Genetic variations in merozoite surface antigen genes of *Babesia bovis* detected in Vietnamese cattle and water buffaloes

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ABSTRACT

The genes that encode merozoite surface antigens (MSAs) in *Babesia bovis* are genetically diverse. In this study, we analyzed the genetic diversity of *B. bovis* MSA-1, MSA-2b, and MSA-2c genes in Vietnamese cattle and water buffaloes. Blood DNA samples from 258 cattle and 49 water buffaloes reared in the Thua Thien Hue province of Vietnam were screened with a *B. bovis*-specific diagnostic PCR assay. The *B. bovis*-positive DNA samples (23 cattle and 16 water buffaloes) were then subjected to PCR assays to amplify the MSA-1, MSA-2b, and MSA-2c genes. Sequencing analyses showed that the Vietnamese MSA-1 and MSA-2b sequences are genetically diverse, whereas MSA-2c is relatively conserved. The nucleotide identity values for these MSA gene sequences were similar in the cattle and water buffaloes. Consistent with the sequencing data, the Vietnamese MSA-1 and MSA-2b sequences were dispersed across several clades in the corresponding phylogenetic trees, whereas the MSA-2c sequences occurred in a single clade. Cattle- and water-buffalo-derived sequences also often clustered together on the phylogenetic trees. The Vietnamese MSA-1, MSA-2b, and MSA-2c sequences were then screened for recombination with automated methods. Of the seven recombination events detected, five and two were associated with the MSA-2b and MSA-2c recombinant sequences, respectively, whereas no MSA-1 recombinants were detected among the sequences analyzed. Recombination between the sequences derived from cattle and water buffaloes was very common, and the resultant recombinant sequences were found in both host animals. These data indicate that the genetic diversity of the MSA sequences does not differ between cattle and water buffaloes in Vietnam. They also suggest that recombination between the *B. bovis* MSA sequences in both cattle and water buffaloes might contribute to the genetic variation in these genes in Vietnam.

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1. Introduction

Babesia bovis is an intraerythrocytic protozoan parasite that infects cattle and buffaloes (Bock et al., 2004). The parasite is transmitted by ixodid ticks and is predominantly prevalent in tropical and subtropical regions of the world (Smith et al., 2000). *B. bovis* often causes severe clinical disease in cattle, and is therefore considered a virulent species of *Babesia* (Ristic, 1981). In addition to the fever, anemia, and jaundice that are commonly associated with *Babesia* infections in host animals, *B. bovis*-infected cattle can

display neurological signs and a respiratory disease syndrome (Everitt et al., 1986). Failure of early diagnosis and treatment may lead to deaths among affected animals (Bock et al., 2004). Therefore, the disease has serious economic implications for cattle farming. Live attenuated vaccines are commercially available in a number of endemic countries, with which susceptible cattle populations can be immunized against this parasite (Shkap et al., 2007), but live attenuated vaccines are not without risks.

One of the major problems associated with the use of live vaccines is vaccine breakthrough, which is thought to be attributable to differences between the vaccine strains and field isolates (Bock and de Vos, 2001). In Australia, analysis of the merozoite surface antigen genes (MSAs) of vaccine strains and outbreak isolates have revealed marked differences between them (Berens et al., 2005; Leroith et al., 2005). Furthermore, recombinant MSA-1 antigens

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prepared according to the nucleotide sequences of outbreak isolates and vaccine strains failed to react with sera collected from the vaccinated animals and from animals that had experienced the outbreaks, respectively (Leroith et al., 2005). These observations have prompted researchers to conclude that the genetic diversity of MSAs might play a role in strain-dependent immunity to *B. bovis* (Berens et al., 2005; Leroith et al., 2005). Therefore, immune control strategies against *B. bovis* infection must be designed with reference to their genetic diversity. The MSAs are also recognized as candidate antigens for the development of sub-unit vaccines (Brown and Palmer, 1999; Hines et al., 1992; Palmer and McElwain, 1995). Therefore, analysis of the genetic makeup of these genes in different isolates should generate important data to evaluate the implications of using MSAs as vaccines.

Against this background, the genetic diversities of MSAs (*MSA-1*, *MSA-2b*, and *MSA-2c*) have been analyzed in several *B. bovis*-endemic countries in the recent past (Altangeral et al., 2012; Genis et al., 2008, 2009; Sivakumar et al., 2013b). Studies conducted in Australia (Berens et al., 2005; Leroith et al., 2005), Sri Lanka (Sivakumar et al., 2013b), the Philippines (Tattiyapong et al., 2014), Thailand (Simking et al., 2013), Israel (Molad et al., 2014), Brazil (Nagano et al., 2013), and Mexico (Borgonio et al., 2008; Genis et al., 2008, 2009) have found that *B. bovis* isolates in these countries are genetically diverse, according to their MSA gene sequences.

Buffaloes are thought to play an important role in the epidemiology of *B. bovis* (da Silva et al., 2013; Elsify et al., 2015; He et al., 2012; Sivakumar et al., 2013a). However, although MSA genes have been widely used for strain verification in *B. bovis*, they have rarely been compared between cattle and buffaloes.

Vietnam is an agriculturally rich country, and several species of *Babesia* and *Theileria* are reported in its livestock populations, including cattle and water buffaloes (Geurden et al., 2008; Khukhuu et al., 2011; Sivakumar et al., 2013a). Although Sivakumar et al. (2013a) recently analyzed the genetic diversity of the *B. bovis* *MSA-2b* gene in Vietnam, only a few gene sequences were examined in that study. Therefore, in the present study, we determined larger numbers of *MSA-1*, *MSA-2b*, and *MSA-2c* gene sequences in *B. bovis* derived from Vietnamese cattle and water buffaloes, and analyzed their genetic diversity.

2. Materials and methods

2.1. Blood sampling and DNA extraction

Blood samples were collected from 258 cattle and 49 water buffaloes reared in Thua Thien Hue province of Vietnam during the period from January to March 2013. The blood samples were drawn from the jugular veins of the animals into Vacutainer tubes containing an anticoagulant. All the cattle and water buffaloes were apparently healthy at the time of sampling. DNA was extracted from all the blood samples with a commercial kit (QIA-amp DNA Blood Mini Kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA samples were then stored at -30°C until analysis.

2.2. PCR detection of *B. bovis*

All the DNA samples were screened for *B. bovis* DNA using a previously described nested PCR assay based on the rhoptry-associated protein 1 (*RAP-1*) gene (Figuerola et al., 1993). The reaction mixtures and cycling conditions were essentially as described by Sivakumar et al. (2012b). The PCR products were then analyzed with agarose gel electrophoresis followed by ethidium bromide staining, and visualized under UV light. The detection of a band close to 298 bp was considered positive for *B. bovis*.

2.3. Amplification and sequencing of the *MSA-1*, *MSA-2b*, and *MSA-2c* genes

All the *B. bovis*-positive DNA samples collected from the cattle and water buffaloes were subjected to PCR assays to amplify the *MSA-1*, *MSA-2b*, and *MSA-2c* genes, as described previously (Sivakumar et al., 2013b). Two forward primers (*MSA-1* F1 and *MSA-1* F2) were used to amplify the *MSA-1* gene in two separate PCR assays, while *MSA-2b* and *MSA-2c* genes were amplified using single forward primers (*MSA-2a1/2a2/2b* F and *MSA-2c* F, respectively), as described by Sivakumar et al. (2013b). In addition, a single reverse primer (*MSA* R) was used to amplify all three genes (*MSA-1*, *MSA-2b*, and *MSA-2c*) (Sivakumar et al., 2013b). The PCR reaction mixtures and cycling conditions have been described in previous reports (Sivakumar et al., 2013b; Tattiyapong et al., 2014). The amplified gene fragments were gel-extracted and cloned as described previously (Sivakumar et al., 2012a). For each cloned product, three clones were picked and both DNA strands of each were sequenced.

2.4. Sequence analysis

The genetic diversity of the sequences determined in the present study was analyzed with the DnaSP v5 software (Librado and Rozas, 2009). The identity values among the nucleotide sequences were calculated using the MatGAT software program (Campanella et al., 2003). In addition, *MSA-1*, *MSA-2b*, and *MSA-2c* gene sequences were converted to amino acid sequences and then subjected to B-cell epitope prediction, as described by Kolaskar and Tungaonkar (1990) (<http://tools.immuneepitope.org/bcell/>). Possible recombination events among the gene sequences were detected with the RDP4 Beta 4.39 software (Martin and Rybicki, 2000), which uses seven automated recombination detection methods: RDP (Martin and Rybicki, 2000), Geneconv (Padidam et al., 1999), Bootscan (Martin et al., 2005), MaxChi (Maynard Smith, 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs et al., 2000), and 3Seq (Boni et al., 2007). Only the recombinations detected by at least three of these programs were considered to reflect possible recombination events. For each recombination event detected with the software, phylogenetic trees were constructed with the RDP4 Beta 4.39 program using the portions of the sequence between the recombination breakpoints, and were analyzed to confirm the positions of the major and minor parental sequences in relation to the recombinant sequence.

2.5. Phylogenetic analyses

The newly determined *MSA-1*, *MSA-2b*, and *MSA-2c* gene sequences, together with the relevant gene sequences already available in GenBank, were used to construct three separate phylogenetic trees. For the phylogenies of *MSA-2b* and *MSA-2c* genes, only the representative (Vietnamese) sequences were used, if the sequences from a single PCR amplicon shared high identity values. The online MAFFT software program (Katoh et al., 2002) was used to construct neighbor-joining phylogenetic trees (Saitou and Nei, 1987) based on the Jukes–Cantor substitution model (Jukes and Cantor, 1969).

3. Results

Of the 258 cattle and 49 water buffalo blood DNA samples, 23 (8.9%) and 16 (32.7%) were positive for *B. bovis*, respectively, according to the *RAP-1*-based nested PCR assay. All the *B. bovis*-positive DNA samples were subjected to PCR assays to amplify

Table 1GenBank accession numbers for the *MSA-1*, *MSA-2b*, and *MSA-2c* gene sequences identified in the present study.

Animal type	Animal ID	GenBank accession numbers		
		<i>MSA-1</i>	<i>MSA-2b</i>	<i>MSA-2c</i>
Cattle	C22			LC004339
	C28	LC004298, LC004299	LC004307, LC004308	LC004340, LC004341, LC004342
	C63		LC004309, LC004310	LC004343, LC004344, LC004345
	C78		LC004311, LC004312, LC004313	
	C79		LC004314, LC004315, LC004316	LC004346, LC004347, LC004348
	C109	LC004300	LC004317, LC004318, LC004319	LC004349, LC004350
	C122		LC004320, LC004321	LC004351, LC004352, LC004353
	C124		LC004322	LC004354, LC004355
	C164			LC004356, LC004357
	C176	LC004301, LC004302, LC004303	LC004323, LC004324	LC004358, LC004359
	C187		LC004325, LC004326	LC004360, LC004361
	C202		LC004327, LC004328, LC004329	LC004362
Water buffalo	B21			LC004363, LC004364
	B23	LC004304, LC004305	LC004330	LC004365, LC004366, LC004367
	B25		LC004331, LC004332	LC004368
	B27	LC004306	LC004333, LC004334	LC004369, LC004370
	B28		LC004335, LC004336	LC004371, LC004372, LC004373
	B34		LC004337, LC004338	
Total		9	32	35

the *B. bovis* *MSA-1*, *MSA-2b*, and *MSA-2c* genes. From 18 of the 39 *B. bovis*-positive DNA samples, at least one target gene was amplifiable with these PCR assays (12 from cattle and six from water buffaloes) (Table 1). Whereas 15 and 16 *B. bovis*-positive DNA samples were positive for *MSA-2b* and *MSA-2c*, respectively, the *MSA-1* gene was amplified from only five DNA samples. Of the two forward primers used to amplify the *MSA-1* gene (Sivakumar et al., 2013b), only *MSA-1* F1 generated PCR amplicons. Three clones were sequenced for each PCR product, and only representative sequences were submitted to GenBank, as shown in Table 1. Sequencing three clones per DNA sample often yielded different variants of an *MSA* gene, and identical sequences were rarely obtained from all three clones derived from a single DNA sample. Therefore, nine *MSA-1* gene sequences (accession numbers LC004298–LC004306), 32 *MSA-2b* gene sequences (accession numbers LC004307–LC004338), and 35 *MSA-2c* gene sequences (accession numbers LC004339–LC004373) were registered with GenBank (Table 1).

The *MSA-1* and *MSA-2b* gene sequences were 823–901 bp and 789–936 bp in length, respectively, whereas all the *MSA-2c* sequences were 798 bp. The nucleotide diversity per site (P_i), calculated with the DnaSP v5 software, was relatively higher for the *MSA-1* (0.23575) and *MSA-2b* sequences (0.15728) than for *MSA-2c* (0.05025) (Table 2). The P_i value for the *MSA-1* gene sequences from cattle (0.23571) was higher than that for the water-buffalo-derived sequences (0.19834), whereas the P_i values for cattle- and water-buffalo-derived *MSA-2b* (0.15331 and 0.15856, respectively) and *MSA-2c* (0.04902 and 0.05022, respectively) were similar in both host animals. Similarly, the sequence identities of the *MSA-1* (63.5%–99.9%) and *MSA-2b* sequences (68.7%–100.0%) were relatively lower than those of the *MSA-2c* sequences (91.4%–99.9%) (Table 2 and Figs. S1–S3). In contrast, the sequence identities of cattle- and water-buffalo-derived *MSA-1* (63.6%–99.9% and 63.5%–99.9%, respectively), *MSA-2b* (68.9%–100% and 72.3–99.9%, respectively), and *MSA-2c* (both 92.0%–99.9%) were similar in the two hosts. In agreement with the P_i values and sequence identity scores, the B-cell epitopes predicted among Vietnamese *MSA-2c* amino acid sequences were relatively conserved, while those of *MSA-1* and *MSA-2b* sequences were diverse (Table 3).

In the phylogenetic analyses, the *MSA-1* gene sequences determined in this study occurred in four separate clades (clades 1, 3,

Table 2Nucleotide diversities and identities of the *MSA-1*, *MSA-2b*, and *MSA-2c* gene sequences from cattle and water buffaloes in Vietnam.

Gene	Source	Numbers	$P_i \pm SD$	Identity (%)
<i>MSA-1</i>	Cattle	6	0.23571 ± 0.05087	63.6–99.9
	Buffaloes	3	0.19834 ± 0.09314	63.5–99.9
	Cattle and buffaloes	9	0.25375 ± 0.02426	63.5–99.9
<i>MSA-2b</i>	Cattle	23	0.15331 ± 0.00935	68.9–100
	Buffaloes	9	0.15856 ± 0.01381	72.3–99.9
	Cattle and buffaloes	32	0.15728 ± 0.00550	68.7–100
<i>MSA-2c</i>	Cattle	24	0.04902 ± 0.00262	92.0–99.9
	Buffaloes	11	0.05022 ± 0.00617	92.0–99.9
	Cattle and buffaloes	35	0.05025 ± 0.00255	91.4–99.9

 P_i , nucleotide diversity per site.

SD, standard deviation.

6, and 8) (Fig. 1). Two of these clades (clades 1 and 6) were formed exclusively by sequences determined in this study, whereas the other two clades (clades 3 and 8) included *MSA-1* sequences from Vietnam, Sri Lanka, and the Philippines. With the exception of clade 6, the cattle- and water-buffalo-derived Vietnamese sequences were dispersed in separate clades (i.e., cattle-derived sequences in clades 1 and 8 and water-buffalo-derived sequences in clade 3). However, the water-buffalo-derived Vietnamese sequences in clade 3 clustered together with those reported from cattle in Sri Lanka and the Philippines (Fig. 1). In contrast, the *MSA-2b* sequences determined in this study occurred in eleven (clades 1, 5, 7, 10–14, and 20–22) of the twenty-two clades on the phylogenetic tree (Fig. 2). The Vietnamese *MSA-2b* sequences often clustered with the sequences from several other countries. Among the *MSA-2b* sequences determined in the present study, water-buffalo-derived sequences were found in five separate clades (clades 1, 7, 11, 20, and 21), and two of them (clades 1 and 21) included cattle-derived sequences as well. However, in addition to the water-buffalo-derived *MSA-2b* sequence (LC004331) generated in the present study, two previously determined sequences (Sivakumar et al., 2013a) sourced from a Vietnamese cattle (AB786920) and a water buffalo (AB786923) were also found in clade 20 (Fig. 2). The phylogenetic tree of the

Table 3

The diversity of amino acid residues in the B-cell epitopes predicted from Vietnamese MSA-1, MSA-2b, and MSA-2c sequences.

MSA-1		MSA-2b		MSA-2c	
Epitopes ^a	Conserved a aa/total aa	Epitopes	Conserved a aa/total aa	Epitopes	Conserved a aa/total aa
FLMTAVCCVVSFAT ^b	10/14	KIFLLTACCASLLSVSAS ^b	16/19	FNITVAFCSILFTYTLASP ^b	13/20
DSIVVPPTHVPAASSRLLFED	6/21	IANLITYL	3/8	NGSHLLFD	7/8
NLMVKLDSS	4/9	DCSRDALKALKDILVLKEE	5/20	HDALKAVKQLIKTD	14/14
MDSVIQIYHKT	8/11	FKSLLEVLIIKK	4/13	TLDLEYLSG	9/9
FKEHVFDISK	3/9	YKKHISA	4/7	LLKLLIEAI	7/9
SVRVGYA	2/7	VKDYTLVKFCNDY	9/14	LSNLYKAFI	6/9
FVSIVSTAGLYFK	3/13	MKIYKAF	3/7	ALRIASE	5/7
SSSVVSES	0/8	EELVKKK	3/7		
KQPAPQSPSAD	0/11				

^a Amino acid residues in the epitopes predicted from the deduced amino acid sequences of LC004298 (MSA-1), LC004333 (MSA-2b), and LC004339 (MSA-2c) were analyzed for their diversity among the Vietnamese sequences.

^b Epitope detected within the signal peptide.

Fig. 1. Phylogenetic tree of the MSA-1 gene. A neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was constructed based on the Jukes–Cantor model (Jukes and Cantor, 1969), using the MAFFT online software (Katoh et al., 2002). The sequences determined in the present study are shown in boldface letters. Note that the Vietnamese sequences are found across multiple clades and that clade 6 contains sequences from a Vietnamese cattle and a water buffalo.

MSA-2c gene divided the sequences into three major clades, and all of the sequences from Vietnamese cattle and water buffaloes occurred in a single clade (clade 3) (Fig. 3).

Recombination was investigated in all of the cattle- and water-buffalo-derived MSA-1, MSA-2b, and MSA-2c gene sequences with automated methods hosted by RDP4 Beta 4.39. Seven potential recombination events were detected by three or more of the recombination detection methods used (Table 4). Among these, five were associated with MSA-2b recombinant sequences and the remaining two recombination events involved MSA-2c recombinant sequences. Except for event numbers 9 and 13, all the events were detected with more than three methods. Most of the recombination events involved recombination between sequences of the same gene, but in event 13, the MSA-1 sequence was detected as the minor parental sequence of the recombinant MSA-2b sequences. Recombination between the cattle- and water-buffalo-derived sequences was frequently detected, and the resultant recombinant sequences were found in cattle and/or water buffaloes (Table 4).

4. Discussion

Genetic diversity is thought to be a survival strategy of protozoan parasites (Deitsch et al., 2009). Antigenic variations arising from this genetic diversity result in different immune profiles in their host animals. The involvement of genetic diversity in the immune evasion of *B. bovis* is well documented in the literature (Berens et al., 2005; Leroith et al., 2005). Consequently, the genetic diversity among the MSA genes has been studied in several endemic countries (Altangeral et al., 2012; Berens et al., 2005; Borgonio et al., 2008; Genis et al., 2008, 2009; Leroith et al., 2005; Molad et al., 2014; Nagano et al., 2013; Sivakumar et al., 2013b; Tattiyapong et al., 2014). Almost all these studies invariably focused on the genetic diversity of the MSA genes of *B. bovis* derived from cattle, whereas *B. bovis* in buffaloes has rarely been investigated, although this host species is considered to play a significant role in its epidemiology (da Silva et al., 2013; Elsify et al., 2015; He et al., 2012; Sivakumar et al., 2013a). Therefore, in this study, we investigated the diversity in the *B. bovis* MSA-1,

Fig. 2. Phylogenetic tree of the MSA-2b gene. A neighbor-joining phylogenetic tree was constructed based on the Jukes–Cantor model, using the MAFFT online software. The sequences determined in the present study are shown in boldface letters. Note that the Vietnamese sequences determined in the present study are found in eleven different clades (clades 1, 5, 7, 10–14, and 20–22) and that the water-buffalo-derived sequences often cluster together with those from cattle.

MSA-2b, and MSA-2c genes from both cattle and water buffaloes bred in Vietnam.

According to previous studies, *B. bovis* is endemic in Vietnamese cattle and water buffaloes (Hau et al., 1999; Sivakumar et al., 2013a). Consistent with those studies, we found that both the cattle and water buffalo populations surveyed in the present study included *B. bovis*-positive animals. The MSA-1, MSA-2b, and MSA-2c genes were amplified and sequenced. Although 39 DNA samples were positive for *B. bovis*, the MSA genes were amplified from only 18 samples. The differences in the sensitivity of the PCR assays that we used could explain this discrepancy, because the diagnostic screening was based on a highly sensitive nested PCR assay (Figueroa et al., 1993), whereas the MSA genes were amplified with single-step PCR assays. The lowest number of sequences was determined for the MSA-1 gene. Both the glycosylphosphatidylinositol-anchor and signal-peptide regions are conserved in MSA-2b and MSA-2c, whereas the signal-peptide region of the MSA-1 gene is not strictly conserved (Sivakumar et al., 2013b). Therefore, based on the currently available sequences, two forward primers were used to amplify MSA-1 (Sivakumar et al., 2013b). However, there might be other MSA-1 variants that cannot be amplified with the primer sets used in

this study, which could explain the low numbers of MSA-1 gene sequences determined here.

A previous study detected several MSA-1 variants in individual carrier animals, suggesting that these animals were co-infected with more than one *B. bovis* strain (Lau et al., 2010). Tick vectors feed on such carriers might become co-infected, and their progenies may transmit multiple strains of *B. bovis*, simultaneously. This might explain why most of the *B. bovis*-infected animals in the present study were detected with multiple MSA variants, although the parasite-positive rate among the cattle was very low. On the other hand, although the sequences determined from a single PCR amplicon were often non-identical, most of them shared high identity scores. The DNA polymerase that we used to amplify the MSA genes is not a high fidelity enzyme, and therefore, the replication errors that could have resulted in minor variations among such sequences cannot be entirely ruled out. However, together with the field DNA samples, the plasmids containing the inserts of MSA-1, MSA-2b, and MSA-2c genes with known nucleotide sequences were also amplified, cloned, and sequenced. The sequences of these controls were identical to those of previously determined, suggesting that the replication errors were unlikely.

Fig. 3. Phylogenetic tree of the *MSA-2c* gene. A neighbor-joining phylogenetic tree was constructed based on the Jukes–Cantor model, using the MAFFT online software. The sequences determined in the present study are shown in boldface letters. Note that all the Vietnamese sequences from cattle and water buffaloes occur in a single clade (clade 3).

The nucleotide identities among the *B. bovis* *MSA* genes were similar in the cattle and water buffaloes in Vietnam. The *Pi* values were also similar for the cattle- and water-buffalo-derived sequences of the *MSA-2b* and *MSA-2c* genes. However, the *Pi* value for the *MSA-1* sequences was lower in water buffaloes than in cattle, possibly because fewer sequences ($n = 3$) were amplified from water buffaloes. Nevertheless, these observations suggest that the levels of genetic diversity in the *B. bovis* *MSA* genes are similar in the cattle and water buffaloes of Vietnam. The *MSA-2c* gene is diverse in Australia (Berens et al., 2005), Sri Lanka (Sivakumar et al., 2013b), the Philippines (Tattiyapong et al., 2014), and Israel (Molad et al., 2014), whereas this gene is conserved in Mongolia (Altangeral et al., 2012) and Brazil (Ramos et al., 2012). In addition, the sequences isolated in Argentina, Mexico, and USA shared high similarities, suggesting that the *MSA-2c* from distant geographical locations may also sometimes be conserved (Dominguez et al., 2010; Florin-Christensen et al., 2002). In Vietnam, the genetic diversity of the *MSA-2c* gene seems lower than that of the *MSA-1* and *MSA-2b* genes, at least among the sequences analyzed in the present study. Consistent with the sequencing analyses, the Vietnamese *MSA-1* and *MSA-2b* sequences were dispersed across several clades on the phylogenetic trees, whereas the *MSA-2c* sequences were detected in a single clade. In the phylogenetic analyses, the sequences of the *MSA-2b* and *MSA-2c* genes from cattle and water buffaloes were often detected in the same clades,

suggesting that the phylogenetic positions of the *B. bovis* *MSA* gene sequences do not differ between the cattle and water buffaloes in Vietnam.

The high genetic variation among the *B. bovis* *MSA* genes is thought to be driven by genetic recombination in the tick vectors (Berens et al., 2005; Simuunza et al., 2011). The gene sequences identified in the present study were analyzed with seven automated methods of detecting recombination, using RDP4 Beta 4.39 (Martin and Rybicki, 2000). Seven potential recombination events were detected, involving 19 recombinant sequences of *MSA-2b* and *MSA-2c*. Recombination was also detected between the cattle- and water-buffalo-derived sequences, with no host specificity, and the resultant recombinants were found in both species. *B. bovis* is usually transmitted by one-host tick species, and therefore the parental sequences for recombination arguably derive from a single host animal. So that, the detection of recombination events between the cattle- and water-buffalo-derived sequences infers the existence of water-buffalo-derived sequences in cattle or cattle-derived sequences in water buffaloes in Vietnam. Cattle and water buffaloes in the surveyed locations are used to be in close contact during grazing, allowing the tick-transmission of *B. bovis* between these host animals. Therefore, it is unlikely, at least in Vietnam, that the *MSA* variants in cattle and water buffaloes differ. Six of seven recombination events were detected between homologous gene sequences. In contrast, event 13 involved *MSA-2b* genes as the recombinant and the major parental sequences, but the *MSA-1* gene was the minor parental sequence. However, because the similarity values between the recombinant and parental sequences were low (data not shown), the reliability of this recombination event is dubious. In contrast, no recombinant sequences were found among the Vietnamese *MSA-1* gene sequences. Similar to our findings, no recombination sites were detected among the *MSA-1* gene sequences analyzed in a previous study (Leroith et al., 2005). We believe that the analysis of large numbers of *MSA-1* gene sequences might shed some light on the role of recombination in generating the diversity observed in this gene.

The high genetic diversity of *MSA* gene sequences might be a limitation for the use of these antigens as recombinant vaccines. LeRoith et al. (2005) and Berens et al. (2005) demonstrated that the genetic variations of *MSA* gene sequences might be linked to the lack of protection by the vaccine strains. In contrast, Suarez et al. (2000) showed that the antibodies raised against recombinant *MSA-1* of *B. bovis* R1A strain effectively inhibit the erythrocyte invasion of Mo7 strain, despite the low similarity shared between the *MSA-1* amino acid sequences from these strains. Therefore, additional studies using immunoblot assays and neutralization tests are essential to explore the possibility of using these antigens as vaccines in Vietnam. On the other hand, although the B-cell epitopes predicted from *MSA-1* and *MSA-2b* sequences were found to be diverse, these epitopes were relatively conserved within a particular clade (data not shown), suggesting that the inclusion of antigens representing different clades in the phylogeny might overcome this constraint.

In summary, in this study, an analysis of the genetic diversity among the *B. bovis* *MSA-1*, *MSA-2b*, and *MSA-2c* genes showed that the patterns of diversity do not differ between the cattle- and water-buffalo-derived sequences in Vietnam. Our findings also suggest that recombination might be a common event between the *MSA-2b* and the *MSA-2c* gene sequences derived from cattle and water buffaloes, and could contribute to the genetic diversity of these genes in Vietnam.

Table 4
Recombination events detected among the MSAs gene sequences determined in this study.

Event No.	Recombinant			Major parent			Minor parent		Average P value						
	Gene (Acc. No.)	Host	Breakpoint positions	Gene (Acc. No.)	Host	Gene (Acc. No.)	Host	Host	RDP	GENECONV	Bootscan	MaxChi	Chimaera	SiScan	3Seq
1	MSA-2b (LC004331)	Buffalo	11 ^a –560 ^a	MSA-2b (LC004307)	Cattle	Unknown (LC004335) ^b			–	2.279 E-02	2.302 E-05	7.201 E-05	5.031 E-05	1.195 E-20	2.671 E-19
3	MSA-2c (LC004363) ^c	Buffalo	245–738 ^a	MSA-2c (LC004339)	Cattle	MSA-2c (LC004361)	Cattle		–	–	–	4.866 E-04	2.552 E-04	3.490 E-10	2.960 E-09
4	MSA-2c (LC004340)	Cattle	74–549 ^a	MSA-2c (LC004369)	Buffalo	MSA-2c (LC004356)	Cattle		–	–	1.597 E-02	1.026 E-04	1.261 E-04	9.742 E-12	6.425 E-08
5	MSA-2b (LC004326)	Cattle	599–668 ^a	MSA-2b (LC004333)	Buffalo	MSA-2b (LC004319)	Cattle		–	1.883 E-07	–	1.297 E-04	1.261 E-04	–	1.154 E-07
7	MSA-2b (LC004332)	Buffalo	572–697 ^a	MSA-2b (LC004333)	Buffalo	MSA-2b (LC004310)	Cattle		6.883 E-07	–	–	1.423 E-04	2.095 E-04	1.003 E-08	5.867 E-05
9	MSA-2b (LC004307) ^d	Cattle	355–533 ^a	MSA-2b (LC004331)	Buffalo	Unknown (LC004328) ^b			–	–	–	1.660 E-03	3.485 E-03	–	2.018 E-04
13	MSA-2b (LC004314) ^e	Cattle	686–738	MSA-2b (LC004319)	Cattle	MSA-1 (LC004305)	Buffalo		6.482 E-04	1.431 E-02	–	–	9.101 E-04	–	–

^a Breakpoints were not determined.

^b The sequences were used to infer the existence of a missing minor parent.

^c Another possible recombinant sequence is the water-buffalo-derived LC004364.

^d Other possible recombinant sequences are cattle-derived LC004317 and LC004325 and water-buffalo-derived LC004330, LC004335, and LC004336.

^e Other possible recombinant sequences are cattle-derived LC004315, LC004316, LC004318, LC004320, and LC004327–LC004329.

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