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Original scientific paper

ASSESSMENT OF 17 MICROSATELLITE LOCI FOR THEIR USE IN PARENTAGE VERIFICATION AND INDIVIDUAL IDENTIFICATION IN THE BALKAN DONKEY BREED

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The aim of this study was to assess a panel of 17 microsatellites for parentage verification and individual identification in the endangered Balkan donkey breed. Allele frequencies for 17 microsatellite loci (*AHT4*, *AHT5*, *ASB2*, *ASB17*, *ASB23*, *CA425*, *HTG4*, *HTG6*, *HTG7*, *HTG10*, *HMS1*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *LEX3* and *VHL20*) were determined in a 77 unrelated Balkan donkeys. Three loci (*ASB2*, *HMS1* and *ASB17*) proved to be unsuitable and had been excluded from the investigation. Analysis of the remaining 14 loci revealed varied levels of polymorphism (three to 12 alleles), while the total number of observed alleles was 118 with an average of 8.42 per locus. Average values of observed heterozygosity and polymorphic information content (PIC) were 0.712 and 0.650, respectively. Twelve out of 14 microsatellite markers were highly informative with PIC values higher than 0.5. Only four loci were in HWE (*HMS2*, *HMS6*, *HMS7* and *HTG6*). The obtained value of combined power of exclusion

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(0.9999) confirms usefulness of this microsatellite panel for parentage verification, while the value of combined power of discrimination of 0.9941 clearly approves the reliability of the panel for individual identification in Balkan donkeys.

Keywords: allele frequency, Balkan donkey, equine microsatellites, parentage testing

INTRODUCTION

Domestic donkey (*Equus asinus*) is a livestock species that was significantly affected by the agriculture industrialization (KUGLER *et al*., 2008). In some rural areas, where donkeys as pack animals are no longer required, a substantial decrease in the population size and even depopulation of donkeys were observed (IVANKOVIC *et al*., 2000, 2002; BORDONARO *et al*., 2012). Therefore, an increasing number of local donkey breeds are under threat of extinction and valuable traits are at risk of being lost. Despite this alarming situation, there are no sufficient scientific data on genetic variability in different donkey breeds, which hampers efforts for their conservation (KUGLER *et al*., 2008). Parentage testing programs present an important element of conservation of genetic variation in livestock species, including donkeys. They enable verification of identities, confirmation of familial relatedness, validation of studbooks, and, thus, are essential for breed registry authorities and their management plans (SCHLÖTTERER, 2004; GROENEVELD *et al*., 2010; FORNAL *et al*., 2013).

Short tandem repeats (STR) or microsatellites are genetic markers commonly used for genetic variability studies and paternity testing in different animal species and breeds (ARANGURES-MENDEZ *et al*., 2001; JORDANA *et al*., 2001; VAN DE GOOR *et al*., 2010; DIMITRIJEVIC *et al*., 2013; DA SILVA *et al*., 2014). They are used both as in-house assembled and optimized microsatellite panels (REIS *et al*., 2008; MITTMANN *et al*., 2010) or as kits originally developed for paternity testing and forensic applications (FORNAL *et al*., 2013). Genotyping of donkeys is most commonly based upon using equine STR loci (BLASI *et al*., 2005; CIAMPOLINI *et al*., 2007; BORDONARO *et al*., 2012; ZHU *et al*., 2013; MATASSINO *et al*., 2014). The International Society for Animal Genetics (ISAG) recommends set of twelve basic microsatellites loci (*AHT4, AHT5, ASB2, HMS2, HMS3, HMS6, HMS7, HTG10, HTG4, VHL20, ASB17* and *ASB23*) and five additional microsatellites loci (*HTG6, HTG7, CA425, HMS1* and *LEX3*) for phylogenetic and forensic analyses in horses (VAN DE GOOR *et al*., 2010; EQUINE GENETICS AND PARENTAGE ANALYSIS WORKSHOP, 2012). Majority of these markers have already been used in previous studies focused on genetic characterization of donkey breeds (ARANGURES-MENDEZ *et al*., 2001; IVANKOVIC *et al*., 2002; CIAMPOLINI *et al*., 2007; BORDONARO *et al*., 2012; ZHU *et al*., 2013).

The Balkan donkeys are medium size donkeys, with slightly elongated rectangular format of the body and grey, brown, dark-grey and reddish-brown colour. Historically, Balkan donkeys were kept in Serbia as valuable animals for work, milk and meat, but industrialization and urbanization inevitably led to dramatic decrease in their population size. The number of sexually mature Balkan donkeys in Serbia is currently estimated to be 250 -300 (STANISIC *et al*., 2014). Therefore, the Balkan donkey is an endangered autochthonous breed and as such represent both a heritage and a genetic resource of Serbia and Balkan region that deserves to be conserved (OFFICIAL GAZETTE OF RS; KUGLER *et al*., 2008). The initial study we performed encompassed morphological, biochemical and hematological characterization of this breed (STANISIC *et al*., 2015). Controlled mating and prevention of hereditary diseases through implementation of effective breeding strategy require a reliable parentage verification and identification system. Therefore, the objective of this study was to assess an “easy to use”

commercially available equine panel of 17 microsatellites for parentage verification and individual identification in the Balkan donkey breed. To our knowledge, this is the first study evaluating paternity testing based on DNA polymorphism in this donkey breed.

MATERIALS AND METHODS

Sample collection and DNA extraction

The study population included 77 unrelated animals: 41 donkeys from Special Nature Reserve „Zasavica“ (44°57′32,2″ N 19°31′32,7″ E), 16 donkeys from Stara planina Mt. (43°06′37″ N 22°57′14″ E) and 20 donkeys located in Kovilj village (45°25′16,7″ N 19°83′69,4″ E). Three major Balkan donkey populations in Serbia are sited in these localities. The blood sampling was performed at the localities over the period May to August 2013. The samples were collected from clinically healthy donkeys by venepunction of the jugular vein. The blood was collected in EDTA tubes and chilled on ice. DNA was extracted using the GeneJET Whole Blood isolation kit (Thermo Scientific), according to the manufacturer's protocol. DNA extracts were quantified using the BioPhotometer spectrophotometer system (Eppendorf). The genomic DNA was diluted after extraction to 1-2ng/µl, in accordance to the manufacturer's recommendations for the Thermo Scientific Equine Genotypes Panel 1.1. The DNA samples were stored at –20°C prior to further analysis.

PCR Amplification and Capillary Electrophoresis

The microsatellite markers analyzed were 17 loci (Table 1) recommended by the ISAG for equine parentage and identification testing (LEAR *et al*., 1999; DIMSOSKI, 2003; AZOR *et al*., 2007; VAN DE GOOR *et al*., 2010; EQUINE GENETICS AND PARENTAGE ANALYSIS WORKSHOP, 2012). Microsatellites were co amplified with the Equine Genotypes Panel 1.1 (Thermo Scientific), under conditions recommended by the manufacturer. The reactions were performed in the programmable thermal cycler MultiGene Gradient (Labnet International Inc.). The fluorescent tagged PCR products were separated by capillary electrophoresis conducted on the ABI Prism 310 Genetic Analyzer (Applied Biosystems), using the GeneScan-350 ROX Size Standard (Applied Biosystems) in accordance with the manufacturer's specifications. DNA fragment size was analyzed by GeneScan® and Genotyper® softwares (Applied Biosystems).

Statistical analyses

Basic diversity indices such as number of alleles, allele frequencies, observed and expected heterozygosity (Ho and He, respectively), were computed for all markers tested using the Arlequine ver. 3.1 (EXCOFFIER *et al*., 2006). Possible deviations from Hardy-Weinberg equilibrium (HWE) were determined using the exact test (GUO and THOMPSON, 1992). Corrections for multiple significance tests were performed by applying a Bonferroni-Holm correction (HOLM, 1979). Polymorphic information content (PIC) of different microsatellites used in the present study was estimated according to the formula reported by BOTSTEIN *et al*. (1980). Power of exclusion (PE) and power of discrimination (PD) were calculated for each microsatellite marker, while combined PE (CPE) and combined PD (CPD) were calculated for the whole set of markers. PE was calculated with an assumption of knowing one parent (JAMIESON and TAYLOR, 1997). PE and PD were determined by using PowerStats ver. 1.2 freeware (Promega Corporation). Experiments were performed according to the ISAG guidelines (BUDOWLE *et al*., 2005).

Table 1. Locus description for seventeen equine microsatellite loci (Dimsoski 2003; Van de Goor et al., 2010)

Locus	Chromosome	Repeat motif	Reference	Size range
VHL20	30	$(TG)_n$	Van Haeringen et al. (1994)	70-90
HTG4	9	$(TG)_nAT(AG)_5AAG(GA)_5$,$ACAG(AGGG)_3$	Ellegren et al. (1992)	133-139
AHT4	24	$(AC)_nAT(AC)_n$	Binns et al. (1995)	140-166
HMS7	1	$(AC)_2(CA)_n$	Guerin et al. (1994)	168-174
HTG6	15	$(TG)_n$	Ellegren et al. (1992)	74-82
AHT5	8	$(GT)_n$	Binns et al. (1995)	122-146
HMS6	4	$(GT)_n$	Guerin et al. (1994)	152-174
ASB23	3	$(TG)_n$ and $(TG)_nTT(TG)_4$	Irvin et al. (1998)	183-203
HTG10	21	$(TG)_n$ and $TATC(TG)_n$	Marklund et al. (1994)	78-100
HTG7	4	$(GT)_n$	Marklund et al. (1994)	117-139
HMS3	9	$(TG)_2(CA)_2TC(CA)_n$;$(TG)_2(CA)_2TC(CA)_n$ $GA(CA)_5$	Guerin et al. (1994)	141-171
HMS2	10	$(CA)_n(TC)_2$	Guerin et al. (1994)	218-238
LEX3	X	$(TG)_n$	Coogle et al. (1996)	136-164
CA425	28	$(GT)_n$	Eggleston-Stott et al.(1997)	224-238
ASB2	15	$(GT)_n$	Breen et al. (1997)	237-269
ASB17	2	$(AC)_n$	Breen et al. (1997)	104-116
HMS1	15	$(TG)_n$	Guerin et al. (1994)	166-178

RESULTS

Out of 17 microsatellites analyzed, the following 14 showed useful data in study population of the Balkan donkey: *AHT4, AHT5, ASB23, CA425, HTG4, HTG6, HTG7, HTG10, HMS2, HMS3, HMS6, HMS7, LEX3, VHL20* (Table 2). The *ASB2, ASB17* and *HMS1* loci were excluded from the analysis. The *ASB2* locus did not provide data for reliable analysis since it exhibited three alleles in only 42 individuals and did not amplify in remain 35 tested individuals. The locus *HMS1* failed to amplify in all samples, while the *ASB17* marker was monomorphic (88bp) for all examined animals of Balkan donkey population.

Among 14 tested loci, the number of alleles varied from 3 (*HTG4*) to 13 (*AHT5*), while the total number of observed alleles in our study population was 118 with an average of 8.42 per

locus. The mean H_o in the Balkan donkey was estimated to be 0.712, while mean H_e was 0.688 (Table 2).

Table 2. Characteristics of the 14 investigated microsatellite loci in the Domestic Balkan donkey

Locus	n_A	FNA	*Ho*	*He*	HWE	PIC
VHL20	7	0.532	0.59740	0.63908	0.00000	0.59
HTG4	3	0.534	0.79310	0.53660	0.00007	0.42
AHT4	11	0.230	0.86842	0.84873	0.00000	0.82
HMS7	4	0.961	0.05195	0.07631	0.05908	0.07
HTG6	5	0.466	0.44828	0.60165	0.02038	0.64
AHT5	13	0.337	0.58140	0.84460	0.00000	0.82
HMS6	8	0.455	0.80519	0.72116	0.03293	0.68
ASB23	8	0.286	0.94805	0.81470	0.00000	0.78
HTG10	12	0.253	1.00000	0.83017	0.00000	0.80
HTG7	10	0.313	0.91667	0.83770	0.00000	0.81
HMS3	12	0.243	0.94595	0.86229	0.00000	0.84
HMS2	8	0.440	0.65333	0.67714	0.00737	0.62
LEX3	8	0.649	0.40260	0.54826	0.00000	0.52
CA425	9	0.308	0.95890	0.80576	0.00000	0.77
Mean	8.42	0.429	0.71223	0.68886		0.65

nA = number of alleles; FNA = frequency of the most frequent allele; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphism information content;*P < 0.005, significantly deviated from Hardy-Weinberg equilibrium (HWE).

Table 3. Values of power of exclusion (PE), combined power of exclusion (CPE), power of discrimination (PD) and combined power of discrimination (CPD) for the examined 14 loci in the Domestic Balkan donkey

No.	Microsatellite marker	PE	PD
1	*VHL20*	0.288	0.764
2	*HTG4*	0.586	0.350
3	*AHT4*	0.731	0.912
4	*HMS7*	0.002	0.124
5	*HTG6*	0.203	0.854
6	*AHT5*	0.269	0.898
7	*HMS6*	0.609	0.854
8	*ASB23*	0.894	0.901
9	*HTG10*	0.974	0.901
10	*HTG7*	0.830	0.861
11	*HMS3*	0.890	0.869
12	*HMS2*	0.360	0.822
13	*LEX3*	0.116	0.688
14	*CA425*	0.888	0.861
		CPE=0.9999	CPD=0.9941

Tests for deviation from HWE showed highly significant ($P < 0.005$) difference between observed and expected genotype frequencies at all loci except for *HMS2*, *HMS6*, *HMS7* and *HTG6*. The lowest PIC value was established for the locus *HMS7* (0.07), while the maximum PIC of 0.84 was noted for *HMS3*. The mean value of this parameter was 0.65. Twelve out of 14 microsatellite markers were highly informative with PIC values of more than 0.5 (Table 2). Paternity parameters included values of PE and PD, where PE values ranged from 0.002 (*HMS7*) to 0.974 (*HTG10*). The CPE value of 14 microsatellite loci in the Balkan donkey breed was 0.9999, while the CPD value was 0.9941 (Table 3).

DISCUSSION

In this study, 17 microsatellite markers were evaluated for their information content and their ability to discriminate individuals and verify parentage in the Balkan donkey population in Serbia. Amplification of the 17 microsatellites was performed using The Equine Genotypes™ Panel 1.1 (Thermo Scientific). The kit has been recommended for routine horse parentage testing (DIMSOSKI, 2003; ZABEK and FORNAL, 2009; VAN DE GOOR *et al*., 2010), but its accuracy and reliability may be variable in donkey breeds. Standardized kits are supposed to enable reliable parentage testing results and do not require as stringent optimization as in-house panels of genetic markers. Positive data from this investigation will indicate the applicability of this panel in donkeys enabling easier testing necessary for conservation purposes.

Out of 17 microsatellite loci analyzed, three loci (*ASB2*, *ASB17* and *HMS1*) were found to be ineffective for application in the Balkan donkey breed. This finding is largely consistent with the results of previous studies using the same microsatellite loci in various donkey breeds. In our study, the *ASB2* locus exhibited only three alleles in 42 individuals, which was insufficient for further analysis. The low number of *ASB2* alleles has already been reported in previous studies. This locus showed 2 alleles in Italian Amiata donkey (CIAMPOLINI *et al*., 2007), while it failed to amplify in five Spanish donkey breeds (ARANGUREN-MENDEZ *et al*., 2001).When it comes to *HMS1* is concerned, it failed to amplify in Balkan donkey population tested. This locus was monomorphic in three Italian (BLASI *et al*., 2005) and five Spanish donkey breeds (ARANGURES-MENDEZ *et al*., 2001), but CIAMPOLINI *et al*. (2007) found four alleles for *HMS1* in Amiata donkey population. The third ineffective locus, ASB17 was monomorphic (88bp) in all heads of Balkan donkey population studied. This locus has not been previously used for parentage verification and individual identification in donkey breeds (ARANGURES-MENDEZ *et al*., 2001; IVANKOVIC *et al*., 2002; CIAMPOLINI *et al*., 2007; BORDONARO *et al*., 2012; ZHU *et al*., 2013; MATASSINO *et al*., 2014). Therefore, usefulness of the locus *ASB17* for these purposes in donkey breeds remains to be established. The mean number of alleles (8.42) found in the Balkan donkey breed was higher than previously reported in Chinese Yang Yuang donkey (6.83) (ZHU *et al*., 2013), Sicilian donkey breeds (6.07) (BORDONARO *et al*., 2012), Amiata donkey (5.61) (CIAMPOLINI *et al*., 2007) and Catalonian donkey breed (7.7) (JORDANA *et al*., 2001). The *HTG4* and *HMS7* loci (3 and 4 alleles, respectively) showed the lowest level of polymorphism in the Balkan donkey breed, which is in accordance with previous results of JORDANA *et al*. (2001) and BORDONARO *et al*. (2012). The most polymorphic loci were: *AHT4* (11 alleles), *AHT5* (13 alleles), *HTG10* (12 alleles), *HTG7* (10 alleles), *HMS3* (12 alleles) and *CA425* (9 alleles). The number of highly informative loci established in our study (that appeared the most informative in our study) was higher than in other characterized donkey breeds (BELLONE *et al*., 1998;

ARANGURES-MENDEZ *et al*., 2001; JORDANA *et al*., 2001; IVANKOVIC *et al*., 2002; BLASI *et al*., 2005; CIAMPOLINI *et al*., 2007; BORDONARO *et al*., 2012; ZHU *et al*., 2013; MATASSINO *et al*., 2014). High polymorphism at the loci *HTG10, AHT4* and *HMS3* was also reported by CIAMPOLINI *et al*. (2007), BORDONARO *et al*. (2012), ZHU *et al*. (2013) and MATASSINO *et al*. (2014), although number of alleles at these loci were higher in Balkan donkey.

The results of microsatellite polymorphism revealed relatively high degree of heterozygosity in the Balkan donkey population investigated in this study. The mean H_e (0.688) in Balkan donkey was lower than the H_e found in Yang Yuang donkey (ZHU *et al*., 2013) and Catalonian donkey (JORDANA *et al*., 2001), while the higher values of H_e were established in Italian donkey breeds (CIAMPOLINI *et al*., 2007; BORDONARO *et al*., 2012). On the other hand, the mean H_o (0.712) for the Balkan donkey was consistently higher than the H_o values found in all other previously analyzed donkey breeds (BELLONE *et al*., 1998; ARANGURES-MENDEZ *et al*., 2001; JORDANA *et al*., 2001; IVANKOVIC *et al*., 2002; BLASI *et al*., 2005; CIAMPOLINI *et al*., 2007; BORDONARO *et al*., 2012; ZHU *et al*., 2013; MATASSINO *et al*., 2014). This is an important finding which indicates that there are appreciable differences in the level of genetic variability among the Balkan donkeys.

Out of the 14 polymorphic loci, 10 microsatellites gave significant deviations from HWE showing a significant heterozygote deficit in studied Balkan donkey population. The remaining four loci, *HMS2*, *HMS6*, *HMS7* and *HTG6*, were in HWE. The possible reasons for deviation from HWE may be the gradual decrease in the population size, presence of loci under selection and "null alleles" (nonamplifying alleles). In addition, population sub-structuring probably occurs within this breed, since the animals originate from different geographical locations. This has already been shown in other donkey breeds studied (JORDANA *et al*., 2001; ZHU *et al*., 2013).

The most polymorphic loci *AHT4*, *AHT5*, *ASB23*, *HTG10*, *HTG7*, *HMS* and *CA425*, exhibited high number of alleles, Ho, He and PIC values. In addition, frequency of the most frequent allele (FNA) across these loci was well below 0.5 (Table 1).

The high CPE and CPD values obtained for the Balkan donkey population investigated indicate the suitability of equine microsatellite markers for paternity testing and individual identification in small donkey populations. It is noteworthy that the CPE value of 0.9999 established for 14 loci tested in the Balkan donkey is higher than the CPE value of 0.9995 proposed by the International Stud Book Committee (ISBC). Therefore, the results of our study show that the panel of 14 equine microsatellite markers (*AHT4*, *AHT5*, *ASB23*, *CA425*, *HTG4*, *HTG6*, *HTG7*, *HTG10*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *LEX3* and *VHL20*) has a great capacity for paternity discrimination in Balkan donkeys in Serbia. To our knowledge, the number of loci employed in this study is higher than in all other investigations of donkey breeds.

Utilization of commercially available panel of equine STRs in different donkey breeds should contribute to their preservation, which has been unfairly neglected over the last decades. In addition, multiplex marker systems can also be used for genotyping of donkey breeds, for assessing their population history, structure, diversity, and for reconstructing relationships among breeds. For more comprehensive genetic characterization of donkey breeds as many markers as possible need to be involved. It would be of great interest to further pursue that line of research in other donkey breeds worldwide.

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PROCENA EFIKASNOSTI 17 MIKROSATELITA U INDIVIDUALNOJ IDENTIFIKACIJI I UTVRĐIVANJU SPORNIH RODBINSKIH ODNOSA KOD RASE BALKANSKI MAGARAC

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Izvod

Cilj ovog rada bio je procena efikasnosti panela od 17 mikrosatelita za individualnu identifikaciju i utvrđivanje roditeljstva kod ugrožene rase balkanski magarac. U ispitivanoj populaciji od 77 magaraca određeni su broj i učestalost alela 17 mikrosatelitskih lokusa (*AHT4*, *AHT5*, *ASB2*, *ASB17*, *ASB23*, *CA425*, *HTG4*, *HTG6*, *HTG7*, *HTG10*, *HMS1*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *LEX3*, *VHL20*). Tri lokusa (*ASB2*, *HMS1*, *ASB17*) su se pokazala kao nepodobna i isključena su iz istraživanja. Analiza preostalih 14 lokusa pokazala je različite nivoe polimorfizma (tri do 12 alela), dok je ukupan broj posmatranih alela bio 118 sa prosekom od 8.42 po lokusu. Prosečne vrednosti za dobijenu heterozigotnost i informativni sadržaj polimorfizma (PIC) iznosile su 0.712, odnosno 0.650. Dvanaest od 14 mikrosatelitskih markera je pokazalo visoku informativnost sa PIC vrednostima većim od 0.5. Samo četiri lokusa je bilo u HW ravnoteži (HMS2, HMS6, HMS7, HTG6). Dobijena verovatnoća isključenja (0.9999) potvrđuje korisnost ovog panela za verifikaciju spornih rodbinskih odnosa, dok moć diskriminacije od 0.9941 potvrđuje pouzdanost panela za individualnu identifikaciju kod balkanskog magaraca.

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