Journal of Biochemistry and Molecular Biology, Vol. 33, No. 3, May 2000, pp. 208-212

JOURNAL OF
Biochemistry
and
Molecular Biology

A Genetic Marker for the Korean Native Cattle (Hanwoo) Found by an Arbitrarily Primed-Polymerase Chain Reaction (AP-PCR)

Ji Seon Lee, Chang Hee Lee, Doo Hyun Nam*, Young-Ja Jung† and Jung Sou Yeo††

Division of Pharmacy, College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea

†Division of National Medicine Evalution, Korea Food and Drug Administration, Eunpyung-ku, Seoul 122-704, Korea

††Department of Animal Sciences, College of Natural Resources, Yeungnam University, Kyongsan 712-749, Korea

Received 21 December 1999, Accepted 6 March 2000

In order to develop a specific genetic marker for the Korean native cattle (Hanwoo), an arbitrarily-primed polymerase chain reaction (AP-PCR) analysis of 6 different cattle breeds was attempted. Eight different arbitrary primers, each longer than 20-mer nucleotides, were used. In comparison to the AP-PCR patterns, several distinctive DNA bands that are specific for a certain breed were detected. When the primer Kpn-X was employed, a 280bp DNA fragment was found to be specific only for Hanwoo. In an individual analysis of Hanwoo, this AP-PCR marker was observed in 123 head of cattle among the 153 that were tested (80.4%). Nucleotide sequencing revealed that this fragment has a short microsatellite sequence of tandem repeat, $A(G)_{1-2}(C)_{1-3}AGAG$. According to the analysis of AP-PCR band patterns, Hanwoo was discovered to be genetically most closely-related with Holstein among the various cattle breeds.

Keywords: AP-PCR, DNA polymorphism, Genetic marker, Hanwoo, Korean native cattle.

Introduction

For the generation of molecular markers based on DNA polymorphism, several strategies have been developed. These include: DNA fingerprinting (DFP) by a restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) analysis with arbitrary primers, and an amplified fragment length polymorphism (AFLP) analysis (Nam *et al.*, 1995; Weising *et al.*, 1995; Kang *et al.*, 1999).

DFP by RFLP was first used to detect the DNA polymorphism that hybridized with multiple tandem repetitive sequences, or hypervariable minisatellite (Jefferys *et al.*, 1985). Even though this method has been widely employed in a genetic analysis of a variety of organisms, it has suffered from the requirements of a large quantity of sample DNA and radioisotope handling.

Another method for genomic polymorphism was developed based on the DNA amplification by a polymerase chain reaction (PCR) using primers of arbitrary sequences (Williams *et al.*, 1990). The advantage of this RAPD analysis method is its speed and ease of use. It does not require the radioisotopes, even though a small amount of sample DNA is employed. Recently, a more sensitive and accurate detection method of amplified DNAs, such as capillary zone electrophoresis, has been developed to make it more practical (Valentini, *et al.*, 1996)

In order to increase the number of DNA bands that appear by DNA amplification using the PCR technique, an alternative approach has been made by employing two separate PCR cycles. The initial low stringency amplification is thus followed by a high stringency amplification (Welsh and McClelland, 1990). Compared to a simple RAPD analysis, this type of arbitrarily-primed PCR (AP-PCR) allowed to show higher DNA polymorphisms, even when amplifying genomic DNA with the longer arbitrary primers.

In the case of cattle, a lot of effort has been made to study DNA polymorphisms between cattle breeds using RAPD analysis (Buitkamp *et al.*, 1991; Bardin *et al.*, 1992; Mannen and Tsujii, 1993; Cho and Han, 1994; Gawakisa, *et al.*, 1994; Kemp and Teale, 1994; Antoniou and Skidmore, 1995; Chung *et al.*, 1995; Kantanen *et al.*, 1995; Kwon *et al.*, 1995, Teale *et al.*, 1995, Rao *et al.*, 1996). Genetic markers for native cattle in various countries were particularly strongly investigated for the improvement of their native cattle breeds. This included the Japanese Black cattle (Wagyu) (Mannen and Tsujii, 1993), German native cattle (Buitkamp *et al.*, 1991), Zebu cattle (Gawakisa, *et al.*, 1994), *Bos indicus* and *Bos taurus* (Kemp and Teale, 1994). Several Korean researchers also tried to find

*To whom correspondence should be addressed.
Tel: 82-53-810-2825; Fax: 82-53-811-3871
E-mail: dhnam@ynucc.yeungnam.ac.kr

Table 1. AP-PCR primers used in this study.

Primer	Sequence (5'→3')	Abbreviation	Reference
M13 reverse sequencing primer	GGAAACAGCTATGACCATG	M13 reverse	Welsh and McClelland (1990)
T7 sequencing primer	GTAATACGACTCACTATAG	T7	Welsh and McClelland (1990)
pBS reverse sequencing primer	GGAAACAGCTATGACCATGA	pBS	Welsh and McClelland (1990)
T3 sequencing primer	GCAATTAACCCTCACTAAAG	T3	Welsh and McClelland (1990)
Kpn-X primer	CTTGCGCGCATACGCACAAC	Kpn-X	Welsh and McClelland (1991)
Kpn-M primer	CTTGCGCGCATGTACATGAC	Kpn-M	Welsh and McClelland (1991)
universal M13 sequencing primer	TTATGTAAAACGACGGCCAGT	M13 universal	Welsh *et al.* (1991)
Kpn-R primer	CCAAGTCGACATGGCACRTGTATACATAYGTAAC	Kpn-R	Welsh and McClelland (1990)

the genetic markers for Korean native cattle (Hanwoo) by the RAPD approach (Cho and Han, 1994).

In this study an AP-PCR amplification method was employed for detecting DNA polymorphisms between cattle breeds. It will be also used for evaluating more specific and reproducible genetic markers for Korean native cattle (Hanwoo) in comparison with other foreign imported cattle breeds.

Materials and Methods

Preparation of Genomic DNA The blood samples of Hanwoo were graciously supplied by the Korean Federation of Animal Improvement (Seoul, Korea). The Holstein were taken at the Animal Farm of Yeungnam University (Kyongsan, Korea). White blood cells collected from 10 ml of blood samples were lysed by SDS and proteinase K. The genomic DNAs were extracted by the phenol-chloroform method (Kwon *et al.*, 1995). The genomic DNA samples from 15 heads of Brahman, Charolais and Simmental were graciously provided by Dr. Young Hoon Yang at the Cheju National University (Cheju, Korea).

AP-PCR Amplification The arbitrary primers used in the DNA amplification (Table 1) were obtained by the custom service of Bioneer Co. (Cheongwon, Korea). Ten ml of the reaction mixture, containing 0.025 units of *Taq* polymerase, 1 μl 10 × *Taq* polymerase buffer (50 mM KCl, 10 mM Tris, pH 9.0), 4 mM $MgCl_2$, 200 μM dNTP, 10 mM of the above primers and 100 ng of genomic DNA, was subjected to 3 cycles at low stringency for the initial DNA amplification; 94°C for 5 min to denature, 40°C for 5 min for annealing of the primer and 74°C for 5 min for the extension using the Gene ThermoUnit (Model No. GTU-16, TAITEC Corporation, Japan). This was followed by 15 cycles of high stringency; 94°C for 1 min to denature, 60°C for 1 min for annealing and 74°C for 2 min for extension. At the end of the reaction, 0.5 units of *Taq* polymerase and 200 μM dNTP in 20 μl of 1 × *Taq* polymerase buffer were supplemented and an additional 30 cycles of the high stringency amplification was continued (Welsh *et al.*, 1990). The amplified DNA samples were separated on 1.4% agarose gels and visualized by staining with ethidium bromide.

DNA Cloning and Sequencing The DNA band that appeared on the agarose gel was extracted using a JET sorb kit (GENOMED Inc., NC, USA) and was re-amplified by a PCR reaction with the same primer. The re-amplified PCR product was further purified with a JET pure kit (GENOMED Inc., NC, USA). This DNA fragment was ligated with pGEM-T vector (Promega Corporation, CA, USA) by T4 DNA ligase and the ligated mixture was transformed into *E. coli* JM109 (Sambrook *et al.*, 1989). From the white colonies on the LB (Luria-Bertani) media (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) containing 50 μg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) and 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), the plasmid was isolated and confirmed to have an insert DNA by carrying out the PCR with the same primer. The nucleotide sequence of insert DNA was determined using universal sequencing primers by the custom service of the Bioneer Co. (Cheongwon, Korea). The determined sequence was further compared by a BLAST search program (http://www.ncbi.nlm.nih.gov/).

Statistical Analysis In order to estimate the degree of similarity between the AP-PCR patterns of the two different breeds, a bandsharing coefficient (BS) was calculated by $BS = 2N_{ab}/(N_a+N_b)$, where N_{ab} is the number of bands commonly present in both breeds, N_a is the number of bands observed in one breed, and N_b is the number of bands observed in the other breed (Nei and Li, 1979).

Results and Discussion

DNA Polymorphism of Various Cattle Breeds by the AP-PCR Method The rationale of the AP-PCR method is

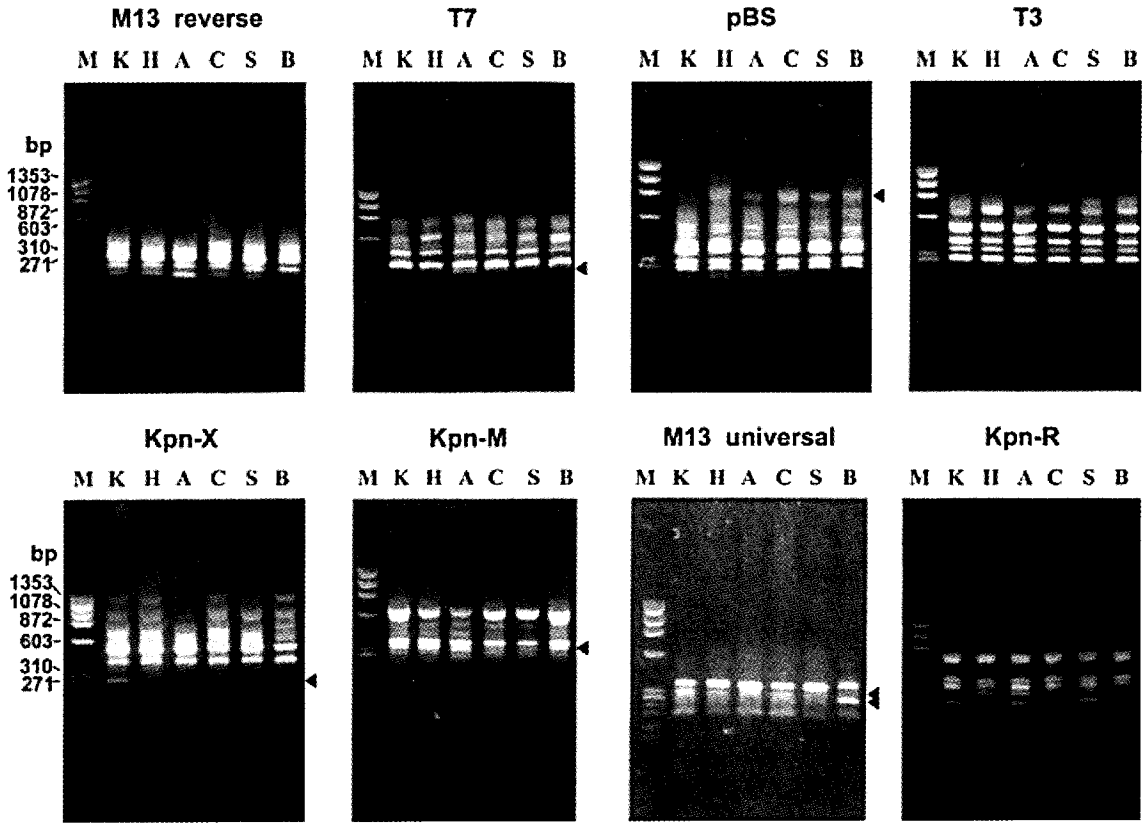

Fig. 1. AP-PCR band patterns of cattle breeds amplified with arbitrary primers. Each primer employed was designated as the abbreviations in Table 1. Using each primer, the genomic DNA of cattle breeds was amplified as described in Materials and Methods, and the resulting mixtures were subjected to 1.4% agarose gel electrophoresis. Arrows indicates DNA markers specific for a certain cattle breed. Lane 1; Molecular marker (ΦX174 DNA/*Hae*III), lane 2; Korean native cattle (Hanwoo), lane 3; Holstein, lane 4; Angus, lane 5; Charolais, lane 6; Simmental, and lane 7; Brahman.

based on the phenomenon that the arbitrary primers can be expected to anneal many sequences of genomic DNA with a variety of mismatches under an initial low stringency condition of amplification. Some of sequences, within a few hundred base pairs, will be further amplified under the high stringency of PCR. Thus the oligonucleotide primers that were longer than 20-mer can provide a distinct genomic fingerprint of the AP-PCR pattern.

In this study, eight arbitrary primers that were designed for other purposes have been employed for the AP-PCR (Welsh and McClelland, 1990; 1991; Welsh *et al.*, 1991). These are: 19-mer of M13 reverse sequencing primer and T7 sequencing primer, 20-mer of pBS reverse sequencing primer, T3 sequencing primer, Kpn-X primer and Kpn-M primer, 21-mer of universal M13 sequencing primer, and 34-mer of Kpn-R primer (Table 1).

By using these primers, the genomic DNAs of 6 different cattle breeds were subjected to AP-PCR amplification in order to investigate their DNA polymorphisms. These included: Hanwoo (Korean native cattle), Angus, Brahman, Charolais, Holstein and Simmental. As seen in Fig. 1, several distinct DNA bands that are specific for a certain breed were detected. In particular when the Kpn-X primer (5-CTTGCGCGCATAC GCACAAC-3) was employed, a 280 bp of DNA fragment was specifically amplified only in the Hanwoo. The distinct DNA band of 260 bp was also found in the genomic DNA of the Brahman when the M13-universal primer was employed; whereas, the Brahman genomic DNA did not show a 310 bp band when amplified with a M13-universal primer. The DNA bands of 310 bp appeared in the Hanwoo, Holstein, Angus and Brahman, but did not appear in the Charolais and Simmental, when amplified with a Kpn-M primer.

In comparison with the RAPD analysis, the AP-PCR approach produced fewer bands, but they were specific bands for DNA polymorphism. The previous workers (Cho and Han, 1994; Chung *et al.*, 1995) reported that they found 2 to 10 RAPD bands that were dependent upon primers. However, our results showed that of the AP-PCR bands less than 5 were observed during the analysis of cattle DNA polymorphism. This means that the AP-PCR amplification technique could be successfully and specifically employed, instead of a RAPD analysis, as a tool for the identification and detection of genetic markers, based on the DNA polymorphism of various cattle breeds.

Table 2. Matrix of pairwise marker differences expressed as bandsharing coefficients of cattle breeds

	Hanwoo	Holstein	Charolais	Angus	Simmental	Brahman
Hanwoo	1.00	0.67	0.38	0.48	0.38	0.48
Holstein		1.00	0.62	0.62	0.53	0.43
Charolais			1.00	0.62	0.62	0.33
Angus				1.00	0.62	0.53
Simmental					1.00	0.53
Brahman						1.00

Table 3. Genetic frequency of the 280bp AP-PCR marker in individuals of Korean native cattle (Hanwoo) when amplified with the Kpn-X primer.

Male			Female			Total		
Tested	Positive	Frequency (%)	Tested	Positive	Frequency(%)	Tested	Positive	Frequency(%)
85	69	81.2	68	54	79.4	153	123	80.4

Band Sharing and Genetic Relatedness Between Cattle Breeds The AP-PCR amplification patterns of each cattle breed were statistically analyzed for the evaluation of genetic similarity. The bandsharing coefficients in Table 2 show that Hanwoo was genetically related the closest to Holstein among the various cattle investigated. In contrast, Brahman showed the farthest genetic distance from other breeds. This result is consistent with a RAPD analysis of cattle breeds by previous workers (Cho and Han, 1994; Kwon *et al.*, 1995). It means that the DNA polymorphism analysis by the AP-PCR technique provides the same results as that by the RAPD method.

A Genetic Marker Specific for Hanwoo and its DNA Sequence In order to confirm whether or not 280 bp of a DNA fragment (among the AP-PCR pattern) is a genetic marker specific for Hanwoo, the genomic DNAs of 153 Hanwoo cattle (consisting of 85 male and 68 female) were amplified with a Kpn-X primer. As seen in Table 3, 81.2% of the male Hanwoo and 79.4% of the female Hanwoo showed

CTTGCGCGCATACGCACAACCAAAATCCCTTGACACCAACCCACTCCCAAGCAGAGA
CAGCCAGAGATGATGTGAGGCCCAGAGTCAAAAAAAAATTTTGTTTTAAATATTCAAT
CTCTAGTCTATGTAGGAATAGCAACCTCCTGTGTCTGGAGGACTGGATCAGGCCTTTT
TATCCAGACTAATAAGCAAATGAGCCAGGATCTGCTTTAAGTCTGCTGGGCACATCC
ACTGATGACCATTATGAACTCTCTAAAACTGGA***GTTGTGCGTATGCGCGCAAG***

Fig. 2. The DNA sequence of the 280bp AP-PCR genetic marker specific for Korean native cattle (Hanwoo) when amplified w: Kpn-X primer. Thick letters at both ends show the Kpn-X primer, and the letters in gray boxes are sequences regarded as tande repeats of microsatellite, $A(G)_{1-2}(C)_{1-3}AGAG$.

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Marker      1 CTTGCGCGCATACGCACAACCAAAATCCCTTGACACCAACCCACTCCCAAGCAGAG   56
cB13C9 24240 TGGCCATGTGCGTACGCAACCAAAATCCCTGGACACCAACAGCGCCCCAT-CAGAG 24294

Marker     57 ACAGCCAGAGATGATGTGAGGC-CCAGAG-TCAAAAAAAATTTTGTTTT--AAATA   108
cB13C9 24295 AGTGCCGGAGGTGACAAGAGGCTCCAGAGTTTAAAAGTAAAATTATTTTTAAAATA 24349

Marker    109 TTCAATCTCTAGTCTATGTAGGAATAGCAACCTCCTGTGTCTGGA--GGACTGGAT   162
cB13C9 24350 TTCAATCTCAAGTCTTTGCAAGAATAACAA---TGTGTGTCTGGAAATGTCTGAAT 24402

Marker    163 CAGGCCTTTTTATCCAGACTAATAAGCAAATGAGCCAGGATCTGCTTTAAGTCTGC   218
cB13C9 24403 CAGGCCGTAGCA-ACAGAATCCATTTTAAGTCTGCTG---TACACTTTCAGAGAGA 24454

Marker    219 TGGGCACATCCACTGATGACCATTATGAACTCTCTAAAACTGGAGTTGTGCGTATG   275
cB13C9 24455 ATTCTTGTTAGCTCTTTAAAAAAAAAAAGTTGGTTGGACTTTTTTTTTATCAAAGT 44511

Marker    276 CGCGCAAG   283
cB13C9 24512 GATCACAT 24519
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Fig. 3. Comparison of the DNA sequence in the 280bp AP-PCR genetic marker specific for Korean native cattle (Hanwoo) (Fig. 2) with that of human cB13C9 clone from chromosome 22. The homologous regions on the base sequences were shown in gray boxes. There was a 76.9% homology between base 15 and 170 of the compared sequences.

this specific DNA band on AP-PCR amplification. Based on the breeding history of Hanwoo during the Korean 1960s industrialization period, when the Hanwoo breed had not been well maintained, it could be assumed that this is a specific marker commonly present in Hanwoo.

Further, this 280 bp-DNA band was cloned into a pGEM-T vector of *E. coli,* and sequenced by a dideoxy chain termination method. The determined nucleotide sequence is shown in Fig. 2. In the middle of the cloned DNA, a short microsatellite sequence of tandem repeats, $A(G)_{1-2}$ $(C)_{1-3}$ AGAG, was found.

Using the BLAST search program, this DNA sequence specific for Hanwoo was compared with 453,356 known sequences. The searching result showed that the amplified sequence has some homologies with only a few human genome sequences. For example, the sequence between base 15 and base 170 of the sequence has 76.9% homology with the human cB13C9 clone from chromosome 22 (Accession No. Z82172), as shown in Fig. 3. However, any homologous sequences that originated from cattle were not found in the known sequences.

This implies that the identified AP-PCR marker can be successfully employed in evaluating and identifying the DNA polymorphism of cattles, especially as a specific DNA marker for Hanwoo. If this marker can be linked with a quantitative trait loci for economically important traits, it would be successfully employed in marker-assisted programs for Hanwoo breeding.

Acknowledgments This work was kindly supported by the Agricultural Research Promotion Center as a part of the Hi-Tech Agricultural Technology Development Program.

References

Antoniou, E. and Skidmore, C. J. (1995) A bovine Y-specific marker amplified by RAPD. *Anim. Genet.* **26**, 444-445.

Bardin, M. G., Bandi, C., Comincini, S., Damiani, G. and Rognoni, G. (1992) Use of RAPDs markers to estimate genetic variation in bovine populations. *Anim. Genet.* **23**, (Suppl. 1), 57.

Buitkamp, J., Zischler, H., Epplen, J. T. and Geldermann, H. (1991) DNA fingerprinting in cattle using oligonucleotide probes. *Anim. Genet.* **22**, 137-146.

Cho, B. W. and Han, J. Y. (1994) Development of RAPD marker specific for Korean cattle (Hanwoo). *Korean J. Anim. Sci.* **36**, 263-270.

Chung, E. R., Kim, W. T. and Han, S. K. (1995) Analysis of DNA polymorphism and genetic characteristics in Holstein dairy cattle using RAPD-PCR technique. *Korean J. Anim. Sci.* **37**, 455-466.

Gwakisa, P. S., Kemp, S. J. and Teale, A. J. (1994) Characterization of Zebu cattle breeds in Tanzania using random amplified polymorphic DNA markers. *Anim. Genet.* **25**, 89-94.

Jeffreys, A. J., Wilson, V. and Thein, S. L. (1985) Individual-specific 'fingerprints' of human DNA. *Nature* **316**, 76-79.

Kang, J. H., Kim, K. H., Maeng, C.-Y. and Kim, K. L. (1999) Asymmetric polymerase chain reaction-single-strand conformation polymorphism (asymmetric PCR-SSCP) as a simple method for allele typing of HLA-DRB. *J. Biochem. Mol. Biol.* **32**, 529-534.

Kantanen, J., Vikki, J., Elo, K. and Makitanila, A. (1995) Random amplified polymorphic DNA in cattle and sheep: application for detecting genetic variation. *Anim. Genet.* **26**, 315-320.

Kemp, S. J. and Teale, A. J. (1994) Randomly primed PCR amplification of pooled DNA reveals polymorphism in a ruminantrepetitive DNA sequence which differentiates *Bos indicus* and *B. taurus. Anim. Genet.* **25**, 83-88.

Kwon, Y. M., Yim, D. S., Lee, H. J., Cho, K. K., Choi, Y. J. and Baik, M. G. (1995) Random amplified polymorphic patterns within Holstein cows and among various cattle breeds. *Mol. Cells* **5**, 393-396.

Mannen, H. and Tsujii, S. (1993) DNA fingerprinting for individual identification and parentage test in Japanese Black cattle using five different mini- and one microsatellite probes. *J. Anim. Genet.* **21**, 62-68.

Nam, H. S., Kim, E., Yoon, W. H. and Lee, K.-J. (1995) Detection of fragment length polymorphism of the VNTR loci D1S80 and D2S123 by PCR amplification and silver staining. *J. Biochem. Mol. Biol.* **28**, 359-362.

Nei, M. and Li, W. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA.* **76**, 5269-5273.

Rao, K. B., Bhat, K. V. and Totey, S. M. (1996) Detection of species-specific genetic markers in farm animals through random amplified polymorphic DNA (RAPD). *Genet. Anal.* **13**, 135-138.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning,* 2nd Ed., Cold Spring Harbor Laboratory, NY.

Teale, A. J., Wambugu, J., Gwakisa, P. S., Stranzinger, G., Bradley, D. and Kemp, S. J. (1995) A polymorphism in randomly amplified DNA that differentiates the Y chromosomes of *Bos indicus* and *Bos taurus. Anim. Genet.* **26**, 243-248.

Valentini, A., Timperio, A. M., Cappuccio, I. and Zolla, L. (1996) Random amplified polymorphic DNA (RAPD) interpretation requires a sensitive method for the detection of amplified DNA, *Electrophoresis* **17**, 1553-1554.

Weising, K., Nybom, H., Wolff, K. and Meyer, W. (1995) DNA fingerprinting in plants and fungi, CRC Press, Boca Raton.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nuc. Acid Res.* **18**, 7213-7218.

Welsh, J. and McClelland, M. (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. *Nuc. Acid Res.* **19**, 5275-5279.

Welsh, J. Peterson, C. and McClelland, M. (1991) Polymorphisms generated by arbitrarily primed PCR in the mouse: Application to strain identification and genetic mapping. *Nuc. Acid Res.* **19**, 303-306.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nuc. Acid Res.* **18**, 6231-6235.