Development of a tandem repeat-based multilocus typing system distinguishing Babesia bovis geographic isolates

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ABSTRACT

Mini- and microsatellite sequences have proven to be excellent tools for the differentiation of strains and populations in several protozoan parasites due to their high variability. In the present work we have searched the genome of the tick-transmitted bovine hemoprotozoan Babesia bovis for tandem repeats (TRs) that could be useful for a multilocus typing system. Hundred and nineteen sequences were shortlisted and tested in five common B. bovis reference isolates originating from distinct geographic locations of North and South America: Texas, USA (T2Bo), Mexico (RAD and Mo7), and Santa Fe and Salta, Argentina (R1A and S2P, respectively). Satellite sequences were PCR-amplified using specific primers, separated by polyacrylamide gel electrophoresis, visualized by silver staining and sized. Fourteen TR sequences could be reliably amplified in all isolates and displayed length polymorphism. All primers used were specific for B. bovis and did not amplify genomic DNA from the bovine host or from Babesia bigemina, the principal co-infecting bovine parasite in the Americas, allowing their future use in field surveys. The 14 satellite markers identified are distributed throughout the four chromosomes of B. bovis as follows: chromosome 1 (n = 3), chromosome 2 (n = 2), chromosome 3 (n = 5), and chromosome 4 (n = 4). Within the five B. bovis isolates we identified nine satellite marker loci with two alleles, three with three alleles, one with four and another with five alleles. In comparison to Theileria parva, a bovine hemoprotozoan that pertains to the same piroplasmida order and owns a genome of similar size, the number of polymorphic TRs and the average number of alleles per TR locus seem to be significantly reduced in the B. bovis genome. Furthermore, the ratio of micro- to minisatellites in both B. bovis and T. parva is considerably lower than in other eukaryotes, as confirmed by bioinformatic analysis. The multilocus genotype of the five B. bovis isolates was assessed and the genetic distance between each other determined followed by cluster analysis based on neighbor joining. The resulting phenogram showed that B. bovis isolates segregated into three clusters according to their geographic origin. The presented marker system is suitable to explore various parameters of B. bovis.
populations such as genetic diversity, infection dynamics and their structure under different epidemiological situations, which are of crucial importance for improved control strategies.

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1. Introduction

Bovine babesiosis is a major constraint of cattle production causing great economic losses in many tropical and subtropical regions worldwide (Montenegro-James, 1992; Bock et al., 2004). Infection with the intracellular hemoproteozoon Babesia bovis is the most virulent cause of the disease and often fatal for susceptible animals. The parasite displays a heteroxenous life cycle between cattle and tick vector: infective sporozoites are transmitted by the Rhipicephalus microplus tick into the bovine host blood and invade erythrocytes. After development into the merozoite stage, a propagation cycle proceeds comprising binary fission followed by release from and reinvasion of erythrocytes. Merozoites may later be taken up during a tick blood meal. Similar to other apicomplexan hemoproteozoan parasites such as Plasmodium sp. and Theileria sp., B. bovis has a haploid life cycle with only a brief diploid phase as a zygote (kinese) which is formed by fusion of male and female gametes in the vector host. Subsequent meiotic division eventually leads to the formation of haploid infective sporozoites (Mehlhorn and Schein, 1984; Kakoma and Mehlhorn, 1994). For some Babesia species (B. bigemina, B. canis, and B. divergens) the haploid/diploid content of different parasite stages has been demonstrated by DNA measurements (Mackenstedt et al., 1990, 1995).

Recently, evidence has been presented that ticks are able to be co-infected with different B. bovis strains during their blood meal, a prerequisite for homologous interstrain recombination (Beren
d
e et al., 2007). Although genetic recombination between B. bovis strains has so far not been demonstrated, it has been suggested that it may contribute to the diversity of the variable merozoite surface antigens (VMSA) (Jasmer et al., 1992; Florin-Christensen et al., 2002; Berens et al., 2005; LeRoith et al., 2006).

Using a panel of micro- and minisatellite markers, it has recently been shown that multiple Theileria parva genome variants are generated by co-infection of the vector tick Rhipicephalus appendiculatus with different parasite isolates, providing evidence that homologous recombination is common in this closely related parasite (Katzer et al., 2006). Apart from their application in the direct demonstration of recombination, micro- and minisatellite markers have also been employed to quantitatively assess the rate of genetic exchange in Theileria sp. populations and in other pathogenic hemoproteozoans like Plasmodium sp. (Anderson et al., 2000; Oura et al., 2003, 2004, 2007; Razakandrainibe et al., 2005; Odongo et al., 2006; Annan et al., 2007; Weir et al., 2007; Beck et al., 2009). Due to these studies, increasing insight has been gained on the structure of Theileria sp. and Plasmodium sp. populations, but comparable investigations for B. bovis are lacking, although bovine babesiosis is considered the economically most important arthropod-transmitted pathogen of live-stock on a global scale (Bock et al., 2004). It must be emphasized that knowledge on the population structure of pathogenic hemoproteozoa is not solely of academic interest but does provide valuable information allowing a more rational application of control measures. Thus, it may enable to assess the risk of vaccine breakthroughs that have been reported to occur following the use of B. bovis live vaccines (Bock et al., 1992, 1995; Lew et al., 1997b).

In Plasmodium sp., it is accepted that high rates of inbreeding resulting in a clonal population structure promote the spread of haplotypic chloroquine resistance in regions with high infectivity (Curtis and Otoo, 1986; Dye and Williams, 1997; Hastings, 1997; Wootton et al., 2002). In contrast, low rates of inbreeding seem to lead to a slow spread of resistance in regions with high infectivity (Dye and Williams, 1997; Hastings, 1997). Accordingly, the incidence of transfer of virulent/pathogenic factors to an attenuated/non-virulent parasite is likely influenced by the rate of inbreeding and genetic exchange in B. bovis populations.

For T. parva it has been discussed that the use of live vaccines may actually not contain but even spread the disease (McKeever, 2007). Evidence has been provided that alleles associated with the vaccine strain emerge in non-vaccinated co-grazing cattle (Oura et al., 2004, 2007). However, it has been also suggested that the substantial recombination demonstrated in complex T. parva populations results in transient existence of defined parasite strains and, likely, also of vaccine strains in the field (McKeever, 2007). In B. bovis, the spread of live vaccines or its genetic components in the field has not been investigated. As for Theileria sp., a marker system for B. bovis would allow to investigate such a phenomenon and, correspondingly, by assessing the rate of inbreeding and recombination in populations of this parasite, provide clues as to the significance of these events.

We present here a set of micro- and minisatellite markers that will facilitate such investigations for B. bovis. In the report at hand, these markers have been applied to characterize and compare five common B. bovis reference isolates.

2. Materials and methods

2.1. Parasite material and DNA preparation

The B. bovis parasite stocks used in this study are detailed in Table 1. The pathogenic strain B. bigemina S2P was used to check for amplification of PCR primers. Merozoites were multiplied in vitro up to a parasitemia of 6% and then purified as previously described (Rodriguez et al., 1986). Subsequently, genomic DNA was extracted with phenol/chloroform according to standard procedures (Sambrook et al., 1989).
The tandem repeat finder (TRF) was used to screen for TR sequences in the B. bovis and Theileria annulata genomes (Brayton et al., 2007; Benson, 1999). TRF is one of the most used TR finder programs and has been successfully applied for the development of micro- and minisatellite markers in other apicomplexan parasites (Mallon et al., 2003; Weir et al., 2007). In contrast to most other algorithms, TRF can generate more comprehensive and comparative surveys as it (i) allows to screen for micro- and minisatellites, and (ii) permits a high flexibility of parameter adjustment enabling the announcement of varying degrees of imperfect repeats. Recently, the TRF has been integrated into the Tandem Repeats Database (TRDB). TRDB is a public database on TRs in genomic DNA that contains many tools for their analysis (Gelfand et al., 2006), including query and filtering capabilities for finding particular repeats of interest. These tools allowed the selection of micro- (period size between 2 and 5 bp) and minisatellites (period size between 6 and 21 bp) exceeding a copy number of 7. The selected sequences were tested for polymorphism using the panel of B. bovis isolates by PCR amplification.

2.3. PCR amplification of micro- and minisatellite loci

Primers were designed to specifically bind the regions flanking the TR sequences. PCR was performed to test these primers for reliable generation of amplicons and length polymorphism. The amplification reaction was carried out in a final volume of 25 μl and the reaction mix consisted of PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 200 μM of each dNTP, 0.8 μM of forward and reverse primers, and 0.75 units of Taq polymerase, to which 40 ng of genomic template DNA was added. Cycling conditions consisted of a first denaturing step of 3 min at 94 °C, followed by 30 cycles each composed of 94 °C for 30 s, 57 °C (or alternatively 60 °C) for 45 s, and 72 °C for 45 s. The final extension step was set at 5 min at 72 °C, after which samples were held at 4 °C until being processed. To check for successful amplification, 5 μl of the PCR reaction was separated on 2% agarose gels. Gels were stained with ethidium bromide and the DNA bands were visualized on a UV transilluminator and photographed.

2.4. Separation of marker amplicons

Amplification products were separated on 8% polyacrylamide gels under non-denaturing conditions or on 5% polyacrylamide/7 M urea denaturing gels and detected by silver staining (Bio-Rad, Hercules, CA). The size of each band was estimated by means of either a 25 bp or a 10 bp DNA ladder (Invitrogen, Carlsbad, CA), respectively, in adjacent lanes of the gel. Under non-denaturing conditions, amplicons differing in 5–10 bp could be confidently distinguished while denaturing gels were able to distinguish size differences of up to 1 nt.

2.5. Data analysis

Bands detected after separation were considered alleles. Letters were alphabetically assigned with increasing size of these alleles. In case two or more bands were observed, the most intense allele band was scored to unequivocally determine the single predominant haplotypic multilocus genotype. In a singular case where two alleles showed a similar intensity, the one corresponding to the predicted allele size was considered. Based on the alleles found, the respective multilocus genotype was assessed. A distance matrix was established by determining the number of different alleles between each isolate pair. The Clustering calculator was used (http://www.biology.ualberta.ca/jbrzusto/cluster.php) to carry out a cluster analysis based on the neighbor-joining method (Saitou and Nei, 1987). The genetic difference between isolates was visualized by constructing a phenogram with Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

3. Results and discussion

In this work we developed a micro- and minisatellite marker system for B. bovis and applied it to compare common reference isolates of this parasite. Using the tandem repeat finder program (TRF) the B. bovis genome was searched for tandem repeats (TRs) by adjustment of relaxed parameter settings (alignment gap penalties for matches 2, mismatches 3, and indels 5; a minimum alignment score of 50, and a maximal period size of 50) that detect perfect and imperfect repeats (Benson, 1999; Brayton et al., 2007). Altogether 3885 TR loci were announced and further filtered for TR candidates most qualified for inclusion in the subsequent PCR test by using query tools of the Tandem Repeats Database (TRDB, Gelfand et al., 2006). Acceptance criteria considered were (i) contribution to an even marker distribution along the genome, (ii) a repeat unit size of less than 22 bp so that the PCR amplicons would be smaller than 400 bp and thus be adequately resolved by gel electrophoresis, and (iii) a high copy number (≥8), as this has been shown to be linked with increased polymorphism (Imwong et al., 2006; Russell et al., 2006).
<table>
<thead>
<tr>
<th>Marker</th>
<th>Chr</th>
<th>PCR primera</th>
<th>Tmbb</th>
<th>Consensus repeat sequencec</th>
<th>Allele size rangeed</th>
<th>Number of allelesf</th>
<th>Primer binding site</th>
<th>Closest CDS</th>
<th>Location in chr/contig</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>I</td>
<td>GGGCCCTTTATAGAAACCTTTCA GCCGTTATGTAAGAACCTTCA</td>
<td>57</td>
<td>(AACCTTTA) 7.8</td>
<td>150-170</td>
<td>3</td>
<td>Intergenic</td>
<td>BBOV_I005120</td>
<td>NW001820854 790580-735</td>
</tr>
<tr>
<td>MS-2</td>
<td>I</td>
<td>ACTAGTGAGGAGGGTCAAGG AATGCAACTTTGCTTAACCTTA</td>
<td>60</td>
<td>(ACTAAGGGG) 10.6</td>
<td>240-260</td>
<td>3</td>
<td>Exon</td>
<td>BBOV_I005140</td>
<td>NW_001820854 798322-567</td>
</tr>
<tr>
<td>MS-3</td>
<td>I</td>
<td>ACTACACTCCTTGTAAGAATTCA CGTGGAAACGCTGAGATATT</td>
<td>60</td>
<td>(GTGGTA) 6.4</td>
<td>125-150</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_I001140</td>
<td>NW_001820853 27324-430</td>
</tr>
<tr>
<td>MS-4</td>
<td>II</td>
<td>GCACTAGTTGGTCTAGGTAAG GTAATCTTCCGTCCTTATATCTT</td>
<td>57</td>
<td>(GCCGTTGCAAT) 7.3</td>
<td>110-150</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_II001410</td>
<td>NC_010574 338623-779</td>
</tr>
<tr>
<td>MS-5</td>
<td>II</td>
<td>AAAAGAAAGATCAGACACCAG CCTTATGCTCTTTGGAAAGGAAT</td>
<td>60</td>
<td>(GAAGAT) 16.2</td>
<td>225-230</td>
<td>3</td>
<td>Exon</td>
<td>BBOV_II006510</td>
<td>NC_010574 1453220-447</td>
</tr>
<tr>
<td>MS-6</td>
<td>III</td>
<td>TTTATGATGTGATCTGATTGAAATG GCAGAGATCTCCTCCTTTA</td>
<td>60</td>
<td>(TAGGTTTA) 15.7</td>
<td>250-310</td>
<td>2</td>
<td>Intergenic</td>
<td>BBOV_II000040</td>
<td>NC_010575 13029-306</td>
</tr>
<tr>
<td>MS-7</td>
<td>III</td>
<td>TCACATGAGATCCATCTTACG TAGTTGCAGGTTTATCTGTTT</td>
<td>60</td>
<td>(TACGGTGG) 6.3</td>
<td>210-250</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_II000680</td>
<td>NC_010575 171520-755</td>
</tr>
<tr>
<td>MS-8</td>
<td>III</td>
<td>CAACACAGAGAACACCTCCT CTTGAGGCTGTTGAAACCTTTA</td>
<td>60</td>
<td>(ACCTGAAGCCG) 7.4</td>
<td>280-320</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_II002020</td>
<td>NC_010575 475511-792</td>
</tr>
<tr>
<td>MS-9</td>
<td>III</td>
<td>GTACTGCCCCGACAAATGTT GTGTGACATGCACAGCTGTGTT</td>
<td>60</td>
<td>(ATTATTACTGTGTCT) 10.5</td>
<td>225-250</td>
<td>2</td>
<td>Intergenic</td>
<td>BBOV_II002310</td>
<td>NC_010575 541873-2122</td>
</tr>
<tr>
<td>MS-10</td>
<td>III</td>
<td>CAAAATACGGGAGCGACCA CTGGAACCTCTGCTGTTTACAGCT</td>
<td>60</td>
<td>(AAGAGGAAG) 13.8</td>
<td>200-225</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_II006480</td>
<td>NC_010575 1393270-481</td>
</tr>
<tr>
<td>MS-11</td>
<td>IV</td>
<td>CATGACTGCTCCTGCTCTGTT ACCTGCCATTTTCCTTACTTACGTTT</td>
<td>60</td>
<td>(GACCGTGAAG) 14.3</td>
<td>225-275</td>
<td>4</td>
<td>Ex-intron</td>
<td>BBOV_IV002980</td>
<td>NW_001820855 677829-8106</td>
</tr>
<tr>
<td>MS-12</td>
<td>IV</td>
<td>AAAGACATATGCTGAGAACGATTCGCTGAGATTGG TAGGTTACCTGTGTTT</td>
<td>57</td>
<td>(GCGATAAGGATG) 8</td>
<td>150-300</td>
<td>5</td>
<td>Exon</td>
<td>BBOV_IV007920</td>
<td>NW_001820857 895975-6262</td>
</tr>
<tr>
<td>ms-1</td>
<td>IV</td>
<td>TTGAGAACCTCCGCTTTACAT CAATGGTACATTCTGGTGTTT</td>
<td>60</td>
<td>(GAT) 10</td>
<td>265-270</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_IV008970</td>
<td>NW_001820857 1195396-659</td>
</tr>
<tr>
<td>ms-2</td>
<td>IV</td>
<td>GGTGAAACAGTACGACAAAAACACATTGTATTGATCACTTAGCATT</td>
<td>60</td>
<td>(GAA) 20</td>
<td>280-300</td>
<td>2</td>
<td>Exon</td>
<td>BBOV_IV010740</td>
<td>NW_001820857 1485917-6212</td>
</tr>
</tbody>
</table>

a The upper forward and lower reverse primer is given.
b Annealing temperature of primer pairs.
c The consensus repeat sequence is given in brackets and the copy number as index.
d Allele size range as estimated by polyacrylamide electrophoresis of amplicons; index gives allele size as expected for T2Bo.
e Alleles observed in investigated samples.
Hundred and nineteen TRs were found to satisfy the above criteria. PCR primers were designed to bind to flanking regions and then tested for reliable amplification and size polymorphism of amplicons using genomic DNA from B. bovis reference isolates (Table 1). Twenty TR loci were shortlisted as their amplification showed differences in size and/or number of generated amplicons. After a second rigorous selection with regard to ease of allele pattern interpretation, and location in the genome, a final assortment of 14 satellite markers remained. In Table 2, primer sequences of each of these markers, their repeat unit, copy number, observed size range and number of their alleles, genomic location and closest coding sequence are shown. For each of the presented micro- and minisatellite markers, the size of alleles amplified from genomic DNA of B. bovis T2Bo was found to be consistent with that expected as based on the published genome of this strain, providing evidence that the desired allele had been amplified (Brayton et al., 2007). Furthermore, primer pairs were found to be specific for B. bovis since they neither amplified genomic DNA of the bovine host nor of B. bigemina (data not shown). This primer specificity allows their potential future use in field samples since B. bigemina is the principal co-infecting parasite in the Americas (Montenegro-James, 1992). The presented 14 TR marker loci should be adequate for population genetic analyses as their number well exceeds the considered lower limit of 7 loci should be adequate for population genetic analyses as their number well exceeds the considered lower limit of 7.

Table 3

Frequency range of tandem repeats in the B. bovis and T. parva genome reported by TRF.

<table>
<thead>
<tr>
<th>Piroplasmid</th>
<th>Frequency range of tandem repeats* (TR = \sum_{ms}^{MS} MS)</th>
<th>Frequency range of microsatellites* (MS)</th>
<th>Frequency range of minisatellites (MS)</th>
<th>Ratio of ms/MS$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. bovis</td>
<td>93–25,399</td>
<td>0–7,128</td>
<td>93–18,271</td>
<td>0/93 to 1/2.6</td>
</tr>
<tr>
<td>T. parva</td>
<td>688–69,171</td>
<td>2–13,368</td>
<td>686–55,803</td>
<td>1/343 to 1/4.2</td>
</tr>
</tbody>
</table>

In all cases, the first value given was determined by the TRF program at the most stringent parameter settings (alignment gap penalties for matches 2, mismatches 2, and indels 7 and an minimum alignment score of 150) and the second value at the most relaxed parameter settings (second value, alignment gap penalties for matches 2, mismatches 3, and indels 5 and a minimum alignment score of 20).

* Tandem repeats (TRs) defined by a period size of 2–50 bp.
* Microsatellites defined by a period size of 2–5 bp.
* Minisatellites defined by a period size 6–50 bp.
* Number of tandem repeats per megabase genome.
* Ratio of micro- to minisatellites (in brackets the corresponding percentages are given).
2006). Thus, the low ration of micro- to minisatellites observed for *B. bovis* and *T. parva* is intriguing. Standardized direct comparisons with different other non-piroplasmid species may be needed since diverse search algorithms were used in the investigations published so far, and TR subsets of different repeat lengths, and/or exclusively perfect TR sequences were compared.

The identified micro- and minisatellites are rather evenly distributed along the four chromosomes (Fig. 1), rendering it possible to investigate the reassortment of inter- and intra-chromosomal regions across the whole genome. With respect to their location to coding regions, five of the fourteen markers are located within members of the variant erythrocyte surface antigen (ves) gene family (MS-1, MS-2, MS-3, MS-4, MS-12) and four in genes encoding hypothetical proteins (MS-5, MS-8, ms-1, ms-2). Three other markers are each located in the gene encoding the eukaryotic initiation factor 4G middle domain (MS-11), spherical body protein 2-truncated copy (MS-10), and a putative membrane protein (MS-7). The remaining two markers (MS-6, MS-9) are located in non-coding regions (Table 2). Accordingly, 11 of the 14 satellite markers are located in exons, one of which is straddling an intron, while the remaining three are situated in intergenic regions (Table 2). Not surprisingly, most markers are located in coding sequences since non-coding regions constitute only 30% of the 8.2 Mb *B. bovis* genome (Brayton et al., 2007). The finding that five of the fourteen markers are seated in members of the ves genes suggests that a considerable amount of TR loci polymorphism may be contained in the ves multigene family which encodes about 120 highly variable surface antigens (Brayton et al., 2007). This supposition was supported by a subsequent genomic examination which revealed that 22 of the 119 candidate TR loci identified in this study are located in ves genes.

In Fig. 2, selected non-denaturing polyacrylamide gels are shown to exemplify allele separation. To substantiate the evaluated allele patterns, samples were also run on denaturing polyacrylamide gels. Due to the higher resolution of this method, allele patterns displayed by marker loci MS-5 and ms-1, that could not be exhaustively resolved in non-denaturing polyacrylamide electrophoresis, could be unambiguously resolved by this method (data not shown). Interestingly, the multilocus genotypes of the two Mexican strains Mo7 and RAD, each of which represents a biological clone, exhibit identical alleles for all but marker MS-12 (Table 4). The clonal composition of

![Fig. 1.](image1) *B. bovis* chromosomes showing the location of the micro- (ms) and minisatellite markers (MS) identified in this work.

![Fig. 2.](image2) Marker analysis of *B. bovis* isolates. Silver stained polyacrylamide gels run under non-denaturing conditions to separate PCR-amplified alleles of mini- and microsatellite markers MS-4 (A), MS-8 (B), MS-10 (C), MS-11 (D), MS-12 (E), and ms-2 (F) are shown. Amplicons were generated from genomic DNA of *B. bovis* isolates T2Bo (lane 1), Mo7 (lane 2), RAD (lane 3), R1A (lane 4), and S2P (lane 5).
both lines was confirmed by the finding that amplification of each of the 14 markers resulted in the appearance of a single allele. It is known that these lines are independent clones, though it cannot be excluded that both originate from the same paternal isolate (Juan Mosqueda, pers. communication).

In contrast, isolates T2Bo, S2P, and R1A are likely comprised of a mixture of parasite variants, as for some markers amplification resulted in the appearance of up to two (S2P, R1A) or three (T2Bo) alleles of lesser intensity. In these instances the most intensive allele was scored while in a single occasion, in which the intensity of two alleles could not be distinguished for isolate T2Bo (marker MS-10, Fig. 2B), the appropriate allele could be identified since it had to correspond in size to that predicted by the published T2Bo genome. As shown in Table 4, a unique predominant multilocus genotype could be unambiguously determined for all isolates by following this procedure, a prerequisite to carry out population genetic studies like the below performed cluster analysis. In average 2.5 alleles per TR locus (35 alleles/14 TR loci) were found in the five investigated _B. bovis_ isolates originating from North, and South America. In comparison, 17 of the 60 TR marker identified in _T. parva_, have been used to assess an average of 3.5 alleles in a group of five _T. parva_ isolates from East and Southern Africa (Oura et al., 2003). Thus, in addition to a substantially decreased number of polymorphic TR loci in _B. bovis_ as outlined above, also the allelic polymorphism (number of alleles per TR marker loci) seems to be lower in this parasite as compared to _T. parva_.

A phenogram visualizing the genetic distance between the five isolates is shown in Fig. 3. Three principal clusters can be observed. The first cluster comprises the _B. bovis_ isolate from Texas (T2Bo) that shows a similar genetic distance to the Mexican and to the Argentinian isolates. A second cluster comprises the cloned Mexican parasites Mo7 and RAD while isolates R1A and S2P from Argentina segregate in a third cluster. Thus, based on their multilocus genotype differences, _B. bovis_ reference isolates can be clearly linked to their geographic origin. Although these findings might be indicative of a population sub-structuring that is based on geographic isolation, more comprehensive future investigations using larger study groups will be needed to show whether this notion holds true.

Genotyping of Australian _B. bovis_ isolates by three different PCR methods has been reported by Lew et al. (1997a). PCR assays of the BvVA1 and Bv80 genes were established and amplification products defining parasite stocks were either identified by size, by hybridization with specific probes, or by enzymatic restriction. In another report American _B. bovis_ strains were characterized by PCR-RFLP assay of the _msa2-a/b_ genes (Wilkowsky et al., 2008). These methods, based on polymorphism of single surface antigen genes, are able to discriminate between isolates and in the case of the Australian study have been applied to studies of vaccine breakthroughs in the field (Lew et al., 1997b; Bock et al., 2000). Also the use of a RAPD assay to distinguish _B. bovis_ isolates has been reported (Lew et al., 1997a; Carson et al., 1994). RAPD proved to distinguish isolates with a higher sensitivity than assays based on single gene polymorphism, and the observed “fingerprint” differences were shown to correlate with the physical/geographic distance of isolate origins (Carson et al., 1994). However, this method suffers from several drawbacks, as observed differences are difficult to reproduce and interpret, and can usually not be linked

![Fig. 3. Phenogram visualizing genetic distances between _B. bovis_ isolates.](image-url)
to specific genotypes. Furthermore, RAPD does not allow estimating the clonal composition of an isolate. A genomic multilocus genotyping system as presented in this work overcomes the restrictions posed by single locus genotyping and/or RAPD as it combines the advantages of a high resolution with the possibility to link observed differences to specific genotypes and estimate the clonal composition of isolates. In addition, single recombination events can be observed as well as the frequency of genetic exchanges in parasite populations under different epidemiological situations assessed.

In Argentina, water buffaloes are reported to be *B. bovis* carriers and their numbers have exponentially grown in the last decade. To ensure the success of the ongoing tick control and eradication campaign, it has been therefore proposed to include them into sanitary surveilances (Ferreri et al., 2009). Preliminary investigations using markers MS-8 and ms-2 show that allele composition from field samples of parasite-infected water buffaloes could be unambiguously assessed using a nested PCR approach (data not shown). This demonstrates that the presented multilocus typing system is principally applicable to determine the genotypic composition of *B. bovis* populations in water buffaloes and compare it with that in cattle grazing on the same field. Likewise, the investigation of the structure of *B. bovis* populations in white tailed deer as compared to cattle would be desirable since these ungulates have also been suggested to act as carriers for this parasite (Cantu et al., 2007). The reintroduction of *R. microplus*-ticks from Mexico to the USA, where it has been eradicated, represents a continuous threat to cattle herds (Montenegro-James, 1992; Cantu et al., 2008). Such studies might reveal differences of the parasite populations in cattle and sympatric carrier animals providing valuable information on the interdependence and complex infection dynamics between them.

In summary, the developed multilocus typing system will enable to determine genetic diversity, infection dynamics and structure of *B. bovis* populations. As these parameters have a strong impact on epidemiology and pathogenicity, their appraisal will lead to an improvement of current disease control and vaccination strategies.

**Conflict of interest**

The authors declare no conflicts of interest.

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**References**


