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Original Scientific Article

APPLICABILITY ASSESSMENT OF A STANDARDIZED MICROSATELLITE MARKER SET IN ENDANGERED BUSHA CATTLE

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Summary: This study is focused on evaluating 12 microsatellite markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, BM1818, ETH3, ETH225, BM1824) as recommended by International Society of Animal Genetics (ISAG) for paternity testing in Busha cattle in Serbia. A herd of 47 heads of Busha cattle were sampled for blood. The number of alleles ranged from 6 (ETH10) to 16 (TGLA122), with a mean value of 9.5. Total number of alleles at all 12 analyzed loci was 114. PIC (Polymorphism Information Content) values ranged from 0.513 (in BM1818) to 0.905 (TGLA53). Power of exclusion (PE) for single markers ranged from 0.228 (BM1818) to 0.607 (BM2113) and the power of discrimination (PD) from 0.75 (BM1818) to 0.96 (TGLA227). The combined power of exclusion and discrimination was very high (0.999 and 0.995 respectively) when all 12 markers were used in combination. Cervus software performed 96% successful paternity assignments. These results show that the 12 markers set recommended by ISAG can be used with high confidence for forensic purposes in Busha cattle.

Key words: microsatellite; paternity testing; buša; busha

Introduction

According to FAO and UNEP data (1), about one third of the world's recognized 5000 livestock and poultry breeds are endangered but in both developed and developing countries they represent a unique resource to meet present and future breeding objectives (2). Busha (also Buša or Buscha), is a collective term for small, robust and long-living cattle, the withers' height at around 100 cm (3). Busha cattle are autochthonous in the Balkan area. Being a mixed type of cattle with relatively low productive traits, Busha has been slowly pushed into extinction as farming became more intensive and demanding. Today this breed is classified as endangered by the Republic of Serbia Law on Animal Husbandry (4). Still, busha population in the Republic of Serbia has diminished in past years. One of the larger heards of busha cattle still exists in the mountain region of Dimitrovgrad, in the south-east of the Republic of Serbia (geographic coordinates 43°01′N 22°47′E). The heard was founded in 2005 and since then the cattle was managed under natural breeding conditions. Animals in this heard originate from

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Bujanovac, Trgoviste, Bosilegrad, Sjenica and Tutin areas of the Republic of Serbia. Several color varieties are represented in the heard: gray, dark-gray, black, yellow and gray with tiger stripes.

Accurate parentage identification is essential in establishing a breeding program tailored to conserve genetic variability in endangered cattle breeds. Conservation strategies can be categorized as either conserving animals in-situ (in the environment in which they were developed), or ex-situ (in all other cases). The latter can be further divided into ex-situ *in vivo* conservation and cryogenic storage (5). Since *in vivo* conservation strategies include pedigree recording as an integral step it is clearly important for conservation models that all identified genetic relationships are correct. Given that minimization of the loss of genetic variation is equivalent to minimization of the rate of inbreeding in a population, the rate of increase of inbreeding (ΔF, F representing the coefficient of inbreeding) is the most important parameter in programs that maintain genetic diversity (5). Previous studies show that introduced errors in paternity testing lead to changes in inbreeding coefficient in cattle populations (6).

Traditionally, pedigree verification in cattle was based on blood groups and biochemical polymorphism analyses. Pedigree data are confirmed based on antigens of 12 blood group systems using over 80 test sera (7). However, a high frequency of incorrect cattle paternity was obtained using traditional markers. In addition, blood typing cannot be done retrospectively, e.g. after a sire is dead (8).

Since first described in cattle by Fries et al (9) microsatellites have been used increasingly in population genetics studies and paternity analyses in cattle. To date, there are several thousands of microsatellite markers described in cattle, a great number of which have been proven informative for parentage testing in a large number of cattle breeds. Accessibility of thousands of microsatellite markers for cattle resulted in the creation of different only partly overlapping sets of markers used for parentage analysis (8). In 2006, International Society for Animal Genetics-ISAG (10) recommended 9 microsatellite loci (BM2113, BM1824, SPS115, TGLA227, TGLA126, TGLA122, ETH10, ETH225 and INRA23) as a minimum set of markers for cattle parentage testing. In the current standard set of 12 microsatellites, additional three loci (ETH3, TGLA53 and BM1818) were included in a standard panel for routine testing (11).

To determine the degree of usefulness of microsatellites for parentage testing in a certain population, the number and frequency of their alleles must be evaluated (12). To date there is no information available on applicability of microsatellite markers for paternity testing in Busha cattle breed, despite the fact that Busha is considered a valuable genetic resource, protected under the Law as an endangered autochthonous breed.

In this study a set of 12 microsatellites (BM2113, BM1824, SPS115, TGLA227, TGLA126, TGLA122, ETH10, ETH225, INRA23, ETH3, TGLA53, and BM1818) as recommended by ISAG was evaluated for their use for paternity testing and pedigree verification in an autochthonous breed of cattle – Busha.

Materials and methods

Twelve microsatellites used in this study are recommended by ISAG for cattle paternity testing. Blood samples were taken from 47 heads of Busha cattle. The samples originated from a single heard of animals from south-eastern part of the Republic of Serbia, the region of Dimitrovgrad (43°01′N 22°47′E). Animals sampled were maintained under natural mating conditions. Taking care of the representativeness of sample, animals included in the study were unrelated two generations in the past. The information on the method of reproduction and relatedness of animals was obtained in an interview with the owner. Samples of blood were collected from the coccygeal vein. Genomic DNA from blood was isolated following standard protocol for organic extraction of nucleic acids (13), and kept frozen at –18°C until further processing. Microsatellites were amplified using the "Bovine Genotypes Panel 1.2" (Thermo Fisher Scientific Inc.) in a single multiplex reaction as recommended by FAO (14). The reactions were carried out according to the manufacturer recommendations. The reactions were performed in a programmable thermal cycler MultiGene Gradient (Labnet International Inc.). The fluorescent labeled PCR products were submitted to fragments analysis by capillary electrophoresis, with an automated sequencer ABI PRISM 310 (Applied Biosystems), using the GeneScan-350 ROX® Size Standard (Applied Biosystems), according to the

manufacturer's specifications. Results were read and interpreted using GeneScan® and Genotyper® software, respectively.

Standard statistical procedures were used to assess the informativeness of selected microsatellite markers. The number of alleles (nA), frequency of the most frequent allele (FNA), observed and expected heterozygosity (Ho and He), polymorphism information content (PIC), power of discrimination (PD) and power of exclusion (PE) were calculated for each microsatellite marker. Combined power of discrimination (CPE) and combined power of exclusion (CPE) were calculated for the whole set of studied markers (15, 16). Allele frequencies, PIC, PD and PE were determined by the PowerStatsV12 freeware, Promega Corporation, USA (17). Observed and expected heterozygosity as well as calculations for Hardy-Weinberg Equilibrium (HWE) were performed in Arlequine ver. 3.1 (18) according to Guo and Thompson (19).

To additionally confirm the applicability of the marker set, a simulation of parentage analysis was performed in CERVUS software (20) using the following assumptions: the number of candidate fathers was 46 and proportion of the sampled fathers was 1, since we assume that all the sires were sampled within this population; proportion of loci typed was set to 90% to account for missing or unreadable data, and proportion of loci mistyped was set to 1% to account for eventual mutations and/or mistakes in genotyping. Minimal number of loci typed was set to 9 given that our dataset had missing data in 3 loci. Finally, 100 000 offspring assignments were done.

Results

Informativness of the analyzed marker set assessed by basic diversity indices and forensic parameters is shown in Table 1 and Table 2, respectively.

The results obtained from Cervus software analysis showed that 96% of offsprings can be assigned to a certain father within the population at the strict confidence level of 95%. More precisely, 4452 out of 100 000 assignments were unsuccessful.

Results show high applicability of the tested marker set for individual identification and paternity verification in Busha cattle.

None of the markers deviated significantly from Hardy-Weinberg equilibrium.

Discussion

This study was focused on evaluation of a microsatellite set of 12 markers recommended by ISAG for their applicability in forensic science. Although Busha cattle breed is considered endangered in several countries and definite measures are being employed with the aim of population restitution, data on performance of microsatellite markers in parentage testing and individual identification is not available for this breed. When assessing a certain set of markers for forensic purposes, several guidelines should be followed: the markers should be characterized by high polymorphism, well-balanced frequency of alleles, PIC, H and PE values exceeding 0.5, high electrophoretic separation of alleles and repeatability of results during sizing (21). Further, ideally markers should be amplifiable in a single multiplex reaction to facilitate work and provide a streamlined analysis.

The marker set recommended by ISAG consists of 12 markers distributed on 12 different autosomes. The set is amplified in a single reaction, and detected through three different channels, therefore not requiring a large amount of manual labor in preparation and results reading.

Overall all markers showed robustness in amplification, as the percentage of missing data in the tested population was 1.99%.

However, all of the markers consist of dinucleotide repeats. This could influence the reliability of data obtained if two or more alleles in a genotype differ by a single repeat.

In the tested Busha population, the number of alleles ranged from 6 (ETH10) to 16 (TGLA122), with a mean value of 9.5. This is a relatively high value when compared to similar studies in different cattle breeds. In 2010, Stevanovic et al. (8) tested a marker set of 11 microsatellites for forensic purposes in YU Simmental cattle showing a mean value for number of alleles to be 8.273. The number of alleles was higher in our study in 10 out of 11 markers tested. When compared to findings of Simčić et al. (22), average number of alleles was higher both for Slovenian Cika and Croatian Busha cattle where this value was 8 and 5, respectively, although a different Busha population was sampled and a smaller number of animals was tested. In a study on Polish cattle breeds by Radko et al. (23) the average number of

Table 1: Forensic parameters

Locus	PIC	PE	PD
TGLA227	0.857	0.461	0.96
BM2113	0.821	0.607	0.95
TGLA53	0.905	0.310	0.95
ETH10	0.668	0.456	0.86
SPS115	0.672	0.456	0.87
TGLA126	0.685	0.529	0.88
TGLA122	0.812	0.422	0.94
INRA23	0.789	0.379	0.93
BM1818	0.513	0.228	0.75
ETH3	0.691	0.358	0.88
ETH225	0.735	0.389	0.91
BM1824	0.765	0.329	0.92
		CPE=0.999	**CPD=0.995**

PIC – Polymorphysm Information Content; PE – Power of Exclusion; PD – Power of Discrimination; CPE – Combined Power of Exclusion; CPD – Combined Power of Discrimination

Table 2: Diversity indices

Locus	N	nA	Ho	He	AR	G-W	FNA
TGLA227	86	13	0.72093	0.88044	26	0.48148	19.8%
BM2113	92	10	0.80435	0.84854	40	0.24390	27.2%
TGLA53	78	15	0.61538	0.92374	30	0.48387	14.1%
ETH10	92	6	0.71739	0.72432	12	0.46154	35.9%
SPS115	92	7	0.71739	0.72838	14	0.46667	37.0%
TGLA126	92	7	0.76087	0.73435	14	0.46667	40.2%
TGLA122	92	16	0.69565	0.83564	38	0.41026	34.8%
INRA23	90	10	0.66667	0.82247	18	0.52632	27.8%
BM1818	92	7	0.54348	0.56450	20	0.33333	62.0%
ETH3	92	8	0.65217	0.73316	18	0.42105	44.6%
ETH225	92	7	0.67391	0.77783	14	0.46667	34.8%
BM1824	92	8	0.63043	0.79814	40	0.19512	37.0%
Mean	90.1670	9.5	0.6832	0.7810	23.667	0.41307	
s.d.	4.2180	3.398	0.0693	0.0940	10.782	0.10271	

N – Number of gene copies; nA – Number of Alleles; Ho – Observed heterozygosity; He – Expected heterozygosity; AR – Allelic range; G-W – Garza-Williamson index statistic; FNA – Frequency of the most frequent allele

alleles is reported as 118 loci in 11 microsatellite markers in five breeds. Compared to these results the mean number of alleles in Busha cattle is higher than in each of Polish breeds individually. The most polymorphic marker with 16 alleles is TGLA122, which concurs with findings of Radko et al. (23) where 16 alleles were found in total in all five breeds and Simčić et al. (22) where this marker was most polymorphic in Cika cattle with a total of 10 alleles.

When compared to different studies where several strains of busha cattle were analysed with a larger microsatellite set, in this study the average number of alleles was relatively high – 9.5 as compared to 8.52 found in Croatian Busha (24). Further the value found in this study was higher than values found in other Busha strains such as: 8.76 (Red Metohian Busha), 8.17 (Illyrian Mountain Busha), 7.69 (Macedonian Busha), 7.61 (Gray Gacko Busha) or 7.57 (Illyrian Lowland Busha) (3).

Allelic range was widest in BM2113 and BM1824 where alleles spanned 40 base pairs (bp), whilst was relatively wide in TGLA122, as expected given the high number of alleles. Frequency of the most frequent allele (FNA) was the highest in marker BM1818 and was calculated to 62%. The same allele exhibited lowest values across the marker set not only for He and Ho, but also for PIC.

BM2113 marker showed the highest value for observed heterozygosity, but TGLA53 showed the highest value for expected heterozygosity. Both markers showed very high PIC values, TGLA53 being the marker with the highest PIC value within this marker set. Similar results were obtained in Red-and-White cattle breed for TGLA53 marker (23).

PIC values in Busha cattle population ranged from 0.513 (in BM1818) to 0.905 (TGLA53). The marker with the highest PIC value was TGLA53. This marker has a very high number of alleles (fifteen) with relatively wide distribution (30 bp) and a relatively low frequency of the most frequent allele – 14.1%. All these attributes distinguish TGLA53 as one of or the most informative marker in this set. When compared to different breeds of Polish cattle, PIC value for this marker is quite high. Mean PIC value across the marker set was 0.743. These values are higher than those found in many high-production breeds such as YU Simmental cattle (8), Czech Pied cattle, Slovakian Pied cattle (25) and Simmental cattle from Poland (26).

In this study, power of exclusion (PE) for single markers ranged from 0.228 (BM1818) to 0.607 (BM2113) and the power of discrimination (PD) from 0.75 (BM1818) to 0.96 (TGLA227). Most importantly, the combined power of exclusion and discrimination was very high (0.999 and 0.995 respectively) when all 12 markers are used in combination.

Given that the input parameters of the Cervus software mimic the real state of the tested population, and that 96% of paternity assignments were successful, the 12 markers tested proved sufficient for accurately identifying parentage even when taking missing data into account. These results further confirm that the marker set is highly applicable for paternity testing in Busha cattle.

Even though all of the markers within this marker set are consisted of dinucleotide repeats, markers have high PIC, PE and PD values and the whole set performed excellent for forensic purposes in the tested population.

In conclusion, the results of this study show that the analyzed marker set can be used with high confidence in parentage testing and individual identification in Busha cattle.

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OCENA UPORABNOSTI STANDARDIZIRANIH MIKROSATELITSKIH OZNAČEVALCEV PRI OGROŽENI VRSTI GOVEDA BUŠA

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Povzetek: V študiji smo želeli preveriti uporabnost 12 mikrosatelitskih označevalcev (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, BM1818, ETH3, ETH225, BM1824), ki jih priporoča Mednarodno združenje za živalsko genetiko (ISAG), za testiranje očetovstva pri govedu buša v Srbiji. Analizirali smo vzorce krvi 47 živali. Skupno število alelov je bilo med 6 (ETH10) in 16 (TGLA122), s srednjo vrednostjo 9,5. Skupno število alelov vseh dvanajstih analiziranih lokusov je bilo 114. PIC (*angl. Polymorphism Information Content*) vrednosti so bile med 0,513 (v BM1818) in 0,905 (TGLA53). Moč izključitve (PE) se je za posamezne označevalce gibala od 0,228 (BM1818) do 0,607 (BM2113) pri moči diskriminacije (PD) od 0,75 (BM1818) do 0,96 (TGLA227). Skupna moč izključevanja in diskriminacije je bila zelo visoka (0,999 in 0,995), ko smo uporabili kombinacijo vseh 12 označevalcev. S programom Cervus smo opravili analizo staršev in s 96% uspešnostjo določili očetovstvo. Ti rezultati kažejo, da se 12 označevalcev, ki jih ISAG priporoča, lahko z visokim zaupanjem uporablja za forenzične namene pri govedu buša.

Ključne besede: mikrosateliti; testiranje očetovstva; govedo buša