GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

MULTINDELS-BOV: Zebu traceback method based on DNA insertion-deletion polymorphisms

M. Groenner-Penna[1,2], E.F.D. Croce[1], C.G. Pimenta[1], H.M.S. Bicalho[1] and S.D.J. Pena[1,2]

[1]Gene-Genealógica Central de GenoTipagem de Animais, Belo Horizonte, MG, Brasil
[2]Laboratório de Genômica Clínica, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Corresponding author: S.D.J. Pena
E-mail: spena@dcc.ufmg.br

Genet. Mol. Res. 13 (4): 9171-9186 (2014)
Received October 29, 2014
Accepted November 3, 2014
Published November 7, 2014
DOI http://dx.doi.org/10.4238/2014.November.7.4

ABSTRACT. Brazil is a major producer and exporter of beef, with a herd of approximately 210 million animals. For the meat industry, a reliable animal traceback from its origin to the consumer market is paramount. Of all available identification systems, DNA is the only one that survives the slaughterhouse and reaches the dish of the consumer. DNA polymorphisms are already used for cattle traceback, but primarily for the subspecies *Bos taurus taurus*. However, in Brazil, another subspecies, *B. taurus indicus* predominates. We describe here the development of a DNA traceback method designed primarily for *B. taurus indicus* (Zebu), without leaving *B. taurus taurus* aside. We used insertion/deletion (indel) polymorphisms, which have the advantage of being simple and easily automatable, since in most cases, the variable loci are biallelic. We studied 94 indels, with a difference of two or more

base pairs, in DNA pools of 60 Zebu and 60 taurine animals. A set of 22 indels with heterozygosity greater than 0.3 were selected and used to construct two multiplex PCRs. On the basis of the allelic frequency of these indels, the probability of random match was calculated to be 1.12×10^{-8} for *B. taurus indicus* and 1.60×10^{-6} for *B. taurus taurus*. Moreover, we estimated that an analysis would cost less than US$15.00 per animal. Thus, this system (MULTINDELS-BOV) is perfectly suited for building large genetic databases and offering viable prospects of a national system for cattle traceback DNA in Brazil.

Key words: Cattle; Traceback; Indel; Polymorphism; *Bos taurus indicus*

INTRODUCTION

Brazil is one of the major producers and exporters of beef in the world, featuring a herd of approximately 210 million animals (FAOSTAT, 2011). For the beef trade, a reliable animal identification from its origin to the consumer market is of crucial importance. There are several ways to identify the animals physically, such as iron markings, earrings, subcutaneous chips, and retina reading (Lima et al., 2006; Bowling et al., 2008). Nevertheless, all these types of markings are lost in the slaughterhouse. DNA is the only tag that cannot be removed from the meat and persists up to the dish of the consumer.

Currently, single nucleotide polymorphisms (SNPs) and microsatellites are the two classes of DNA polymorphisms most commonly used for animal identification (Pascoal et al., 2005; Shackell et al., 2005; Karniol et al., 2009; Orrù et al., 2009; Allen et al., 2010). However, both present some problems: SNPs require expensive equipment for typing, and microsatellites are not optimal for multiplexing because they are multi-allelic. Another relevant fact is that current DNA traceback methods have been developed for testing animals of the *Bos taurus taurus* subspecies. Nevertheless, in Brazil and also some other important producing countries, *B. taurus indicus* predominates (Groeneveld et al., 2010), and there is a need to develop methods for traceback that are efficient for this subspecies.

Thus, in the development of a DNA traceback method for use in Brazil, we focused our attention primarily to user-friendly inexpensive molecular methods and on the subspecies *B. taurus indicus* (Zebu or Indicine cattle), however without neglecting the *B. taurus taurus* subspecies (European or taurine cattle). For the development of such method, we decided to use insertion/deletion (indel) polymorphisms, which have the advantage of being simple, rapid and easily automatable, since in most cases the variable loci have only two alleles. Moreover, the size of the amplicons can be reduced to 50-60 bp if needed, permitting the typing of degraded DNA (Bastos-Rodrigues et al., 2006).

MATERIAL AND METHODS

Samples

The samples used in this study belonged to the company Gene-Genealógica Central de GenoTipagem de Animais Ltda. which has a bank of genetic material extracted from hair

follicles, blood, semen, and tissue of animals genotyped on the request of breeders and breed associations throughout Brazil. To assess the insertion and deletion polymorphisms in the two subspecies of cattle, DNA pools were made from 60 *B. taurus indicus* animals (including 10 samples of each of the races Gyr, Nellore, Guzerat, Brahman, Sindhi, and Tabapuan) and 60 *B. taurus taurus* animals (including 20 samples of each of the races Holstein, Jersey, and Angus). These pools of DNA were used in PCR for the identification of polymorphic loci and the determination of allelic frequencies.

DNA extraction

Genomic DNA was extracted from four carefully selected intact hair follicles, using the proteinase K method (50 µL Madisen solution, 0.1 M Tris-HCl, pH 8.0, 0.04 M EDTA, pH 8.0, 1 M NaCl, 0.2% SDS, and 2 µL 20 mg/mL proteinase K, with incubation for 3 h at 56°C), giving an efficiency of 30 ng/mL. The procedure for extracting DNA from blood and semen was performed with 5% Chelex100®. The DNA samples were stored at room temperature.

Insertion-deletion polymorphisms (indels)

For this study, 75 indels were selected from dbSNP, from NCBI (National Center for Biotechnology Information) because they showed a difference of 2 or more bp between alleles, which facilitated the detection of the polymorphism. We also examined the relevant literature and found another 21 indels (Hills et al., 2003; Sander et al., 2004; Nakatsu et al., 2004; Siadkowska et al., 2006; Ferraz et al., 2006; Cargill and Womack, 2007; Sasazaki et al., 2007; Hoashi et al., 2007; Seabury et al., 2008). Since our primer design did not work properly for two indels (which would be Ibov68 and Ibov69 in Tables 1 and 2), they were discarded and thus, altogether, 94 indels previously reported as polymorphic in *B. taurus taurus* were analyzed in the Zebu animals (Table 1).

Analysis of indels

The primers used for amplification of the indel region were designed in the Primer-BLAST program (Ye et al., 2012) and are shown in Table 2. PCR was carried out with: 0.36 µL 1 µM primer F, 0.18 µL 10 µM primer R, 0.18 µL 10 µM M13-FAM or M13-HEX, 6.8 µL universal pre-mix (1.32X *Taq* DNA polymerase buffer, mix of dATP, dCTP, dTTP, and dGTP in 0.36 µM 4.63 µM $MgCl_2$), 0.2 µL *Taq* DNA polymerase, 0.28 µL H_2O, and 1.0 µL 30 ng/µL DNA or H_2O. A stepdown PCR program was used in a thermocycler: 95°C for 1 min, 2 cycles at each annealing temperature, 95°C for 45 s, touchdown from 61 to 53°C for 45 s and 72°C for 1 min; 25 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min. The amplicons were detected on the MegaBACE 1000® following the guidelines of the manufacturer (GE). Reading the results that were generated in MegaBACE 1000® was performed with MegaBACE Fragment Profile 1.2 from Amersham Biosciences (GE). Starting with PCR using 96 primer pairs, tests were performed pooling DNA from 60 Zebu and 60 taurine animals. The height of the curves obtained for each amplicon was used as a reference for quantification of each indel allele and for calculating the frequency of each allele.

Table 1. Indel loci known to be polymorphic in *Bos taurus taurus* analyzed for the estimation of variability in *Bos taurus indicus*.

Indel name	Indel size	dbSNP rs id	Reference
IBov01	3	rs29003887	
IBov02	3	rs29002112	
IBov03	3	rs17870616	
IBov04	3	rs29002187	
IBov05	3	rs29003113	
IBov06	3	rs29003131	
IBov07	3	rs29002704	
IBov08	3	rs29003043	
IBov09	3	rs29003907	
IBov10	4	rs29004154	
IBov11	4	rs17870234	
IBov12	4	rs29002299	
IBov13	4	rs29002184	
IBov14	4	rs29003128	
IBov15	4	rs29001780	
IBov16	4	rs29003986	
IBov17	4	rs29002819	
IBov18	4	rs29002278	
IBov19	4	rs29002520	
IBov20	4	rs29003512	
IBov21	4	rs29003233	
IBov22	5	rs17870560	
IBov23	5	rs41257489	
IBov24	5	rs29002827	
IBov25	5	rs41255797	
IBov26	6	rs17870558	
IBov27	6	rs29003033	
IBov28	9	rs17870280	
IBov29	9	rs29002813	
IBov30	9	rs41257431	
IBov31	10	rs41257476	
IBov32	10	rs29003426	
IBov33	12	rs29004129	
IBov34	13	rs41255830	
IBov35	15	rs29002795	
IBov36	15	rs41257558	
IBov37	15	rs29003697	
IBov38	18	rs29003341	
IBov39	19	rs29002493	
IBov40	22	rs29002500	
IBov41	25	rs55617435	
IBov42	3	rs17873919	
IBov43	3	rs17874005	
IBov44	4	rs17873113	
IBov45	4	rs17873935	
IBov46	16		Cargill and Womack, 2007
IBov47	3		Cargill and Womack, 2007
IBov48	4		Cargill and Womack, 2007
IBov49	4		Cargill and Womack, 2007
IBov50	82		Cargill and Womack, 2007
IBov51	4		Cargill and Womack, 2007
IBov52	12		Hills et al., 2003
IBov53	23		Sander et al., 2004
IBov54	14		Sander et al., 2004
IBov55	24		Hills et al., 2003
IBov56	5		Sasazaki et al., 2007
IBov57	4		Siadkowska et al., 2006
IBov58	13		Nakatsu et al., 2004
IBov59	84		Hoashi et al., 2007
IBov60	3		Seabury et al., 2007
IBov61	49		Hills et al., 2003
IBov62	7		Hills et al., 2003
IBov63	3		Hills et al., 2003
IBov64	5		Hills et al., 2003
IBov65	3		Ferraz et al., 2006

Continued on next page

Table 1. Continued.

Indel name	Indel size	dbSNP rs id	Reference
Ibov66	2	rs41257498	
Ibov67	2	rs41257516	
Ibov70	2	rs29002108	
Ibov71	2	rs41255863	
Ibov72	2	rs17870457	
Ibov73	2	rs29004160	
Ibov74	2	rs29002345	
Ibov75	2	rs29002207	
Ibov76	2	rs29002409	
Ibov77	2	rs29002264	
Ibov78	2	rs17870298	
Ibov79	2	rs29004149	
Ibov80	2	rs29004145	
Ibov81	2	rs29004094	
Ibov82	2	rs29003872	
Ibov83	2	rs29002731	
Ibov84	2	rs41255780	
Ibov85	2	rs29002611	
Ibov86	2	rs29003044	
Ibov87	2	rs17870220	
Ibov88	2	rs17870219	
Ibov89	2	rs29004134	
Ibov90	2	rs29003567	
Ibov91	2	rs17871656	
Ibov92	2	rs29002556	
Ibov93	2	rs29002550	
Ibov94	3	rs108452982	
Ibov95	3	rs110022434	
Ibov96	2	rs68268241	

Multiplex PCR

The 22 indel loci with heterozygosity closest to 0.5 were selected for multiplex PCR amplification. New primers were designed for multiplexing using the muPlex software (Rachlin et al., 2005) (Table 4). These primers were grouped into two multiplexes of 11 indels each for use in two PCR analyses (Table 5). Two primer mixes were made according to the following conditions: 2.3 µL 1 µM of each primer F, 2.3 µL 10 µM of each primer R, 25.3 µL 10 µM M13-FAM or M13-HEX. From these primer mixes, we used 1 µL in the PCR with 6.8 µL universal pre-mix (1.32X *Taq* DNA polymerase buffer, mix of dATP, dCTP, dTTP, and dGTP in 0.36 µM 4.63 µM $MgCl_2$), 0.2 µL *Taq* DNA polymerase, 0.28 µL H_2O, and 1.0 µL DNA or H_2O. The thermocycler program used was the same as described before. The amplified fragments were analyzed as described previously.

Indel analysis in the Structure program

The Structure program, version 2.3.4 (Pritchard et al., 2000), was used to determine if the indel panel could identify the *Bos* subspecies. K = 2 (k is the number of populations) was used. Although Zebu cattle also have some taurine ancestry (Kumar et al., 2003), this is relatively small and can be ignored for this purpose. The admixture model was used with correlated allele frequencies. Each run consisted of 50,000 burn-in steps, followed by 250,000 Markov chain Monte Carlo interactions.

Table 2. PCR primers for amplification of indels in *Bos taurus indicus*.

Primer name	Primer sequence (5'-3')
Ibov01F	TGGTCTGCCTTACTCTGGTC
Ibov01R	GTCATACTGTGTAGCCAATAGCC
Ibov02F	CTATCCCAAAGCCTCAGATCC
Ibov02R	GTCATTGGCTGTTGCTCTTC
Ibov03F	CTTCTAGTATCTAGTGGAGGCCA
Ibov03R	CAGCATGGCGTAGCTCTCATAC
Ibov04F	CTTGGGTGAAGGGGAACTTCG
Ibov04R	GGGGCTGCAAATAAAGACAGAC
Ibov05F	CGCTATGAATGAGGATGTAGTCC
Ibov05R	CCTCCCCTTCACACTTTCTACTAC
Ibov06F	CTCTACTTGATTGGAGCTGGAAG
Ibov06R	CCCCATGCTTTCTGTGAGAG
Ibov07F	CACTAACCTTAGCTCCTCTCAC
Ibov07R	GCTTTTCCAAGGACTCACAC
Ibov08F	CTAATCCAGTCAGTGTACCTCC
Ibov08R	CGCTGAACAGTATTCGAGTTAC
Ibov09F	CACTACTCCACCTTGTATACCTTG
Ibov09R	GACAGAAAGGGAATCGCTGAG
Ibov10F	GGCCATAAAACAGATTGTCTGAG
Ibov10R	CCAGAGAGATTGGCTAGTATGTC
Ibov11F	CTAGCAGAGGAGATGCCACG
Ibov11R	GCCTCAGAGCCTCACACATG
Ibov12F	GCTGTTAGCCATCAGGCAAG
Ibov12R	GGCATAGACTAATTTCTGTCTGCATC
Ibov13F	CTGTTTAACAGTTTTGGGAGCTG
Ibov13R	GTATACAAGGCAATGAGGCC
Ibov14F	GTCAAGTCTAGCCTGTGCTG
Ibov14R	GGATGCTACCATTTGGTCAG
Ibov15F	GAAGAGCAATAGCCCAGTGC
Ibov15R	GCTGAGGAATTCTCTGATTATCTCC
Ibov16F	CCTTACAGCTTCTTCTAAATGGAG
Ibov16R	GATTGCACCTTACTATCAGAAAGG
Ibov17F	CAGTCTGTTCTCACCTTCCAG
Ibov17R	CTGCCAGAAGGTAATGAGACG
Ibov18F	CCATTGGCACTGACTCTAAGTC
Ibov18R	GTTTGGTCTTCAGGTCAGAAAG
Ibov19F	CGCCAATGCAAGAGATGTAAG
Ibov19R	CCTCATTCCTGGGGAAACTC
Ibov20F	CACGCACTGCAACAACTAAG
Ibov20R	GAGGACTGAAAGAACTTCCCC
Ibov21F	TCTGCATTGCTAGAGATGGAAG
Ibov21R	GGGTCAAAAACAGATTCCAGAG
Ibov22F	GGAGGGAGACCTCAATTTCAC
Ibov22R	CTCTCTGTGTTTGTCCTGAGG
Ibov23F	CAGGGTGAAGGTCACAGAAC
Ibov23R	GCCCCTTTTGTACAGATGGAG
Ibov24F	AGAAACTGCAAAAGTACAAACAAG
Ibov24R	TGAAGTCGCTCAGTCGTGTC
Ibov25F	GGAGCTGAGACTGTGACTG
Ibov25R	GGGAACCATGAATTCTGCTG
Ibov26F	CCAATGAAGGTGAAGCGTCC
Ibov26R	CTGAGGCTGCTCTAAGAGTTC
Ibov27F	CAGGAGAGCTGATTTCTAGTTCTG
Ibov27R	CTATTCATAGTGGAGCTGGGC
Ibov28F	GTAGAGAGACCCTGAAGCAGC
Ibov28R	ACAGCCTTGCTGGTGACATC
Ibov29F	CACCTAGCGGTGCAACATATC
Ibov29R	GGCATTGACTCCATCTGCAGTA
Ibov30F	TGAGATGACCCTCTTTGAAGGAAC
Ibov30R	GGATGGCTGCTTGGCTTATAG
Ibov31F	GCTAATTCTCTTGACTTGCAGGC
Ibov31R	CGTGGTGCCTCCGTTCTATG
Ibov32F	GATACCGACAGCAGAGAGAGG

Continued on next page

Table 2. Continued.

Primer name	Primer sequence (5'-3')
Ibov32R	TCCTAAACGCTTTGTCATCCTGT
Ibov33F	GAAAGACATAATCAGGCTCTCCAG
Ibov33R	CTAGGGTCTTAATTGTATGTTCCTG
Ibov34F	GTAGAAGTTCTACCACTGCCAG
Ibov34R	GCCACACCTATTGCTCTATATGAA
Ibov35F	CTCCCCTTCTTTCAGGACAC
Ibov35R	CCAGAGAGAGACAAGCAAGC
Ibov36F	GCAGAGGCTGGTCACCTAAG
Ibov36R	CCATTCCAGGCACACCTACT
Ibov37F	GGCAGAGGAGAGTCAAGTCC
Ibov37R	CAGAGGCTGGTCACCTAAGG
Ibov38F	TAGCTCTGAGAGAGAGGAAAAGG
Ibov38R	TGTAGGGACACCACTTTTCTGC
Ibov39F	GAAGGACCAATCCTCCCCTG
Ibov39R	CCAAGCAATCCTGAGTCCTG
Ibov40F	GAGAGCACACATGTCTTCTGTC
Ibov40R	CTGTGGTTGTCCTCTGGTGTC
Ibov41F	CTGGCAGGCAGATTCGTAAC
Ibov41R	GGGTGATTTGTGGTAGGGTTTC
Ibov42F	GAGGTTCACAATAGGATTCACGC
Ibov42R	CGGTAAGCAACCGAATGAAGC
Ibov43F	CACACCATGAAATTCCAGCTC
Ibov43R	CCATGCTGAAGACACAGGAG
Ibov44F	CAGAACAAGAGAGAAGCTGGTC
Ibov44R	GCTGTGTGTGAGTCAGCTATC
Ibov45F	GCTCACTGTAAATGGTTCGCTC
Ibov45R	TTTGTGATTTCATGGCAGGG
Ibov46F	GAACTCTCCTCTCTTGCATCCTTC
Ibov46R	GGTATCTAAGGCCAAGGGATGC
Ibov47F	GGCAACCAGTGTTAGAATTTGG
Ibov47R	CTTAGCCACTAGATCGCCAG
Ibov48F	GTGATGGGCTGGTAACAGAG
Ibov48R	CAGTTTAGTCGCTCAGTCGTG
Ibov49F	CTGAGCGACTAAACTGAATCTAATC
Ibov49R	GAAGGCAGGAGAGAAGGAATC
Ibov50F	CCTATTTCAGGCCTTTTGTTACC
Ibov50R	GTACGTTTTACCCTTTCACAACTG
Ibov51F	CTCACCCATGTGTTGCCAGAAGAGC
Ibov51R	CCACATCCTCTACTTGCCACCCTTC
Ibov52F	GAATCGGATTGGTGGGAGGC
Ibov52R	GGCTAGATTCCTACACACCACC
Ibov53F	GTTCTGCACTGTTATTACTGGC
Ibov53R	CCAGGGGATTTACATGACCTAG
Ibov54F	CAGTACACCTGATTTTCAAGTCC
Ibov54R	GTAGGCCAAAGAGTTGGACAG
Ibov55F	TGGAGGCAACCGTTATCCAC
Ibov55R	GGCTTACTGGGTTTGTTCCATTG
Ibov56F	ATGGGGCCAACAGAATCTTAG
Ibov56R	CATGGTAAGAGGAGCCCAAG
Ibov57F	CTGGGTGGAGCAGTGAACAC
Ibov57R	GTGGGCTTGTCTGTTCAGATCA
Ibov58F	GATTTCCTTCGATGCTTCATGC
Ibov58R	CATTCACAAGTATGGCACTCATG
Ibov59F	CACAACGCCATCGAGAAACGCTAC
Ibov59R	GTGGGAGGGAGATGGCACAAGTG
Ibov60F	CCCAGATATGGAAACTGAACCAG
Ibov60R	GCTGGAAGGTAAGGGAGGAG
Ibov61F	CAGACACGTATCTCCCTGTAG
Ibov61R	CTCATCTTCCTGCCATCTCTG
Ibov62F	GAGTGGGTGCAATCTGATGAC
Ibov62R	ATTCCTGACCAAGGGAAGACC
Ibov63F	CAGTCCTGAGTCAGGCAATG

Continued on next page

Table 2. Continued.

Primer name	Primer sequence (5'-3')
Ibov63R	AGGAACCCTCATTATGCTGTC
Ibov64F	CTAGGTCATGTAAATCCCCTGG
Ibov64R	CAGCACCTTCTCCATACACTG
Ibov65F	GGTCCTTGCATAAATGTATAGAGC
Ibov65R	GTCCTGGGATCCTGGAATTG
Ibov66F	CTGGGTCCTGATTGGTCACT
Ibov66R	GGTGCAGAGCACAAAAATCA
Ibov67F	TTCTCATGTCTCCCTCTGGG
Ibov67R	AACCTCCCCTGTGTCTCGTT
Ibov70F	CCAATATTTTGGGGGATCTCT
Ibov70R	GATGGAGATGGGGAGTGGTA
Ibov71F	TCCTTGCCTTCATGGGATAG
Ibov71R	GACCTGCCCTTCCTTACCTC
Ibov72F	TTTGTTTGGCATTCAGAGCA
Ibov72R	CGAAGTGTTTGACTCCGGTT
Ibov73F	GGCTTTTGTAACCTCGCTTG
Ibov73R	TCTCTCTCTGGGAACAGCGT
Ibov74F	AGCTTTTCCCAACAGCTTCA
Ibov74R	TCTGGGCTTTTAGCTGCATT
Ibov75F	GAAAGTTGCAGGCAACCACT
Ibov75R	TGAACTTGCCATGTGCTCTC
Ibov76F	TGTGTGAGGATTTAATAGGGCA
Ibov76R	TGCCTGAATCTCTGGTGTCA
Ibov77F	GCATGGTGGTTTGGTTTTCT
Ibov77R	ACCATCTTTGGGGAAAAAGC
Ibov78F	GGGATCCTCAAGATCATGTCA
Ibov78R	TGTGATCTAAATTTATGTGGCATT
Ibov79F	CCAAACGATTTTCAAGAAAGG
Ibov79R	CTCTGTGTCCCTTTATGTGGG
Ibov80F	TGGGAGCTCACCAAAAGAAT
Ibov80R	TTGCTTCTATGGCAAAGCCT
Ibov81F	AACCACCCAAATCCCATGTA
Ibov81R	AACACAAACTCTGTTTTAAATCTTACG
Ibov82F	TCGGGACCTCTTCATTTGAG
Ibov82R	TGTTTGTGCAATTTGAGCATT
Ibov83F	TGCTACCCTGGTAACCAACA
Ibov83R	GAAAGGAAATTAGGGGCCAG
Ibov84F	CCCCAACAATTCTGTCCATT
Ibov84R	TGTCAAGCATTTCCAAAGCA
Ibov85F	TTCTACCTACATTGCCCCCA
Ibov85R	TGTCAAGCATTTCCAAAGCA
Ibov86F	TTGCCATGTAAATTTCCCAGA
Ibov86R	CTCGAATACTGTTCAGCGCA
Ibov87F	CATAACTGAGAGCCATTGGGA
Ibov87R	GGGATAACAGGCATGACACC
Ibov88F	TCAAACCATCGTGTGAAAGAA
Ibov88R	TGAAGCAGAGGACTCCATGA
Ibov89F	CTCACCACAACCAGTTCCCT
Ibov89R	GGTAGCCTTTTGGTTAGCCC
Ibov90F	GGGGTAGAAGGCCTGAAACT
Ibov90R	CGACCCAAGAGCATTTGTTT
Ibov91F	TTCTCATGTCTCCCTCTGGG
Ibov91R	CTCCCCTGTGTCTCGTTTTT
Ibov92F	CCTAAAGCCATCCAAAGAGC
Ibov92R	ACCGACTCGAAGGACATTTG
Ibov93F	AATGCCAGTTTGGCAGTCAG
Ibov93R	ATCAGTGAGCCCCAGACAAC
Ibov94F	GCTCCACCCAGGGACTAAA
Ibov94R	GGATCAGGATGAGAACCCAA
Ibov95F	AAGACCAGACCCATCTGCAC
Ibov95R	TTCAGCATGTGCTTCAAAGG
Ibov96F	GCTGTCCAAGGGACTTTCAA
Ibov96R	ATCCATGATTGGTCCTGCTC

Analysis of indel polymorphisms in silver-stained high-resolution polyacrylamide gels

To make indel analysis less expensive and independent of sophisticated equipment, we successfully adapted our primers and multiplex systems for genotyping in long thin denaturing polyacrylamide gels with silver staining, exactly as described previously (Pena and Pena, 2012). These gels can be easily run and fully automated with a simple large scanner and commercial software as described by Pena and Pena (2012).

RESULTS AND DISCUSSION

Identification of polymorphic loci and estimation of allele frequencies

We initially tested 94 indel polymorphisms with two or more base pair differences between alleles using as samples two DNA pools of 60 Zebu and 60 taurine animals, respectively. From the amplification of these indels, we identified 50 indels that showed two or more identifiable alleles, i.e., that were polymorphic in *B. taurus indicus* (Table 3).

We then amplified each locus using individual samples of 70 Zebu and 70 taurine animals to estimate the frequency of each allele (Table 3) to select the 22 indels with heterozygosity closest to 50% (bold type in Table 3). The allelic sizes of the chosen indels are shown in Table 4.

Table 3. Allele frequencies of polymorphic insertion/deletion loci identified in *Bos taurus indicus*.

Locus	Allele 1	Allele 2	Allele 3	Locus	Allele 1	Allele 2	Allele 3
Ibov01	**0.24**	**0.76**	**-**	Ibov47	0.14	0.86	-
Ibov02	0.87	0.13	-	Ibov50	0.36	0.64	-
Ibov03	0.48	0.52	-	**Ibov52**	**0.21**	**0.79**	**-**
Ibov05	0.51	0.49	-	Ibov55	0.09	0.91	-
Ibov08	0.20	0.80	-	Ibov56	0.84	0.16	-
Ibov10	0.20	0.39	0.41	**Ibov60**	**0.71**	**0.29**	**-**
Ibov13	0.07	0.93	-	Ibov61	0.39	0.61	-
Ibov15	**0.61**	**0.39**	**-**	Ibov62	0.26	0.24	0.50
Ibov18	**0.32**	**0.68**	**-**	Ibov66	0.69	0.31	
Ibov22	0.10	0.90	-	**Ibov67**	**0.26**	**0.44**	**0.30**
Ibov27	**0.42**	**0.58**	**-**	**Ibov72**	**0.54**	**0.46**	**-**
Ibov30	0.90	0.10	-	Ibov73	0.25	0.37	0.38
Ibov31	0.48	0.52	-	**Ibov74**	**0.52**	**0.48**	**-**
Ibov32	0.12	0.88	-	**Ibov76**	**0.38**	**0.62**	**-**
Ibov34	0.81	0.19	-	Ibov77	0.78	0.22	-
Ibov37	**0.52**	**0.48**	**-**	**Ibov78**	**0.44**	**0.56**	**-**
Ibov38	0.76	0.24	-	**Ibov79**	**0.50**	**0.50**	**-**
Ibov39	**0.55**	**0.45**	**-**	Ibov80	0.28	0.72	-
Ibov40	**0.50**	**0.50**	**-**	**Ibov83**	**0.33**	**0.35**	**0.32**
Ibov41	**0.76**	**0.24**	-	**Ibov87**	**0.25**	**0.46**	**0.29**
Ibov42	**0.53**	**0.47**	**-**	Ibov89	0.80	0.20	-
Ibov43	0.73	0.27	-	Ibov90	0.89	0.11	-
Ibov44	0.30	0.70	-	**Ibov91**	**0.23**	**0.39**	**0.38**
Ibov45	**0.14**	**0.61**	**0.25**	Ibov93	0.67	0.33	-
Ibov46	0.54	0.46	-	**Ibov96**	**0.53**	**0.47**	**-**

The 22 indels with heterozygozity closest to 50% (bold type) were selected to be included in the multiplex sets.

Table 4. Indel allele size.

Multiplex 1			Multiplex 2		
Indel	Allele	Size (bp)	Indel	Allele	Size (bp)
Ibov01	1	236	Ibov41	1	269
	2	238		2	274
Ibov15	1	129	Ibov45	1	212
	2	133		2	214
Ibov18	1	161		3	218
	2	165	Ibov67	1	127
Ibov27	1	208		2	129
	2	215	Ibov72	1	159
Ibov37	1	176		2	161
	2	191	Ibov74	1	136
Ibov39	1	279		2	138
	2	298		3	185
Ibov40	1	101		4	187
	2	124	Ibov76	1	117
Ibov42	1	117		2	119
	2	120	Ibov78	1	105
Ibov52	1	75		2	107
	2	87	Ibov83	1	94
Ibov60	1	103		2	96
	2	106		3	98
Ibov79	1	93	Ibov91	1	151
	2	95		2	152
Ibov87	1	150		3	153
	2	152	Ibov93	1	144
				2	146

Having selected the indels with the highest heterozygosity in *B. taurus indicu*s, the next step was to be able to multiplex them. Accordingly, new primers were designed using the muPlex software (Rachlin et al., 2005) (Table 5) to achieve multiplexing of 11 indels for use in each of two PCR analyses (Table 6). An example of multiplex PCR amplification and resolution by capillary electrophoresis and fluorescence detection in the MegaBACE 1000 automatic DNA sequencer is shown in Figure 1. The system of analysis of bovine DNA samples with these two multiplexes was called MULTINDELS-BOV.

Calculation of match probabilities

The probability of random match (match probability) can be easily calculated using the multiplication rule from the frequencies of the 22 indel markers (National Research Council, 1996). In our case, the probability of random match of the two multiplexes for analysis in an automated sequencer was estimated at 1.12×10^{-8} (1 in 89 million) for *B. taurus indicus* and 1.60×10^{-6} (1 in 620 thousand) for *B. taurus taurus*. These very low match probabilities make MULTINDELS-BOV well suited to serve as a simple and effective traceback method for cattle in Brazil.

Indel panel to differentiate *Bos* subspecies

Using the data obtained from the indel genotyping, the Structure program was used to analyze the capacity of the indel multiplexes (MULTINDELS-BOV) to discriminate individual animals according to subspecies. Thirty-three animals from each of the subspecies were

Table 5. Multiplex PCR primers.

Primer name	Primer sequence (5'-3')
Ibov03mF	GTGAGGAGAGAATTGCTTCTA
Ibov03mR	TGCCCTCACTGTCCTATC
Ibov05mF	TCCTGTAGCAGCCATACTT
Ibov05mR	CCAATGAGACGTGACAAGA
Ibov08mF	CAGGCTGTGGAAATCTAATC
Ibov08mR	CAAGGAAATGTGTGGCTT
Ibov10mF	ATAGGAATGAGTGGCCATA
Ibov10mR	CCCATTCACTGAAACAAAC
Ibov15mF	GGAAGAGCAATAGCCCAG
Ibov15mR	TTGTTGCTTATACCATCATCA
Ibov27mF	GATCCATGCTTCTCTGTTATT
Ibov27mR	GCAATCCTTGTGTGAAGTAGT
Ibov30mF	AAGGAACCAATTGTTACGAC
Ibov30mR	GCTGCTTGGCTTATAGGAT
Ibov31mF	CACGGCCATCATAGGTAG
Ibov31mR	TGGAGTAATTCCGCATCA
Ibov34mF	TTCACACTGCACTTCAATATC
Ibov34mR	TTTTCAAGATCTGAAGGAATTGAAA
Ibov37mF	CCCTACTCCCTGGAGTCT
Ibov37mR	TGAGGACAAACTCTGGCTA
Ibov38mF	GGTGGACAAACACCAAAT
Ibov38mR	AACTCTTCACCTGGATCTAGA
Ibov39mF	CCCTGCTCCATATATACCTG
Ibov39mR	AAATACCAAGCAATCCTGAG
Ibov40mF	CGTGTGTGTGTCCATAATATC
Ibov40mR	CAAGAAACTCAACATGGGAG
Ibov42mF	ACATGGGAGGTTCACAATA
Ibov42mR	TCACCTGTATTTACAGCCG
Ibov44mF	CAGATACCTCATGAAAGAGAAA
Ibov44mR	TTGACTCTGCCTCTAGGTAAGAGT
Ibov45mF	GTAAATGGTTCGCTCTGAC
Ibov45mR	AGCACTGAATGCTGTAACTG
Ibov46mF	TTGACCCATTATAAAGCAAA
Ibov46mR	CAATTCAAGCAAAGTGCTG
Ibov50mF	GGTTACTGAGGCCTATGAAA
Ibov50mR	TCACAACTGAAATAGCATTGTA
Ibov52mF	TGGTTAGGAGAGCTCCATT
Ibov52mR	CCTACACACCACCCACATA
Ibov60mF	CTCTTCTTGTTTCTTTCTTTCT
Ibov60mR	TATACCTGTGGCGGATTC
Ibov65mF	AGGACCCTGAGTCGTCTG
Ibov65mR	AGGAGGTTCTAAATTATCCAT
Ibov66mF	GAGATAGCAGCGAGCCAATC
Ibov66mR	GGTGCAGAGCACAAAAATCA
Ibov72mF	TTGGCATTCAGAGCATAAT
Ibov72mR	CAAAGGCATGTGTTTCTAGA
Ibov74mF	CCAAGGACAACTCCACAG
Ibov74mR	ATCGACTTCTCTCTCTCCTG
Ibov76mF	GCTATAGTTTCATGACACAGTAA
Ibov76mR	CTTCTCATTTATCAAGGCAC
Ibov78mF	CCTACTTCATGCTTGTGTGA
Ibov78mR	ACGTCATCATCAACAACAAA
Ibov79mF	AGAAGCCACTAAATACTTTCTTA
Ibov79mR	TCCTTCTTTATGTGGGTACA
Ibov80mF	TCAAAAATGCTACTGACATGGAA
Ibov80mR	TGGCTTTGTCCTGTCTTTTG
Ibov83mF	CAACTATACATCGCACACAAA
Ibov83mR	CTGCCTCCTCGTCTTCTA
Ibov87mF	AACCATCGTGTGAAAGAATA
Ibov87mR	GCAAGGAAACAGAACTGATC
Ibov91mF	GGAGAGACTTGAATCTGAATAC
Ibov91mR	ACAAGGTCCTATTGTATAGCAC
Ibov93mF	TGGCTTGATTCAATTTCTAC
Ibov93mR	GCTACATCTTGGAATGGAA
Ibov96mF	ATCAGTCCTCCAATGAATATT
Ibov96mR	TTCAGTGGTGGTCTACCTG

analyzed, under the premise of the existence of two populations (k = 2). The results showed that MULTINDELS-BOV is able to absolutely differentiate the Zebu animals from the taurine animals as shown in Figure 2.

Table 6. Distribution of the indel loci in the multiplex PCRs.

Multiplex reaction 1	Multiplex reaction 2
Ibov01	Ibov41
Ibov15	Ibov67
Ibov18	Ibov72
Ibov27	Ibov74
Ibov37	Ibov76
Ibov39	Ibov78
Ibov40	Ibov79
Ibov42	Ibov83
Ibov45	Ibov87
Ibov52	Ibov91
Ibov60	Ibov93

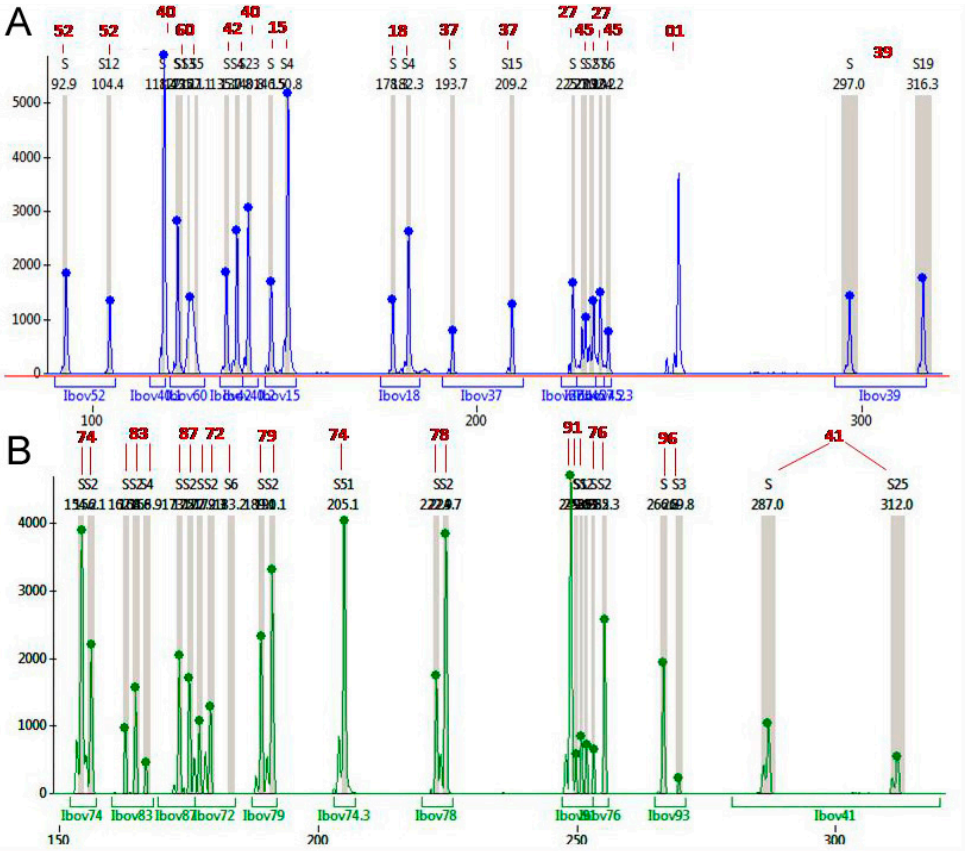

Figure 1. Allelic profile of the two multiplex amplifications using a DNA pool of 60 *Bos taurus indicus* animals as sample, showing all alleles. **A.** Multiplex 1; **B.** Multiplex 2.

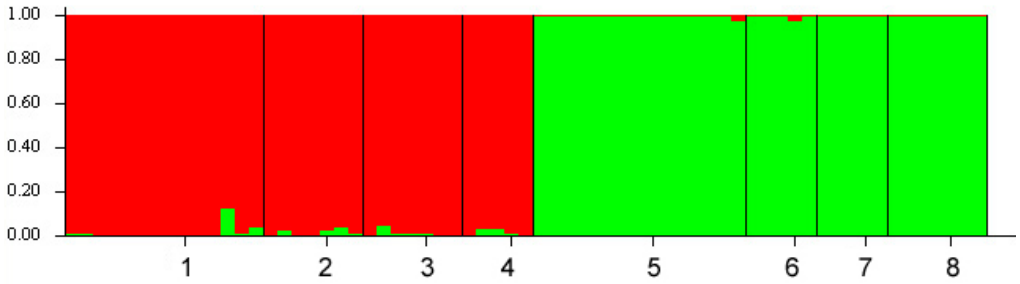

Figure 2. Bar plot generated in the Structure program. The red color designates Zebu ancestry and the numbers 1-4 indicate the races: 1 = Nellore, 2 = Gyr, 3 = Guzerat, and 4 = Brahman. The green color designates taurine ancestry and the numbers 5-8 indicate the races: 4 = Holstein, 5 = Jersey, 6 = Hereford, and 7 = Angus.

Analysis of indel polymorphisms in silver-stained high-resolution polyacrylamide gels

Developing countries such as Brazil are technological mosaics. While some regions of the country have state-of-the-art laboratories, other poorer regions may lack the budget for the large expenses involved in purchasing and operating equipment such as fluorescent DNA analyzers. In such "low-tech" laboratories, the simplicity of running and analyzing the simple diallelic indel multiplexes would be especially helpful.

Thus, we successfully developed a parallel multiplex system (SILVER MULTINDELS-BOV) for genotyping the 22-indel panel in long thin denaturing polyacrylamide gels with silver staining, which can be highly useful as a low-cost adjunct to the more technologically complex fluorescent typing of the same loci in automatic DNA sequencers. For that, it was necessary to design special PCR primers, which are listed in Table 7. The analysis using polyacrylamide gel electrophoresis allowed good discrimination of the amplicons (Figure 3).

The great advantage of this new system of typing is its low cost. Besides a PCR thermocycler, all the special laboratory equipment needed is an electrophoresis DNA sequencing apparatus for long thin denaturing gels, a high wattage power supply, and a large flat scanner. The total cost for this equipment is less than US$10,000. The unlabeled primers and all other supplies are very inexpensive, so genotyping of an individual animal will cost less than US$15.00.

CONCLUSION

Through the use of multiplexed indel polymorphisms, MULTINDELS-BOV proved to be advantageous for cattle traceback and provided excellent results as shown above. The method is simple, rapid, and readily automatable, since in the vast majority of cases, there are only two alleles at each locus. Moreover, the size of the amplification products can be reduced, facilitating the analysis of degraded DNA samples.

Thus, we feel that the MULTINDELS-BOV indel panel that we developed should be capable of providing traceability of cattle in Brazil, generating greater import market confidence, applications in veterinary forensics in the identification of stolen animals (even if they have been slaughtered), and as a bonus, the capacity to estimate the relatedness and to identify the subspecies of cattle using simple admixture genetics (Bicalho et al., 2006).

Table 7. PCR primers for analysis in denaturing polyacrylamide gel.

Primer	Primer sequence (5'-3')
Multiplex 1	
Ibov15mPF	GGAAGAGCAATAGCCCAG
Ibov15mPR	TTGTTGCTTATACCATCATCA
Ibov18mPF	ATAGGGTCGCAAAGCATCAG
Ibov18mPR	CCAGCATTGCATGATTTGTT
Ibov27mPF	GATCCATGCTTCTCTGTTATT
Ibov27mPR	GCAATCCTTGTGTGAAGTAGT
Ibov37mPF	CCCTACTCCCTGGAGTCT
Ibov37mPR	TGAGGACAAACTCTGGCTA
Ibov39mPF	CCCTGCTCCATATATACCTG
Ibov39mPR	AAATACCAAGCAATCCTGAG
Ibov40mPF	CGTGTGTGTGTCCATAATATC
Ibov40mPR	CAAGAAACTCAACATGGGAG
Ibov42mPF	ACATGGGAGGTTCACAATA
Ibov42mPR	TCACCTGTATTTACAGCCG
Ibov52mPF	TGGTTAGGAGAGCTCCATT
Ibov52mPR	CCTACACACCACCCACATA
Ibov60mPF	CTCTTCTTGTTTCTTTCTTTCT
Ibov60mPR	TATACCTGTGGCGGATTC
Ibov01mPF	AGGTTAAAGAGCAGCACTG
Ibov01mPR	TGTGTGAACAAAGAATGTAAAG
Ibov79mPF	AGAAGCCACTAAATACTTTCTTA
Ibov79mPR	TCCTTCTTTATGTGGGTACA
Ibov87mPF	AACCATCGTGTGAAAGAATA
Ibov87mPR	GCAAGGAAACAGAACTGATC
Multiplex 2	
Ibov41mPF	GGGTAGACAGCATGAAGAGA
Ibov41mPR	CCAGACCAGGGATGAAAC
Ibov72mPF	TTGGCATTCAGAGCATAAT
Ibov72mPR	CAAAGGCATGTGTTTCTAGA
Ibov74mPF	CCAAGGACAACTCCACAG
Ibov74mPR	ATCGACTTCTCTCTCTCCTG
Ibov76mPF	GCTATAGTTTCATGACACAGTAA
Ibov76mPR	CTTCTCATTTATCAAGGCAC
Ibov78mPF	CCTACTTCATGCTTGTGTGA
Ibov78mPR	ACGTCATCATCAACAACAAA
Ibov83mPF	CAACTATACATCGCACACAAA
Ibov83mPR	CTGCCTCCTCGTCTTCTA
Ibov91mPF	GGAGAGACTTGAATCTGAATAC
Ibov91mPR	ACAAGGTCCTATTGTATAGCAC
Ibov67F	TTCTCATGTCTCCCTCTGGG
Ibov67R	AACCTCCCCTGTGTCTCGTT
Ibov93mPF	TGGCTTGATTCAATTTCTAC
Ibov93mPR	GCTACATCTTGGAATGGAA
Ibov45mPF	GTAAATGGTTCGCTCTGAC
Ibov45mPR	AGCACTGAATGCTGTAACTG

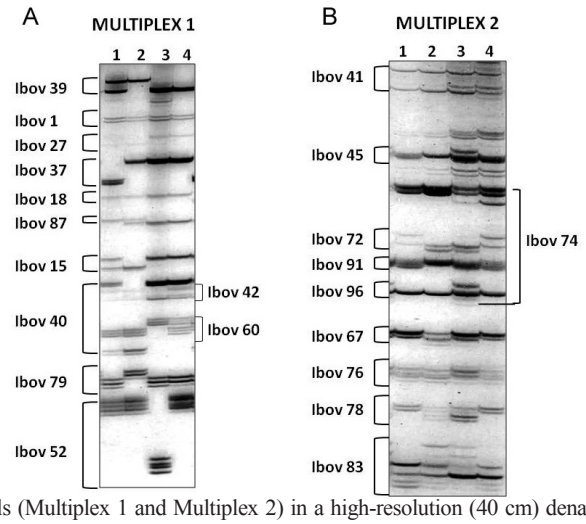

Figure 3. Profile of indels (Multiplex 1 and Multiplex 2) in a high-resolution (40 cm) denaturing polyacrylamide gel stained with silver nitrate. **A.** Multiplex 1: *lanes 1* and *2* = Nelore animal; *lane 3* = Jersey animal; *lane 4* = Holstein animal. **B.** Multiplex 2: *lanes 1* and *2* = Nelore animal; *lane 3* = Jersey animal; *lane 4* = Holstein animal.

ACKNOWLEDGMENTS

Research supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil.

REFERENCES

Allen AR, Taylor M, McKeown B, Curry AI, et al. (2010). Compilation of a panel of informative single nucleotide polymorphisms for bovine identification in the Northern Irish cattle population. *BMC Genet.* 11-5.

Bastos-Rodrigues L, Pimenta JR and Pena SDJ (2006). The genetic structure of human populations studied through short insertion-deletion polymorphisms. *Ann. Hum. Genet.* 70: 658-665.

Bicalho HM, Pimenta CG, Mendes IK, Pena HB, et al. (2006). Determination of ancestral proportions in synthetic bovine breeds using commonly employed microsatellite markers. *Genet. Mol. Res.* 5: 432-437.

Bowling MB, Pendell DL, Morris DL, Yoon Y, et al. (2008). Identification and traceability of cattle in selected countries outside of North America. *PAS* 24: 287-294.

Cargill EJ and Womack JE (2007). Detection of polymorphisms in bovine toll-like receptors 3, 7, 8, and 9. *Genomics* 89: 745-755.

FAOSTAT (2011). Production: Live Animals, Cattle, Brazil. Available at [http://faostat3.fao.org/home/index.html#VISUALIZE]. Accessed January 7, 2014.

Ferraz AL, Bortolossi JC, Curi RA, Ferro MI, et al. (2006). Identification and characterization of polymorphisms within the 5' flanking region, first exon and part of first intron of bovine GH gene. *J. Anim. Breed. Genet.* 123: 208-212.

Groeneveld LF, Lenstra JA, Eding H, Toro MA, et al. (2010). Genetic diversity in farm animals - a review. *Anim. Genet.* 41 (Suppl 1): 6-31.

Hills D, Schlaepfer J, Comincini S, MacLean I, et al. (2003). Sequence variation in the bovine and ovine PRNP genes. *Anim. Genet.* 34: 183-190.

Hoashi S, Ashida N, Ohsaki H, Utsugi T, et al. (2007). Genotype of bovine sterol regulatory element binding protein-1 (SREBP-1) is associated with fatty acid composition in Japanese black cattle. *Mamm. Genome* 18: 880-886.

Karniol B, Shirak A, Baruch E, Singrün C, et al. (2009). Development of a 25-plex SNP assay for traceability in cattle. *Anim. Genet.* 40: 353-356.

Kumar P, Freeman AR, Loftus RT and Gaillard C (2003). Admixture analysis of South Asian cattle. *Heredity* 91: 43-50.

Lima VMB, Bornstein CT and Cukierman HL (2006). The Brazilian bovine traceability system - a critical appraisal. *Estud. Soc. Agric.* 14: 49-87.

Nakatsu Y, Yamada K, Ueda J, Onogi A, et al. (2004). Genetic polymorphisms and antiviral activity in the bovine MX1 gene. *Anim. Genet.* 35: 182-187.

National Research Council (1996). The Evaluation of Forensic Evidence. National Academy Press, Washington, 31.

Orrù L, Catillo G, Napolitano F, De Matteis G, et al. (2009). Characterization of a SNPs panel for meat traceability in six cattle breeds. *Food Control* 20: 856-860.

Pascoal A, Prado M, Calo P, Cepeda A, et al. (2005). Detection of bovine DNA in raw and heat-processed foodstuffs, commercial foods and specific risk materials by a novel specific polymerase chain reaction method. *Eur. Food Res. Technol.* 220: 444-450.

Pena HB and Pena SD (2012). Automated genotyping of a highly informative panel of 40 short insertion-deletion polymorphisms resolved in polyacrylamide gels for forensic identification and kinship analysis. *Transf. Med. Hemother.* 39: 211-216.

Pritchard JK, Stephens M and Donnelly P (2000). Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Rachlin J, Ding CM, Cantor C and Kasif S (2005). muPlex: A multi-objective approach to multiplex pcr assay design. *Nucleic Acids Res.* 33: W544-W547.

Sander P, Hamann H, Pfeiffer I, Wemheuer W, et al. (2004). Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds. *Neurogenetics* 5: 19-25.

Sasazaki S, Mutoha H, Tsurifunea K and Mannena H (2007): Development of DNA markers for discrimination between domestic and imported beef. *Meat Sci.* 77: 161-166.

Seabury CM, Cargill EJ and Womack JE (2007). Sequence variability and protein domain architectures for bovine Toll-like receptors 1, 5, and 10. *Genomics* 90: 502-515.

Siadkowska E, Zwierzchowski L, Oprządek J, Strzałkowska N, et al. (2006). Effect of polymorphism in IGF-1 gene on production traits in Polish Holstein-Friesian cattle. *Anim. Sci. Pap. Rep.* 24: 225-237.

Shackell GH, Mathias HC, Cave VM and Dodds KG (2005). Evaluation of microsatellites as a potential tool for product tracing of ground beef mixtures. *Meat Sci.* 70: 337-345.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, et al. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13: 134.

