Animal Genetics, 2000, **31**, 360–366

Validation of 15 microsatellites for parentage testing in North American bison, *Bison bison* and domestic cattle

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Summary

Fifteen bovine microsatellites were evaluated for use in parentage testing in 725 bison from 14 public populations, 178 bison from two private ranches and 107 domestic cattle from five different breeds. The number of alleles per locus ranged from five to 16 in bison and from five to 13 in cattle. On average, expected heterozygosity, polymorphism information content (PIC) and probability of exclusion values were slightly lower in bison than in cattle. A core set of 12 loci was further refined to produce a set of multiplexed markers suitable for routine parentage testing. Assuming one known parent, the core set of markers provides exclusion probabilities in bison of 0·9955 and in cattle of 0·9995 averaged across all populations or breeds tested. Tests of Hardy-Weinberg and linkage equilibrium showed only minor deviations. This core set of 12 loci represent a powerful and efficient method for determining parentage in North American bison and domestic cattle.

Keywords: bison, cattle, likelihood, microsatellite, parentage

Introduction

Bison once numbered in the millions in North America but because of the population bottleneck experienced in the late 1800s, bison numbers were reduced to no more than 300 individuals by 1880 (Coder 1975; Dary 1989). Almost all of the bison alive today can be traced back to five populations that were used to repopulate most of the extant public and private herds (Coder 1975). Current semi-wild bison populations are fragmented among public parks and sanctuaries throughout the US and Canada. However, the vast majority of bison today reside on private ranches where they are raised for meat production. Recently, Mommens *et al.* (1998) demonstrated that bovine microsatellites are better suited for parentage testing in bison than conventional blood typing because of a greater degree of variation. However, their sample was limited to a single herd located in Belgium, which probably does not represent the actual genetic variation found in bison in North America.

Currently, parentage testing in domestic animals is based on exclusionary techniques using genetic markers. An offspring is tested assuming one known parent and one or a limited number of candidate parents. If only one candidate parent is left non-excluded, that parent is assigned parentage to the offspring. Although one non-excluded parent may be the true parent, there exists the possibility that other non-excluded candidate parents exist in the population but were not considered. A likelihood-based testing procedure is more appropriate for situations in which there are many candidate parents and obtaining a known parent is difficult. Using likelihood-based procedures, all potential parents are considered as candidates and there is no need to identify a known parent prior to testing.

The purpose of this study was to characterize, standardize and provide validation for a set of highly polymorphic microsatellites for use in routine parentage testing in North American bison and domestic cattle.

Materials and methods

DNA source

Fourteen public bison herds, two private bison herds and five cattle breeds were sampled. Sample sizes and population locations are listed in Table 1. These herds represent most of the major public herds that have played a role in populating private bison herds around the world. Therefore, the majority of the genetic variation present in extant bison herds should be contained within these public herds.

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Accepted 17 July 2000

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DNA extraction

Genomic DNA was isolated from white blood cells by proteinase K treatment followed by phenol:chloroform extraction (Sambrook *et al.* 1989) or by using the SUPER QUICK-GENE DNA Isolation kit (Analytical Genetic Testing Center, Inc. Denver, CO, USA). DNA was also extracted from hair follicles using the following procedure. Approximately eight to 12 hair follicles were cut from the switch of the tail using a razor blade and digested for 4 h at 55 °C in 200 μl lysis buffer (500 mM KCl, 100 mM Tris–HCl pH 8·0, 0·1 μg/ml gelatin, 0·45% Triton X-100, 0·45% Tween-20, 0·5 mg/ml proteinase K). After digestion, samples were centrifuged at 5000 **g** for 2 min. The clear aqueous layer was then transferred to a new tube and 0·5 μl of 10 mg/ml RNase A was added. The sample was then extracted once using phenol/chloroform/isoamyl alcohol (25:24:1) followed by a chloroform extraction. DNA was ethanol precipitated then resuspended in 50 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8·0).

Loci

Bovine microsatellites were chosen from the USDA cattle mapping database (http://sol.marc.usda.gov) that fulfilled the following set of criteria in cattle:

1. High PIC values, high heterozygosity and a large number of alleles.
2. Lack of known null alleles.
3. Loci non-syntenic or separated by more than 40 cM.
4. Allele size range.
5. Suitability for multiplex PCR.

Primer sequences flanking 15 microsatellites that fulfilled these criteria were synthesized with a fluorescent label attached to the 5′ end of each forward primer (Table 2).

Multiplex PCR

Based on the results of genotyping approximately 500 bison and 50 cattle for these 15 loci, a core set of 12 loci were selected. These could be amplified in two PCR reactions and co-

Table 1. Bison populations and domestic cattle breeds sampled

	Abbreviation	Location	Sample size
Public herds			
Antelope Island State Park	AI	Utah	67
Custer State Park	CSP	South Dakota	37
Elk Island National Park (woods)	EIW	Alberta	25
Elk Island National Park (plains)	EIP	Alberta	24
Fort Niobrara National Wildlife Refuge	FN	Nebraska	24
Finney Game Refuge	GC	Kansas	50
Henry Mountains	HM	Utah	21
Caprock Canyon State Park	CCSP	Texas	33
Mackenzei Bison Sanctuary (woods)	MBS	Canada	40
Maxwell Game Refuge	MX	Kansas	35
National Bison Range	NBR	Montana	38
Wind Cave National Park	WC	South Dakota	152
Wood Buffalo National Park (woods)	WBNP	Canada	21
Yellowstone National Park	YNP	Wyoming	158
Total			725
Private herds			
Arrowhead Buffalo Ranch, Ltd.	ABR	Ohio	135
Hidden Hollow Preserve	HHP	Kentucky	43
Total			178
Cattle breeds			
Angus	AN		54
Hereford	HE		16
Holstein	HO		12
Shorthorn	SH		12
Texas Longhorn	TLH		13
Total			107

Table 2. Chromosomal location and fluorescent dye used for each of the 15 loci selected from the USDA database

Locus	Chromosome*	Position*	Dye†
BM1225	20	8·0	TET
BM1706	16	80·6	6FAM
BM17132	19	58·6	6FAM
BM1905	23	64·3	TET
BM2113	2	106·2	6FAM
BM4440	2	55·0	TET
BM720	13	38·6	TET
BMS1117	21	9·9	HEX
BMS1172	4	27·3	6FAM
BMS1862	24	32·8	HEX
BMS2639	18	57·0	6FAM
BMS410	12	0·0	TET
BMS510	28	22·1	HEX
BMS527	1	55·9	6FAM
RM372	8	19·1	HEX

*Bovine chromosome and relative position (cM).
†ABI fluorescent label used with forward primer.

loaded in a single lane of an ABI Prism 377 sequencer or a single injection on an ABI Prism 310 capillary-based Genetic Analyzer (PE Biosystems, Foster City, CA, USA). Core multiplex A consists of *BMS510, BMS410, BM17132, RM372* and *BMS527*. Core multiplex B consists of *BM4440, BM2113, BMS1862, BM1905, BM720, BM1706* and *BM1225*. PCR conditions for core multiplexes A and B are as follows: 25–100 ng template DNA, 10 mM Tris–HCl (pH 9·0), 50 mM KCl, 1% Triton®-X, 3·0 mM $MgCl_2$, 500 μm dNTPs, 0·05–0·3 μm each primer, 1 × MasterAmp PCR enhancer (Epicentre Technologies, Madison, WI, USA), and 0·5 U *Taq* DNA polymerase (Promega, Madison, WI, USA) in a 5 μl reaction. Thermal cycle parameters for core multiplex A and B were 2 min 96 °C followed by 35 cycles of (15 s 96 °C, 15 s 54 °C, 5 s 72 °C) using a final extension step of 20 min at 72 °C using a GeneAmp® PCR 9700 thermocycler (PE Biosystems).

Genotyping

PCR products were separated on an ABI Prism 377 DNA Sequencer (ABI377) or an ABI Prism 310 Genetic Analyzer (ABI310) (PE Biosystems) and sized relative to an internal size standard (GS500, PE Biosystems or MAPMARKER LOW, Bioventures). Fluorescent signals from the dye labelled microsatellites were detected using GENESCAN 3·1 software (PE Biosystems). Genotypes were assigned using Genotyper 2·0 software (PE Biosystems) by assigning both an integer value and the actual decimal value (called size) to each peak. After the allelic ladder was developed, the ladder was included on each gel (ABI377) or with each group of samples (ABI310) and genotypes were assigned relative to the actual sequence sizes of the allelic ladder. Previous samples that were genotyped without the allelic ladder were re-assigned genotypes based on the true sequence size of each allele.

Cloning and sequencing

Approximately one half of the bison alleles at each locus were cloned and sequenced. Samples were amplified individually and cloned using either the Original TA Cloning Kit or the Topo TA Cloning kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. Approximately 10–20 positive clones from each ligation were picked and grown overnight in 3 ml Terrific Broth containing 50 μg/ml ampicillin. A standard alkali-lysis mini-prep procedure was used to recover plasmid DNA (Sambrook *et al.* 1989). Plasmid DNA was diluted 1:50 with TE buffer and used as a source of template DNA for PCR. Each positive clone was amplified via PCR and genotyped using the ABI310. Clones that sized identical to one of the original alleles of the animal were used to make glycerol stocks. Cloned alleles were sequenced using the Big-dye™ terminator cycle sequencing kit (PE Biosystems) and an ABI377 automated sequencer. Sequenced alleles were submitted to Genbank and have accession numbers AF213181 to AF213246. An allelic ladder was constructed by mixing equimolar amounts of DNA from the sequenced plasmids into a DNA mastermix. The combined plasmids were used as template DNA for the allelic ladder in each PCR multiplex.

Data analysis

Expected heterozygosity (Nei, 1987), exclusion probabilities and polymorphism information content (PIC) (Botstein *et al.* 1980) were calculated for each marker within each population. Two exclusion probabilities were calculated which correspond to different scenarios. Exclusion probability one (PE1) assumes genotypes are known for the offspring and a putative parent, but genotypes are not available for a known parent (one parent missing). Exclusion probability two (PE2) assumes genotypes are known for the offspring, one confirmed parent, and one putative parent (both parents genotyped). PE1 and PE2, as well as combined exclusion probabilities were calculated according to

Jamieson & Taylor (1997). Tests of Hardy–Weinberg equilibrium (HWE) were performed using the program GENEPOP version 3·1d (Raymond & Rousset, 1995). Exact *P*-values were calculated for loci that had four alleles or less in a population. For loci that had more than four alleles present in a population, an unbiased estimate of the exact HW probability was calculated using the Markov chain method of Guo & Thompson (1992). Unbiased estimates of genotypic disequilibrium were calculated with GENEPOP using the Markov chain method. Parameters used for all Markov chain procedures were: dememorization of 10000 steps, 125 batches and 40000 iterations per batch for a total Markov chain length of 5 million steps.

Parentage inference

Parentage testing was performed on the two pedigreed private bison herds to evaluate the actual effectiveness of the loci for determining parentage, verify Mendelian inheritance and check for the presence of null alleles. The accuracy of these pedigrees has previously been verified by genotyping over 200 microsatellites in these herds (unpublished data). The ABR sample contained 92 offspring and 44 potential parents. The HHP sample contained 29 offspring and 22 potential parents. Likelihood based parentage testing was performed using the program CERVUS 1·0 (Marshall *et al.* 1998) after the procedures outlined in the program. Analysis parameters used for simulations were as follows: 10000 cycles, 45 candidate parents for the ABR herd and 22 candidate parents for the HHP herd, 95% of the candidate parents sampled, 100% of the loci typed, 1% typing error, 80% relaxed confidence and 95% strict confidence.

Results

Unbiased expected heterozygosity, PIC, exclusion probabilities, allele frequencies, repeat length and the standard deviation in allele size calling are located in Appendix A which can be obtained via the internet at http://www.cvm.tamu.edu/derrlab/index.html. A total of 138 and 151 alleles were found in bison and domestic cattle, respectively. The number of alleles per locus ranged from five to 16 in bison and from five to 13 in cattle. Of the 15 loci tested, five had a greater number of alleles in bison than in domestic cattle. For four of these loci, *BM2113*, *BM1706*, *BMS1172* and *BMS2639*, this result is probably a result of the limited number of cattle tested. According to published results, all four of these loci have an equal number of alleles or more in cattle compared with that observed in bison (Stone *et al.* 1995; Bishop *et al.* 1994). The exception is *BM1225* in which 16 alleles were observed in bison but cattle are reported to only have 11 alleles. Excluding *BM4440* for the CCSP population, which was monomorphic, expected heterozygosities in bison ranged from 84·2 to 6·0% and from 85·4 to 39·8% for cattle. The overall mean heterozygosity across all populations and all markers was 62·17% for bison and 70·16% for cattle (Table 3). The only populations that failed to reach the 99% threshold for PE2 were Antelope Island and the CCSP population. This is most likely a result of the fact that both of these herds were founded by a small number of individuals and have remained genetically isolated for much of their history (Popov & Low 1950; Coder 1975).

Hardy–Weinberg and genotypic disequilibrium tests

Calculation of genotype frequencies and exclusion probabilities from allele frequencies depend on the underlying assumptions of HWE. However, the errors associated with using allele frequencies to calculate genotype frequencies and exclusion probabilities should be minimal as long as there is approximate agreement with HW expectations.

In order to test Hardy–Weinberg assumptions, three distinct tests of HWE were performed with the difference being the alternate hypothesis to equilibrium. Each locus within each population was checked for HWE for a total of 314 comparisons (CCSP was monomorphic at *BM4440*). Eight per cent (25/314) of the locus/population combinations showed significant departure from HWE at $P < 0{\cdot}05$ for the probability test. In order to more precisely identify these deviations, score tests (U-tests) (Rousset & Raymond 1995) were performed with the alternative hypothesis of either heterozygote excess or deficiency. When the alternative hypothesis was heterozygote excess, 4·1% (13/314) of the locus/population combinations showed significant deviations from HWE at $P < 0{\cdot}05$. When the alternative hypothesis was heterozygote deficiency 10·5% (33/314) of the locus/population combinations were significant at $P < 0{\cdot}05$. There was no consistency between the three tests to indicate any specific locus/population was in disequilibrium.

Non-random association of gametes to form genotypes could also affect using allele frequencies to calculate genotype frequencies. In natu-

ral populations this is most likely a result of population sub-structuring. Tests of genotypic disequilibrium within populations resulted in 1876 comparisons. Three populations (AI, ABR and HHP) were not tested for genotypic disequilibrium as all three of these populations use a limited number of breeding bulls. When these populations were eliminated from consideration because of known breeding structure, 6·4% (120/1876) of the combinations were significant at $P < 0{\cdot}05$. Tests of genotypic disequilibrium across populations resulted in 105 comparisons. No locus pairs showed significant disequilibrium across populations at $P < 0{\cdot}05$ (Bonferoni corrected).

Parentage inference

Both the ABR and HHP pedigrees were used to check the inheritance of the markers and to evaluate the effectiveness of both the markers and the likelihood testing procedure in a production setting. A total of 121 offspring were used to evaluate the test's effectiveness. For each offspring, every reproductively capable animal in the population was considered as a potential parent, allowing for the possibility of missing parents. The first cycle of parentage analysis resulted in eliminating all potential parents that showed incompatibilities at more than one locus. Parents that showed mismatches at one locus were considered as potential parents to allow for the possibility of either a mutation or a genotyping error. The potential parents that were left were then considered as known parents and this additional information was used to re-test the offspring against the original set of potential parents. After the second round of parentage analysis in the ABR herd, 87% (80/92) of the offspring were unambiguously assigned parentage to the correct sire and dam. A total of 12 offspring were not assigned parentage after the second cycle because these 12 cows were purchased as bred heifers; therefore, the sires were unavailable. After inspecting the results from the first cycle of parentage analysis, the correct dam was assigned with >95% confidence in each case where the sire was unavailable. Parentage analysis results for the HHP population were much the same as for ABR. Every offspring was unambiguously assigned parentage except two offspring whose sire was not sampled, in which case, the correct dam was assigned with >95% confidence.

Table 3. Mean expected heterozygosity across all 15 loci and combined average exclusion probabilities for all 15 loci and the core set of 12 loci

Population	Mean expected heterozygosity	All loci		Core set	
		PE1	PE2	PE1	PE2
AI	0·4496	0·8622	0·9858	0·8612	0·9820
CSP	0·6818	0·9953	0·9999	0·9901	0·9997
EIP	0·6666	0·9938	0·9999	0·9894	0·9997
EIW	0·5541	0·9663	0·9989	0·9506	0·9974
FN	0·6602	0·9943	0·9999	0·9860	0·9996
GC	0·6521	0·9923	0·9999	0·9828	0·9994
HM	0·5757	0·9777	0·9991	0·9599	0·9973
CCSP	0·4160	0·8457	0·9796	0·7768	0·9570
MBS	0·6334	0·9893	0·9998	0·9838	0·9996
MX	0·6729	0·9956	0·9999	0·9916	0·9998
NBR	0·6542	0·9902	0·9998	0·9714	0·9988
WBNP	0·6759	0·9950	0·9999	0·9886	0·9997
WC	0·6630	0·9943	0·9999	0·9859	0·9995
YNP	0·6340	0·9898	0·9998	0·9813	0·9993
ABR	0·6981	0·9972	1·0000	0·9920	0·9998
HHP	0·6591	0·9956	0·9999	0·9920	0·9995
AN	0·6907	0·9965	1·0000	0·9934	0·9999
HE	0·6359	0·9839	0·9995	0·9662	0·9981
HO	0·7279	0·9978	1·0000	0·9930	0·9999
SH	0·7029	0·9971	1·0000	0·9930	0·9999
TLH	0·7507	0·9993	1·0000	0·9984	1·0000
Mean bison	0·6217	0·9734	0·9976	0·9610	0·9955
Mean cattle	0·7016	0·9949	0·9999	0·9888	0·9995

Discussion

The goal of the present study was to characterize, standardize and provide validation for a set of polymorphic microsatellites for use in routine parentage testing in North American bison and domestic cattle. Table 3 demonstrates that the exclusion probabilities found in bison and cattle for these loci are comparable with other loci previously described (Glowatzki-Mullis *et al.* 1995; Heyen *et al.* 1997; Mommens *et al.* 1998; Peelman *et al.* 1998). Additionally, in cattle the core set of markers produces similar exclusion probabilities to the commercially available StockMarks™ kit (PE Biosystems). However, in bison the core set of markers offer higher exclusion probabilities than either the StockMarks™ kit or the ISAG approved set of markers (Mommens *et al.* 1998).

In order to validate the use of these markers for parentage testing in bison, the guidelines set forth for selecting loci for human parentage testing were followed (Parentage Testing Committee and American Association of Blood Banks, 1997). A total of 121 offspring were tested from two separate private populations. In every case the loci exhibited normal co-dominant Mendelian inheritance with no evidence of null alleles or mutations.

The development of an allelic ladder, which is used for each genotyping run, fulfills the requirement of a known DNA control and makes it possible to directly compare samples that are run at different points in time or even on different machines. In the case of parentage testing of domestic animals, this is a desirable feature as the offspring will be tested years apart and re-running parents each year would be inefficient and costly. An allelic ladder also increases consistency between laboratories as each genotype is assigned relative to a known standard. Locus *BMS510* exhibited single base pair differences in bison. Normally this would preclude this locus from being used as a marker for parentage testing because of the difficulty in allele assignment reproducibility. However, this problem was overcome by sizing alleles relative to the allelic ladder. The minimum and maximum standard deviation of allele sizes for this locus was 0·06 and 0·25 bp, respectively, with an average over all the alleles of 0·09 bp. These values represent between-gel deviations. The within-gel standard deviation, averaged across alleles, is reduced to only 0·06 bp. Smith (1995) demonstrated that values in this range were highly unlikely to produce incorrect allele assignment when an allelic ladder is used.

In order to use allele frequencies to calculate genotype frequencies and exclusion probabilities, allele frequencies from the populations tested must be consistent with HW expectations. Bison (903) from 14 public populations and two private populations represent an adequate sample with which to estimate allele frequencies. Tests of HWE, although showing minor deviations for some locus population combinations, did not yield consistent deviations for the testing methods employed. The number of HW deviations observed in this study is similar to other studies in humans. Hammond *et al.* (1994) found 11·5% (18/156), Edwards *et al.* (1992) found 11·6% (7/60) and Thomson *et al.* (1999) found 5·5% (2/36) of the possible locus-population-test combinations showed deviations, which is comparable to the 7·5% (71/942) found in the current study. The lack of consistency in the observed deviations leads us to conclude that these loci are in HWE for the populations tested.

Tests of genotypic disequillibrium showed no consistent deviations in the populations which were not expected *a priori* to show deviations. In a production setting such as with the HHP and ABR populations, genotypic disequillibrium is expected because a limited number of bulls are used for breeding. However, in these cases typically the entire population will be tested and departures from genotypic equilibrium will have little effect on the final parentage analysis. Therefore, we conclude that the observed deviations in HWE and genotypic equilibrium are small enough that they will not significantly affect the calculations of genotypic frequencies or multilocus probabilities from allele frequencies.

The advantages of likelihood-based parentage assignment over exclusionary methods have been demonstrated by Slate *et al.* (2000) for natural populations and extended to captive production populations in the current study. Indeed, even with highly developed sets of markers such as those presented here, genotyping errors occur. A likelihood-based system to assign parentage allows the laboratory to identify potential errors or mutations and make corrections before parentage is rejected.

PCR based methods in conjunction with highly variable microsatellite loci and fluorescent based genotyping provide the technologies needed to establish a new standard for parentage testing. The core set of 12 microsatellites presented here offers a powerful battery of markers for both parentage testing and individual identification. These markers combined with likelihood-based parentage testing will

help to refine breeding programmes and allow for improved genetic management by accurate determination of pedigrees in both bison and cattle.

Acknowledgements

We would like to thank the managers of the public and private bison herds for providing samples. In addition, we would like to thank S. Davis, J. Caldwell and R. Brenneman for providing the domestic cattle samples and Natalie Halbert for genotyping the Wind Cave and Yellowstone Populations.

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