CHAPTER 6

Analysis of the gene encoding the *Theileria parva* polymorphic immunodominant molecule (PIM) reveals evidence of the presence of cattle-type alleles in South Africa

"There's two possible outcomes: if the result confirms the hypothesis, then you've made a discovery. If the result is contrary to the hypothesis, then you've made a discovery." ***Enrico Fermi***

6.1 Abstract

Analysis of two *T. parva* genes coding for the antigenic proteins p67 and p104, revealed the presence of cattle-type alleles identical to those of *T. parva* Muguga (a stock that causes ECF in Kenya) from *T. parva* samples collected from three cattle from a farm in Ladysmith. In addition, variants of p67 allele 1 and p104 allele 1, characteristic of the cattle-type alleles, were identified from *T. parva* samples obtained from buffalo from four game parks in South Africa. Consequently, polymorphic immunodominant molecule (PIM) PCR-RFLP profiles and inferred amino acid sequences were analyzed to confirm the presence of cattle-type alleles in *T. parva* samples obtained from cattle and buffalo in South Africa. PIM PCR-RFLP profiles similar to that of the *T. parva* Muguga stock were obtained from three of the six cattle samples from the Ladysmith farm and the inferred amino acid sequences of the PIM gene from two of these samples (Lad 02 and Lad 10) were almost identical to the *T. parva* Muguga PIM sequence. This finding supports recent studies in which p67 and p104 alleles similar to those of the *T. parva* Muguga stock were identified from the same Ladysmith samples. None of the PIM gene sequences obtained from *T. parva* field samples characterized in this study were identical, providing further evidence that the PIM gene evolves at an extremely high rate. Cattle-type PIM alleles were not identified from buffalo *T. parva* samples. In addition to sequences similar to known PIM alleles, for the first time, 'mixed' alleles consisting of cattle- and buffalo-type amino acid motifs were identified. The significance of *T. parva* parasites carrying 'mixed' PIM alleles will have to be established and their risk to cattle evaluated. With the extent of genetic diversity that has been demonstrated by the three characterization studies presented in this thesis, the epidemiology of theileriosis in South Africa needs to be further investigated.

6.2 Introduction

The tick transmitted apicoplexan protozoan parasite, *Theileria parva*, is by far the most pathogenic and economically significant *Theileria* sp. in eastern, central and southern Africa (Mukhebi *et al*., 1992). Infections by *T. parva* are associated with high mortality, primarily in exotic and crossbred cattle, but also in indigenous calves and adult cattle in endemically stable areas (Perry and Young, 1995). This places major constraints on cattle production and the expansion of the dairy industry. The Cape buffalo *(Syncerus caffer*) is the natural reservoir host of the parasite.

Tick transmission of the parasite from infected buffalo to susceptible cattle results in a disease syndrome called Corridor disease, while *T. parva* parasites that can circulate in cattle cause two disease syndromes, East Coast fever (ECF) and January disease (Theiler, 1904; Neitz, 1955; Lawrence, 1992). To distinguish between the different types of *T. parva* parasites, parasites that circulate in cattle and cause ECF and January disease are referred to as cattle-derived *T. parva* and parasites that originate from buffalo and cause Corridor disease are known as buffalo-derived *T. parva* (Perry and Young, 1993). East Coast fever was introduced into South Africa at the end of the 19th century and eradicated in the early 1950s (Anonymous, 1981). Although ECF was eradicated in southern Africa, its principal vector, the three-host ixodid tick *Rhipicephalus appendiculatus,* is still widespread. Corridor disease is a controlled disease in South Africa and sporadic outbreaks still occur.

Studies on two *T. parva* genes, p67 and p104, presented in the previous two chapters, revealed the presence of cattle-type alleles identical to those of *T. parva* Muguga (a stock that causes ECF in Kenya) from *T. parva* samples collected from three cattle from a farm in Ladysmith, South Africa (Chapter 4; Chapter 5; Sibeko *et al*., 2010). In addition, variants of p67 allele 1 and p104 allele 1, characteristic of the cattle-type alleles, were identified from *T. parva* samples obtained from buffalo from four game parks in South Africa, namely, Kruger National Park, Hluhluwe-iMfolozi, Ithala and Mabalingwe (Collins, 1997; Chapter 4; Sibeko *et al*., 2010). Unless these alleles can be associated with parasites that can cause fatal disease, their importance remains unclear. Consequently, another *T. parva* gene encoding an antigenic protein, the polymorphic immunodominant molecule (PIM), was investigated.

The PIM gene has previously been successfully used to differentiate between cattle- and buffalo-derived *T. parva* stocks (Geysen *et al*., 1999; Bishop *et al*., 2001). Although PIM is expressed by both the sporozoite and the schizont stages of the parasite, it is expressed predominantly by the schizont stage and is thus believed to play a role as a target antigen in the induction of the cytotoxic T cell response (Shapiro *et al*., 1987; Toye *et al*., 1991; Katende *et al*., 1998; Shaw, 2003; Graham *et al*., 2007). It is also capable of inducing sporozoite-neutralizing antibodies and has been exploited in discriminatory assays for *T. parva* isolates because of its highly conserved termini flanking a variable region with a highly polymorphic central region (Toye *et al*., 1995a; 1995b; Bishop *et al*., 2001; Geysen *et al*., 1999; 2004; De Deken *et al*., 2007). The variations in the central region of the PIM gene produce the polymorphism that has been exploited for discrimination between *T. parva* stocks (Geysen *et al*., 1999; Bishop *et al*., 2001).

In this study, the PIM gene was characterized to confirm the presence of cattle-type alleles in *T. parva* samples obtained from cattle and buffalo in South Africa. The diversity of the parasite populations circulating in buffalo and cattle in South Africa was also demonstrated.

6.3 Materials and methods

6.3.1 Sample collection

Blood samples were collected from buffalo from different game parks, and from cattle from farms with suspected theileriosis. The samples were collected in EDTA vaccutainer tubes and stored at -20 ºC, for long term storage, or 4 ºC for short term storage, before extraction of DNA.

6.3.2 DNA isolation and selection of *T. parva*-positive samples

Total DNA was extracted from 200 µl of EDTA blood using the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany), according to the method described by the manufacturer, except that extracted DNA was eluted in 100 µl elution buffer. Extracted DNA was stored at 4°C until further analysis. The presence of *T. parva* DNA was determined using the real-time PCR assay as previously described (Chapter 3; Sibeko *et al*., 2008). One hundred and nine *T. parva*-positive samples consisting of 101 field samples collected from buffalo from different game parks, and eight collected from cattle from farms with suspected theileriosis, were investigated (Table 6.1).

Table 6.1 Geographical origin and source of blood samples (n=109) used for characterization of *T. parva* parasites

Geographical location	Province	Sample Name*	Host of blood sample	Date of collection/ References
Hluhluwe-iMfolozi Park (n=38)	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 30, HIP 31, **HIP 32**, HIP 33, HIP 34, HIP 35, HIP 36, HIP 37, HIP 38, **HIP 39**, HIP 42, HIP 49	Buffalo	2004
Kruger National Park (KNP) (n=47)	Mpumalanga	**KNP 39**, **KNP 42**, **KNP 43**, 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 AC10, KNP AD3, KNP B10, KNP B22, KNP D11, KNP D24, KNP E7, KNP E18, KNP F9, KNP G2, KNP G11, KNP H8, KNP J5, KNP L6, KNP L27, KNP M2, KNP M12, KNP M2706, KNP N1, KNP N8, KNP O1, **KNP O11**, 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 06, **Lad 10, Lad 17 Lad M119, Lad I438**	Bovines	2003 Thompson *et al*. (2008)
Mabalingwe Game Reserve (n=6)	Limpopo	**Mab A13, Mab A22**, **Mab B21**, **Mab BB37**, **Mab BB38, Mab BB43**	Buffalo	2004
Ithala Game Reserve (n=10)	KwaZulu-Natal	**Itha 1**, **Itha 2, Itha 3, Itha 4, Itha 5, Itha 6, Itha 7, Itha 8, Itha 9, Itha 10**	Buffalo	2005/6
Schoonspruit (n=1)	Mpumalanga	**Schoonspruit**	Bovine	Neitz (1948)
Bloemfontein (n=1)	Free-State	**Bloe B**	Bovine	2004

*Samples in bold were selected for cloning to produce RFLP profiles from individual clones and only 27 of the 35 were used for sequencing (see Table 6.2).

6.3.3 Amplification of the PIM gene from *T. parva* samples

The variable region of the *T. parva* PIM gene was amplified from *T. parva* positive DNA samples using the semi-nested PCR described by De Deken *et al*. (2007). A nested PCR was performed using primers Pim1 [5' GTG AAT GTT GTG ATC TTA ATC C 3'] and PimR4 [5' CCC ACA ACC GTG GAA TGG CGT A 3'] for the primary PCR and primers PimFm [5' ATT CCA CTG GTT CTT CCG ATS TA 3', where S = C or G] and PimR4 for the secondary PCR. Briefly, 5 µl of total DNA was used in a 25 µl amplification reaction for the primary PCR and half a microlitre 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).

6.3.4 Analysis of the PIM gene from *T. parva* samples using PCR-RFLP

Restriction fragment length polymorphism was performed as described by De Deken *et al*. (2007); briefly, PCR products were digested overnight with the restriction enzyme, *Bcl*I; the digested products were separated on a 10% polyacrylamide gel before DNA detection by SYBR® green (SIGMA-ALDRICH, USA). RFLP patterns were analysed by visual inspection and by using BioNumerics version 5.1 (Applied Maths, Kortrijk, Belgium). Normalisation 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. 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. DNA fragments of less than 100 bp were excluded from the analysis as these could not be accurately estimated using the 100 bp DNA ladder and in some instances had run out of the gel. Samples with similar RFLP profiles obtained from different animals were defined as clusters.

6.3.5 Cloning and sequencing of PIM PCR products

The PIM PCR products from 34 selected *T. parva* samples, including 27 buffalo and seven cattle samples (shown in bold in Table 6.1), were cloned into pCR®2.1-TOPO® cloning vector (Invitrogen, Carlsbad, USA); at least 24 clones were screened for each sample. The presence of inserts in the recombinants was confirmed by colony PCR following the secondary PCR

protocol used above. Amplicons produced from colony PCR were digested with *Bcl*I to produce RFLP profiles for individual clones. Only clones that produced amplicons which successfully digested with *Bcl*I were considered for further analysis. Consequently, clones from 20 *T. parva* samples from buffalo and seven from cattle were sequenced using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA); 300 to 450 ng of plasmid DNA were used in the sequencing reactions. Sequencing was performed by INQABA Biotechnologies in South Africa, using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA).

6.3.6 Sequence analysis

The PIM gene 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). Sequences were aligned with previously published *T. parva* PIM sequences [Muguga (accession number: L06323), Marikebuni (accession number: L41148) and 7104 (accession number: L41833)] using MacClade v4.0 (Maddison and Maddison, 1992); the alignment was adjusted manually because of the highly polymorphic structure of the PIM gene. It was impossible to perform phylogenetic analysis for the PIM sequences because of the polymorphic nature of this gene.

6.4 Results

6.4.1 PIM PCR-RFLP profile analysis

The PIM gene PCR products obtained from *T. parva* positive samples analyzed in this study ranged in size from 0.7 to 1.2 kb (results not shown). Since PIM is a single copy gene (Toye *et al*., 1995b), multiple infections were indicated by multiple PCR products in cases where the amplicon sizes obtained from a single sample varied.

From visual inspection of the PIM PCR-RFLP profiles, profiles from all 47 samples from KNP were heterogeneous (Figure 6.1a). However, the profiles obtained from 23/38 (61%) of the Hluhluwe-iMfolozi samples from buffalo were relatively homogeneous (Figure 1b). Similarly 4/6 (67%) samples from Mabalingwe and all 10 from Ithala produced relatively homogeneous profiles (Figures 6.1c and 6.1d). Further analysis of this result by cluster analysis using BioNumerics was not possible as the PCR-RFLP profiles were too complex, as

a result of mixed infections. The profiles were characterized by multiple bands from multiple PCR products; the PCR products were present at different concentrations resulting in multiple bands of different intensities. It was difficult to distinguish between bands from incompletely digested amplicons and authentic bands. Therefore, 27 samples representative of *T. parva* samples from buffalo that produced homogeneous and heterogeneous profiles as well as seven cattle samples (shown in bold in Table 6.1) were selected for cloning in order to produce RFLP profiles from individual clones.

Mixed infections were evident from 31/34 (91.2%) samples, as more than one profile was obtained from different clones of each of these samples. Three samples from Ladysmith (Lad 02, Lad 06 and Lad 10) were exceptions, as all clones from these samples produced only one profile and this profile was identical to that of *T. parva* Muguga (a *T. parva* stock causing ECF in Kenya) and *T. parva* Schoonspruit (an isolate obtained from a bovine infected during the ECF epidemic in the former Transvaal, now Gauteng Province, in South Africa) (Neitz, 1948) (Figure 6.2). When RFLP profiles obtained from clones produced from samples which had homogeneous overall profiles were visually analysed, it was observed that there were dominant profiles that were responsible for the apparently homogenous overall profile between different samples. For example, among other profiles, three profiles were found to be dominant in clones produced from the 10 samples from Ithala; the three profiles were obtained in, respectively, 20/61 (33%), 14/61 (23%) and 11/61 (18%) clones produced from four different samples.

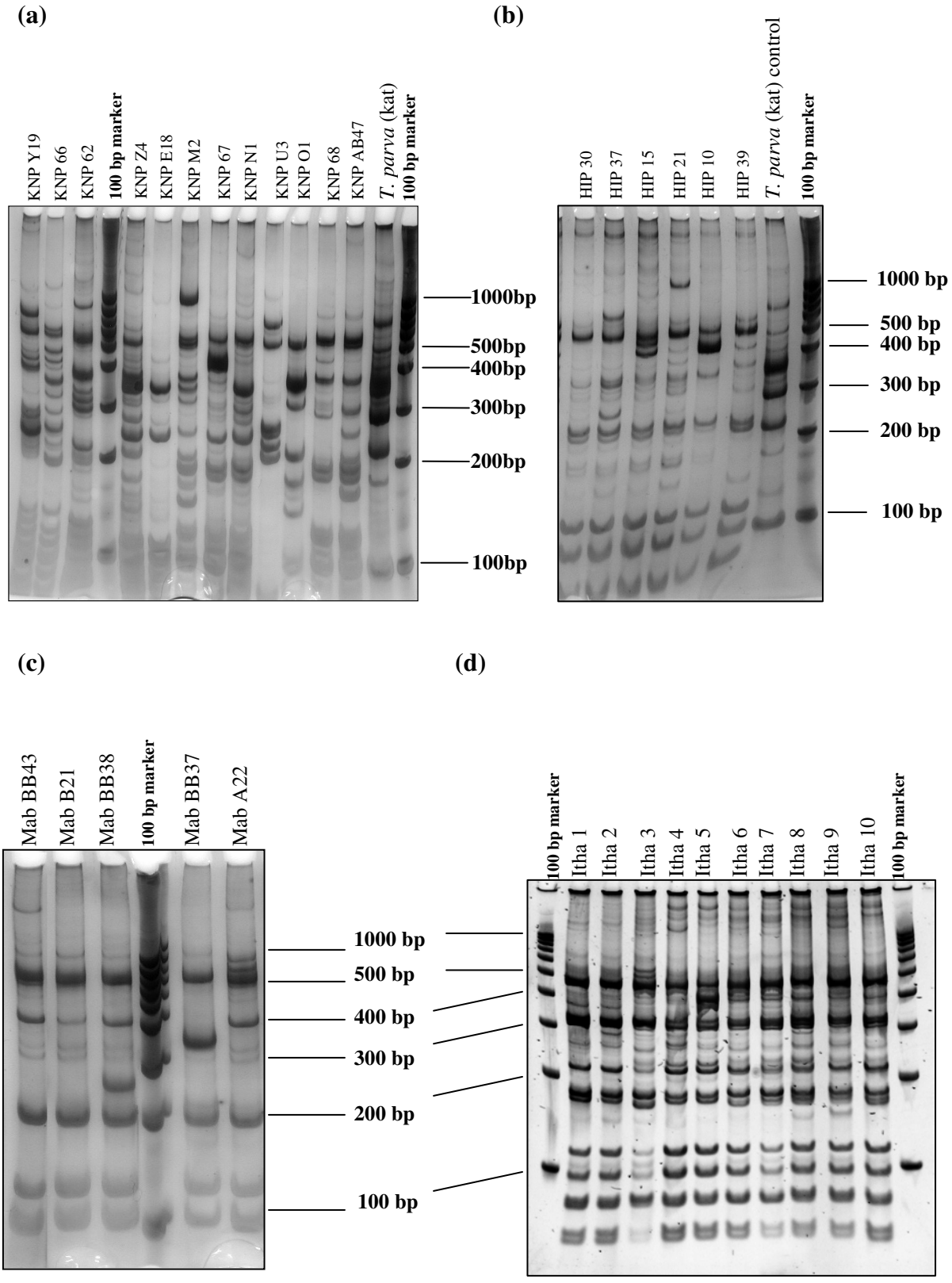


Figure 6.1 Representative PIM gene *Bcl*I PCR-RFLP profiles showing (a) heterogeneous profiles obtained from buffalo *T. parva* samples from KNP, (b), (c) and (d) homogeneous profiles obtained from buffalo samples from Hluhluwe-iMfolozi, Mabalingwe and Ithala, respectively.

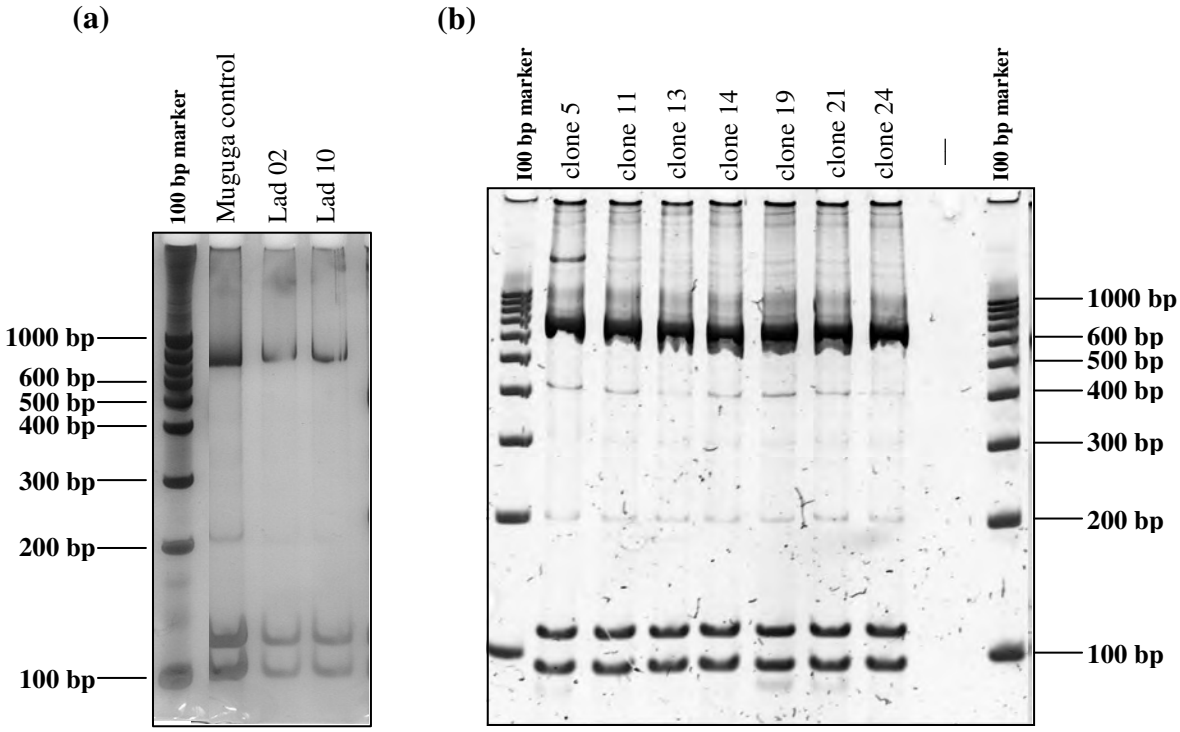


Figure 6.2 PIM gene *Bcl*I PCR-RFLP profiles obtained from (a) cattle *T. parva* samples from Ladysmith and (b) clones produced from cattle sample, Lad 10.

Cluster analysis of PCR-RFLP profiles using BioNumerics identified five cluster groups, A, B, C, D and E, from 261 clones produced from both buffalo and cattle *T. parva* samples (Figures 6.3 and 6.4). Cluster A was the largest group with 105/261 (40%) clones followed by cluster D with 73/261 (28%), then C (42/261, 16%), B (26/261, 10%) and E (15/261, 6%). No correlation with geographic distribution could be established from the major cluster groups. PIM profiles from clones obtained from KNP, Hluhluwe-iMfolozi, Mabalingwe and Ithala buffalo *T. parva* samples were distributed in all five cluster groups on the dendrogram. Profiles from clones produced from cattle samples Lad 02, Lad 06 and Lad 10 grouped with *T. parva* Muguga and *T. parva* Schoonspruit profiles in cluster A (Figures 6.3 and 6.4). Although most PIM profiles in cluster A were obtained from buffalo samples, 42/48 (88%) of the PIM profiles from clones obtained from cattle samples occurred in this group. PIM profiles from clones produced from the other cattle *T. parva* samples from Ladysmith and Bloemfontein grouped closely with profiles obtained from buffalo samples from Hluhluwe-iMfolozi and Ithala in different subgroups within cluster A (Figures 6.3 and 6.4).

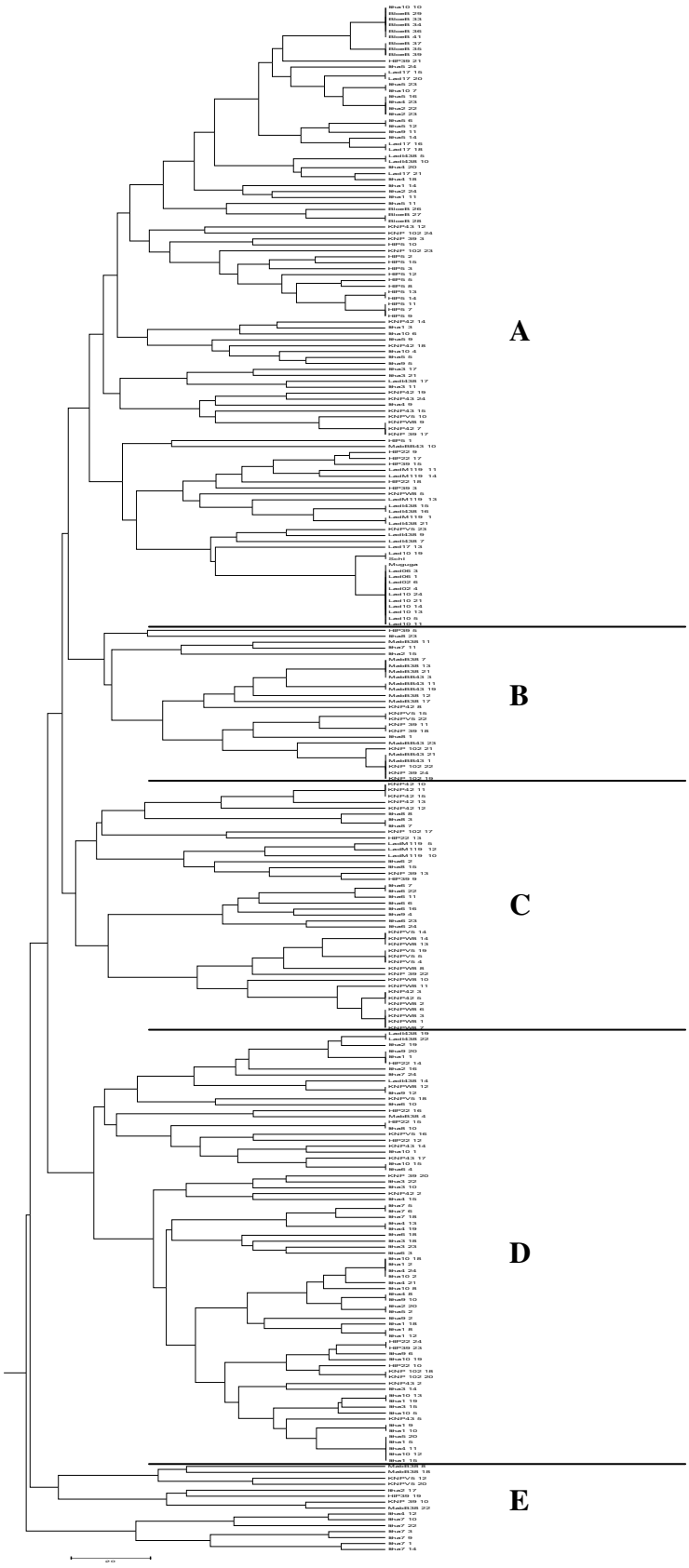
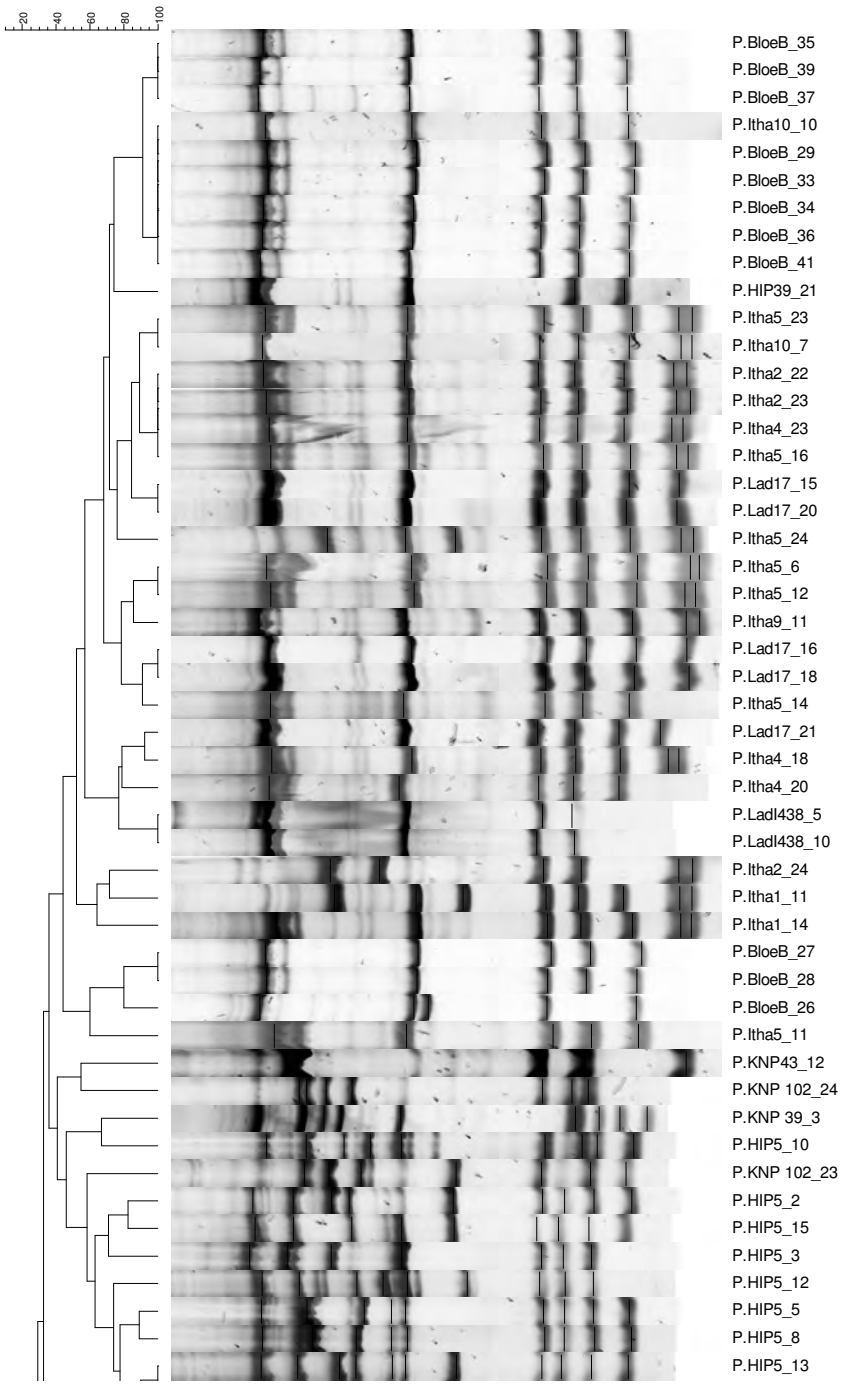
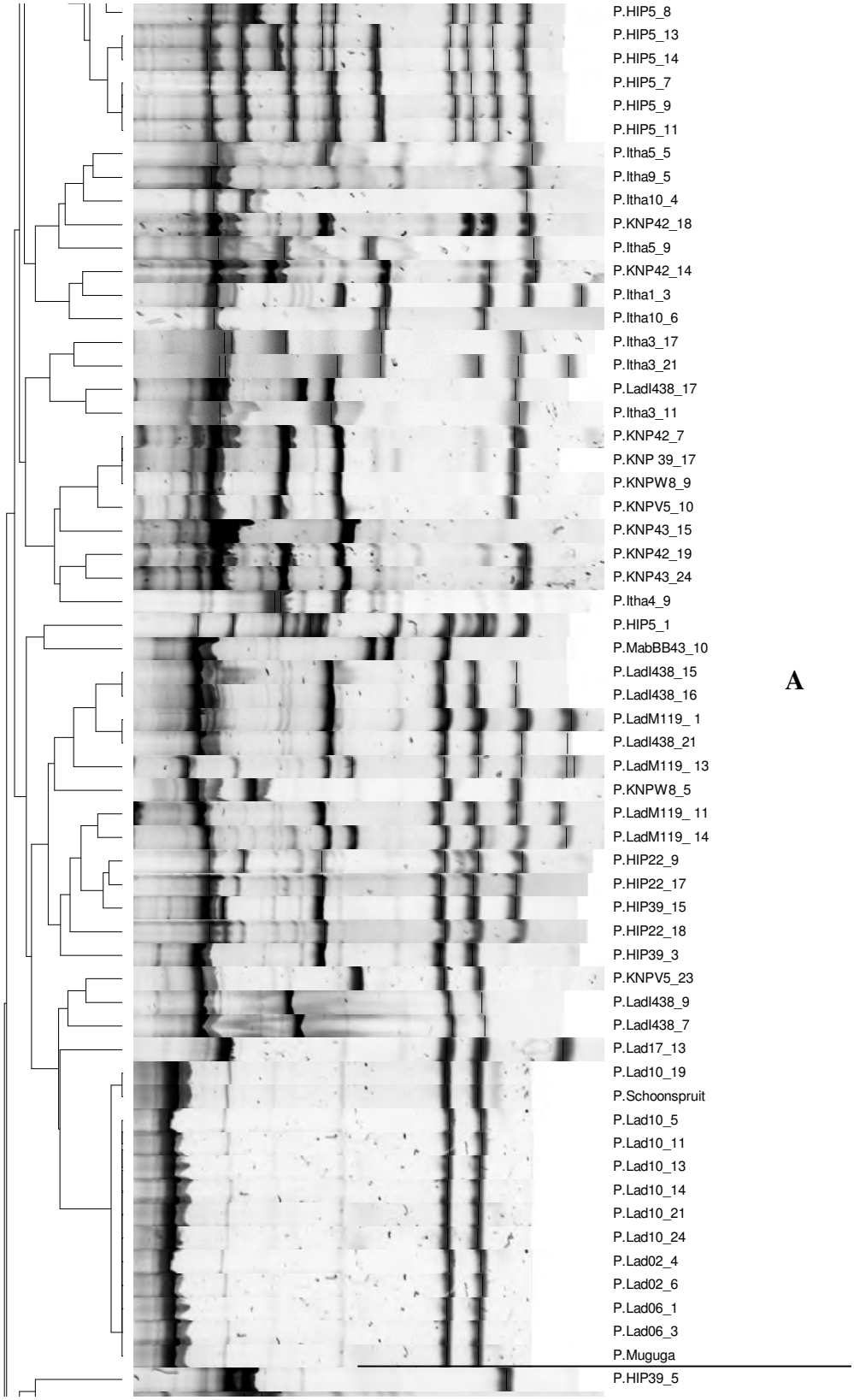


Figure 6.3 A simplified similarity dendrogram generated by BioNumerics v5.1 cluster analysis of PCR-RFLP profiles from cloned PIM amplicons using the Dice Coefficient analysis.



A

Figure 6.4 A detailed similarity dendrogram generated by BioNumerics v5.1 cluster analysis of PCR-RFLP profiles from cloned PIM amplicons using the Dice Coefficient analysis showing actual profiles used to produce the dendrogram. Figure 6.4 continues on pages 136 to 139.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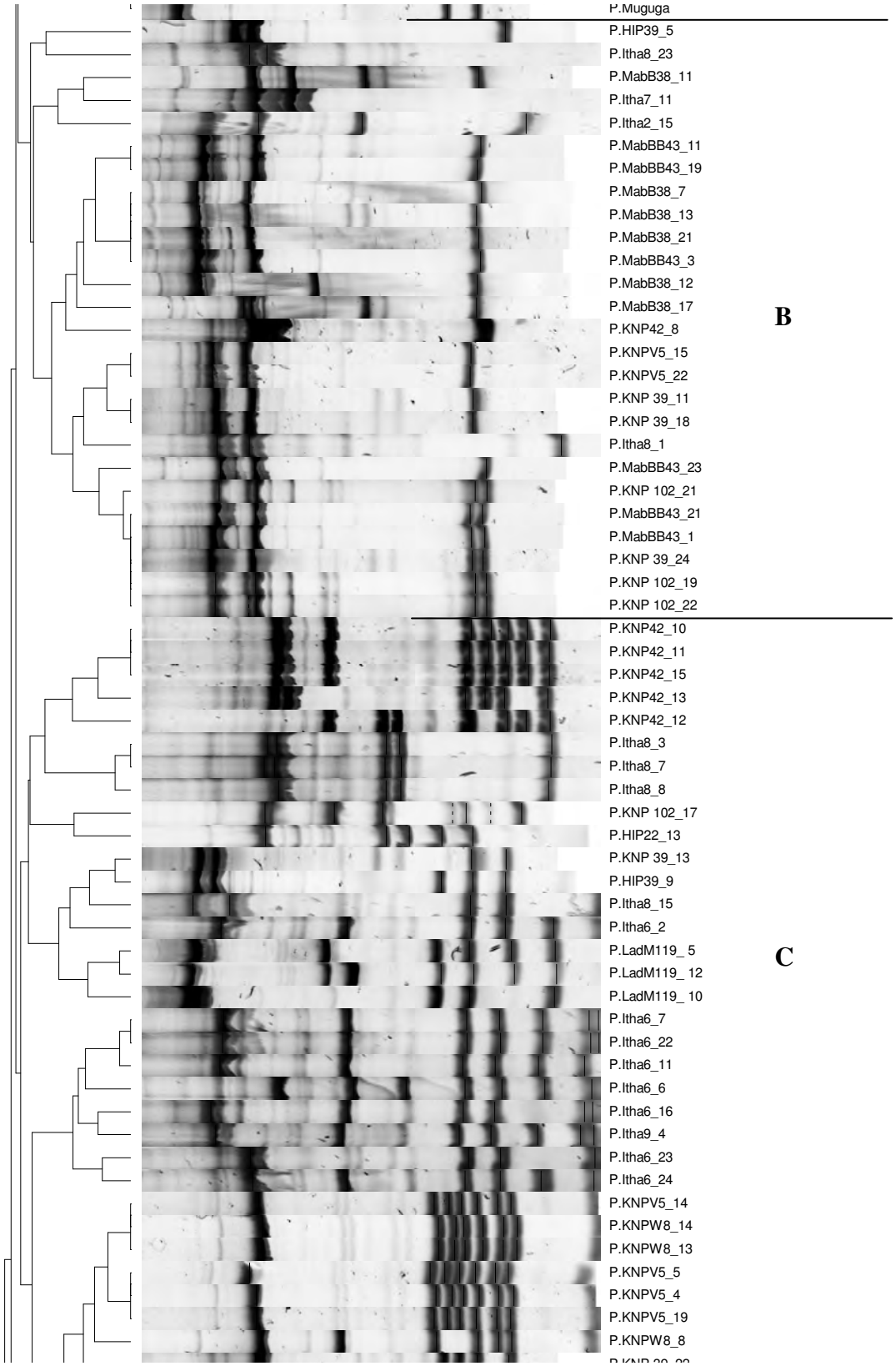
A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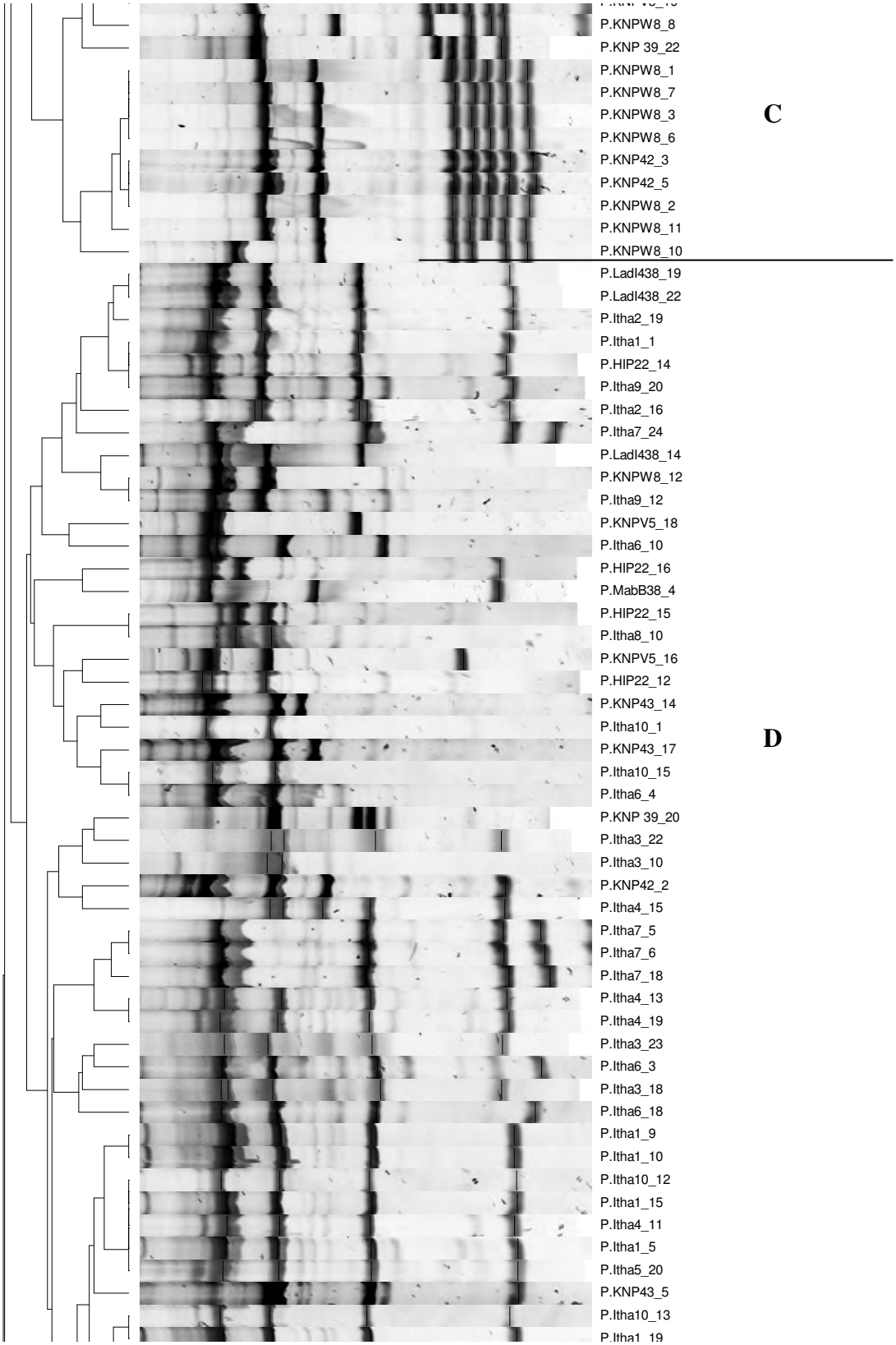
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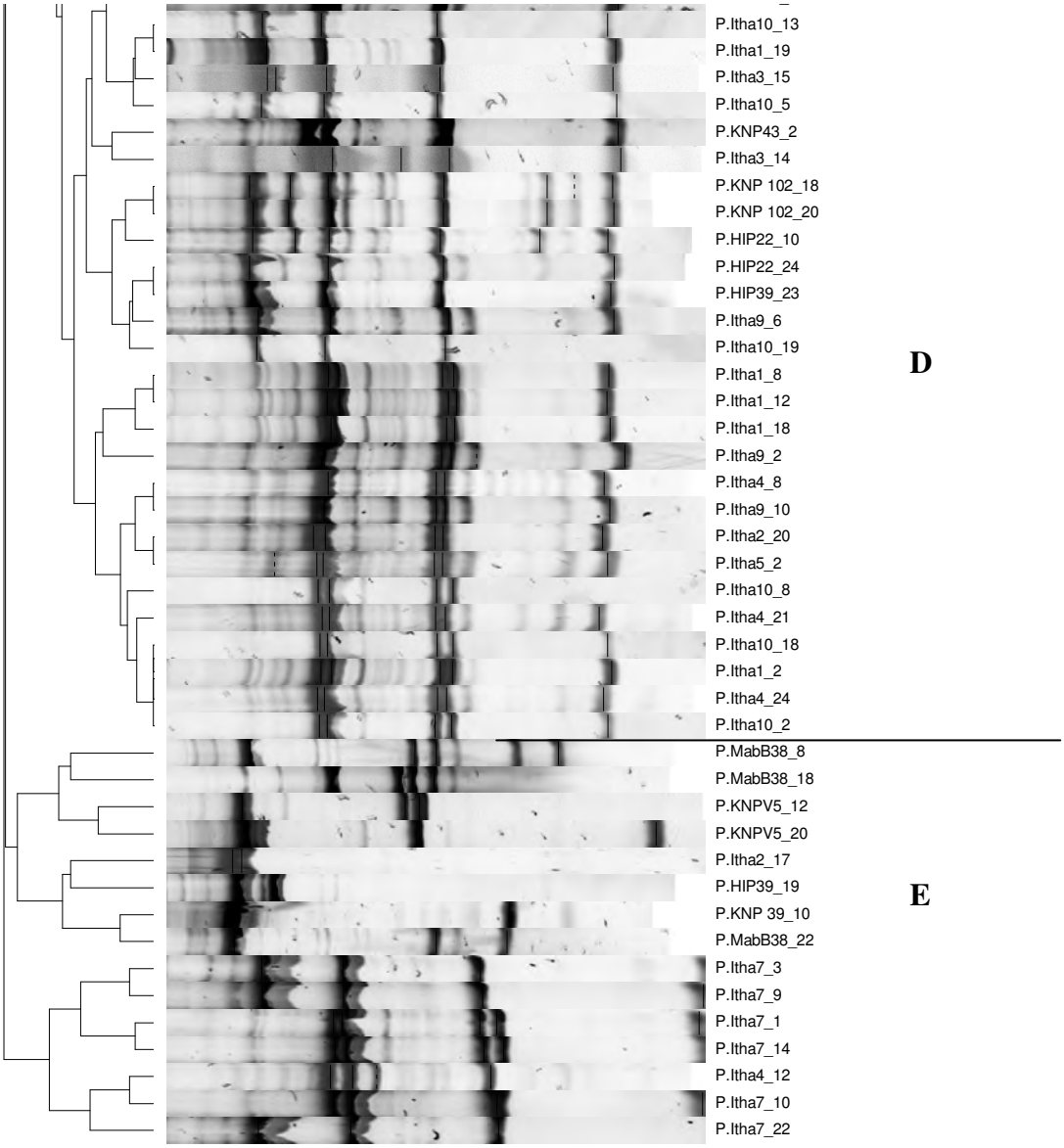
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Specific 'signatures' composed of several small fragments (less than 150 bp) were produced in the PIM PCR-RFLP profiles obtained from some *T. parva* field samples from KNP and Hluhluwe-iMfolozi. Two specific 'signatures' were associated with some RFLP profiles from clones from KNP samples. One of these was defined by five small DNA fragments of sizes ~50, 60, 80, 100 and 120 bp, and the other defined by six fragments of sizes ~50, 60, 80, 100, 120 and 130 bp (Figure 6.5a); both 'signatures' co-occurred with other bands of larger sizes. The Hluhluwe-iMfolozi 'signature' was characterized by four fragments of approximately 50, 80, 100 and 120 bp in size (Figure 6.5b). The KNP 'signatures' were observed in 26/52 (50%) clones from three samples, while the Hluhluwe-iMfolozi 'signature' was observed in almost all the clones (31/36, 86%) from the two Hluhluwe-iMfolozi *T. parva* samples that were analyzed. The Hluhluwe-iMfolozi 'signature' was also apparent in profiles from field samples obtained from buffalo from Ithala (Figure 6.1d), and from three bovines from Ladysmith (Lad 17, Lad I438 and Lad M119) and a bovine from Bloemfontein (results not shown). It was also found in profiles obtained from clones of PIM amplicons from these samples (results not shown).

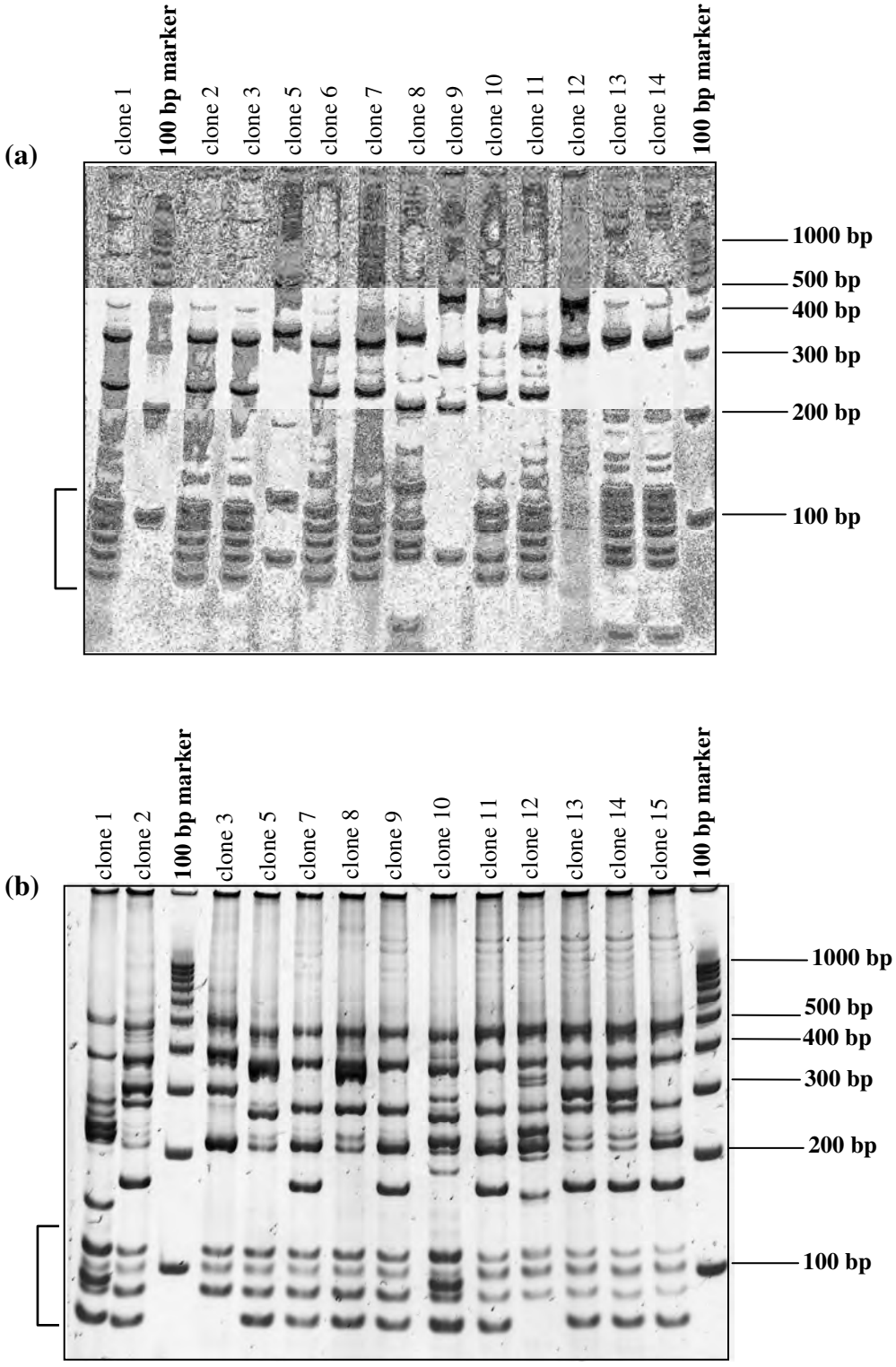


Figure 6.5 Characteristic 'signatures', indicated by brackets ([), were identified in *T. parva* PIM RFLP profiles obtained from cloned PCR products from samples collected from buffalo from Kruger National Park and Hluhluwe-iMfolozi Game Park. (a): PIM RFLP profiles with specific band 'signatures' obtained from clones from sample KNP W8 from Kruger National Park, characterized by five (clones 1, 2, 3, 6, 7 and 11) and six (clones 13 and 14) small DNA fragments of sizes ranging from ~ 50 to 130 bp. (b): PIM RFLP profiles obtained from clones from sample HIP 5 from Hluhluwe-iMfolozi with the 'four band signature' consisting of ~ 50, 80, 100 and 120 bp DNA fragments.

6.4.2 PIM gene sequence analysis

Clones from different cluster groups were selected for sequencing. A total of 97 PIM sequences were obtained from cloned amplicons produced from 27 selected *T. parva*-positive samples (Table 6.2). Analysis of the amino acid alignment of the PIM sequences revealed three groups of PIM sequences, cattle-type, buffalo-type and 'mixed'-type (Figure 6.6).

Cattle-type PIM sequences:

A tetrapeptide repeat, QPEP (position 428-447 shown in a solid-line block in Figure 6.6), in the variable region was previously identified by Toye *et al.* (1995b) as characteristic of cattle-derived *T. parva* PIM sequences. The amino acid sequences between positions 29 and 259 and positions 493 and 497 were also identified as exclusive to cattle-type PIM alleles in this study. In this way, seven PIM sequences obtained from samples investigated in this study were identified as cattle-type *T. parva* PIM sequences (Table 6.2). Six of these were obtained from two *T. parva* cattle samples from a farm in Ladysmith (Lad 02 and Lad 10) and one from *T. parva* Schoonspruit. These sequences had 98% identity to the *T. parva* Muguga PIM sequence, with one to three amino acid differences; the major difference being a deletion of eight amino acids at position 420-427 in the South African sequences (Figure 6.6). Cattle-type PIM sequences were not identified from samples obtained from buffalo in this study.

Table 6.2 Number of different types of PIM sequences obtained from clones produced from 27 selected *T. parva* samples

Origin of sample	Sample designation	Type and number of PIM sequence(s) obtained		
		Cattle-type (7 sequences obtained from 3 samples)	**Buffalo-type** (53 sequences obtained from 20 samples)'	**'Mixed' type** (37 sequences obtained from 12 samples)
Kruger National Park (n=4)	KNP V5	-	1	-
	KNP W8	-	-	4
	KNP 102	-	2	-
	KNP O11	-	2	-
Hluhluwe-iMfolozi Park (n=4)	HIP 5	-	3	-
	HIP 19	-	3	2
	HIP 22	-	1	4
	HIP 32	-	6	4
Ithala Game Reserve (n=9)	Itha 2	-	1	2
	Itha 3	-	5	-
	Itha 4	-	6	1
	Itha 5	-	3	-
	Itha 6	-	1	6
	Itha 7	-	2	-
	Itha 8	-	2	-
	Itha 9	-	4	-
	Itha 10	-	2	-
Mabalingwe Game Reserve (n=3)	Mab A13	-	5	-
	Mab BB38	-	2	-
	Mab BB43	-	-	1
Schoonspruit (n=1)	**Schoonspruit**	1	-	-
Ladysmith (n=5)	**Lad 2**	2	-	-
	Lad 10	4	-	-
	Lad 17	-	-	7
	Lad M119	-	-	1
	Lad I438	-	1	2
Bloemfontein (n=1)	**Bloe B**	-	1	3

All samples in bold were obtained from cattle

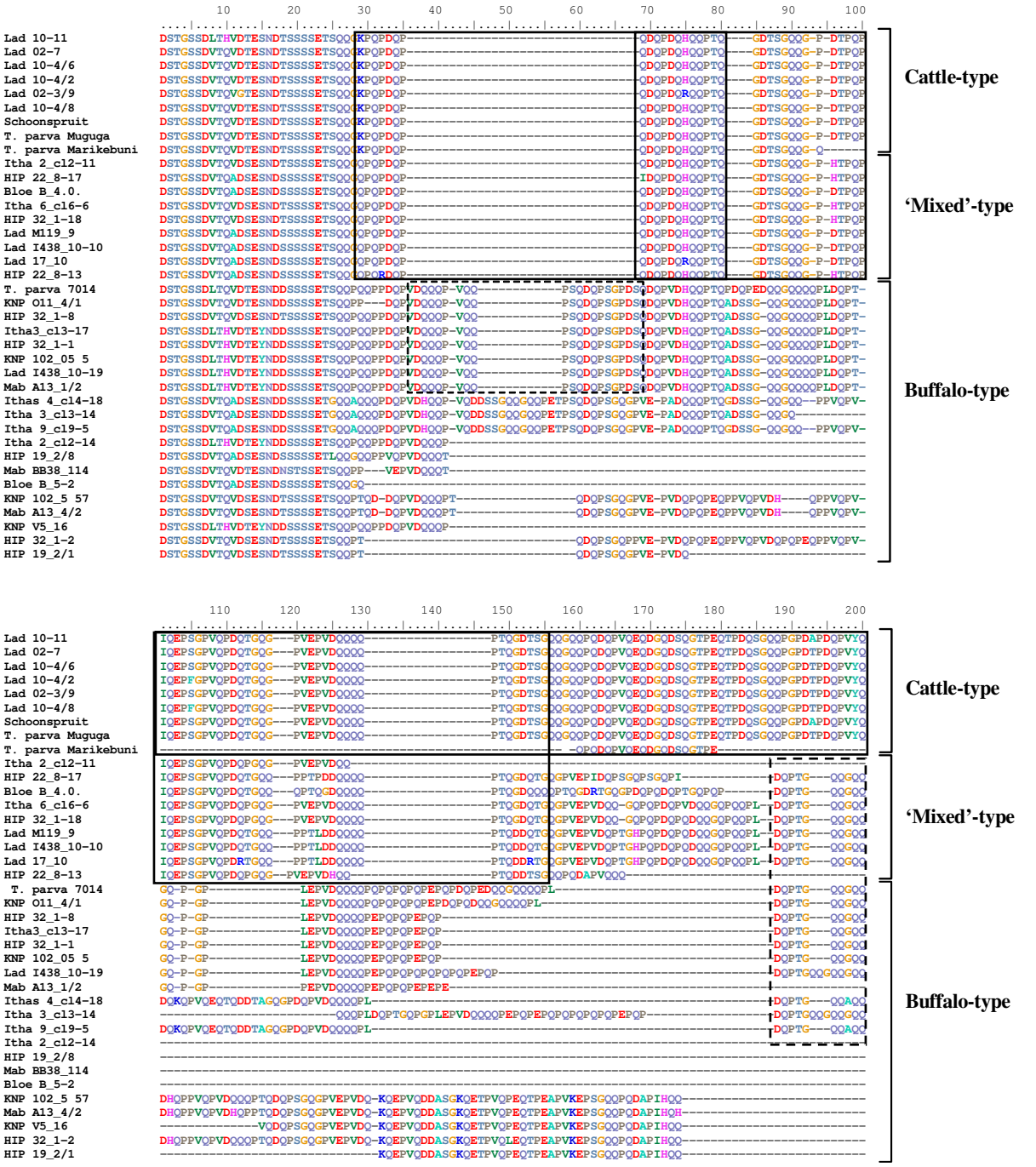
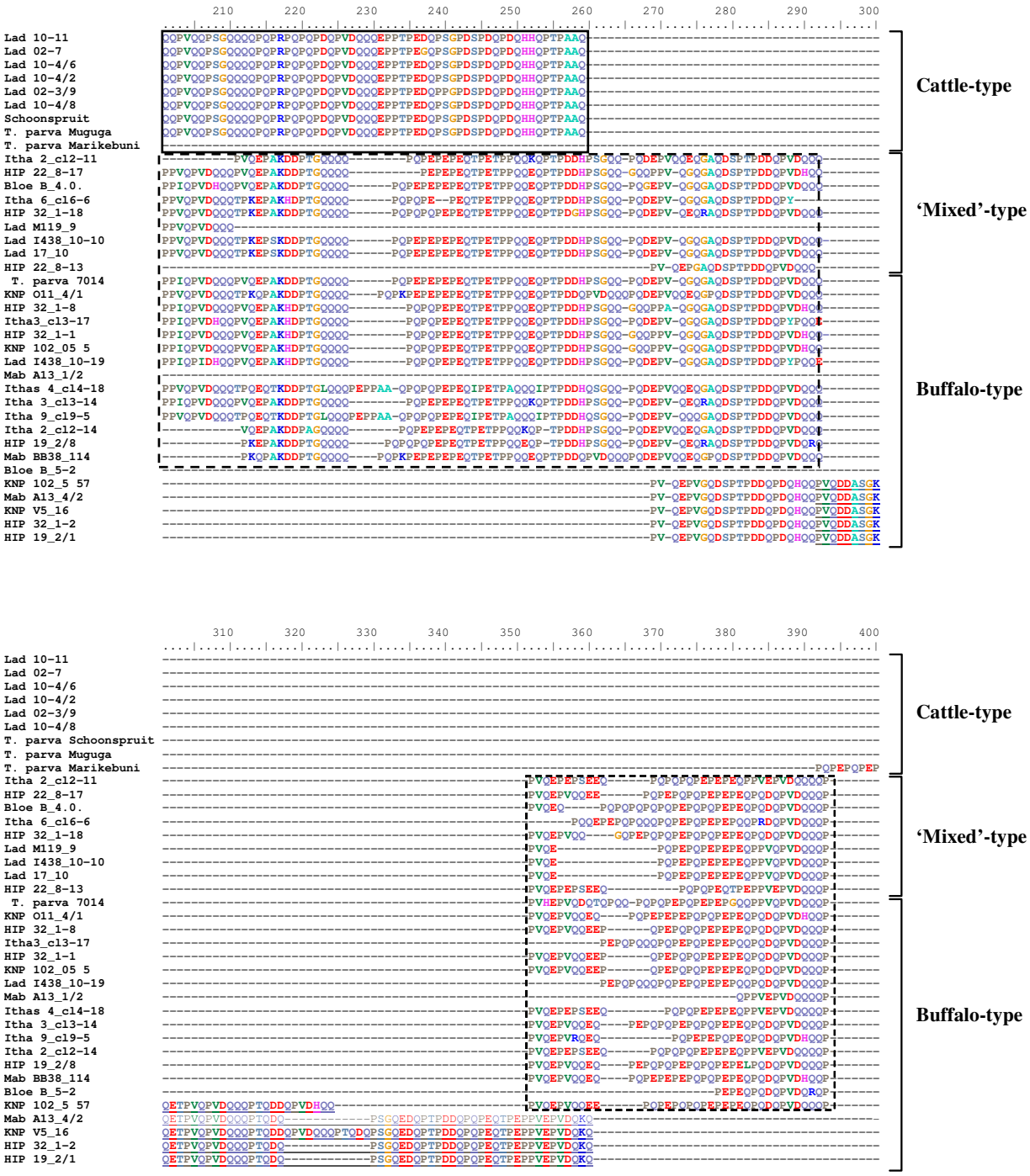
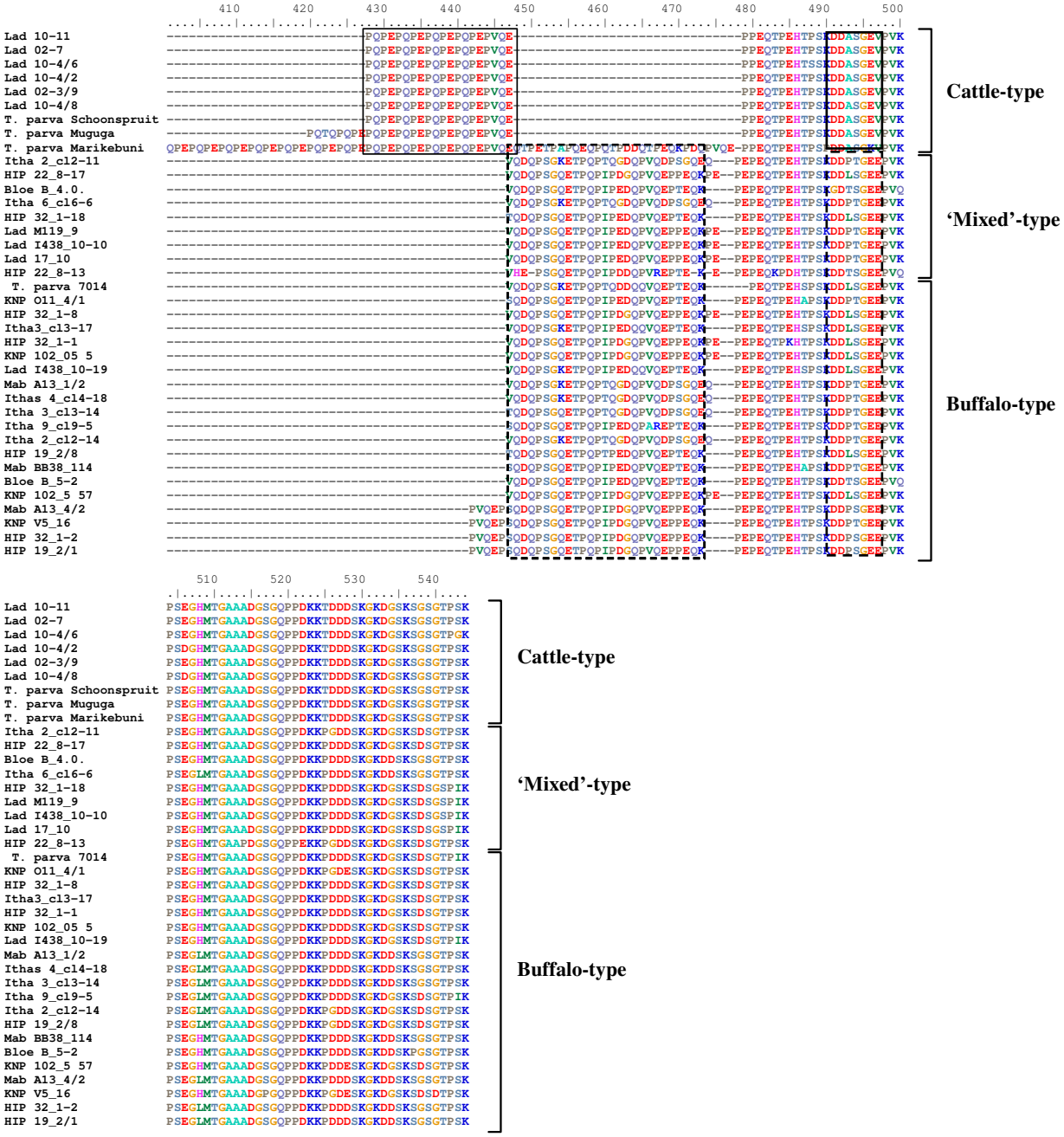


Figure 6.6 Multiple sequence alignment of the inferred PIM amino acid sequences obtained from buffalo and cattle *T. parva* samples collected from different geographical areas in South Africa (Table 6.1). PIM sequences were aligned with previously published *T. parva* PIM sequences, Muguga (accession number: L06323), Marikebuni (accession number: L41148) and 7104 (accession number: L41833). The sequence alignment was constructed manually because of the extreme polymorphism in the central region of the PIM gene. Amino acid motifs characteristic of cattle-type PIM sequence are shown in solid-line blocks including the tetrapeptide repeat characteristic of the central variable region of cattle-derived PIM sequences at positions 428-447. All amino acid motifs characteristic of buffalo-type PIM sequence are shown in broken-line blocks. Figure 6.6 continues on pages 145 and 146.


Cattle-type
'Mixed'-type
Buffalo-type
Cattle-type
'Mixed'-type
Buffalo-type


Cattle-type
'Mixed'-type
Buffalo-type
Cattle-type
'Mixed'-type
Buffalo-type

Buffalo-type PIM sequences:

Toye *et al*. (1995b) identified a 20-amino-acid insert (VDQQQPVQQPSQDQPSGPDS shown in broken-line block at position 36-68 in Figure 6.6) as characteristic to buffalo-type PIM amino acid sequences. In addition to this, two other buffalo-type amino acid motifs occurring at positions 352-393 and 447-473, were identified in this study (shown in broken-line blocks in Figure 6.6). However, some of the PIM sequences obtained from *T. parva* field samples collected from buffalo from KNP, Hluhluwe-iMfolozi, Mabalingwe and Ithala game parks lacked the 352-393 amino acid motif; instead, these sequences contained unique inserts of variable sizes between position 292-360 (underlined in Figure 6.6). The 20-amino-acid insert identified by Toye *et al*. (1995b) from PIM sequences from buffalo-derived *T. parva* 7014 and Hluhluwe stocks PIM sequences, was missing from 9/53 (17%) of the buffalo-type PIM sequences obtained in this study. Furthermore, a different insert between positions 36 and 68 was identified from buffalo-type sequences obtained from Ithala buffalo samples.

Using these motifs, 54.6% (53/97) of the PIM sequences obtained in this study were identified as buffalo-type PIM sequences and were obtained from *T. parva* samples collected from both cattle and buffalo (Table 6.2). One sequence obtained from KNP V5 had a large insert in the central region of the gene which was very different in sequence from that of other PIM sequences (results not shown). Very short PIM sequences, missing most of the variable central region, were obtained from some clones of PIM amplicons obtained from samples originating from buffalo (results not shown). However, the sequences flanking the central region from these alleles were characteristic of buffalo-type PIM sequences as defined in this study.

'Mixed'-type PIM sequences:

The use of the amino acid motifs identified in this study allowed identification of 37 'mixed' PIM sequences (Figure 6.6). It is possible that hybrid PCR products could arise during PCR amplification in samples containing mixed infections, as a result of template switching. These PCR artifacts are identifiable by sequence identities in hybrid sequences when compared with sequences of other amplicons produced in the same amplification reaction. To confirm whether the 'mixed' sequences obtained in this study were authentic, the PIM nucleic acid sequences in the more conserved regions, i.e. sequences flanking the central variable region, were compared to those of other sequences obtained from the same sample. None of the 'mixed' sequences were identical to any of the other PIM sequences obtained from the same

sample in these conserved regions, suggesting that these sequences were therefore genuine and could not have resulted from PCR artifacts.

Two subtypes of 'mixed' PIM sequences were identified, M-I (32/37) and M-II (5/37) (Figure 6.7). Subtype M-I consisted of sequences characteristic of cattle-type *T. parva* PIM sequences at the amino-terminus and buffalo-type sequences at the carboxy-terminus (Figure 6.7). Subtype M-II comprised sequences characteristic of buffalo-type *T. parva* PIM sequences at the amino-terminus and cattle-type sequences at the carboxy-terminus (Figure 6.7).

The 37 'mixed' PIM sequences were obtained from samples collected from both cattle and buffalo (Table 6.2). Of the 22 *T. parva* PIM sequences obtained from seven cattle samples in this study, 31.8% (7/22) were cattle-type, 59.1% (13/22) were 'mixed'-type, and only 9.1% (2/22) were buffalo-type (Table 2). In comparison, no cattle-type PIM sequences were identified from 20 buffalo samples, 32% (24/75) of the PIM sequences obtained from buffalo samples were mixed-type, and 68% (51/75) were buffalo-type.

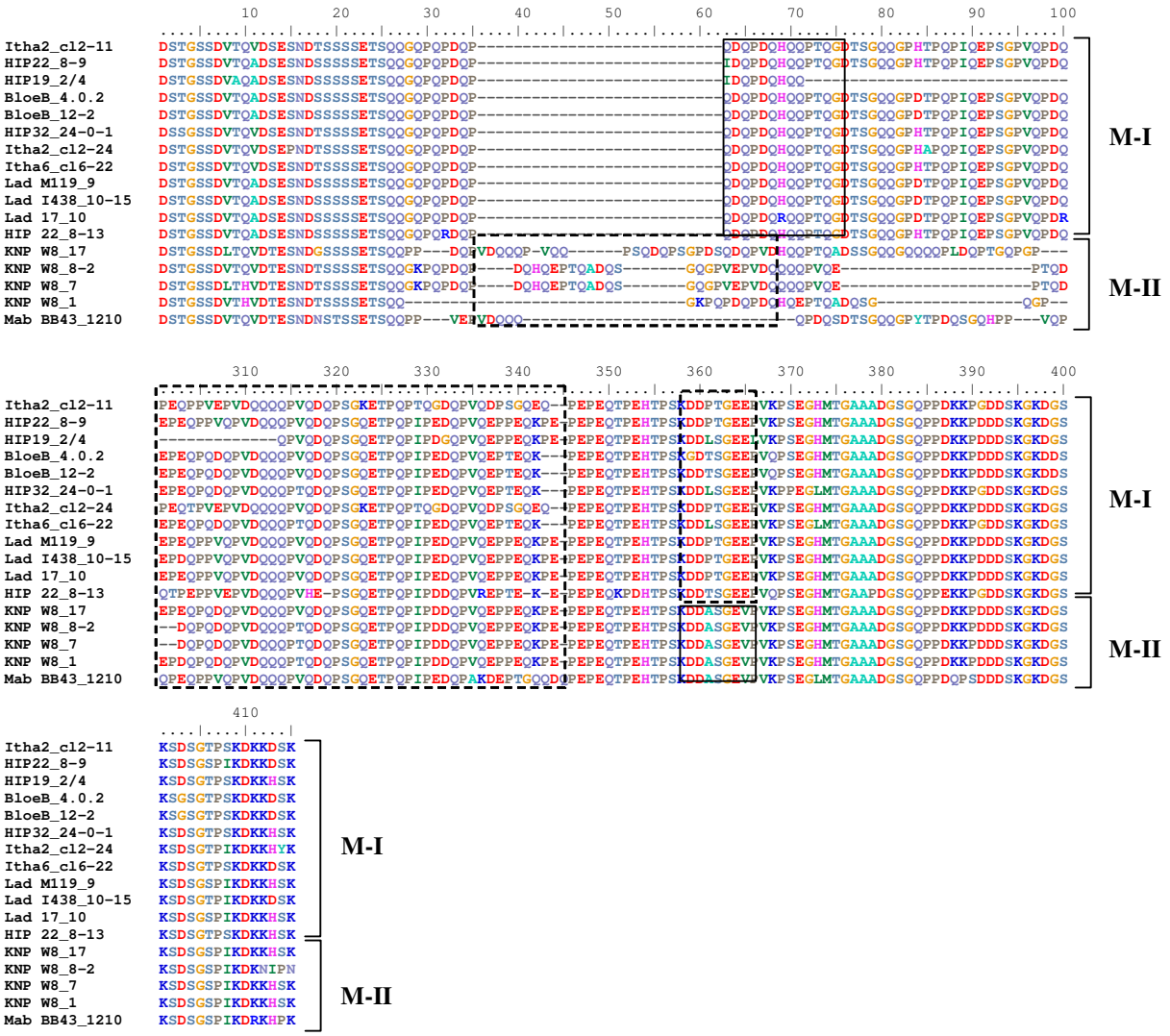


Figure 6.7 Multiple sequence alignment of representative amino acid sequences of 'mixed'-type *T. parva* PIM sequences, showing subtypes M-I and M-II. Regions typical of buffalo-type and cattle-type PIM sequences are indicated in broken-line and solid-line blocks, respectively. This alignment excludes the region between positions 101 and 300, which has buffalo-type PIM sequences in all subtypes.

6.5 Discussion

Previous studies on characterization of South African *T. parva* field samples revealed the presence of cattle-type p67 and p104 alleles (Chapter 4; Chapter 5; Sibeko *et al*., 2010), suggesting the presence of parasites with characteristics of cattle-derived *T. parva* in South Africa. Although ECF has not been reported in South Africa since its eradication in the early 1950s, these findings are a concern. In this chapter, a third gene was investigated to further characterize *T. parva* samples obtained from cattle and buffalo in South Africa to confirm results obtained by analysis of p67 and p104 genes.

Polymorphic immunodominant molecule PCR-RFLP profiles for the majority of *T. parva* field samples obtained from buffalo in this study were complex. Buffalo-derived *T. parva* isolates are expected to be heterogeneous and have previously been shown to display widely variable RFLP profiles because of multiplicity of infections in buffalo compared to single infections in cattle (Geysen, 2000; Geysen *et al*., 2004). Five PIM RFLP cluster groups were identified from BioNumerics cluster analysis of RFLP profiles obtained from cloned PIM amplicons but these showed no correlation to geographic origin of the samples within each group, as was shown for p104.

Surprisingly, relatively homogeneous PIM profiles were observed from samples originating from buffalo from Hluhluwe-iMfolozi, Mabalingwe and Ithala game parks. These findings could suggest that there is limited genetic diversity in *T. parva* parasites in these relatively small buffalo populations. However, RFLP profiles obtained from clones of PIM amplicons indicated that genetic diversity does exist in the Hluhluwe-iMfolozi, Mabalingwe and Ithala *T. parva* populations, as profiles from clones produced from these samples clustered in different groups. Although multiple PIM profiles were identified in cloned PIM amplicons from samples with homogeneous profiles, some profiles occurred more frequently than others, explaining the apparently homogeneous profiles obtained from these *T. parva* populations. In addition, in the Hluhluwe-iMfolozi samples the presence of a common ‘signature’ contributed to the apparent homogeneity of the profiles.

The extensively characterized *T. parva* PIM (Baylis *et al*., 1993; Toye *et al*., 1996) is encoded by a single copy gene and its structure consists of a central variable region, flanked by conserved 5’ and 3’ termini (Toye *et al*., 1995a; 1995b; Geysen *et al*., 2004). Previous studies have identified two groups of PIM sequences, and a number of characteristics could be used

to distinguish PIM sequences from cattle-derived *T. parva* isolates from those from buffalo-derived isolates (Toye *et al*., 1995b; Geysen *et al*., 2004). None of the PIM gene sequences obtained from *T. parva* field samples characterized in this study were identical, providing further evidence that the PIM gene evolves at an extremely high rate (Toye *et al*., 1995b). Extensive sequence heterogeneity among PIM sequences was demonstrated in the South African *T. parva* samples from buffalo examined in this study, confirming the extensive genetic diversity reported previously in *T. parva* parasites in buffalo (Chapter 4; Chapter 5; Collins and Allsopp, 1999; Sibeko *et al*., 2010). Furthermore, sequence variants of buffalo-type PIM sequences were identified which have never been reported before. The extensive polymorphism found in the coding region of the PIM gene is associated with selective pressure from the protective immune response and may confer selective advantage to the parasite (Toye *et al*., 1995a). Geysen *et al*. (2004) reported a high Ns/S (non-synonymous / synonymous substitutions) ratio in the PIM sequence which is an indication of the influence of selective forces on the sequence and the evasion of the host immune responses. Mechanisms responsible for the extensive diversity of the PIM gene and which influence its mosaic structure are not known, although X-like recombination motifs responsible for initiation of gene conversion events have been identified in PIM sequences (Geysen *et al*., 2004) and it is thought that this mechanism is likely to be responsible for the mosaic nature of the PIM gene. Novel alleles have been reported to arise from re-shuffling of important epitopes as a result of gene conversion and reciprocal intergenic exchanges (Dormoy *et al*., 1997). The discriminative characteristics of the PIM gene render it a good candidate for exploitation in discriminatory assays used for *T. parva* isolates (Bishop *et al*., 2001; De Deken *et al*., 2007). Nevertheless, it is advisable that assays based on this gene should be continuously evaluated because of the rapid evolution of the PIM gene.

In this study, for the first time, 'mixed' PIM sequences with characteristics of both PIM sequence types were identified. The combinations of different "blocks" of sequence observed in the PIM gene are reminiscent of the structure that has previously been shown for the precursor to the major merozoite surface antigens (PMMSA) in *Plasmodium falciparum* (Peterson *et al*., 1988). Recombination within the conserved blocks in the PMMSA is thought to result in reassembling of the variable blocks and accounts for much of the antigenic variation in this molecule. Studies of the population structure of *T. parva* parasites in Uganda showed that genetic exchange occurs frequently between isolates of *T. parva*, confirming the existence of a sexual cycle (Oura *et al*., 2005). Evidence for recombination between *T. parva* parasites, in a form of mosaic segments, has previously been observed in the internal

transcribed spacer (ITS) region (Collins and Allsopp, 1999) and sexual recombination between different *T. parva* stocks has been demonstrated in the laboratory (Morzaria *et al*., 1993; Bishop *et al*., 2002). It seems likely that the 'mixed' PIM sequences have arisen through recombination between cattle-type and buffalo-type PIM sequences.

While the 'mixed' PIM sequences identified in this study may well represent recombination events, we do not know the full extent of the recombination in the rest of the genome in these parasites. Recombination between buffalo-type *T. parva* parasites and cattle-type parasites can only occur where there has been contact between buffalo and cattle, and it has been reported that it is unlikely for recombinant parasites to become established in the cattle population (Geysen, 2000; Geysen *et al*., 2004). Nonetheless, 'mixed' PIM sequences were obtained from samples originating from both buffalo and cattle in this study, and the results indicate that 'mixed' PIM sequences are more prevalent in *T. parva* samples from cattle than in *T. parva* samples from buffalo. This could suggest that *T. parva* parasites with the 'mixed' PIM allele might be more likely to establish in cattle. In fact, very few buffalo-type PIM sequences were identified in cattle, suggesting that there may have been selection for both 'mixed'-type and cattle-type PIM sequences in cattle

Interestingly, no cattle-type PIM sequences were obtained from *T. parva* samples collected from buffalo. Buffalo are believed to carry a heterogeneous population of parasites and, as original hosts of the parasite, cattle-derived *T. parva* parasites are thought to have originated in buffalo, so we might have expected to see cattle-type PIM sequences in *T. parva* samples from buffalo. However, given the rapid nature of the evolution of the PIM gene, it is probable that cattle-type alleles are present at a low frequency in *T. parva* parasites in buffalo. It would therefore be necessary to clone the PIM genes from a larger number of buffalo samples in order to identify cattle-type PIM sequences. Findings obtained in a study on characterization of another *T. parva* antigenic gene, p67, revealed the presence of variants of the p67 allele similar to those of cattle-derived parasites in *T. parva* samples collected from buffalo in South Africa (Collins, 1997; Chapter 4; Sibeko *et al*., 2010), although no p67 sequence identical to the cattle-type allele was identified in buffalo samples. The PIM sequences obtained from samples with variants of cattle-type p67 were either buffalo-type (6/25, 24%) or 'mixed'-type (19/25, 76%) showing that other genes in parasites with variants of the cattle-type p67 allele are not necessarily cattle-type alleles. There is therefore a need to establish the significance of these alleles in the epidemiology of theileriosis and the risk they pose to the naïve cattle population in South Africa.

PCR-RFLP profiles similar to that of the *T. parva* Muguga stock were obtained from three of the six cattle samples from the Ladysmith farm and the inferred amino acid sequences of the PIM gene from two of these samples (Lad 02 and Lad 10) were almost identical to the *T. parva* Muguga PIM sequence. This finding supports recent studies in which p67 and p104 alleles similar to those of the *T. parva* Muguga stock were identified from the same Ladysmith samples (Chapter 4; Chapter 5; Sibeko *et al*., 2010). While it is not known if the Muguga-like RFLP profiles or sequences can be associated with the pathogenicity of *T. parva* isolates, findings in these studies strengthen the evidence for the presence of a subpopulation of *T. parva* parasites similar to ECF-causing East African strains in South Africa, at least on one farm. Our results might provide evidence for the selection of a subpoplution of *T. parva* parasites through cattle-to-cattle transmission of *T. parva* on the Ladysmith farm (resulting in parasites with cattle-type p67, p104 and PIM alleles). However, similarity between sequences is not necessarily an indicator of the association of the cattle-type PIM sequence with the disease syndrome, as ECF was not diagnosed on the Ladysmith farm.

Polymorphic immunodominant molecule PCR-RFLP profiles obtained from the remaining three samples from cattle from the Ladysmith farm occurred in three cluster groups, A, C and D. All three had the specific 'signature' which was observed from most of the *T. parva* samples collected from buffalo from Hluhluwe-iMfolozi Park, suggesting that some of the *T. parva* parasites on this farm may have originated from the Hluhluwe-iMfolozi buffalo. Furthermore, buffalo-type p67, p104 and PIM sequences were obtained from these three Ladysmith samples, further supporting the possibility of buffalo-to-cattle transmission on this farm. Unfortunately it was not possible to confirm whether the cattle on this farm had contact with infected buffalo (Thompson *et al*., 2008), but circumstantial evidence and our results indicate that this might have occurred.

The PIM profile obtained from a bovine sample from a farm in Bloemfontein had the 'signature' characteristic of most Hluhluwe-iMfolozi profiles also observed in some of the Ladysmith samples. The RFLP profiles from this sample occurred in cluster A where 28% and 18% of Ladysmith and Hluhluwe-iMfolozi profiles, respectively, also grouped. Moreover, buffalo-type and 'mixed'-type PIM sequences were obtained from the Bloemfontein sample. These results suggest that the *T. parva* parasite characterized from the Bloemfontein bovine sample was similar to *T. parva* parasites from Hluhluwe-iMfolozi, and in fact, the infected buffalo breeding herd on the Bloemfontein farm originated from Hluhluwe-iMfolozi. Therefore, it might be possible to use PIM RFLP profiles to track the

origin of *T. parva* infections, especially when the profiles have a specific signature that characterizes a particular parasite population. This case presents something of a puzzle: it is not known how the bovine on the Bloemfontein farm was infected as the tick vector, *R. appendiculatus*, has not been known to occur in the Bloemfontein area. It is possible that the vector tick was introduced onto the property along with the infected buffalo but did not become established, as intensive tick surveys failed to identify the presence of vector ticks (FT Potgieter, unpublished results).

6.6 Summary

In summary, the findings in this study confirm the presence of a cattle-type PIM allele in the same cattle *T. parva* samples from which p67 and p104 alleles similar to that of *T. parva* Muguga were obtained. Results presented here suggest that there may have been both cattle-to-cattle and buffalo-to-cattle transmission of *T. parva* on the Ladysmith farm. No cattle-type PIM alleles were identified from samples obtained from buffalo in this study. However, if the cattle-type alleles are present at a low frequency in *T. parva* parasites in buffalo, it is possible that insufficient samples were examined in this study and a larger number of samples would have to be investigated before any conclusions could be made. The extensive genetic diversity of *T. parva* parasite populations in South Africa was demonstrated in the identification of novel and 'mixed' PIM sequences. The significance of *T. parva* parasites carrying 'mixed' PIM alleles will have to be established and their risk to cattle evaluated. With the extent of genetic diversity that has been demonstrated by the three characterization studies presented in this thesis, it is clear that the population genetic structure of *T. parva* parasites in South Africa needs to be studied in detail, possibly by using other molecular tools such as mini- and micro-satellite markers (Oura *et al.*, 2003; 2005), to fully establish the parasite populations circulating in the country, and their threat and significance in the epidemiology of theileriosis in South Africa.

6.7 References

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