

Supplementary Figure S1. Clustal omega alignment of ROM4 paralogs of *B. bovis*, T2Bo strain

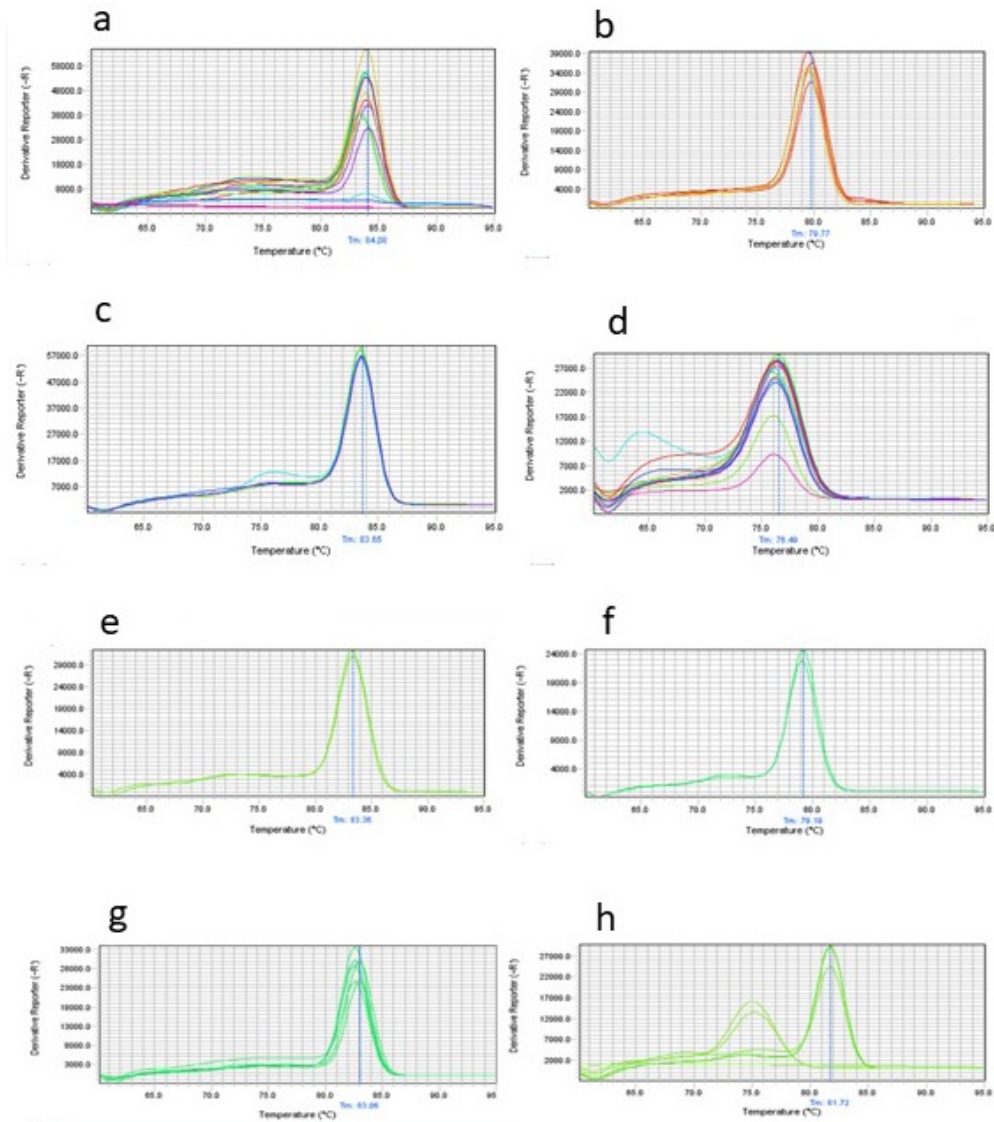
4_1	-----	0
4_2	-----	0
4_5	MGDNDSDNKKANRHSLSNAIKGGFVKDMVNRGFRKKINDSDKNDDSKDKGGEKPSNVSK	60
4_3	MGDNDSDNKKANRHSLSNAIKGGFVKDMVNRGFRKKINDSDKNDDSNVDGSPNDIT----	56
4_4	MGDNDSDNKKANRHSLSNAIKGGFVKDMVNRGFRKKINDSDKNDDSNVDGSPNDIT----	56
4_1	-----	0
4_2	-----	0
4_5	DSSMKAGNVAALRDQFKRAAEKDESKDQNSQAGQSSSLTSKAPLGDPRDQARAKFLAE	120
4_3	DKGHRNA-----GIEDSENILSQA-----QSDTKDKPVSVAKSLAE	92
4_4	DKGHRNA-----GIEDSENILSQA-----QSDTKDKPVSVAKSLAE	92
4_1	-----	0
4_2	-----	0
4_5	KYEKILAARNMVEESASRALQRPATSTPAKIPASVSGVSKEPKDTSEQPRPSKAEPSPST	180
4_3	KYERGLFNRNKVD-----	106
4_4	KYERGLFNRNKVD-----	106
4_1	-----	0
4_2	-----MPCKYP	6
4_5	TPSTSLGPQEPKDTSEQPRPSKAEPSPSTTPSTSLGPEPKDTSEQPRPSKAEPSPSTTP	240
4_3	-----SLISKAESQPSTTP	120
4_4	-----SLISKAESQPSTTP	120
4_1	-----MKDEIEFS	9
4_2	SV-----SGVS--IDR	15
4_5	STSLGPEPKDTSEQPRPSKAESKLSLTLKSLRFSRESRSVIKGTGSGFGSSQVSSNAGR	300
4_3	STSMGS-----SEIKGARAFGSSQVSSNAGR	147
4_4	STSMGS-----SEIKGARAFGSSQVSSNAGR	147
4_1	-----DEEPTQTGEVHLA---SPEIEAPQKVPKTKVIVNRRSPINPLE--KLAYVAGF	59
4_2	WESALYDEKPVKTVEITQCSTAEPEEVSTGA-----EPPSEAMTHRLCKWIFDL	65
4_5	RVHTLADTASTDDASIRQA---MSQSFSSPGV-----SQPEPERASRGRNMFGM	347
4_3	RVHTLADTASTDDASIRQA---MSQSFSSPGV-----SQPEPERASRGRNMFGM	194
4_4	RVHTLADTASTDDASIRQA---MSQSFSSPGV-----SQPEPERASRGRNMFGM	194
	: . . . . : . . . : * : : . :	
4_1	KNNEDNIVKGSDFMLEKNPLGLRPFMLMINFALVLIFFITQLVFNITFNGRCISSVLY	119
4_2	VNKLKCYCIRK---DSADHDVSGKLVLRRTALIIYSTAYICSLVLNKTTFDGKCMGNVDF	121
4_5	RR-RPCWIRI---QVKEKIFPGKMILAISTTILIVVFMFELFINRATFNRCIGEVDI	402
4_3	RR-RPCWIRI---QVKEKIFPGKMVLAISTTILMIVVFLFELLNKTTFNRCIGQVDI	249
4_4	RR-RPCWIRI---QVKEKIFPGKMVLAISTTILMIVVFLFELLNKTTFNRCIGQVDI	249
	. * : . . * : . : : : * : * : * : * : * : * : *	
4_1	PAIQTGREAIKPIVEFGYGAECYNLKTSTANRVFFGESASDEGWPKKEYLQKLVTAASSA	179
4_2	GYGTLG---HPPFSLGYNACTQNTKTKIIEPNFHIHQNTGQVLLVWKENERWH---D	174
4_5	THRHEG---KAILSYLGVGCENNLKTTAERGFGLGAGASDKGYVVDVYHKNLATSLSA	458
4_3	SNRLAG---KSTLSFIGYVGCENNLKTTAERGFGLGAAASDAGYPDDIVTDGRLAHAPA	305
4_4	SNRLAG---KSTLSFIGYVGCENNLKTTAERGFGLGAAASDAGYPDDIVTDGRLAHAPA	305
	* : * : * : * : * : * : * : * : * : * : * : *	
4_1	SSDAPNERVYNLLGGLSTNVMRNYEYRVFWSMFLHSSWKHLFFNVFCIQALWIEPD	239
4_2	RISEPGSQSLTVLGAVDTNHIRIYGEWFRIFTGIIHLHAGWFHLLNNAIHFIVLYIEPE	234
4_5	PPDGPNYRIGTIVGALSANTIRIYGESSRLTSLIFLHGGRWHLNCLNMLNMLLYVIEPD	518
4_3	VVDGPNVRIASIIIGAVSANEVRLYNEKFRILSAIFLHAGFTLFTNCLNMLNMLLYVIEPD	365
4_4	VVDGPNVRIASIIIGAVSANEVRLYNEKFRILSAIFLHAGFTLFTNCLNMLNMLLYVIEPD	365
	. * : . . * : * : * : * : * : * : * : * : * : * : * : *	
4_1	WGFMRMTGLFLISIGGNLTGAVLSPCGLTVGSSGAMYGLYGAMIPYCIYWNTPRPVF	299
4_2	WGFYRTLLGYFVTGCGSYLAGAVFIPCLRQIGSSGVHFGFIGAIAPYCVENWYRMGSPVI	294
4_5	WGFKRTLALYIFGYSANLVHASMSPCPCWASGSLFLSYLGFAPYPTVEHWDNLRSPMA	578
4_3	WGFKRTLALYLVAGIIGGNLVHTSMSPCPCWASGSLFLSYLGFAPYPTVEHWDNLRSPMA	425
4_4	WGFKRTLALYLVAGIIGGNLVHTSMSPCPCWASGSLFLSYLGFAPYPTVEHWDNLRSPMA	425
	** * * * : : . . * : * : * : * : * : * : * : * : * : * : * : *	
4_1	LFCYNIITLIIIGFLMGLAPNVDNYCHIGCVFGLWGFATIKSVSSCDKCTIVERSLLCP	359
4_2	VLSLILLVPLLDLCI--KEADIGIQIHLGGYIFGMLYGFSTIKAVTLFEDKGVYRYFTIKF	353
4_5	LIVIAITISLLEIIL--PGVGSNHAHLGGFAFGLCFGFATLKSVSADFDRGALWSRIALRF	637
4_3	LLLISVAFVSIIEFFS--FVRGVSKHAHLGGFAFGLCFGFATLKSVSADFDRGALWSRIALRF	484
4_4	LLLISVAFVSIIEFFS--FVRGVSKHAHLGGFAFGLCFGFATLKSVSADFDRGALWSRIALRF	484
	: : : : : . . * : * : * : * : * : * : * : * : * : * : *	
4_1	LISWALPQKWKAKRLIIIVFKKDRGRNRQKDMAQKHAVQNGVTPRRIALIKKKFERQGA	419
4_2	FSRWLKESTRN--LYIRKVVKACQIEE---MNRIQYENQAKSSTCGFFMKRLFGVYPL	407
4_5	SSRLSPEAVQK---HQAKVVKSQAQSEE---ILRIKYEQGAKDSANRLRFVVRMFGIYYP	690
4_3	SSRLSPEAVQK---HQAKVVKSQAQSEE---ILRIKYEQGAKDSANRLRFVVRMFGIYYP	537
4_4	SSRLSPEAVQK---HQAKVVKSQAQSEE---ILRIKYEQGAKDSANRLRFVVRMFGIYYP	537
	: * : * : : . . : : : : : : : : * : * : *	
4_1	PPCRMRLREWIFRITSLMMVL-----LFTILGLFLSKPELYAKFPPGGEYKL	467
4_2	GPYRMRPRDWTTRGIFSCLMVI-----LMTFLILMLCFEPLYSFNPVTSVSAF	455
4_5	GPYRMRRLRDVLRVFLCAL-----IAMVFFYLATFYESVYKNIIDRNATILF	738
4_3	GPYRMRRLRDVLRVFLCAL-----IAMVFFYYSTFNERFYTWLNRAGIRFV	585
4_4	GPYRMRRLRDVLRVFLCALVQPVFTYTPQIAMVFFYYSTFNERFYTWLNRAGIRFV	597
	* * * * * : : * : : : : : : : * : * : *	
4_1	SGWQTCCECFITNIEKLFSGHRDIPAQYL--NRKLFWCFNVDDAKHFCGGDYAKTAPMDN	526
4_2	--TQTCICCYIKIRNKSAL---QRLQVLDLAGYFCFRSKEYANRYCF-----	498
4_5	--SRACHCGYFKS--SGDEL---LLNQIGNLAGKFCYFDSEASARDKFCVAVSG-----	783
4_3	--SPLCHCGYLRDLTGPSI---LSKQIGSLAGKYCYQSEALWNVFCEK-----	629
4_4	--SPLCHCGYLRDLTGPSI---LSKQIGSLAGKYCYQSEALWNVFCEK-----	641
	* * : : : * : : : : * : * : * : * : *	
4_1	ILKSSHDAALGLINTVTEAIK	547

4_2	-----	498
4_5	-----	783
4_3	-----	629
4_4	-----	641

**Supplementary Figure S2.** Clustal omega alignment of intergenic regions upstream to *rom4.3* (Int3), *rom4.4* (Int4) and *rom4.5* (Int5)

Int3	TTTGGTGACAGACTAGACTATCAGAATAATGGCCACGAGCACAATCTCACACATATGTAA	60
Int5	TTTGGTGACAGACTAGACTATCAGAATAATGGCCACGAGCACAATCTCACACATATGTAA	60
Int4	TTTGGTACACAACATACTATCAATAATGGCCACGAGCACAATCTCACACATATGTAA	60
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	ACAAAAACCAACACACGCCAAAAATGGCAATCTAGTATATTAGATGATAAAAGTCTGT	120
Int4	ACAAAAACCAACACACGCCGGAAATAGTAATATTTTATAGTAGTCTATAAAGAAATGT	120
Int5	ACAAAAACCAACACACGCCGGAAATAGTAATGTTTATATTAATATAAAGAGATGT	120
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	TATTTTAAAGAATCATTACACAGAGCACTGCACCGTCATAGGAATAAATACGTCGTGCA	180
Int4	GATGTCGATATATTTCGTGAGTTG-CATATCTCGCACATCAACGTGAATAATCAATAATA	179
Int5	AATATTAAGAATCTTCTTTAAA-GTACTGCACCGTCACATGAATAAATAACCCCGTGCA	179
	** *	
Int3	TGCAAGTCTTCCATAAAAGAATAACTAAAAATAAATGACGAAGCCATTTAGTGAACA	240
Int4	CGCATGTAATAGGGATGCAACAC--ACGCCAATTACACATCAAGGGTAAATAGAACA	237
Int5	TGCAAGTCTTCCAGATAAGAATAA--GTAAAAATGAATAATTCGGCTAATCAATAGAACA	237
	** *	
Int3	CATCCATTGATGACACATGTTTTCGTGTTGTATATTAGTTTTACGTGTATACACCCCTGGG	300
Int4	CATCCATTGATGACACATGTTTTCGTGTTGTATATTAGTTTTACGTGTATACACCCCTGGG	297
Int5	CATCCATTGATGACATATGTTTTCGTGTTGGATATTAGTTTTACGTGTATACAGCCTAGG	297
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	TAATATATATACAATCCCATCACACATCTGTCGTATACAAAACGAATATCAGTCGTCTA	360
Int4	TAATATATATACAATCCCATCACACAGCTGCCTATATAAA---ATAATATAAGTCGCCTA	354
Int5	TAATATATCTACAATCCCATCACACAGCTGCCTATATAAA---ATAATATAAGTCGCCTA	354
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	CACACTTTGTAAACAACCGAATGTGTAGTGCATTAGACCGGTAAACACAATGCTAATAGA	420
Int4	CACACTTTGTAAACAACCGAATGTGTAGTGCATTAGACCGGTAAACACAATGCTAATAGA	414
Int5	CACACTTTGTAAACAACCGAATGTGTAGTGCATTAGACCGGTAAACACAATGCTAATAGA	414
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	GGCATAAAACCACAAGGGTCACTGAATAAAAACGGTAGATTACATAT-----	467
Int4	GGCATAAAACCACAAGGGTCACTGAATAAAAACGGTAGATTACATATAACGTGTTATATG	474
Int5	GGCATAAAACCACAAGGGTCACTGAATAAAAACGGTAGATTACATATAACGTGTTATATG	474
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	-----	467
Int4	CCTTGTGGTCAACAACCTTACCACGTTTGTGATTGGCGCCATCCCGTCAATAGTACCT	534
Int5	CCTTGTGGTCAACAACCTTACCACGTTTGTGATTGGCGCCATCCCGTCAATAGTACCT	534
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	-----	467
Int4	TATTTACGACGATATAGCACAGATAATGGTCTAACAAAAGTATGTTACACATTGTTTAC	594
Int5	TATTTACGACGATATAGCACAGATAATGGTCTAACAAAAGTATGTTACACATTGTTTAC	594
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	-----	467
Int4	ATGCTTACGCAAGGAAGATGCGCAGTCGGTTTCGACCCGAGGGCAGAAGTGGTTCAACCA	654
Int5	ATGCTTACGCAAGGAAGATGCGCAGTCGGTTTCGACCCGAGGGCAGAAGTGGTTCAACCA	654
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	-----	467
Int4	GCTAACAGCATTATCTTTCTAATCGCTGATTGACTTTTTACATAGTGATGAATGTGA	714
Int5	GCTAACAGCATTATCTTTCTAATCGCTGATTGACTTTTTACATAGTGATGAATGTGA	714
	***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **	
Int3	-----	467
Int4	TAAGTACCTATGGTTAATACCACTGCTTGCTA	747
Int5	TAAGTACCTATGGTTAATACCACTGCTTGCTA	747

**Supplementary Figure S3.** Melting curve analysis to select an adequate housekeeping gene as normalization control of transcription in *B. bovis*, as determined by qPCR. Dissociation curves were obtained when primer pairs targeting topoisomerase (topo 1: a, e; topo 2: b, f), *gadph* (c, g) or actin (d, h) and plasmid (a-d) or cDNA from AS and SS (e-h), and StepOne Software v2.3 were employed.



**Supplementary Figure S4.** Clustal omega alignment of nucleotide sequences of *rom4.5* alleles of *Babesia bovis* T2B and R1A. Note total conservation along the sequences with the exception of a region of different length which encodes the 29-aa repeats.

R1A	ATGGGAGATAATGATTGCAATGATAAAGAAGATGCTAATAGGCATTCACCTAACGCTATT	60
T2B	ATGGGAGATAATGATTGCAATGATAAAGAAGATGCTAATAGGCATTCACCTAACGCTATT	60
	*****	
R1A	AAAGCGGTTTTGTGAAGGATATGGTGAATCGGGGATTCAGGAAGAAGATTAATGATAGT	120
T2B	AAAGCGGTTTTGTGAAGGATATGGTGAATCGGGGATTCAGGAAGAAGATTAATGATAGT	120
	*****	
R1A	GATAAGAATGATGATAGCAAGAGGATAAAGGTGGAGAAAAACCGAGTAACGTGTCGAAG	180
T2B	GATAAGAATGATGATAGCAAGAGGATAAAGGTGGAGAAAAACCGAGTAACGTGTCGAAG	180
	*****	
R1A	GATTCGTCGCTATGAAAGCTGGTAATGTGGCTGCTTTGAGAGATCAATTCAAGCGTCT	240
T2B	GATTCGTCGCTATGAAAGCTGGTAATGTGGCTGCTTTGAGAGATCAATTCAAGCGTCT	240
	*****	
R1A	GCTGAGAAAGATGAATCTAAAGACCAAAATTCGCAAGCTGGGCAGAGCAGCTTAACCTCG	300
T2B	GCTGAGAAAGATGAATCTAAAGACCAAAATTCGCAAGCTGGGCAGAGCAGCTTAACCTCG	300
	*****	
R1A	AAGGCTCCTCTTGGTGATCCGAGAAGCTGATCAACGAGCAGGCAAAATTCCTCGCAGAG	360
T2Bo	AAGGCTCCTCTTGGTGATCCGAGAAGCTGATCAACGAGCAGGCAAAATTCCTCGCAGAG	360
	*****	
R1A	AAATATGAAAAAATATTGGCTGCCCGTAACATGGTGAAGAGTCCGCCAGTCGTGCATTG	420
T2Bo	AAATATGAAAAAATATTGGCTGCCCGTAACATGGTGAAGAGTCCGCCAGTCGTGCATTG	420
	*****	
R1A	CAGCGCCAGCGACGAGTACACCAGCAAGATACCGGCTCTGTATCTGGTGTTCGAAA	480
T2B	CAGCGCCAGCGACGAGTACACCAGCAAGATACCGGCTCTGTATCTGGTGTTCGAAA	480
	*****	
R1A	GAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACC	540
T2B	GAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACC	540
	*****	
R1A	ACACCATCTACCTCCTTAGGTCACACAAGAACCTAAAGACACCAGCGAACAACCTAGGCCA	600
T2B	ACACCATCTACCTCCTTAGGTCACACAAGAACCTAAAGACACCAGCGAACAACCTAGGCCA	600
	*****	
R1A	TCGAAAGCTGAACCTCGACCTAGTACCACCATCTACCTCCTTAGGTCACACAAGAACCT	660
T2B	TCGAAAGCTGAACCTCGACCTAGTACCACCATCTACCTCCTTAGGTCACACAAGAACCT	627
	*****	
R1A	AAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACCACACCA	720
T2B	-----	627
	-----	
R1A	TCTACCTCCTTAGGTCACACAAGAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAA	780
T2B	-----	627
	-----	
R1A	GCTGAACCTCGACCTAGTACCACCATCTACCTCCTTAGGTCACACAAGAACCTAAAGAC	840
T2B	-----	627
	-----	
R1A	ACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACCACCATCTACC	900
T2B	-----	627
	-----	
R1A	TCCTTAGGTCACACAAGAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAA	960
T2B	-----	627
	-----	
R1A	CCTCGACCTAGTACCACCATCTACCTCCTTAGGTCACACAAGAACCTAAAGACACCAGC	1020
T2B	-----	627
	-----	
R1A	GAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACCACCATCTACCTCCTTA	1080
T2B	-----ACACCATCTACCTCCTTA	645
	*****	
R1A	GGTCCACAAGAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGA	1140
T2B	GGTCCACAAGAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGA	705
	*****	
R1A	CCTAGTACCACCATCTACCTCCTTAGGTCACACAAGAACCTAAAGACACCAGCGAACA	1200
T2B	CCTAGTACCACCATCTACCTCCTTAGGTCACACAAGAACCTAAAGACACCAGCGAACA	765
	*****	
R1A	CCAGATCATCGAAAGCTGAGTCCAAATTGAGTACTACACTATCAAATGAGATTCCTCT	1260
T2B	CCAGATCATCGAAAGCTGAGTCCAAATTGAGTACTACACTATCAAATGAGATTCCTCT	825
	*****	
R1A	CGTGAATCCCGATCAGTGATTAAGGTACGGGCTCTTTGGCGGTTTCATCTCAGGTAAGC	1320
T2Bo	CGTGAATCCCGATCAGTGATTAAGGTACGGGCTCTTTGGCGGTTTCATCTCAGGTAAGC	885
	*****	
R1A	TCCAATGCTGGTCGTCGTTTCACTTTGGCGGACACTGCTCCACTGATGACGCTAGT	1380
T2B	TCCAATGCTGGTCGTCGTTTCACTTTGGCGGACACTGCTCCACTGATGACGCTAGT	945
	*****	
R1A	ATTAGACAGGCTATGTCCCAATCCTTTCCAGCCCTGGTGTAGTCAGCCAGCAACCC	1440
T2B	ATTAGACAGGCTATGTCCCAATCCTTTCCAGCCCTGGTGTAGTCAGCCAGCAACCC	1005
	*****	

R1A	GAGCGTGCCTCGCGTGGCCGTAACATGTTTGGTATGCGTCGCGTCCATGTTGGATTGCT	1500
T2B	GAGCGTGCCTCGCGTGGCCGTAACATGTTTGGTATGCGTCGCGTCCATGTTGGATTGCT *****	1065
R1A	ATTCAAGTGAAGGAGAAGATATTTCCCGGTAAGATGATATTTGGCAATCAGTACTACAATA	1560
T2B	ATTCAAGTGAAGGAGAAGATATTTCCCGGTAAGATGATATTTGGCAATCAGTACTACAATA *****	1125
R1A	CTGATAATTGTTGTTTTTATGTTTGTAGTTATTCATCAACAGAGCTACCTTTAACGGTCGC	1620
T2B	CTGATAATTGTTGTTTTTATGTTTGTAGTTATTCATCAACAGAGCTACCTTTAACGGTCGC *****	1185
R1A	TGTATAGTGAAGTGGATTATACTCACAGACATGAGGGTAAAGCTATCTTATCATACCTG	1680
T2B	TGTATAGTGAAGTGGATTATACTCACAGACATGAGGGTAAAGCTATCTTATCATACCTG *****	1245
R1A	GGTTATGTTGGTGTGAGAATAACCTCAAAACAACCTGCTGCCGAACGTGGATTCCCTGGT	1740
T2B	GGTTATGTTGGTGTGAGAATAACCTCAAAACAACCTGCTGCCGAACGTGGATTCCCTGGT *****	1305
R1A	GCTGGAGCGAGTGATAAAGGCTATCCAGTTGATTATGTGCATAAAGGGAATTTGGCAACT	1800
T2B	GCTGGAGCGAGTGATAAAGGCTATCCAGTTGATTATGTGCATAAAGGGAATTTGGCAACT *****	1365
R1A	TCACTTGCCTCCTCGACGGACCTAATTACCGTATAGGCCTATTTGGCGCACTATCG	1860
T2Bo	TCACTTGCCTCCTCGACGGACCTAATTACCGTATAGGCCTATTTGGCGCACTATCG *****	1425
R1A	GCTAATACAATTCGTATCTATGGAGAGTCCTCTAGTTGCTTACTTCCATATCTTACAT	1920
T2B	GCTAATACAATTCGTATCTATGGAGAGTCCTCTAGTTGCTTACTTCCATATCTTACAT *****	1485
R1A	GGAGGAAGTGGCATCTCTTTGTAATTGTTTGTATGAACATGTTGCTTCTCTATGTGATT	1980
T2B	GGAGGAAGTGGCATCTCTTTGTAATTGTTTGTATGAACATGTTGCTTCTCTATGTGATT *****	1545
R1A	GAGCCGGTAAGTGGCCACTTTTGTGTTTGTCTAATCATATTTAGGACTGGGGATTCAAACG	2040
T2B	GAGCCGGTAAGTGGCCACTTTTGTGTTTGTCTAATCATATTTAGGACTGGGGATTCAAACG *****	1605
R1A	CACATTAGCGCTGTATATATCGGTGGTTACAGTGCCAACTGGTTCACGCCAGTATGTC	2100
T2B	CACATTAGCGCTGTATATATCGGTGGTTACAGTGCCAACTGGTTCACGCCAGTATGTC *****	1665
R1A	TCCTTGTATCCCATGCTGGGGAGCTTCTGGGTCAGTGTAGTTTGTATGGAGCATTGAT	2160
T2B	TCCTTGTATCCCATGCTGGGGAGCTTCTGGGTCAGTGTAGTTTGTATGGAGCATTGAT *****	1725
R1A	TCCTTACACGGTAGAGCATTGGGACAACCTGAGATCACCATGGCTCTAATTGTCATAGC	2220
T2B	TCCTTACACGGTAGAGCATTGGGACAACCTGAGATCACCATGGCTCTAATTGTCATAGC *****	1785
R1A	CATAACAATCTCTCTCTGGAATAATATACCAGGAGTAGGCGTTAGTAATCATGCCCA	2280
T2B	CATAACAATCTCTCTCTGGAATAATATACCAGGAGTAGGCGTTAGTAATCATGCCCA *****	1845
R1A	CTTGGTAGGTTTTTATTGCTATGTTAATGAATACACAGGGTGGTTTTGCGTTTGGTTTG	2340
T2B	CTTGGTAGGTTTTTATTGCTATGTTAATGAATACACAGGGTGGTTTTGCGTTTGGTTTG *****	1905
R1A	TGTTTCGGTTTCGCCACATTGAAATCAGTATCTGCGTTTGACCGTGGTGCCTTGTGGTCA	2400
T2B	TGTTTCGGTTTCGCCACATTGAAATCAGTATCTGCGTTTGACCGTGGTGCCTTGTGGTCA *****	1965
R1A	CGTATAGCACTTCGGTTTTCTAGTAGGCTTTCACCAGAGCCGTCGAGAACACCAAGCC	2460
T2B	CGTATAGCACTTCGGTTTTCTAGTAGGCTTTCACCAGAGCCGTCGAGAACACCAAGCC *****	2025
R1A	AAGGTTGTGAAGAGTCTCAGTCAGAGGAGATTCTGCGTATTAATATGAGCAGGTGCT	2520
T2B	AAGGTTGTGAAGAGTCTCAGTCAGAGGAGATTCTGCGTATTAATATGAGCAGGTGCT *****	2085
R1A	AAGGATAGTGTCTAATCGTCTTCGCTTTGTCAAGCGTATGTTTCGGTATATATCCCTATGGC	2580
T2B	AAGGATAGTGTCTAATCGTCTTCGCTTTGTCAAGCGTATGTTTCGGTATATATCCCTATGGC *****	2145
R1A	CCATACCGTATGAGGCTGCGTGTACTTACTCGCGTTGTATTCCTTTGTGCCCTAGTA	2640
T2B	CCATACCGTATGAGGCTGCGTGTACTTACTCGCGTTGTATTCCTTTGTGCCCTAGTA *****	2205
R1A	AGTCAACCGGTAATTACATACACTCCTGAGCAGATCGCGATGTTTGTATCTTCTATTG	2700
T2B	AGTCAACCGGTAATTACATACACTCCTGAGCAGATCGCGATGTTTGTATCTTCTATTG *****	2265
R1A	GCGACTTCTATGAAAGCGTCTATAAGAATATAGATAGGAATGCAACGACTTTTCTCT	2760
T2B	GCGACTTCTATGAAAGCGTCTATAAGAATATAGATAGGAATGCAACGACTTTTCTCT *****	2325
R1A	CGTGCCCTGCCATTGCGGCTATTTCAAATCATCTGGTGTAGGTTGTTAATCAAATA	2820
T2B	CGTGCCCTGCCATTGCGGCTATTTCAAATCATCTGGTGTAGGTTGTTAATCAAATA *****	2385
R1A	GGCAACCTAGCTGGCAAATTCATTTGTTTCGATTGAGAAAGCGCCAGGGATAAGTTCTGT	2880
T2B	GGCAACCTAGCTGGCAAATTCATTTGTTTCGATTGAGAAAGCGCCAGGGATAAGTTCTGT *****	2445
R1A	GCTGTTCTGGTTGAACCGGTTAACATGTATCAGTACATGCTGAACTTTTCATCATT	2939
T2B	GCTGTTCTGGTTGAACCGGTTAACATGTATCAGTACATGCTGAACTTTTCATCATT *****	2504

**Supplementary Figure S5.** Clustal omega alignment of the N-terminal region of ROM4.5 of *Babesia bovis* T2Bo and R1A. Repeats in the R1A sequence are highlighted with different colors and the aa in the conserved motifs that flank the repeats' region are shown in red.

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Rom4.1_R1A      MGDNDSNDKKNANRHSLNAIKGGFVKDMVNRGFRKKINDSDKNDDSKEDKGGKEKPSNVSK 60
Rom4.1_T2B      MGDNDSNDKKNANRHSLNAIKGGFVKDMVNRGFRKKINDSDKNDDSKEDKGGKEKPSNVSK 60
*****

Rom4.1_R1A      DSSSMKAGNVAALRDQFKRAAEKDESKDQNSQAGQSSLTSKAPLGDPRTDQRARAKFLAE 120
Rom4.1_T2B      DSSSMKAGNVAALRDQFKRAAEKDESKDQNSQAGQSSLTSKAPLGDPRTDQRARAKFLAE 120
*****

Rom4.1_R1A      KYEKILAARNMVEESASRALQRPATSTPAKIPASVSGVSKEPKDTSEQPRPSKAEPRPST 180
Rom4.1_T2B      KYEKILAARNMVEESASRALQRPATSTPAKIPASVSGVSKEPKDTSEQPRPSKAEPRPST 180
*****

Rom4.1_R1A      TPSTSLGPQEPKDTSEQPRPSKAEPRPSTTPSTSLGPQEPKDTSEQPRPSKAEPRPSTTP 240
Rom4.1_T2B      TPSTSLGPQEPKDTSEQPRPSKAEPRPSTTP----- 211
*****

Rom4.1_R1A      STSLGPQEPKDTSEQPRPSKAEPRPSTTPSTSLGPQEPKDTSEQPRPSKAEPRPSTTPST 300
Rom4.1_T2B      ----- 211

Rom4.1_R1A      SLGPQEPKDTSEQPRPSKAEPRPSTTPSTSLGPQEPKDTSEQPRPSKAEPRPSTTPSTSI 360
Rom4.1_T2B      -----STSL 215
*****

Rom4.1_R1A      GPQEPKDTSEQPRPSKAEPRPSTTPSTSLGPQEPKDTSEQPRSSKAESKLSTTL SKLRF 420
Rom4.1_T2B      GPQEPKDTSEQPRPSKAEPRPSTTPSTSLGPQEPKDTSEQPRSSKAESKLSTTL SKLRF 275
*****

Rom4.1_R1A      RESRSVIKGTGSFGSSQVSSNAGRRVHTLADTASTDDASIRQAMSQSFSSPGVSPQPEQP 480
Rom4.1_T2B      RESRSVIKGTGSFGSSQVSSNAGRRVHTLADTASTDDASIRQAMSQSFSSPGVSPQPEQP 335
*****

Rom4.1_R1A      ERASRGRNMFGRRRPCWIRIQVKEKIFPGKM          512
Rom4.1_T2B      ERASRGRNMFGRRRPCWIRIQVKEKIFPGKM          367
*****

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**Supplementary Figure S6.** Analysis of polymorphism and synonymous and non-synonymous substitutions in the repeat region of ROM4.5. **A.** Nucleotide sequence variants encoding for 29 aa-repeats in different *Babesia bovis* geographical isolates. The sequence shown for T2B is 100% conserved in all analyzed American isolates. When different sequences were found in an isolate, they are designated with letters. The results of the SNAP analysis to find the average dN/dS are shown below. **B.** Clustal omega alignment of the 29 aa peptides encoded by the nucleotide sequences shown in A. Asterisks at the bottom mark conserved positions between all sequences, colons mark conserved substitutions and absence of a sign corresponds to non-conserved substitutions.

**A**

T2B	GAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACCACACCATCTACCTCCTTAGGTCCACAA
SA2	GAACCTAAAGACACCAGCGAACAACCTAGGCCATCGAAAGCTGAACCTCGACCTAGTACCACACCATCTACTTCGTTAGGTCCACAA
I3A	GAAATGAAAGGTAGCAGTGAACAACCTAAGCCATCTAAAAGTGAAGTCTCGACCTAGTACTACACCGTCTACTTCGTTAGGTCCACAA
I3B	GAAGTAAAGAAACTAGTGAACAACCTAAGCCATCTAAAAGTGAAGTCTCGACCTAGTACTACACCGTCTACTTCGTTAGGTCCACAA
I3C	GAAGTAAAGAAACTAGTGAACAACCTAGGCCATCTAAAAGTGAAGTCTCGACCTAGTACTACACCGTCTACTTCGTTAGGTCCACAA
I3D	GAAGTAAAGAAACTAGTGAACAACCTAGGCCATCTAAAAGTGAAGTCTCGACCTAGTACTACACCGTCTACTTCGTTAGGTCCACAA
A1A	GAAATGAAAGGTAGCAGTGAAGCAGCCTAGGCCATCGAAAGGTGAACCTCGTCCTAGTACCACACCATCTACTTCCTTAGGTCAACAA
A1B	GAAGCTAAAGACATTAGTGAGCAGCCTAGGCCATCGAAAGGTGAACCTCGTCCTAGTACCACACCATCTACTTCCTTAGGTCAACAA
A1C	GAAGCTAAAGACATCAGTGAAAAGCCTAGGCCATCGAAAGGTGAACCTCGTCCTAGTACCACACCATCTACTTCCTTAGGTCAACAA
A15A	GAAATGAAAGGTAGCAGTGAAGCAGCCTAGGCCATCGAAAGGTGAACCTCGTCCTAGTACCACACCATCTACTTCCTTAGGTCAACAA
A15B	GAAGCTAAAGACATTAGTGAGCAGCCTAGGCCATCGAAAGGTGAACCTCGTCCTAGTACCACACCATCTACTTCCTTAGGTCAACAA
A15C	GAAGCTAAAGACATTAGTGAAAAGCCTAGGCCATCGAAAGGTGAACCTCGTCCTAGTACCACACCATCTACTTCCTTAGGTCAACAA
A15D	GAAGCTAAAGACATTAGTGAGCAACCTAGGCCATCGAAAGGTGAACCTCGACCTAGTACCACACCATCTACTTCCTTAGGTCCACAA

SNAP results: Averages of all pairwise comparisons: dS = 0.3854, dN = 0.0947, dN/dS = 0.24

**B**

```
A1C      EAKDISEKPRPSKGEPRPSTTPSTSLGQQ      29
A15C     EAKDISEKPRPSKGEPRPSTTPSTSLGQQ      29
T2B      EPKDTSEQPRPSKAEPRPSTTPSTSLGPQ      29
SA2      EPKDTSEQPRPSKAEPRPSTTPSTSLGPQ      29
A1B      EAKDISEQPRPSKAEPRPSTTPSTSLGQQ      29
A15B     EAKDISEQPRPSKAEPRPSTTPSTSLGQQ      29
A15D     EAKDISEQPRPSKAEPRPSTTPSTSLGPQ      29
A1A      EMKGSSEQPRPSKGEPRPSTTPSTSLGQQ      29
A15A     EMKGSSEQPRPSKGEPRPSTTPSTSLGQQ      29
I3A      EMKGSSEQPKPSKTESRPSTTPSTSLGPQ      29
I3D      EVKETSEQPRPSKTESRPSTAPSTSLGPQ      29
I3B      EVKETSEQPKPSKTESRPSTTPSTSLGPQ      29
I3C      EVKETSEQPRPSKTESRPSTTPSTSLGPQ      29
          * *  ** : * . *** * **** : ***** *
```

**Supplementary Figure S7.** Clustal omega alignment between N-terminal regions of ROM4.5 of the Mexican Mo7 strain and the Australian A15 strain of *Babesia bovis*. The repeat region is marked by square brackets. The conserved aa motifs flanking the repeats' region are in red.

```
ROM4.5_A15      MGDNDSNEKKNAGRHSLSAIKGGFVKDMVNRGFRKKVNDSDKNDEGKEDKSGDKPTNVSK 60
ROM4.5_Mo7      MGDNDSNEKKNAGRHSLSNAIKGGFVKDMVNRGFRKKVNDSDKNDESKEDKGGDKPTNVSK 60
          ***** . ***** . ***** . *****

ROM4.5_A15      DTSSMKAGNVAALRDQFKRAVEKDESKDQNSQAGQSSLTSKAPLGDPRDQARAKFLAE 120
ROM4.5_Mo7      DTSSMKSGNVAALRDQFKRAVEKDDSKDQNSQAGQSSSTSKAPLGDPRDQARAKFLAE 120
          ***** : ***** : ***** *****

ROM4.5_A15      KYEKILAARNMVEESASRALQRPATNTPAKIIPPSVSGVSKEMKGSSEQPRPSKAEPRPST 180
ROM4.5_Mo7      KYEKILAARNMVEESASRALQRPATSTPAKIIPPSVSGVSKEPKDTSEQPRPSKAEPRPST 180
          ***** . ***** * . *****

ROM4.5_A15      TPSTSLGQQEAKDISEQPRPSKAEPRPSTTPSTSLGQQEAKDISEKPRPSKAEPRPSTTP 240
ROM4.5_Mo7      TPSTSLGPQE----- 190
          ***** **

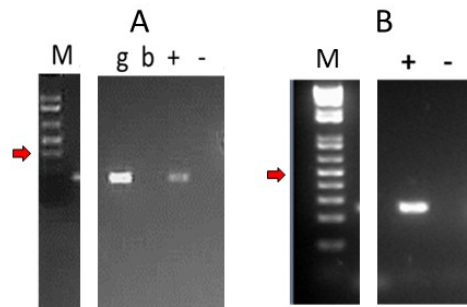
ROM4.5_A15      STSLGQQEAKDISEQPRPSKAEPRPSTTPSTSLGPQEAKDISEQPRPSKAEPRPSTTPST 300
ROM4.5_Mo7      -----PKDTSSEQPRPSKAEPRPSTTPSTSLGPQEPKDTSEQPRPSKAEPRPSTTPST 242
          ** ***** ** *****

ROM4.5_A15      SLGPQEAKDISEQPRPSKAESKLSTALSKLRSSRESRSVIKGTGSFGGSSQVSSSAGRRV 360
ROM4.5_Mo7      SLGPQEPKDTSEQPRPSKAESKLSTTLSKLRFSRESRSVIKGTGSFGGSSQVSSNAGRRV 302
          ***** ** ***** : ***** *****

ROM4.5_A15      HTLADTASTDDASIRQALSQSFSSPGVSEQPERGSRGRNMFGMRRRPCWIRIQVKEKI 420
ROM4.5_Mo7      HTLADTASTDDASIRQAMSQSFSSPDVSHPEQPERASRGRNMFGMRRRPCWIRIQVKEKI 362
          ***** : ***** . ** : ***** . *****

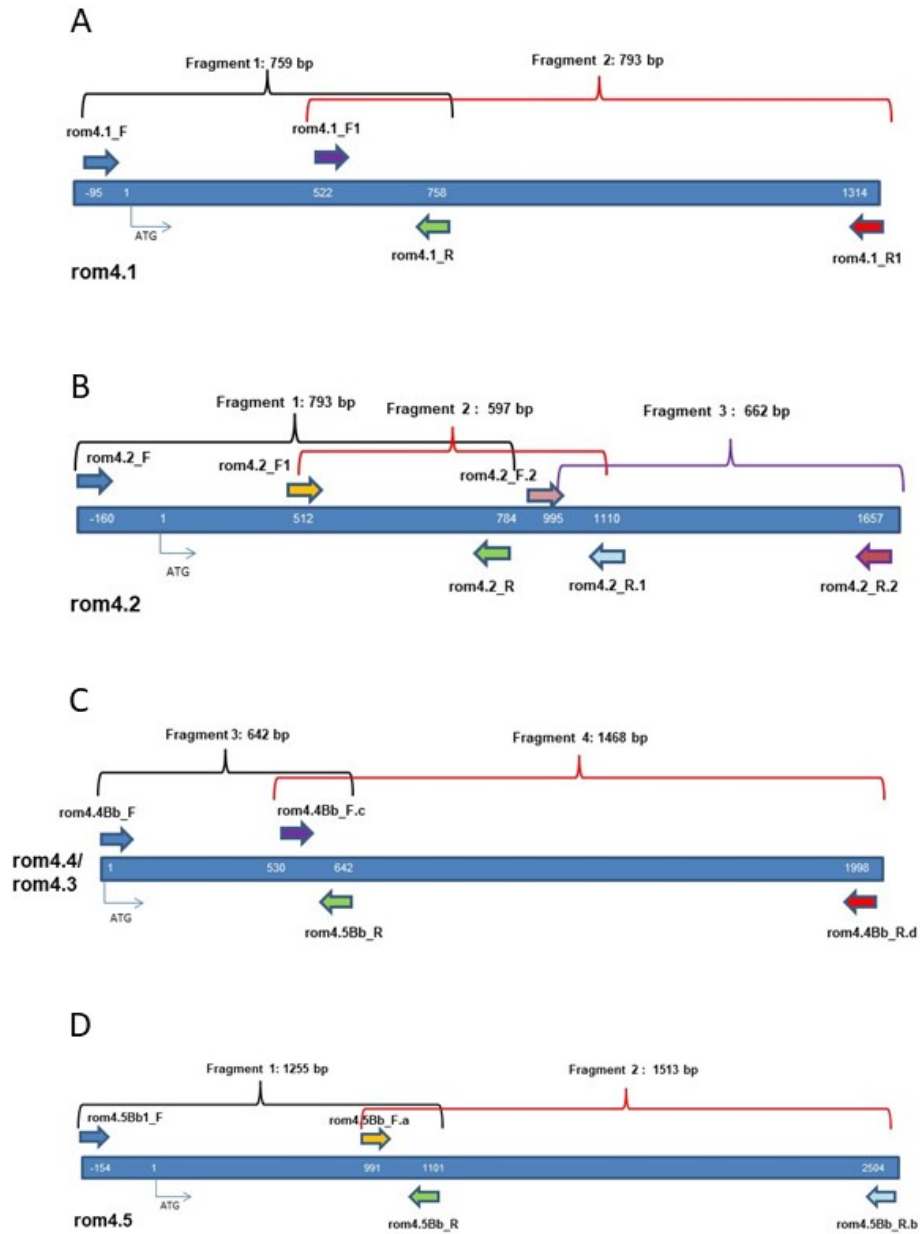
ROM4.5_A15      FPGKM      425
ROM4.5_Mo7      FPGKM      367
          *****
```

**Supplementary Figure S8.** PCR and RT-PCR amplification of *Babesia bovis rom4.3/4.4*. DNA (g) or RNA (+, -) extracted from AS (A) or SS (B) were used as templates for PCR and RT-PCR amplification of *rom4.3/4.4* genes or transcripts, respectively. M: DNA ladder; the arrows mark the 500 bp band; b: blank without DNA template; + reaction with reverse transcriptase; - reaction without reverse transcriptase. A band corresponding to the expected size (259 bp) was only obtained when reverse transcriptase was added to the RT-PCR reaction, showing that the AS and SS RNA preparations were free of DNA contamination.





**Supplementary Figure S9.** Scheme of PCR amplification of *rom4* alleles for polymorphism analysis. A: *rom4.1*, B: *rom4.2*, C: *rom4.3/4.4*, D: *rom4.5*



**Supplementary Table S1.** Oligonucleotides used in qRT-PCR

Gene	Primer name	Sequence	T (°C)	Length (bp)
<i>rom4.1</i> BBOV_II006100	rom4.1_F rom4.1_R	CACTCATATCGTGGGCATTG CATGTTTTTGGGCCATATCC	59.9 59.8	125
<i>rom4.2</i> BBOV_II006070	rom4.2_F rom4.2_R	CAGGCGTACATTTTGGGTTT AGGCGATCCCATCCTATACC	59.9 59.7	74
<i>rom4.4</i> BBOV_II005940/ <i>rom4.3</i> BBOV_II005950	rom4.2/3_F rom4.2/3_R	TCGCATTCGTTAGCATTGAG CGGTCAAACGCAGATACTGA	59.9 59.8	126
<i>rom4.5</i> BBOV_II005930	rom4.5_F rom4.5_R	GGCAACTTCACTTGCTCCTC TAGTGCGCCAACAATAGTGC	60 59.9	64
<i>topoisomerase II</i> BBOV_III004820	topo1_F topo1_R	GGGATAGGTACAGGATTCAGC TCCGTTTTTCATCTAGCCACTG	61 64	197
	topo2_F topo2_R	CAGGACAGAGGAAGGTGCTC CCATGGTGGTATGCAGAGTG	60 60	109
<i>actin</i> BBOV_IV009790	actin_F actin_R	ACCAGGCTGAACCGCTACTA TCATCAAAAACGGAAGCTCCT	60 60	240
<i>gadph</i> BBOV_II002540	gadph_F gadph_R	TTGGTTCTCCGTGCTTCTTT GGAATCTGAGAAGGCTCACG	60 60	209

**Supplementary Table S2.** Ct and efficiency values obtained by qPCR targeting potential reference genes of *Babesia bovis*. The housekeeping genes *topoisomerase*, *gadph* and *actin* were tested by qPCR using specific primers and (A) plasmid DNA and (B) AS or SS cDNA as template. The primers used are shown in Table S1. Each determination was done in triplicates (Ct1, Ct2 and Ct3). Efficiency was calculated using the absolute fluorescence increase method with LinRegPCR software.

**A**

Primer pair	Ct 1	Ct 2	Ct 3	Mean Ct	Mean efficiency	Mean efficiency %
topo 1	24.537	24.469	23.960	24.32	1.825	82.5
topo 2	18.009	18.367	18.153	18.18	1.881	88.1
gadph	22.199	21.832	22.207	22.08	1.807	80.7
actin	25.168	26.055	25.497	25.57	1.868	86.8

**B**

Primer pair	Mean CT of AS	Mean CT of SS	SS/AS ratio	Mean efficiency	Mean efficiency %
topo 1	32.99	30.72	0.93	1.936	93.6
topo 2	33.38	32.99	0.99	1.931	93.1
gadph	34.8	25.64	0.74	1.934	93.4
actin	31.65	35.52	1.12	1.934	93.4

**Supplementary Table S3.** Information on *Babesia bovis* strains and isolates used to study *rom4* sequence polymorphism

Isolate/strain name	Region and/or country of origin	Characteristics	Reference
T2Bo	Texas, USA	Pathogenic <sup>a</sup>	[51]
Mo7	Mexico	Pathogenic <sup>a</sup>	[52]
R1A	Salta, Argentina	Attenuated <sup>a</sup>	[53,54]
S2P	Salta, Argentina	Pathogenic <sup>a</sup>	[54,55]
M2P	Corrientes, Argentina	Mildly pathogenic	[54]
B4P	Rio Grande do Sul, Brazil	Pathogenic	[56]
I3	Gonen, Israel	Attenuated	[57]
A1 (F71 plus1)	North Queensland, Australia	Mildly pathogenic	[58]
A15 (H92)	Central Queensland, Australia	Pathogenic	[58]
SA2 (RLB18/061)	South Africa	unknown	-

**Supplementary Table S4.** Primers used to amplify *rom4* segments for polymorphism studies

Primer	Sequence	Length (bp)	Tm (°C)
rom4.1_F	CCTCCATAGTAGTTGGAAGC TC	579	58
rom4.1_R	ATGCGACGCGGAGTAACAC		61
rom4.1_F1	CTACGGCTAATCATCGTGTC	793	57
rom4.1_R1	CGGGCATGTAGTTCGTAATC		56
rom4.2_F	GATTGTATTGCGAACGGCACTG	793	61
rom4.2_R	CTCAACACAGTACGGTGCAA 3		58
rom4.2_F1	GGTTGTGGAAGCTATCTAGCTG	597	61
rom4.2_R1	GTGAATCGGTAGTATGGAATCC		58
rom4.2_F2	TGGCATAACGACACAATATTGTT	662	57
rom4.2_R2	ACAACGAAAGAGAACCAGGA		57
rom4.4Bb_F	ATGGGAGATAATGATTCGAATG	642	62
rom4.5Bb_R	CATCTTACCGGGAAATATCTTC		54
rom4.4Bb_F.c	TCAGCCAGAGCAACCCGAG	1468	62
rom4.4Bb_R.d	TTATTTCTCACAAAAACATTCCA		54
rom4.5Bb1_F	TGCGAGATATGCAACTCACGA	1255	60
rom4.5Bb_R	CATCTTACCGGGAAATATCTTC		54
rom4.5Bb_F.a	AGCCAGAGCAACCCGAGCG	1513	68
rom4.5Bb_R.b	AATGATGAAAAGTTCAGCATGTA		55