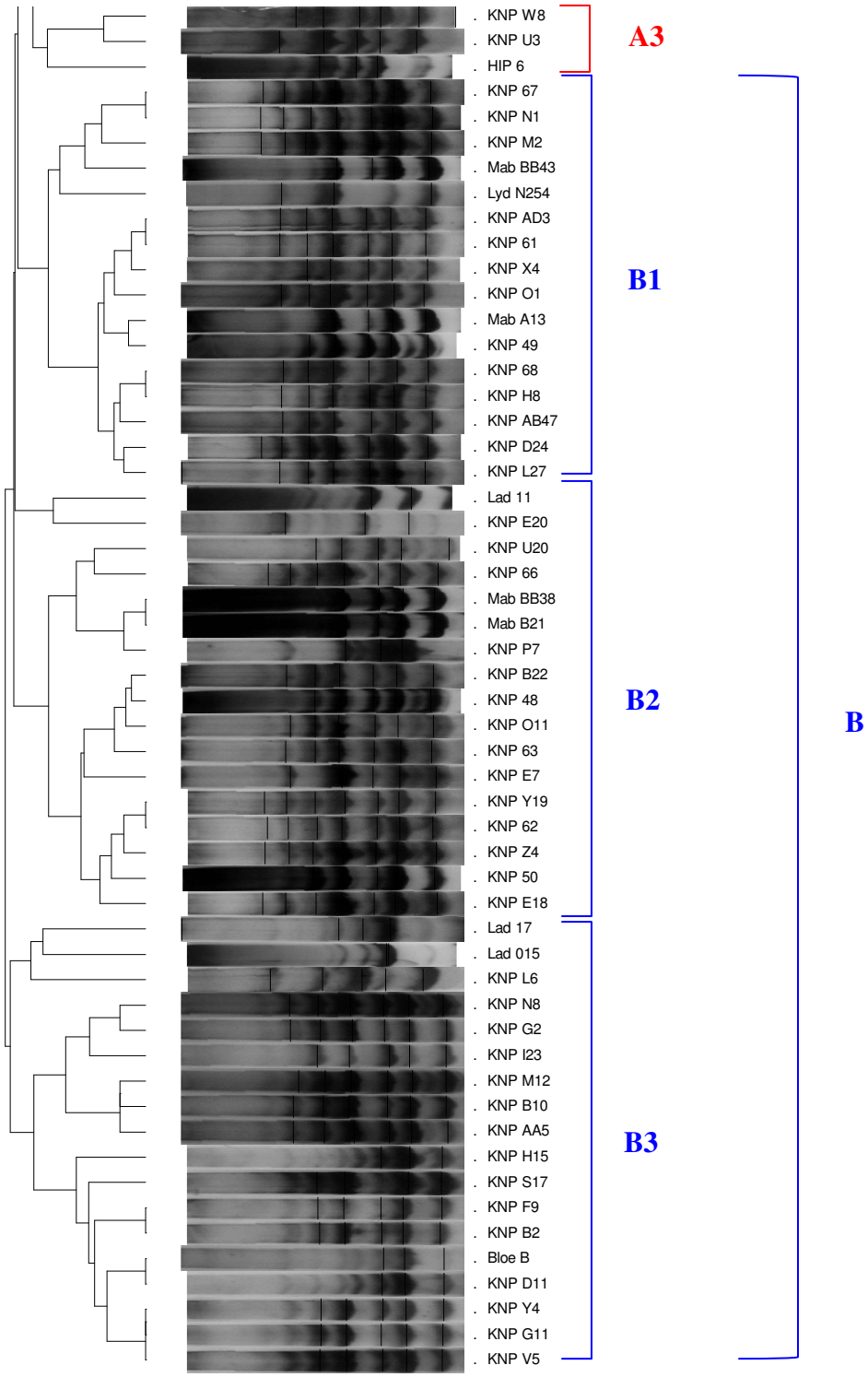


Figure 5.3 A detailed similarity dendrogram for p104 PCR-RFLP profiles cluster analysis based on the Dice Coefficient analysis produced using BioNumerics v5.1 showing actual profiles used to create the dendrogram. Figure 5.3 continues on page 110.


KNP W8
KNP U3
HIP 6
KNP 67
KNP N1
KNP M2
Mab BB43
Lyd N254
KNP AD3
KNP 61
KNP X4
KNP O1
Mab A13
KNP 49
KNP 68
KNP H8
KNP AB47
KNP D24
KNP L27
Lad 11
KNP E20
KNP U20
KNP 66
Mab BB38
Mab B21
KNP P7
KNP B22
KNP 48
KNP O11
KNP 63
KNP E7
KNP Y19
KNP 62
KNP Z4
KNP 50
KNP E18
Lad 17
Lad 015
KNP L6
KNP N8
KNP G2
KNP I23
KNP M12
KNP B10
KNP AA5
KNP H15
KNP S17
KNP F9
KNP B2
Bloe B
KNP D11
KNP Y4
KNP G11
KNP V5
A3
B1
B2
B3
B

5.4.2 p104 gene sequence analysis

To confirm results obtained by RFLP profile cluster analysis and to establish p104 alleles that occur in the different subgroups, PCR products from representatives of each cluster group were cloned and sequenced. Fifty-three good quality p104 sequences were obtained from clones produced from amplicons of the 19 selected *T. parva* samples. Except for two, the p104 amino acid sequences obtained from *T. parva* field samples analyzed in this study were not identical to the p104 sequences previously reported by Skilton *et al*. (2002) although they were very similar with sequence identities raging from 92-99% in the region analyzed. Skilton *et al*. (2002) identified four p104 alleles representing p104 amino acid sequences obtained from different *T. parva* stocks of cattle and buffalo in East Africa. According to Skilton *et al*. (2002) allele 1 (accession number: M2954) represents the *T. parva* Muguga p104 amino acid sequence (Iams *et al*., 1990); allele 2 (accession number: AY034069) is found in the Marikebuni and Uganda *T. parva* stocks and alleles 3 (accession number: AY034070) and 4 (accession number: AY034071) represent the *T. parva* Boleni and 7014 p104 amino acid sequences, respectively. Muguga, Marikebuni, Uganda and Boleni are cattle-derived *T. parva* stocks whereas 7014 is a buffalo-derived *T. parva* stock.

Both maximum parsimony and Bayesian analyses produced similar groupings of p104 sequences; however, the maximum parsimony analysis did not resolve the differences within groups containing alleles 2 and 3. Therefore only the phylogenetic tree based on Bayesian analysis is presented (Figure 5.4). Of the 53 South African p104 sequences, one sequence from the buffalo *T. parva* sample MabA13 (clone 6) had a deletion of 17 amino acids in the region analysed. The p104 sequences obtained in this study could be grouped into two main clades, A and B, and each of the major clades could be divided into two sub-clades, A1 and A2, and B1 and B2 (Figure 5.4). Alleles 2 and 3 grouped in sub-clade A2 and alleles 1 and 4 in sub-clade B1. Two p104 amino acid sequences obtained from samples collected from cattle grouped closely to the *T. parva* Muguga p104 sequence and both had sequence identity of 100% (Lad10 and *T. parva* Schoonspruit) to that of the *T. parva* Muguga p104 amino acid sequence. In addition to these, a p104 sequence obtained from a buffalo *T. parva* sample, KNP B10_3.0.2, had 97% sequence identity to that of *T. parva* Muguga and grouped closely to p104 sequences from Lad10, *T. parva* Schoonspruit and *T. parva* Muguga in sub-clade B1. Apart from *T. parva* Katete, no sequences grouped closely to allele 2; similarly no sequences grouped closely with allele 3. Sequence identities of 96-97% were obtained from sequences that grouped closely with p104 allele 4 (KNP102 8 3, KNPW8 8.0.2, KNPAB47 8.0.1).

The groups obtained from PCR-RFLP cluster analysis did not correlate with the clades identified by phylogenetic analysis of p104 amino acid sequences (Table 5.2). Samples with sub-clade B1 p104 sequences were found in almost all RFLP subgroups. Unfortunately, DNA from samples of reference sequences was not available for PCR-RFLP analysis and it was therefore not possible to determine where the p104 profiles from these samples would fit in the cluster analysis.

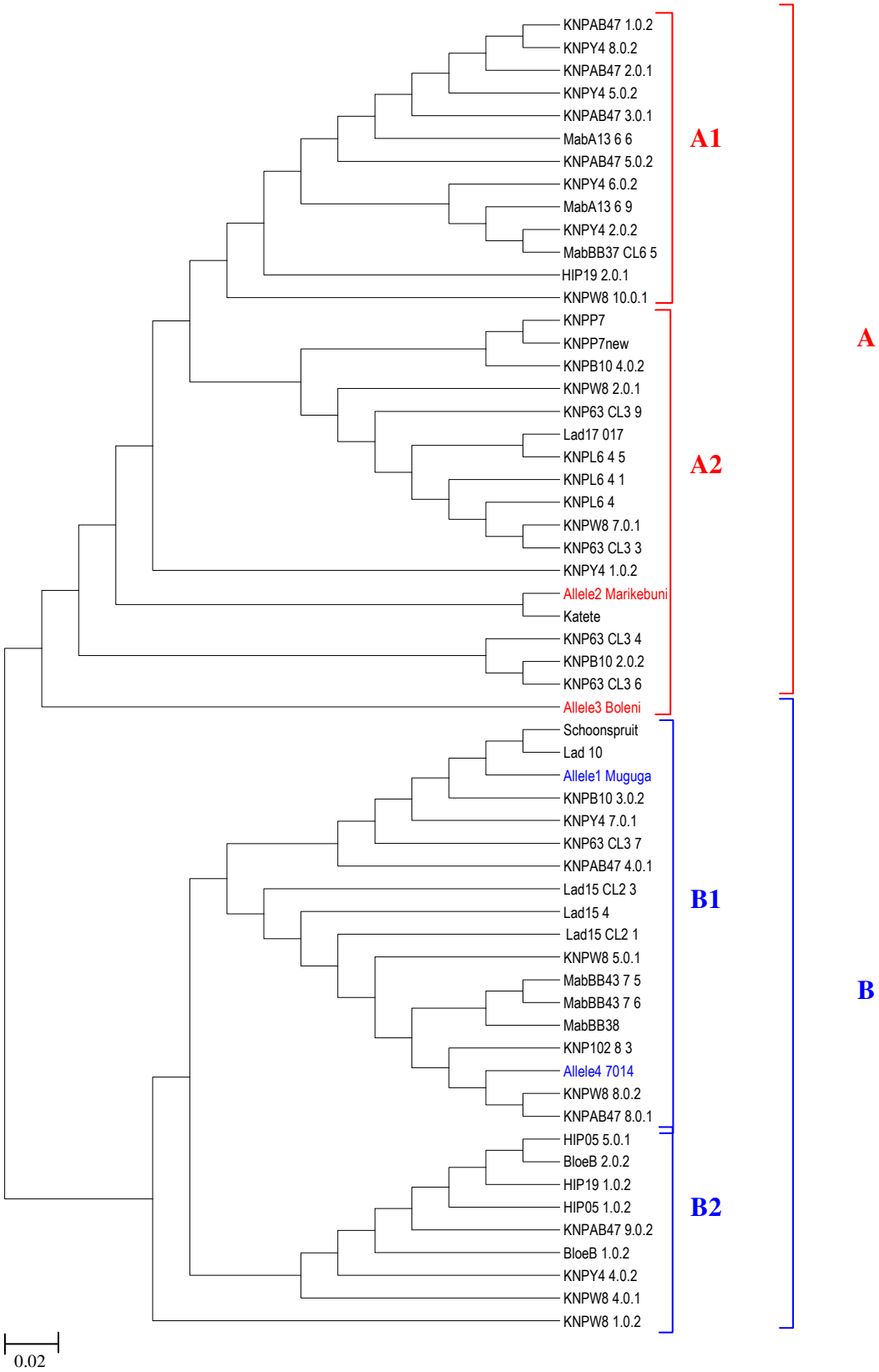


Figure 5.4 Phylogenetic relationship of *T. parva* strains as revealed by p104 amino acid sequence analysis. The phylogenetic tree was calculated by Bayesian analysis; the first 10,000 trees were discarded as burnin and the majority-rule consensus tree was generated.

Table 5.2 Comparison of p104 PCR-RFLP cluster analysis and phylogenetic analysis of p104 amino acid sequences from 19 selected *T. parva* samples

Major PCR-RFLP cluster group	PCR-RFLP cluster subgroup	Sample name	p104 phylogenetic analysis sub-clade
A	A1	HIP 5	B2
		HIP 19	A1, B2
		KNP 102	B1
		Mab BB37	A1
	A2	**Lad 10**	B1
		Schoonspruit	B1
	A3	KNP W8	A1, A2, B1, B2
B	B1	Mab BB43	B1
		KNP AB47	A1, B1, B2
		Mab A13	A1
	B2	KNP P7	A2
		Mab BB38	B1
		KNP 63	A2, B1
	B3	**Lad 15**	B1
		Lad 17	A2
		Bloe B	B2
		KNP B10	A2, B1
		KNP L6	A2
		KNP Y4	A1, A2, B1, B2

Samples shown in bold were obtained from cattle

5.5 Discussion

In South Africa, ECF, caused by the blood parasite *T. parva* was eradicated in 1954 and there have been no reports of ECF since then (Anonymous, 1981). However, the tick vector *R. appendiculatus* and the parasite that causes Corridor disease still occur and therefore this disease is a controlled disease in South Africa. It is not clear if the *T. parva* parasites that cause ECF existed in buffalo before the outbreak of ECF or if these parasites were introduced with the livestock imported from East Africa. This study was performed to establish if cattle-type *T. parva* p104 alleles exist in buffalo in South Africa.

Cluster analysis of *T. parva* p104 RFLP profiles revealed clustering correlating broadly with geographic origin. Samples from Hluhluwe-iMfolozi produced an apparently homogeneous fingerprint, and appeared in the same subgroup (A1), while those from KNP buffalo samples were heterogeneous and appeared in all six subgroups. This finding could suggest that there is a less diverse population of *T. parva* parasites circulating in the Hluhluwe-iMfolozi game park as evidenced by the relatively homogeneous RFLP profiles, while the heterogenous

profiles obtained from *T. parva* samples from buffalo from KNP might be evident of a more diverse population.

The phylogenetic analysis of p104 amino acid sequences revealed two major clades, A and B, with sub-clades A1 and A2, and B1 and B2, respectively. p104 alleles 2 (*T. parva* Marikebuni) and 3 (*T. parva* Boleni) grouped in sub-clade A2. Similarly, alleles 1 (*T. parva* Muguga) and 4 (*T. parva* 7014) grouped in sub-clade B1. From this analysis, allele 1 and allele 4 seem to have evolved from a common ancestor. A separate speciation event appears to have given rise to the cattle-type p104 allele in the cattle-derived *T. parva* isolates Marikebuni and Katete. These results support data obtained from analysis of the p67 gene (Chapter 4; Sibeko *et al*., 2010), and provide further evidence to suggest that *T. parva* was originally a buffalo parasite, and that strains that circulate in cattle are likely to originate from buffalo. Novel p104 sequences which were not similar to known p104 alleles were identified in sub-clades A1 and B2. It is not clear if sequences in these two sub-clades are variants of the known sequences; analysis of the complete p104 gene sequence from alleles 1, 2, 3 and 4 and 'new' alleles would be required for comparison studies to confirm this.

There was no significant correlation between data obtained from PCR-RFLP cluster analysis and the phylogeny obtained from sequence analysis. This is probably because field samples usually contain mixed infections. The PCR-RFLP cluster analysis therefore represents an overall p104 profile of all the *T. parva* strains in the sample, whereas each p104 sequence represents a single *T. parva* strain. Therefore, with mixed infections, several individual sequences can be obtained from clones from a single sample and these can be distributed in different clades. For example, p104 sequences obtained from clones prepared from KNPW8 (a buffalo sample) occurred in all different clades on the phylogenetic tree. Furthermore, in cases of mixed infections, PCR-RFLP cluster groups would be overwhelmed by profiles of dominant parasites, whereas profiles from less dominant parasites, which will probably not be apparent on the profile, might have grouped differently. The PCR-RFLP cluster analysis of profiles produced from clones would probably give a different picture and would be more likely to correlate better with the sequence data analysis.

Three cattle samples from a farm in Ladysmith and *T. parva* Schoonspruit had a cattle-type *T. parva* p104 RFLP profile identical to that of *T. parva* Muguga, and the p104 amino acid sequences obtained from Lad 10 and *T. parva* Schoonspruit were identical to the *T. parva* Muguga p104 sequence in the region analyzed. Since *T. parva* Schoonspruit was isolated

during the ECF outbreak (Neitz, 1948), these results provide evidence that the *T. parva* parasites present in South Africa during the ECF epidemic were similar to those that cause ECF in East Africa. Interestingly, in a separate study, analysis of the p67 gene from Lad 10 revealed the presence of a p67 allele identical to that of *T. parva* Muguga (Chapter 4; Sibeko *et al.*, 2010). Taken together, these findings suggest that there may have been selection of cattle-type *T. parva* parasites in cattle on this farm. However, classical ECF was not diagnosed in cattle on the Ladysmith farm and reports of cattle-to-cattle transmission could not be substantiated, although *T. parva* carrier cattle were identified (Thompson *et al.*, 2008). While the presence in South African cattle of a parasite apparently similar to *T. parva* Muguga is a concern, there is no evidence to suggest that genotypes at individual loci such as p104 and p67, can be linked to the pathogenicity of the isolate.

p104 PCR-RFLP fingerprints obtained from other cattle samples from the Ladysmith farm (Lad 11, Lad 15 and Lad 17), grouped with p104 profiles from buffalo samples. The p104 amino acid sequences obtained from Lad 15 and Lad 17 grouped in sub-clades B1 and B2, respectively. The PCR-RFLP analysis and the sequence analysis therefore suggest that *T. parva* parasites circulating in some of the cattle on the Ladysmith farm may have been transmitted from buffalo to cattle, but there was no evidence of contact between these cattle and buffalo (Thompson *et al.*, 2008).

p104 PCR-RFLP fingerprints obtained from other cattle samples (Bloe B, Lyd 254) also grouped with profiles from buffalo samples suggesting that these *T. parva* parasites may have been transmitted from buffalo to cattle. These results were supported by the sequence analysis; the p104 sequence obtained from Bloe B grouped in sub-clade B2 with other p104 sequences from *T. parva* parasites obtained from buffalo. In South Africa, contact between cattle and buffalo is strictly controlled. However, in some areas cattle are grazed in pastures adjacent to game parks where buffalo are kept. If cattle break into or buffalo break out of the park where the vector tick is present, naive cattle are likely to be infected by *T. parva* infected buffalo. This may have been the case with these animals.

None of the p104 sequences obtained from buffalo *T. parva* samples in this study were identical to any of the previously reported p104 sequences (Skilton *et al.*, 2002). The sequence identities of p104 sequences obtained from buffalo in this study, when compared with published sequences, ranged from 94 to 97%. This finding suggests that *T. parva* p104

alleles in South African buffalo are more diverse and that variations in the p104 gene may not be as limited as previously thought (Geysen *et al*., 1999).

Sequences obtained from three buffalo *T. parva* samples from KNP grouped closely with the p104 allele 4 sequence. One of these sequences came from a *T. parva* sample obtained from buffalo KNP 102; this isolate has previously been used in a tick transmission experiment where the infected bovine eventually died from classical Corridor disease (Chapter 3; Sibeko *et al*., 2008). Characterization of the p67 gene from this isolate showed the presence of sequences similar to both cattle- and buffalo-type p67 alleles, and, in addition to these, two novel p67 alleles (Chapter 4; Sibeko *et al*., 2010). Although the KNP 102 *T. parva* isolate could cause Corridor disease, when a mix of parasites exists in an isolate it is impossible to determine which of these were responsible for inducing the disease.

Although alleles 2 and 3 grouped in sub-clade A2, none of the p104 sequences obtained in this study grouped closely with either of these alleles. Allele 2 was obtained from *T. parva* Marikebuni, which causes ECF in Kenya. Allele 3 was identified in *T. parva* Boleni, which was isolated in Zimbabwe and is a heterogeneous isolate that has both mild and pathogenic strains; the mild stock is used for vaccination against ECF (Irvin *et al*., 1989). Previous studies have shown that *T. parva* Boleni is genetically completely different at the p104, p150, p32 and PIM loci from the classical *T. parva* genotypes found in cattle, suggesting that it is a buffalo-type parasite, isolated from cattle (Dirk Geysen, pers. comm.). Interestingly, a sequence similar to p104 allele 1 was obtained from a buffalo sample, KNP B10. Variants of p67 allele 1 were also identified from buffalo samples from KNP (Chapter 4; Sibeko *et al*., 2010). These results could suggest that parasites with characteristics similar to cattle-derived *T. parva* might occur in *T. parva* populations circulating in buffalo in KNP. However, it is not clear if parasites that possess these alleles can cause disease and their significance in the epidemiology of theileriosis in South Africa needs to be established.

5.6 Summary

In summary, we have demonstrated that p104 amino acid sequences very similar to those of *T. parva* Muguga were present in *T. parva* parasites on a farm in South Africa where cattle theileriosis was reported. These findings, corroborated by the results obtained when p67 was used to characterize *T. parva* parasites from this farm (Chapter 4; Sibeko *et al*., 2010), remain a concern even though ECF has not been reported in South Africa since its eradication in

1954. While the cattle-type p104 alleles 2 (*T. parva* Marikebuni) and 3 (*T. parva* Boleni) grouped with p104 sequences from buffalo in South Africa, none of the South African p104 sequences obtained in this study grouped closely with either of these alleles. However, variants of p104 allele 1 (*T. parva* Muguga) were identified in buffalo. The significance of these parasites in the epidemiology of theileriosis in South Africa will have to be determined and the risk of disease evaluated. However, from the data obtained in this study, sequence variation in the region of the p104 gene analysed cannot be used to differentiate reliably between the cattle-type and the buffalo-type alleles because of the high sequence similarity between the two. The challenge to identify more reliable and specific markers that can be directly associated with different disease syndromes caused by *T. parva* still remains. This is crucial for the design and implementation of preventative measures in South Africa to protect livestock against disease caused by *T. parva* infections.

5.7 References

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