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Full Length Research Paper

Development of a high-throughput microsatellite typing approach for forensic and population genetic analysis of wild and domestic African Bovini.

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Conservation management and forensic traceability of African buffalo and cattle rely on the timely provision of unbiased and accurate genetic information. An approach in which 17 cattle microsatellite markers are co-electrophoresed, following amplification in three core multiplex reactions was established for this purpose. Mean allelic richness per locus was 8.24 and 6.47, for buffalo and Bonsmara cattle, respectively, whilst an unbiased match probability of $6.5x10^{-17}$ and 1.03×10^{-16} was obtained for each. These results confirm the usefulness of this rapid, cost-effective typing approach for forensic, paternity and fine-scale genetic analyses of wild and domestic African Bovini tribe members.

Key words: African buffalo, microsatellite, forensic, traceability, typing, Bovini, Africa.

INTRODUCTION

African buffalo, *Syncerus caffer*, occur throughout sub-Saharan Africa, and rainfall and disease are two important factors that influence their distribution and abundance in the region (Skinner and Chimimba, 2005). Diseases such as foot-and-mouth disease (FMD) and bovine tuberculosis (BTB), which are readily transmitted between buffalo and cattle, are a serious impediment to international trade, and drastic steps have been imposed in many countries to limit transmission (Condy, 1979; Taylor and Martin, 1987). The disease status of African buffalo has resulted in a burgeoning disease-free buffalo breeding and testing industry aimed at populating game parks within the FMD-free zone of South Africa (Winterbach, 1998). Large differences in the monetary value between 'clean' and 'infected' animals has subsequently developed (Winterbach, 1998), which in turn has become a major driver for illegal trade in these animals. The availability of forensic techniques that permit accurate traceability, are therefore required to deter illegal trade and to assist in the prosecution of offenders (Dziuk, 2003).

Breeding of disease-free buffalo from a limited number of 'clean' animals can potentially lead to reduced genetic variation in the founder population. Accurate estimates of genetic variation and paternity verification are therefore important for ensuring that selection is based on sound genetic parameters. From a conservation and disease epidemiology viewpoint, it is furthermore important to be able to discern variable individual dispersal in the wild. This is of particular importance in South Africa where BTB is prevalent in the Kruger National Park and Hluhluwe-iMfolozi Park (De Vos et al., 2001; Vosloo et al., 2001) and having marked spill-over effects in other species (Keet et al., 1996).

Previous studies have shown that African buffalo can

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be successfully profiled with cattle markers (O'Ryan et al., 1998; Simonsen et al., 1998; Van Hooft et al., 2000). However, the panel sizes used in these studies were generally low: seven, six and fourteen, respectively and each locus was amplified and run individually making past approaches expensive, laborious and time consuming, especially when typing numerous samples. The aim of the research presented here was to develop an automated profiling system using a panel of 17 autosomal bovine microsatellite markers. The markers were size-selected and labelled to permit co-amplification in three multiplex reactions prior to simultaneous electrophoresis. This approach circumvents the financial and time constraints associated with extensive typing of numerous individuals, whilst meeting the growing need for both paternity verification and individual identification for forensic purposes.

MATERIALS AND METHOD

Samples

DNA was extracted from whole blood of 60 buffalo sampled at random from 30 geographically separated herds (2 individuals from each herd) throughout the Kruger National Park (KNP) in South Africa. Extractions were performed with the High pure PCR template preparation kit (Roche Applied Science) according to supplier specifications. In addition, DNA was extracted from hair samples of 34 unrelated Bonsmara cattle, a South African developed beef cattle breed (Bergh and Gerhard, 2000).

Multiplex PCR conditions

Selection of the panel of 17 microsatellite markers was primarily based on polymorphism, ability to be co-amplified, ease of scoring and allelic size ranges. Three core multiplexes were set up as follows: Multiplex M1 comprised loci TGLA227, BM1824, ETH225, ETH10 and SPS115; multiplex M2 included loci TGLA57, DIK020, INRA006, TGLA263, BM4028 and INRA128; multiplex M3 contained loci BM3517, BM719, ILSTS026, BM3205, CSSM19 and TGLA159 (Table 1). Prior to amplification of the entire sample set in the three core PCR multiplex format, single locus amplifications were carried out on individual animals in order to control for artefacts associated with multiplex PCR. In addition, reaction conditions were optimised to ensure that levels of amplification product generated for the different loci (scored in terms of peak heights) were balanced.

Each core multiplex was amplified separately in a final reaction volume of 10 µl containing 50 – 100 ng of genomic DNA, 2 units of *Taq* DNA polymerase (Super Therm Gold, Southern Cross Biotechnology), between 0.09 and 0.57 pmol of each primer, 1.5 mM $MgCL_2$, 300 µM dNTP's and 1X Super Therm Gold buffer (Southern Cross Biotechnology). Following enzyme activation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 45 s, primer-annealing at 58°C for 45 s and extension at 72°C for 1 min, were carried out on an Eppendorf Mastercycler, prior to a final extension step at 72°C for 1 h. The core multiplexes were diluted 5 fold with water, and 1 µl of each diluted core multiplex was pooled and added to 7 µl of a loading mix containing 0.0125 µl Liz size standard for every 1 µl of formamide. Following heat denaturation at 94°C for 4 min, reactions were chilled on ice and loaded as a single injection on an ABI 3100 DNA sequencer (Applied Biosystems). Labelled amplification products were analysed and sized using Genescan Analysis software version 3.7 and Genotyper 3.7 (Applied Biosystems, Foster City).

Statistical analysis

Observed and expected heterozygosities, exclusion probabilities and sex-chromosome linkage and allelic richness were calculated with CERVUS 2.0 (Marshall et al., 1998), while deviation from Hardy-Weinberg equilibrium (HWE) proportions, heterozygote deficiencies, inbreeding coefficients (F_{IS}) and the non-random associations between alleles were carried out using Genepop 3.3. (Raymond and Rousset, 1995) and FSTAT (Goudet, 2001). Unbiased probabilities of identity (PI) were calculated using GIMLET (Valière, 2002) and sequential Bonferroni corrections, aimed at compensating for the increased chance of a Type I error when conducting multiple significance tests, were applied using FSTAT.

RESULTS AND DISCUSSION

Indices of diversity for both the buffalo and the Bonsmara are shown in Table 2. The number of alleles per locus for the buffalo varied between 2 and 15, with the mean number of alleles per locus (allelic diversity) being 8.24 (SD: 4.12). For the Bonsmara cattle, the number of alleles varied from 3 to 10, while the mean allelic diversity was 6.47 (SD: 2.1). The mean expected heterozygosity (Nei's unbiased gene diversity) across all loci was 0.64 (SD: 0.05) and 0.67 (SD: 0.03) for buffalo and Bonsmara cattle, respectively. The polymorphic information content (PIC) per locus ranged from 0.137 to 0.876 (mean: 0.615; SD: 0.23) for buffalo and from 0.228 to 0.788 for the Bonsmara cattle (mean: 0.630; SD: 0.15).

Co-electrophoresis of the 17 Msat markers revealed that there were no overlapping allele size ranges for buffalo (Figure 1). For the Bonsmara cattle however, loci BM3517 and BM4028 did show overlapping allelic size ranges, pointing to a need to either change the fluorescent label of one of the loci (e.g. BM4028 could be labelled with the green fluorescent label VIC), or to add a few bases to one of the primer pairs in question in order to ensure adequate separation of the allelic ranges of these two loci. Comparison of results obtained with monoplex versus multiplex amplification confirmed that artefacts such as allelic dropout or the production of false alleles, which may manifest during multiplexing (Luikart et al., 1999), did not occur with the high quality DNA obtained from blood and hair samples.

No significant deviation from HWE was observed for buffalo when an analysis was carried out across all loci (F_{IS} = 0.028, P = 0.074). A per locus analysis revealed that five loci (BM1824, TGLA227, TGLA159, ETH10 and INRA128) may deviate from HWE due to a heterozygote deficit ($P < 0.05$), but these were not significant after Bonferroni correction. Similarly, for the Bonsmara cattle, deviation from HWE across all loci, was not observed (F_{IS} = 0.037, P = 0.080). Locus CSSM19 did reveal a significant heterozygote deficit (p = 0.0066) prior to Bonferroni correction, but the significance was lost after a Bonferroni correction was applied. Although these obser-

Table 1. Summary of the 17 cattle markers used to type African buffalo *Syncerus caffer* detailing the bovine chromosome marker location and primer sequence, label and concentration used for each multiplex.

Msat ID	Fluorescent label	Multiplex ID	Cattle chromosome Number	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Primer conc. (nM)
[1]BM 1824	Fam	M1	1	gAg CAA ggT gTT TTT CCA ATC	CAT TCT CCA ACT gCT TCC TTg	20
[1]ETH 225	Vic	M1	29	gAT CAC CTT gCC ACT ATT TCC T	ACA TgA CAg CCA gCT gCT ACT	10
[4]ETH 10	Ned	M1	5	gTT CAg gAC Tgg CCC TgC TAA CA	CCT CCA gCC CAC TTT CTC TTC TC	17
[7]SPS 115	Pet	M1	15	AAA gTg ACA CAA CAg CTT CTC Cag	AAC gAg TgT CCT AgT TTg gCT gTg	50
[2]TGLA 227	Fam	M1	18	CgA ATT CCA AAT CTg TTA ATT TgC T	ACA gAC AgA AAC TCA ATg AAA gCA	67
[1]BM 4028	Ned	M2	29	ACg gAA gCA gCA TCT CTT AC	ATg gAA ACA Tgg TCT CCT gC	20
[1]INRA 006*	Fam	M2	3	Agg AAT ATC TgT ATC AAC CTC AgT C	CTg AgC Tgg ggT ggg AgC TAT AAA TA	30
[3]DIK 020*	Vic	M2	10	AAC CAg TAA TCg TgA gAg gA	AAg AAA gTC CCT ACC ATg Ag	50
[7]TGLA 263*	Pet	M2	3	CAA gTg CTg gAT ACT ATC TgA gCA	TTA AAg CAT CCT CAC CTA TAT ATg C	80
[5]INRA 128*	Ned	M2	1	TAA gCA CCg CAC AgC AgA TgC	AgA CTA gTC Agg CTT CCT AC	35
[2]TGLA 057*	Vic	M2	1	gCT TTT TAA TCC TCA gCT TgC Tg	gCT TCC AAA ACT TTA CAA TAT gTA T	35
[1]BM 3205*	Pet	M3	1	TCT TgC TTC CTT CCA AAT CTC	TgC CCT TAT TTT AAC AgT CTg C	25
[1]BM 3517*	Ned	M3	20	gTg TgT Tgg CAT CTg gAC Tg	TgT CAA ATT CTA TgC Agg ATg g	30
[1]BM 719*	Ned	M3	16	TTC TgC AAA Tgg gCT AgA gg	CAC ACC CTA gTT TgT AAG Cag C	30
[2]CSSM 19*	Fam	M3	1	TTg TCA gCA ACT TCT TgT ATC TTT	TgT TTT AAg CCA CCC AAT TAT TTg	30
[6]ILSTS026*	Pet	M3	2	CTg AAT Tgg CTC CAA Agg CC	AAA CAg AAg TCC Agg gCT gC	55
[2]TGLA 159*	Vic	M3	4	gCA TCC Agg gAA CAA ATT ACA AAC	TTT ATT TCg AAT CTC TTg AgT ACA g	35

*Denotes markers common to this study and that of Van Hooft et al. (2000). Superscripts denote references to chromosomal locations in cattle and primer sequences for the respective loci: [1]Bishop et al., 1994; [2]Barendse et al., 1994; [3]Hirano et al., 1996; [4]Luikart et al., 1999; [5]Vaiman et al., 1994b; [6]Kemp et al., 1995; [7]Mommens et al., 1998.

vations do not rule out the presence of null alleles, the absences of significant deviations from HWE after Bonferroni correction indicate that their frequency must be low. No sex chromosome linkage was observed among the respective loci for either buffalo or cattle, and linkage disequilibrium was not detected among loci after Bonferroni correction.

The total combined exclusionary power was 0.9994 (first parent) and 0.9999 (second parent) for buffalo and 0.9977 (first parent) and 0.9999 (second parent) for the Bonsmara cattle. These values show that paternity verification for both buffalo and Bonsmara cattle can be accurately executed which is a requirement for both buffalo and cattle breeding programmes. The combined cumulative PI, an indication of the resolving power to distinguish between individuals, across all loci was $6.5x10^{-17}$ and 1.03×10^{-16} for the buffalo and cattle, respectively. These numbers greatly exceed minimum levels acceptable for forensic casework studies and identity verification of individual animals for traceability purposes. The PI for the Bonsmara cattle using this panel of markers

Table 2. Genetic parameters obtained for African buffalo with the panel of 17 microsatellites used in this study, with those obtained for Bonsmara cattle being given in brackets in each column.

Locus	A_n	Allelic range (bp)	H_o	H_e	PIC	Excl(1)	Excl(2)	F_{IS}
BM 1824	14 (6)	169-199 (177-191)	0.78 (0.71)	0.87 (0.77)	0.86 (0.73)	0.60 (0.37)	0.75 (0.55)	0.109 (0.076)
CSSM 19	11 (7)	128-154(134-154)	0.78 (0.44)	0.78 (0.57)	0.75 (0.53)	0.41 (0.18)	0.59 (0.35)	0.006 (0.231)
INRA 006	7 (6)	107-119 (102-116)	0.61 (0.64)	0.62 (0.68)	0.57 (0.63)	0.21 (0.26)	0.38 (0.44)	0.007 (0.062)
TGLA 227	4 (10)	70-76 (76-96)	0.35 (0.76)	0.44 (0.81)	0.40 (0.78)	0.10 (0.46)	0.23 (0.64)	0.210 (0.067)
DIK 020	15 (6)	164-208 (172-184)	0.89 (0.78)	0.89 (0.73)	0.87 (0.68)	0.63 (0.31)	0.77 (0.49)	-0.005 (-0.071)
ETH 225	2 (7)	133-137 (135-155)	0.46 (0.69)	0.41 (0.81)	0.32 (0.77)	0.08 (0.44)	0.16 (0.62)	-0.141 (0.15)
TGLA 159	8 (5)	223-237 (208-242)	0.70 (0.46)	0.79 (0.64)	0.76 (0.58)	0.42 (0.22)	0.59 (0.39)	0.124 (0.28)
TGLA 057	7 (5)	75-101 (83-97)	0.78 (0.70)	0.77 (0.64)	0.73 (0.57)	0.38 (0.21)	0.55 (0.37)	-0.005 (-0.102)
BM 3517	7 (10)	84-96 (98-118)	0.53 (0.72)	0.54 (0.72)	0.50 (0.68)	0.16 (0.33)	0.33 (0.52)	0.019 (-0.003)
BM 4028	3 (10)	126-134 (101-123)	0.11 (0.82)	0.14 (0.81)	0.13 (0.78)	0.01 (0.45)	0.07 (0.62)	0.184 (-0.007)
BM 719	12 (8)	136-160 (140-158)	0.86 (0.70)	0.83 (0.74)	0.80 (0.70)	0.49 (0.34)	0.66 (0.52)	-0.038 (0.047)
ETH 10	2 (7)	204-206 (206-218)	0.18 (0.93)	0.26 (0.77)	0.23 (0.73)	0.03 (0.37)	0.11 (0.55)	0.320 (-0.199)
INRA 128	7 (4)	166-182 (174-180)	0.43 (0.76)	0.53 (0.75)	0.51 (0.69)	0.16 (0.32)	0.34 (0.49)	0.196 (-0.012)
BM 3205	12 (3)	198-220 (204-208)	0.83 (0.23)	0.85 (0.24)	0.83 (0.22)	0.54 (0.02)	0.70 (0.12)	0.030 (0.065)
ILSTS026	11 (6)	143-167 (151-165)	0.86 (0.66)	0.86 (0.69)	0.84 (0.63)	0.56 (0.27)	0.72 (0.44)	0.002 (0.035)
SPS 115	12 (4)	223-249 (245-257)	0.90 (0.32)	0.83 (0.36)	0.80 (0.33)	0.50 (0.06)	0.67 (0.19)	-0.082 (0.11)
TGLA 263	6 (6)	114-130 (108-124)	0.60 (0.67)	0.52 (0.68)	0.47 (0.62)	0.14 (0.26)	0.29 (0.42)	-0.151 (0.009)

H_o: Observed heterozygosity; H_e: Expected heterozygosity; PIC: polymorphic information content; Excl (1): exclusion probability with one parent genotyped; Excl (2): exclusion probability with both parents genotyped; bp: base pairs; F_{IS} measures the heterozygote deficit within a sample population; A_n: allelic richness.

was also orders of magnitude higher than the value of 1.17×10^{-13} that was obtained with the ISAG panel (results not shown).

The development of the high-throughput, high-resolution and cost-effective typing system reported here confirms the suitability of this approach for forensic and population genetics studies. In addition, the improved resolution that this technique provided for a South African developed cattle breed, the Bonsmara indicates that this approach not only provides a valuable, supplementary/ alternative typing method but confirms the likeli-

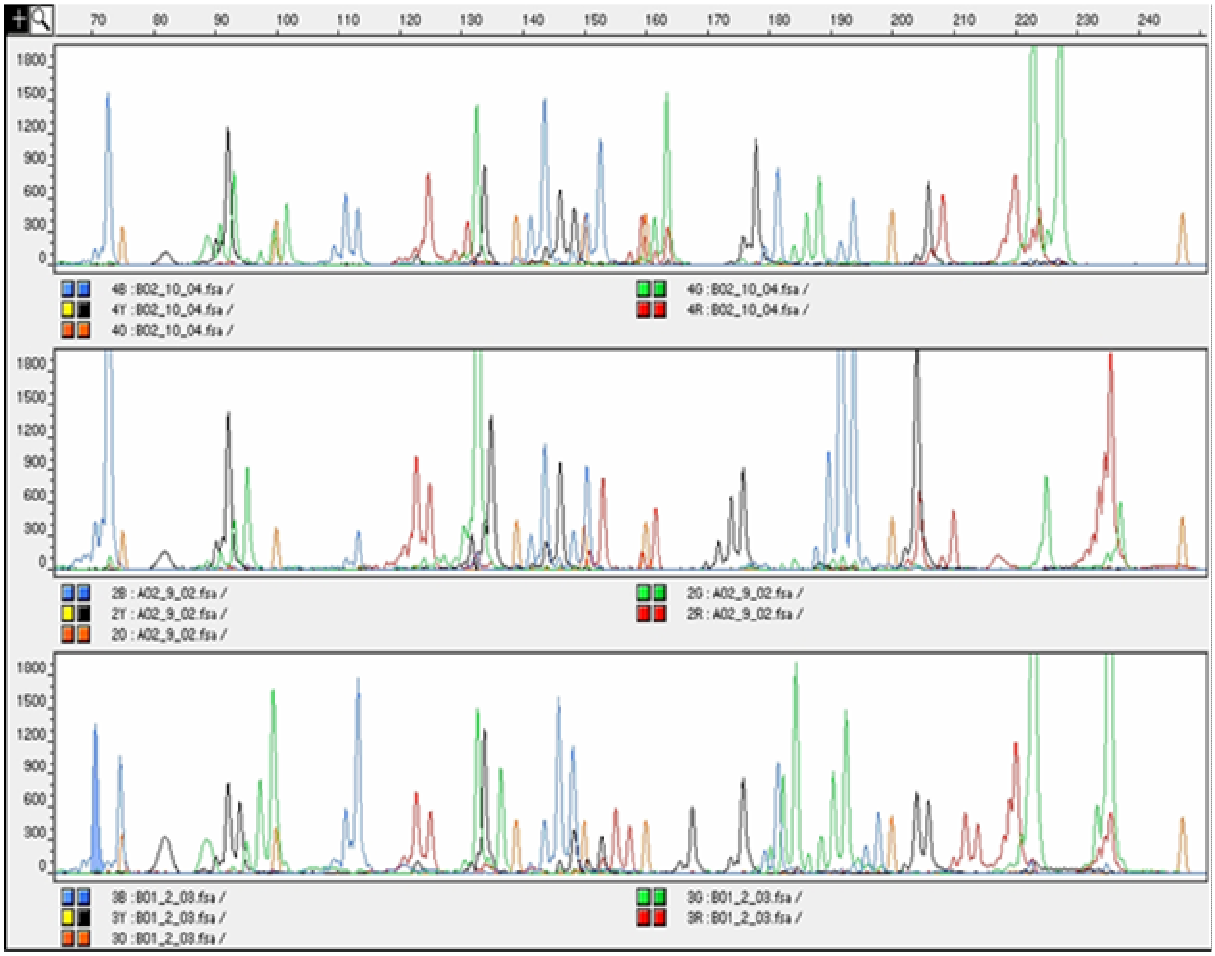

Figure 1. Genotyper 3.7 allelic profiles for three individual buffalo generated by co-electrophoresis of 17 microsatellite markers amplified in three core multiplex reactions.

hood of a broader applicability to other representatives of the Bovini tribe in Africa.

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