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A DNA FINGERPRINTING SCHEME TO ESTABLISH THE IDENTITY OF SEMEN SAMPLES OF CATTLE BREEDS IN ARTIFICIAL INSEMINATION PROGRAMS OF SRI LANKA

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Dairy cattle industry plays a prominent role in Sri Lankan economy because of the very high consumption of dairy milk and dairy products. Artificial Insemination is frequently used to inculcate improved performances such as high milk yield and better adaptability to the cows. However, the accuracy and outcome of Artificial Insemination is sometimes debatable due to the mixed-ups of semen samples belonged to different breeds. This could happen when semen samples are imported and distributed among regional artificial insemination centers. Hence, the availability of a robust DNA based method to identify cattle breeds at the semen stage is very important. Therefore, the present study was conducted to discriminate the five commonly used cattle breeds (Sahiwal, Jersey, Friesian, Ayrshire and Australian Friesian Sahiwal) in artificial insemination programs by using PCR based DNA fingerprinting with cattle specific microsatellite markers.

The semen samples were collected from Ambewela Dairy Farms –Nuwara-Eliya, Sri Lanka (Ayrshire) and Central Artificial Insemination Station-Kundasale, Sri Lanka (Jersey, Friesian, Sahiwal, AFS). DNA was extracted from semen samples and genotypic analysis was done using 14 cattle specific microsatellite markers (*ETH152, ETH225, HEL1, CSSM66, RM180, RM011, RM192, BM6425, BMS1678, BMS1941, BM3517, TGLA304, BMS1747* and *ILSTS011*).

Only four markers generated polymorphic bands (*ETH225, RM011, BM3517* and *BM6425*). The markers *BM3517* and *BM6425* can be suggested for the precise discrimination of five breeds. *BM6425* generated three different bands; size ranging from 123-200 bp. Marker *BM3517* displayed four different bands; size ranging from 100-150 bp. In combination these two markers can be clearly used to authenticate the exact breed in which semen sample is coming and avoid any ambiguities. In addition marker *BM3517* and *RM011* in combination can be used to independently verify the results.

Key words: Cattle breeds, DNA fingerprinting, Artificial Insemination, Sri Lanka

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The domesticated cattle (*Bos Taurus* and *Bos indicus*) play a pivotal role in the economy. The dairy industry has a long history and it is one of the world's most highly regulated food sectors. The dairy cattle industry provides 86% of the milk supply in the world (Pritchard, 2001). Total world annual milk production estimated to grow from 692 million ton in 2010 to 827 million ton in 2020 (Beldman *et al.*, 2014) further highlighting its importance.

The dairy industry is very important in Sri Lanka. Among many livestock sub-sectors, dairy cattle industry has the highest importance in the process of providing the nutrition to population through milk and milk products. The livestock statistics in 2012 indicated that cattle population in the country is 1.23 million and among them 280,250 are milking animals. The annual milk production in 2012 was 238,000 metric tons (Central Bank, Sri Lanka, 2013).

However, the supply of milk and milk products is inadequate to fulfill the current

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demand in Sri Lanka. Currently it is sufficient only to fulfill 46 % of the country's demand (Department of Animal Production and Health, Sri Lanka, 2012). The majority of requirement is covered by importing the dairy products mainly from Australia and New Zealand (Ministry of Livestock and Rural Community Development, Sri Lanka, 2010). In 2012, 84,000 metric tons of milk products were imported with the cost of USD 298 million (Central Bank, Sri Lanka, 2013). There are some major concerns associated with imported milk powder, such as containing of dicyandiamide (DCD), agrochemical residues (Danaher & Jordan, 2013) and the higher market price of milk powder.

The dairy industry in Sri Lanka must be developed to address above issues and the amelioration of dairy industry provides job opportunities and hence alleviates poverty especially in rural communities (Ministry of Livestock and Rural Community Development, Sri Lanka, 2010). The improvement of dairy industry can be achieved through multiple approaches including provision and management of high yielding cows. The artificial insemination (AI) is the technique most frequently practice to improve the genetic background of the dairy cattle (Foote, 2002). In Sri Lanka, imported European cattle breeds; Ayrshire, Jersey and Friesian, an Indian breed; Sahiwal and Tropical European cross breed; Australian Friesian Sahiwal (AFS) are mainly used in the AI programs as sires (Ibrahim *et al.*, 1999). Moreover, imported sexed semen and embryo transfers are also used in very limited capacity.

In AI programs, the verification of imported semen samples is essential because mix-ups of semen samples could happen (Lakmali, 2014). However, precise and authenticated semen samples (i.e. the required breed) must be used to achieve the expected genetic makeup of the calf and this set up the foundation for successful dairy industry. The authentication of semen samples for the respective breed can only be detected through DNA fingerprinting (Van de Goor *et al.*, 2009).

DNA fingerprinting (i.e. DNA profiling or DNA typing) is useful for the identification of individuals, establishment of family relationships, registration of pedigrees and also for the identification of suitable breeds/strains by investigating their genetic compositions. DNA fingerprinting has been used in many livestock sectors to study the genetic polymorphisms. Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR) and Single Nucleotide Polymorphism (SNP) markers have been used in poultry (Farrag *et al.*, 2010), goat, sheep and many other domesticated animals (Buitkamp *et al.*, 1991; Ajmone-Marsan *et al.*, 2001; Crepaldi *et al.*, 2001; Pariset *et al.*, 2006). DNA fingerprinting using simple sequence repeats (SSR) markers is the most robust and easy to use method for identification purposes. SSR markers have been used in many genetic studies of cattle (Usha *et al.*, 1995; Rincon *et al.*, 2000; Kumar *et al.*, 2003; Machado *et al.*, 2003; Metta *et al.*, 2004; Mukesh *et al.*, 2004; Kumar *et al.*, 2006; Pandey *et al.*, 2006; Karthickeyan *et al.*, 2008).

According to our knowledge no DNA fingerprinting studies have been conducted in Sri Lanka for authentication or to identify the any mixed up semen samples. Therefore, the present study was conducted to establish robust DNA fingerprinting scheme using SSR makers to differentiate the semen samples of cattle breeds; Ayrshire, Friesian, Sahiwal, Jersey, AFS that are commonly used as sires in AI programs.

MATERIALS AND METHODS

Cattle breeds

Authenticated breeds used for the semen sample collection,

Friesian, Jersey, Ayrshire, Sahiwal and Australian Friesian Sahiwal (AFS)

Semen samples

Cryopreserved cattle semen samples (Friesian, Sahiwal, Jersey and AFS) were obtained from Central Artificial Insemination Station, Kundasale, Sri Lanka and Ambewela Dairy Farms at Nuwara-Eliya, Sri Lanka (Imported Ayrshire semen).

DNA extraction

The semen samples were used for DNA extraction. Five hundred microliters of semen sample was pipetted out and put into

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labeled sterile micro-centrifuge tube. Sperms were lysed in 500 µl of lysis solution (500 µl TNE buffer (Tris-NaCl-EDTA buffer)- 10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl; 2.5 µl 20mg/ml Proteinase K, 40 µl 0.5 M DTT, 100 µl 5% SDS). The samples were incubated overnight at 56 ^{0}C in a water bath. After the overnight incubation, aqueous layer in the sample was transferred into a new micro-centrifuge tube. Equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the aqueous phase. The two phases were mixed gently and centrifuged at 10,000 rpm for three minutes. Next the viscous aqueous layer was transferred into a new tube and 0.1 volume of Sodium acetate (pH 5.3) was added. Then DNA was precipitated by adding two volumes of ice-cold 100% ethanol and centrifuged for 15 minutes at 15,000 rpm. The DNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for three minutes. The supernatant was discarded and DNA pellet was air dried for 30 minutes. Finally pellet was re-suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Yoshida *et al.*, 1995). Extracted DNA was stored in -37 ^{0}C.

Polymerase Chain Reaction

DNA fingerprinting for five semen samples were conducted using 14 cattle specific microsatellite markers. The markers were; *ETH152 (D5S1), ETH225 (D9S1), HEL1 (D15S10), CSSM66, RM180, RM011, RM192, BMS1941, BM6425, BMS1747, ILSTS011, BMS1678, BM3517 and TGLA304* (Table 1). Polymerase Chain Reaction (PCR) was conducted using these 14 SSR markers to identify the polymorphic markers and breed specific DNA banding patterns.

The PCR was performed using 96 well PCR plates. Amplification was done using 10-20 ng/µl of template DNA in 15 µl reaction volumes. Reaction mixture was comprised with 7.5 µl taq mix (Promega, USA), 4.5-5.5 µl of Nuclease free water and 0.5 µM of each primer. The amplifications were performed in a thermal cycler (Takara OT Sushiga- Japan) using five mins initial denaturation at 94 ºC, followed by 35 cycles of 30 secs. at 94 ºC, 90 secs. at annealing temperatures (Table 1), two mins at 72 ºC and final extension step at 72 ºC for 10 mins. After amplification, 2.5% agarose gel electrophoresis was used to visualize the bands. Five microliter of PCR products run at 60v for 2 hrs. DNA ladder of 50 bp / 123 bp ladder was used to determine the size of the DNA bands. The markers were classified into monomorphic and polymorphic groups based on the banding patterns.

RESULTS

Marker polymorphism and authentication of semen DNA samples

Four SSR markers were polymorphic and 10 SSR markers were monomorphic for the five breeds studied. Four polymorphic markers generated a total of 13 polymorphic alleles in the size range of 150-200 bp (Figure 1).

Five breeds were classified into three groups by the polymorphic bands generated for marker *ETH225*; Group 1: AFS and S, Group 2: F and Group 3: J and A (Figure 1M). Similarly, three breed groups could be obtained using the marker *RM011*; Group 1: AFS, Group 2:-F and J and Group 3: S and A (Figure 1N). The marker *BM6425* was instrumental in creating three breed groups; Group 1: AFS, Group 2: J and Group 3: F, S and A (Figure 1O). The marker *BM3517* generated four groups; Group 1: AFS, Group 2: J and A, Group 3: F and Group 4: S (Figure 1P).

The marker *BM3517* showed the highest polymorphism with four alleles. But it can separate five breeds into only four groups. In order to have five groups (i.e. individual breed identification) the marker *BM6425* in combination with *BM3517* can be used. As another combination the marker *RM011* also can be used in combination with *BM3517* for individual breed identification.

Cluster analysis using four polymorphic marker alleles

In addition to the DNA fingerprinting analysis, all the polymorphic alleles (a total of 13) of the four markers were subjected to cluster analysis to get an inference about the genetic diversity of five breeds studied. The 1 and 0 scores were given according to the presence and absence respectively for the each band in each breed and binary data matrix was prepared. A dendrogram was constructed using the algorithms of

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Table 1 SSR markers used for the authentication of cattle semen samples

Locus	Primer sequence (5'-3') Forward and Reverse Primers	Annealing temperature ºC	Size range of alleles (bp)	Marker/allele data	Source
ETH152 (D5S1)	TACTCGTAGGGCAGGCTGCCTG GAGACCTCAGGGTTGGTGATCAG	60	175	Monomorphic	MacHugh et al., 1997
RM192	ATTTTCACCTGGGAAATCCC CCATGGACTGAGGAGCCAG	55	100-150	Monomorphic	Coppieters *et al.*, 1998
HEL1 (D15S0)	AGTCCATGGGATTGAAAGAGTTG CTTTTATTCAACAGATATTTAACAAGG	55	140	Monomorphic	MacHugh et al., 1997
CSSM66	ACACAAATCCTTTCTGCCAGCTA AATTCAATGCACTGAGGAGCTTG	57	200	Monomorphic	Coppieters *et al.*, 1998
BMS1747	TCTAAGCTCCTTGAAGACAGGC GGCTTTGTATTCCCCTCTCC	57	100	Monomorphic	Coppieters *et al.*, 1998
BMS1678	TCTTCTCTGCACTTTGGTTGC ATAGCTGACATCCACTGGGC	57	148	Monomorphic	Coppieters *et al.*, 1998
ILSTS011	GCTTGCTACATGGAAAGTGC CTAAAATGCAGAGCCCTACC	55	250	Monomorphic	Coppieters *et al.*, 1998
RM180	TGGCCAAGACATCTGCCATTC GGAGTCTGGTGGGTTACAGTCC	57	100-150	Monomorphic	Coppieters *et al.*, 1998
BMS1941	TTCTAAATACTCTGCGGTGCAAGA AGCTTATAGTGCTGTACGAAGGT	55	50-100	Monomorphic	Coppieters *et al.*, 1998
TGLA304	GATCTGTCAACCTTTCAATTGATTC CTAGGTGTAGAACTGAGGAGGGT	57	100-150	Monomorphic	Arranz *et al.*, 1998
ETH225 (D9S1)	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	57	100-200	Polymorphic	MacHugh *et al.*, 1997
BM6425	AGTTGAACCTGGGTCTCCTG TGCAATGGCAGTGAAAAAAG	55	123-200	Polymorphic	Coppieters *et al.*, 1998
BM3517	GTGTGTTGGCATCTGGAGTG TGTCAAATTCTATGCAGGATGG	55	100-150	Polymorphic	Arranz *et al.*, 1998
RM011	AGAAATTGCCCCAAAGAGATGTT GATCAAACCCCTGGAGGAGTC	57	100-150	Polymorphic	Coppieters *et al.*, 1998

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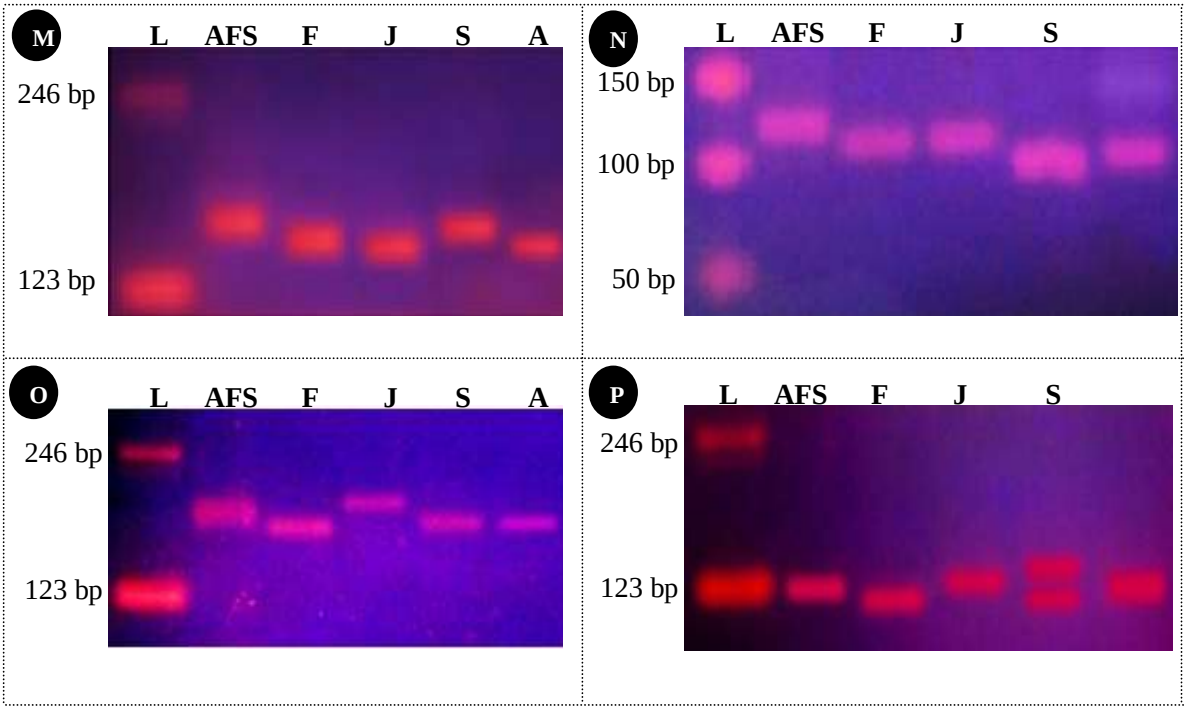

Figure 1 Agarose gel images of the PCR amplicons from semen samples of five breeds; AFS: Australian Frisian Sahiwal, F: Friesian, J: Jersey, S: Sahiwal, A: Ayrshire and L: Ladder DNA. Panel M: *ETH225*, Panel N: *RM011*, Panel O: *BM6425* and Panel P: *BM3517*. The 123 bp ladder was used in Panels M, O and P and the 50 bp ladder was used in Panel N.

McQuitty Linkage and Square Pearson Distance, using the Statistical Package Minitab 16 (Figure S1). According to the dendrogram, genetically most related breeds are Jersey and Ayrshire; they shared more than 67.92% of similarity. The Ayrshire and Jersey are both European breeds. Hence they may have more similar genetic composition among them. AFS breed was more divergent from the other four breeds. It has nearly 3.75% genetic similarity to the other breeds. AFS is a synthetic bred between Friesian and Sahiwal. According to the previous studies, they have 50% genetic compositions each from Friesian and Sahiwal (Taneja, 1999). But this genetic composition may not be present in the actual genomic content. However, in this dendrogram AFS could be considered as an out group because it has very low genetic similarity to the others. The Friesian and Sahiwal breeds showed 35.83% of genetic similarity with the other breeds.

DISCUSSION

A DNA fingerprinting scheme for the identification of semen samples belong to five cattle breeds was established using polymorphic DNA markers. These markers can generate detectable banding patterns for the precise identification of cattle breeds. Marker *BM6425* and marker *BM3517* can be suggested for the DNA fingerprinting scheme in practical uses. By using the combination of DNA markers, *BM6425* and *BM3517*, five breeds can be completely differentiated. This DNA fingerprinting scheme can be used for the identification of cattle breeds at the semen stage and to verify the semen samples for the particular breed. Since only two markers have been used, this can be considered as an inexpensive DNA fingerprinting method ideal for a developing country like Sri Lanka. The cost for the detection of one sample is very low and estimated to be USD 12.00 according to the current market prices of commercial kits and consumables.

In our study, major purpose was to establish an inexpensive DNA fingerprinting scheme to verify the identity of male parents used in AI programs of cattle. We have used only the major exotic breeds currently popular in Sri Lanka. This study can be further developed to obtain comparisons by using indigenous breeds. In addition, all of these markers have tested for the cattle breeds in

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the other countries. Some of the markers have been stated as polymorphic in previous studies ((MacHugh, 1996). But according to our results those are monomorphic (HEL1 and ETH152). Hence this experiment is also important for the identification and validation of the markers which can be used to identify the breeds in Sri Lankan cattle industry.

CONCLUSIONS

BM6425 and *BM3517* markers can be effectively used to distinguish five different Cattle breeds; Ayrshire, Friesian, Jersey, Sahiwal and Australian Friesian Sahiwal. This PCR based DNA fingerprinting scheme can be used to authenticate the semen samples of cattle breeds used as male parents in Artificial Insemination programs. Furthermore, this can be developed to identify cross bred and also the indigenous cattle breeds. As an independent verification of the results from *BM6425* and *BM3517* markers, another combination of markers *BM3517* and *RM011* in combination can be used independently. The results obtained from this study could be used in the field to improve the accuracy of cattle breeding programs. In long term, it would significantly contribute for the development of dairy industry in Sri Lanka.

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