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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 hemoprotozoon *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*

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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 hemoprotozoon *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 hemoprotozoan parasites such as *Plasmodium* sp. and *Theileria* sp., *B. bovis* has a haploid life cycle with only a brief diploid phase as a zygote (kinete) 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 (Berens 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 hemoprotozoans 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 livestock on a global scale (Bock et al., 2004). It must be emphasized that knowledge on the population structure of pathogenic hemoprotozoa 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).

Table 1
Babesia bovis isolates used in this study.

B. bovis stock	Country of origin	Characteristics	Reference
T2Bo	TX, USA	Virulent strain	Hines et al. (1992)
RAD	Mexico	Biological clone, attenuated by irradiation	Rodriguez et al. (1983)
Mo7	Mexico	Biological clone, vaccine strain	Rodriguez et al. (1983), Shkap et al. (1994)
S2P	Salta, Argentina	Virulent strain	Echaide et al. (1993)
R1A	Santa Fe, Argentina	Attenuated by passage through splenectomized calves	Anziani et al. (1993)

2.2. Identification of tandem repeats (TRs)

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 $MgCl_2$, 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 ($x \geq 8$), as this has been shown to be linked with increased polymorphism (Imwong et al., 2006; Russell et al., 2006).

Table 2
Micro- and minisatellite markers for population genetic studies of *B. bovis*.

Marker	Chr	PCR primer[a]	Tm[b]	Consensus repeat sequence[c]	Allele size range[d]	Number of alleles[e]	Primer binding site	Closest CDS	Location in chr/contig
MS-1	I	GGCGCCCTTATAGAACTTTCA GCCGCTATGGTAAAACTCCA	57	$(AACCTTTACTGA)^{7.8}$	$150\text{-}170^{155}$	3	Intergenic	BBOV_I005120	NW001820854 790580-735
MS-2	I	ACTAGTGGAGGGGGTGAAGG AATGCCTTGTGTAGCCCATC	60	$(ACTAAGGGGG)^{10.6}$	$240\text{-}260^{245}$	3	Exon	BBOV_I005140	NW_001820854 798322-567
MS-3	I	ACTCACTGCGGTGTAAAGTTCA CGGTGAAACGCTGAGAGATATT	60	$(GTTGTAGTA)^{6.4}$	$125\text{-}150^{156}$	2	Exon	BBOV_I001140	NW_001820853 273274-430
MS-4	II	GCACTAGTTGGGTGGAGTAGGA TGTTCTCCGGCTCTATATCCTT	57	$(GGCGGTGGCAAT)^{7.3}$	$110\text{-}150^{157}$	2	Exon	BBOV_II001410	NC_010574 358623-779
MS-5	II	AAAAGGAAAAGATCACGACCAG TTCTATGTCTTTGAAAGCGGAAT	60	$(GAAGAT)^{16.2}$	$225\text{-}230^{228}$	3	Exon	BBOV_II006510	NC_010574 1453220-447
MS-6	III	TTTATGTAGTGATCATGGTAGGAAATG GCAGAAGATCTCCCCATTGTTA	60	$(TAGGGTTTA)^{15.7}$	$250\text{-}310^{278}$	2	Intergenic	BBOV_III000040	NC_010575 13029-306
MS-7	III	TCACTATCGCATGCCATTTC ACTGTGACAGGGGTTCCTTG	60	$(TACTGGTGG)^{6.3}$	$210\text{-}250^{235}$	2	Exon	BBOV_III000680	NC_010575 171520-755
MS-8	III	CAACACCAGAAGCACCTCCT CCTGAGCGTTGATGCAAATA	60	$(ACCTGAAGCGGC)^{7.4}$	$280\text{-}320^{281}$	2	Exon	BBOV_III002020	NC_010575 475511-792
MS-9	III	GTACTTGCCCGGACAATAGGT CGTTTGTACGGTATCAAGTCGTT	60	$(ATTATTAGTGTCT)^{10.5}$	$225\text{-}250^{249}$	2	Intergenic	BBOV_III002310	NC_010575 541873-2122
MS-10	III	CAAAATACGGGAGGCAGAAA TCTGGTCCTTTGCACTGATCT	60	$(AAGAGGAAG)^{13.8}$	$200\text{-}225^{211}$	2	Exon	BBOV_III006480	NC_010575 1393270-481
MS-11	IV	CATGACTGCTAGCCTACTTGCTT ACTCGCCCATAAAACGAATG	60	$(GACGCTGAAAAG)^{10.3}$	$225\text{-}275^{277}$	4	Ex-intron-ex	BBOV_IV002980	NW_001820855 677829-8106
MS-12	IV	AAGACACTATTGGAGAAGATTGG CTCCGCTGCCTTCTCTAGT	57	$(GGCTAAGGATG)^{8}$	$150\text{-}300^{288}$	5	Exon	BBOV_IV007920	NW_001820857 895975-6262
ms-1	IV	TTGAGGACCTTCCGTCTTACAT CATTCGTGATTCATTGCTTGTT	60	$(GAT)^{10}$	$265\text{-}270^{273}$	2	Exon	BBOV_IV008970	NW_001820857 1109386-659
ms-2	IV	GGTGAAACACGAATCAGACAAG AACCACCTGATCAGCATTCTTT	60	$(GAA)^{20}$	$280\text{-}300^{295}$	2	Exon	BBOV_IV010740	NW_001820857 1485917-6212

[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 marker loci necessary for such studies (de Meeûs et al., 2007).

A micro- and minisatellite marker system has also been developed for *T. parva* strain typification by Oura et al. (2003). *T. parva* is, as *B. bovis*, a bovine-infecting pathogenic piroplasmid and features a genome of similar size (~8.3 Mb). Our study provides indications that the TR polymorphism in the *B. bovis* genome is of considerable lesser extent than that observed in *T. parva*. As outlined above, fourteen out of hundred and nineteen TR loci (12%) were found to exhibit polymorphism and qualified as genetic markers when tested in five *B. bovis* isolates originating from North and South America. In contrast, sixty out of hundred and thirty-three TR loci (45%) were found to be polymorphic and eligible as markers after PCR screening of only two *T. parva* isolates originating from two locations in Kenya, Muguga and Makebuni (Oura et al., 2003). Thus, although a roughly comparable number of TR loci were screened by PCR for polymorphism in five *B. bovis* isolates originating from a far greater geographic range (the Americas) than the corresponding two *T parva* isolates (Kenya), a significantly lower proportion of polymorphic TR loci was identified (12% vs. 45%). To find out whether, apart from a lower proportion of polymorphic TR loci, the *B. bovis* genome may also exhibit a lower absolute number of TRs, the overall content of TR sequences in the genome of both parasites was determined using TRF at the most stringent and relaxed parameter settings. As shown in Table 3, 8 times less perfect TRs and 2.7 less TRs at relaxed parameter settings were identified for *B. bovis* as compared to *T. parva*. This substantially lower overall content of TR loci in the *B. bovis* genome likely contributes to a considerably decreased total amount of polymorphic TR sequences in this parasite.

Surprisingly, only two of the fourteen markers identified in *B. bovis* are micro- while the remaining twelve are minisatellites, corresponding to a ratio of 1 micro- to 7 minisatellites (Table 2). A roughly similar ratio of 1 micro- to 5 minisatellite markers (11 micro- vs. 49 minisatellite markers) were, as outlined above, identified after 133 candidate TR loci were screened by PCR using two *T. parva* isolates from Kenya (Oura et al., 2003). To determine whether the significantly lower number of identified polymorphic micro- compared to minisatellite sequences may reflect a correspondingly lower presence of this class of TR sequences in the genome of these piroplasmid species, their ratio and frequency was assessed. As can be taken from Table 3, the ratio of about 1 micro- to 2.6 minisatellites (39%) for *B. bovis* and 1 micro- to 4.2 minisatellites (24%) for *T. parva* at relaxed parameter settings roughly approximates the above mentioned ratios for these parasites obtained after PCR testing. On the other hand, when stringent parameters were set to exclusively detect perfect TRs, the reduction in the number of micro- compared to minisatellites in *B. bovis* and *T. parva* genomes was found to be even more pronounced (Table 3). In eukaryotes, microsatellites are usually regarded to be about 10–300 times more abundant than minisatellites (Richard et al., 2008). Also in other apicomplexans such as *Plasmodium* and *Cryptosporidium* spp. microsatellites have been reported to be more frequent than minisatellites, at least when perfect TRs are considered (Su and Wellems, 1999; Toth et al., 2000; Mallon et al., 2003; Sobreira et al.,

Table 3
Frequency range of tandem repeats in the *B. bovis* and *T. parva* genome reported by TRF.

Piroplasmid	Frequency range of				Ratio of ms/MS[e]
	Tandem repeats[a] (TR = ∑ms + MS)	Microsatellites[b] (ms)	Minisatellites[c] (MS)	Tandem repeats per Mb[d] (TR/Mb)	
B. bovis	93–25,399	0–7,128	93–18,271	11–3097	0/93 to 1/2.6 (0.0–39.0%)
T. parva	688–69,171	2–13,368	686–55,803	82–8334	1/343 to1/4.2 (0.3–24.0%)

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).

[a] Tandem repeats (TRs) defined by a period size of 2–50 bp.
[b] Microsatellites defined by a period size of 2–5 bp.
[c] Minisatellites defined by a period size 6–50 bp.
[d] Number of tandem repeats per megabase genome.
[e] Ratio of micro- to minisatellites (in brackets the corresponding percentages are given).

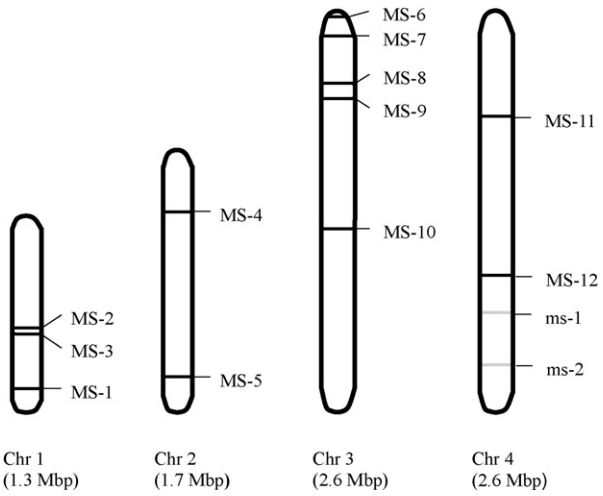

Fig. 1. *B. bovis* chromosomes showing the location of the micro- (ms) and minisatellite markers (MS) identified in this work.

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

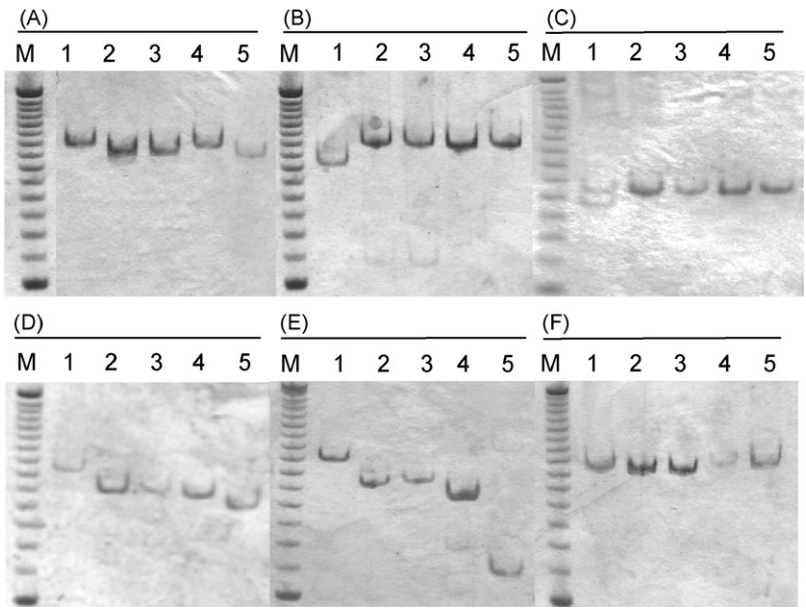

Fig. 2. Marker analysis of *B. bovis* isolates. Silver stained polyacrylamide gels run under non-denaturing conditions to separate PCR-amplified alleles of mini- and microsatellite markers MS-4 (A), MS-8 (B), MS-10 (C), MS-11 (D), MS-12 (E), and ms-2 (F) are shown. Amplicons were generated from genomic DNA of *B. bovis* isolates T2Bo (lane 1), Mo7 (lane 2), RAD (lane 3), R1A (lane 4), and S2P (lane 5).

Table 4
Tandem repeat alleles identified in *B. bovis* reference isolates of different geographic origin.

	Marker loci[a]													
	Chr I			Chr II		Chr III					Chr IV			
Isolates	MS-1	MS-2	MS-3	MS-4	MS-5[b]	MS-6	MS-7	MS-8	MS-9	MS-10[c]	MS-11	MS-12	ms-1[b]	ms-2
T2Bo	B	B	A	A	C	A	A	B	A	B	A	A	A	B
Mo7	C	C	A	B	B	A	B	A	B	A	B	C	B	B
RAD	C	C	A	B	B	A	B	A	B	A	B	B	B	B
R1A	C	C	A	A	A	B	A	A	B	A	C	D	B	A
S2P	A	A	B	B	A	A	A	A	B	A	D	E	B	A
Alleles	3	3	2	2	3	2	2	2	2	2	4	5	2	2

[a] The most intense of two or multiple bands was scored.
[b] Alleles were resolved by denaturing polyacrylamide electrophoresis.
[c] Of two bands with similar intensity of the T2Bo isolate the one corresponding with allele size as expected was scored.

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

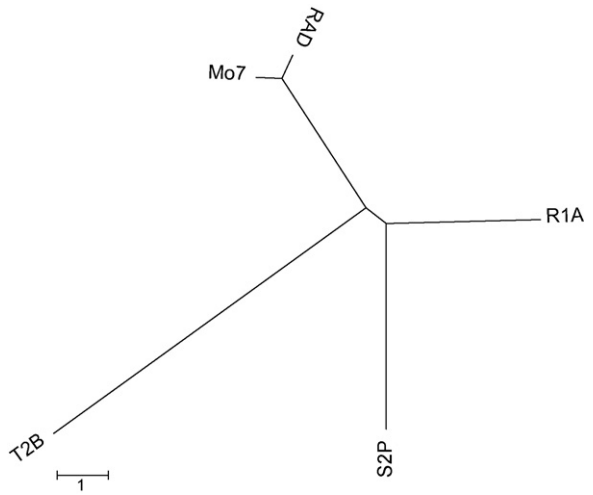

Fig. 3. Phenogram visualizing genetic distances between *B. bovis* isolates. The tree is drawn to scale. The size of the scale bar corresponds to one allele difference across the tested fourteen micro- and minisatellite marker loci.

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 surveillances (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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