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MICROSATELLITES VARIATIONS IN ROMANIAN THOROUGHBRED AND ARABIAN HORSE POPULATIONS

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ABSTRACT. The use of PCR technology for analyzing microsattelites is increasing every day. Microsatellite markers are evenly distributed across genome and can be identified within DNA samples using PCR. Genetic diversity between Thoroughbred and Arabian horse populations were analyzed using 12 microsatellite markers. The DNA loci analyzed – AHT4, AHT5, ASB2, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10 and VHL20 – were chosen based upon the polymorphism detected in other breeds. High levels of polymorphism were observed over populations with the average number of alleles and average of observed heterozygosity at 6.41 and 0.683, respectively. Between the two populations no significant differences were observed in expected heterozygosity suggesting that more intensive breeding practices may have resulted in a further erosion of genetic variability. This technology has great potential for use in horse breeding situations where levels of genetic variation could be monitored and inbreeding controlled in a commercial breeding program.

KEY WORDS: microsatellites, polymorphism, allele frequencies, heterozygosity, Thoroughbred, Arabian.

INTRODUCTION

The recent developments in molecular genetics allowing the detection of genes responsible for economic traits have opened a new area in farm animal selection, including horses. Recently, breeders have turned to molecular biology and use PCR (Polymerase Chain Reaction) for detection of short sequence repeats, which are also

referred to as microsatellites. Microsatellite markers are evenly distributed across genome and highly polymorphic. This technology provides a sensitive method for parentage verification and individual identification. It can also be used to screen for markers linked to performance traits or genetic disorders.

PCR have the attractive features: can be highly automated, require only small amounts of biological samples, are not restricted to fresh blood samples and is simpler and cheaper than other techniques. Microsatellites are highly polymorphic genetic markers with codominantly inherited alleles that are relatively easy to score. They are tandem repeats of short motifs ranging from di- to tetranucleotides with variability in the number of repeats.

One of the difficulties in implementing a selective breeding program in horse stocks is maintaining pedigree information. A number of microsatellite markers are now in use for parentage testing in several horse breeds *(e.g.* Binns *et al*., 1995; Bowling *et al*., 1997; Gralak *et al.*, 1998; Meriaux *et al*., 1998; Wimmers *et al*., 1998) and other breed registries are now in the process of converting to a DNA based technology.

Another difficulty in managing a selective breeding program is loss of genetic variability and increases in inbreeding as a result of the inadvertent mating of related individuals. The effects of inbreeding in horses will result in a decrease in genetic variability, which will limit the potential for genetic gain from artificial selection. Once reliable pedigree information is available, matings can be arranged in order to minimize inbreeding.

MATERIAL AND METHODS

Blood samples were obtained from Cislău haras for Thoroughbred horses and from Mangalia haras for Arabian.. These harases are the only ones being in the official evidence of the Romanian Agricultural Ministry. The isolation of genomic DNA from the white blood cells was performed with Wizard Genomic DNA Extraction Kit (Promega).

The forward and reverse primers are premixed and the PCR conditions optimised so that the loci can be amplified as multiplex reactions, and then loaded with the GeneScan-500 ROX Size Standard on ABI PRISM 310 DNA Genetic Analyser. Amplification of the STR loci was realized by multiplex PCR using StockMarks Paternity PCR Typing Kit (Applied Biosystems), according to the procedure recommended by the manufacturer. The reagents in the kit are used to amplify DNA samples using the fluorescent dye-labelled primers specific to the relevant loci. The kit contains all of the labelled and unlabeled primers, AmpliTaq Gold ® DNA Polymerase, control DNA, dNTPs mix and buffers. Control DNA is provided with each kit to verify the correct amplification and detection of the animal-specific loci. The forward and reverse primers are premixed and the PCR conditions optimised so that the loci can be amplified as multiplex reactions. As the samples migrate past the fluorescence detector, the GeneScan ® Software collects the signal and assigns a base pair size for each sample. Allelic nomenclature is in order by size in base pairs, from smallest to largest.

PCR amplifications were performed in 0.2 mL tubes, in AppliedBiosystem 2700 thermocycler, using 30 cycles with denaturation at 95 ºC (30 s), annealing at 60 °C (30 s) and extension at 72 °C (60 s). The first denaturation step was performed at 95 ºC (10 min) and the last extension was to 60 min at 72 °C.

RESULTS AND DISCUSSIONS

In our experiment we analysed 80/171 Thoroughbred horses from Cislău haras and 80/279 Arabian horses from Mangalia haras. Successful amplification yields allele peaks with the associated PCR stutter bands within a maximum range of eight base pairs from the allele peak. The number of allele peaks depends on whether the individual tested is a heterozygote or homozygote. For this StockMarks Kit all loci are dinucleotide repeats.

Allele frequencies, the observed heterozygosity *(H*o) and expected heterozygosity *(H*e) were calculated using the software program Cervus 1.0 (Marshall *et al*., 1998). The results are present in Table 1 and Table 2.

High levels of polymorphism were observed for populations of horses studied. From twelve microsatellites, ten were highly polymorphic and two (HTG4 and HTG7) present low polimorphism (Table 1, 2). AHT4 and VHL20 were the most variable loci with ten, respectively nine alleles and high heterozygosity. Alleles' size range at individual loci varied between 85 and 253 bp.

A number of loci showed quite different allele frequency distributions between the two horses' populations. For a number of microsatellites, alleles that present frequencies up to 0.35 in one populations were not detected in other population (AHT4, HMS7, HMS2, HTG10, VHL20). A number of alleles displaying a high frequency of (between 0.35 and 0.61) in one population showed a lower frequency (between 0.08 and 0.29) in the other population (Table 2). On AHT4, HTG10 and VHL20 loci the Thoroughbred population exhibited different alleles compared with Arabian population.

AHT5 and HTG6 loci present lower level of heterozygosity in the booth populations of horses. HTG7 locus presents lower level of heterozygosity in Arabian population and HMS2 locus in Thoroughbred population. The highest levels of heterozygosity exist on ASB2 locus, in booth populations.

CONCLUSIONS

Microsatellite markers are more likely than other methods to detect small differences between populations due to their high levels of allelic variation, being able to discriminate in both overall heterozygosity and mean number of alleles. Our study demonstrates differences between Thoroughbred and Arabian horse populations in terms of allelic diversity. Inbreeding is the term for mating systems that involve producing offspring from related animals. The amount of inbreeding obtained is determined by how closely the ancestors are related. The result is an increase in genetic uniformity of progeny within the inbred population caused by the reduction of the genetic variation. The less genetic variation within these lines, the

less their performance will be in crossing. The practice of inbreeding involves the entire genotype, not just the genes of the selected traits. While desirable traits can be improved through inbreeding, undesirable traits can also become more prevalent.

Small effective population sizes and poorly managed breeding programs are generally blamed for loss of genetic diversity in farmed stocks. Maintaining pedigree information on all individuals in the breeding program and using this information to arrange matings will ensure only minimum increases in inbreeding every generation.

In conclusion, this study demonstrates the high levels of polymorphism detectable with microsatellite loci within the two populations of horses from Romania. In horse breeding this technology has the potential to be of great use in monitoring levels of genetic variation within stocks as well as for parentage and relatedness purposes. These results also show that allelic diversity is a more sensitive measure of differences in genetic variation between the two populations.

REFERENCES

ALLENDORF, F.W. 1986. Genetic drift and the loss of alleles versus heterozygosity. Zoo Biol. 5, 181–190.

BERNOCO, D. and BYRNS, G. 1991. DNA fingerprint variation in horses. Animal Biotechnology 2:145-160.

BINNS, M.M, HOLMES, N.G, HOLLIMAN, A. and SCOTT, A.M. 1995. Identification of polymorphic microsatellite loci in the horse and their use in Thoroughbred parentage testing. Br. Vet. J. 151:9-15.

BOWLING, A.T. and RUVINSKY, A. 2000. The Genetics of the Horse**.** CABI Publishing, Wallingford, U.K.

BOWLING, A.T, EGGLESTON-STOTT, M.L, BYRNS, G., CLARK, R.S, DILEANNIS, S. and WICTUM E., 1997. Validation of microsatellite markers for routine horse parentage testing. Anim. Genet. 28:247-252.

BREEN, M., LINDGREN, G., and BINNS, M.M., 1997. Genetic and physical assignments of equine microsatellites first integration of anchored markers in horse genome mapping. Mamm. Genome 8:267-273.

CAETANO, A.R., SHIUE, Y.L., LYONS, L.A., O'BRIEN, S.J., LAUGHLIN, T.F., BOWLING, A.T., MURRAY, J.D., 1999. A comparative gene map of the horse (Equus caballus). Genome Res. Dec 9(12):1239-49.

COOGLE, L., REID, R. and BAILEY, E., 1996. Equine dinucleotide repeat loci *LEX015-LEX024.* Anim. Genet. 27:217-218.

DOUBLE, M.C., COCKBURN, A., BARRY, S.C. and SMOUSE, P.E., 1997. Exclusion probabilities for single-locus paternity analysis when related males compete for matings. Mol. Ecol. 6:1155-1166.

ELLEGREN, H., JOHANSSON, M., SANDBERG, K. and ANDERSON, L., 1992. Cloning of highly polymorphic microsatellites in the horse. Anim. Genet. 23:133-142.

GRALAK, B., KURYL, J. and NIEMCZEWSKI, C. 1998. Usefulness of a set of seven microsatellites for parentage control in Arabian Horses and Thoroughbreds in Poland. Anim. Genet. 29 (Suppl. 1):16.

GOODMAN, S.J., 1997. Rst Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. Mol. Ecol. 6, 881–885.

GUÉRIN, G., BERTAUD, M. and AMIGUES, Y., 1994. Characterization of seven new horse microsatellites: HMS1, HMS2, HMS3, HMS5, HMS6, HMS7 and HMS8. Anim. Genet. 25:62.

JAMIESON, A., 1994. The effectiveness of using co-dominant poly-morphic allelic series for (1) checking pedigrees and (2) distinguishing fullsib pair members. Anim. Genet. 25 (Suppl. 1):37-44.

MARKLUND, S., ELLEGREN, H., ERIKSSON, S., SANDBERG, K. and ANDERSSON, L., 1994. Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. Anim. Genet. 25:19-23.

MARSHALL, T.C., SLATE, J., KRUUK, L.E.B. and PEMBERTON, J.M., 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7:639-655.

MARUYAMA, T., FUERST, P.A., 1985. Population bottlenecks and non-equilibrium models in population genetics: II. Number of alleles in a small population that was formed by a recent bottleneck. Genetics 111, 675–689.

MERIAUX, J.C., ROGNON, X., MAHLA, R., OURAGH, L. and BOSCHER, M.Y., 1998. Usefulness of microsatellite markers for parentage control and phylogenetic relationships in French and Moroccan horse breeds. Anim. Genet. 29 (Suppl. 1):15.

MONTGOMERY, G.W. and SISE, J.A., 1990. Extraction of DNA from sheep white blood cells. N. Z. J. Agric. Res. 33:437-441.

SAKAGAMI, M., T. TOZAKI, S. MASHIMA, K. HIROTA, and H. MUKOYAMA, 1995. Equine parentage testing by microsatellite locus at chromosome 1q2.1. *Anim. Genet.* **26:** 123-124.

SCHLÖTTERER, C. and PEMBERTON, J. 1998. The use of microsatellites for genetic analysis of natural populations - a critical review. In: DeSalle R and Schierwater B (eds) Molecular Approaches to Ecology and Evolution. Birkhäuser Verlag Basel, pp. 71-86.

SHIUE, Y.L., L.A. BICKEL, A.R. CAETANO, L.V. MILLON, R.S. CLARK, M.L. EGGLESTON, R. MICHELMORE, E. BAILEY, G. GUÉRIN, S. GODARD 1999. A synteny map of the horse genome comprised of 240 microsatellite and RAPD markers. *Anim. Genet.* **30:** 1-9.

SLATKIN, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139, 457–462.

SWOFFORD, D.L. and SELANDER, R.B. 1989. *BIOSYS-1 User's Manual, Release 1.7. A computer program for the analysis of allelic variation in population genetics and biochemical systemat-ic*s. Illinois Natural History Survey, Champaign, Illinois.

VALDES, A.M., SLATKIN, M., FREIMER, N.B. 1993. Allele frequencies at microsatellite loci — the stepwise mutation model revisited. Genetics 133, 737–749.

VAN HAERINGEN, H., BOWLING, A.T., LENSTRA, J.A., ZWAAGSTRA, K.A. and STOTT, M.L. 1994. A highly polymorphic horse microsatellite locus VHL20. Animal Genetics 25:207.

WIMMERS, K., PONSUKSILI, S., SCHMOLL, F., HARDGE, T., HATZIPANAGIOTOU, A., WEBER, J., WOSTMANN, S., OLIK, K. and SCHELLANDER, K. 1998. Efficiency of microsatellite markers of the international standard panel for parentage control in German horse population. Zuchtungskunde 70(4):233-241.

Table 1: Details about homozygotes and heterozygotes percent in Thoroughbred and Arabian horse populations from Romania.

Locus	Homozygotes (%)		Heterozygotes (%)		Alleles/Locus	
	Thoroughbred	Arabian	Thoroughbred	Arabian	Thoroughbred	Arabian
HTG7	36,2	42,9	63,8	57,1	3	3
VHL20	31,9	24,5	68,1	75,5	4	6
HTG6	48,9	42,9	51,1	57,1	5	3
HTG4	40,4	34,7	59,6	65,3	4	3
HTG10	23,4	28,6	76,6	71,4	5	5
HMS7	21,3	16,3	78,7	83,7	5	5
HMS6	31,9	34,7	68,1	65,3	4	5
HMS3	21,3	24,5	78,7	75,5	7	7
HMS2	46,8	18,4	53,2	81,6	4	5
ASB2	19,1	14,3	80,9	85,7	7	6
AHT5	44,4	44,9	55,6	55,1	5	6
AHT4	27,7	38,8	72,3	61,2	5	8

Locus/Breed	Alleles and Frequencies											
	144	148	149	150	151	152	155	157	159	161	H (O)	H (E)
AHT4 TH	0,2660	-	0,0957	0,0532	0,1809	-	-	-	0,4043	-	0,723	0,729
AR	0,0918	0,6224	-	0,0612	-	0,0102	0,0102	0,0204	0,1735	0,0102	0,612	0,575
	129	131	133	135	137	139					H (O)	H (E)
AHT5 TH	0,2889	0,3333	-	0,2333	0,1222	0,0222					0,556	0,744
AR	0,6122	0,0408	0,0306	0,0510	0,2449	0,0204					0,551	0,565
	234	239	243	245	247	249	251	253			H (O)	H (E)
ASB2 TH	-	0,1383	0,1064	0,1809	0,0532	0,0106	0,3191	0,1915			0,809	0,804
AR	0,0306	0,2347	0,2449	-	0,2041	-	0,2755	0,0102			0,857	0,774
	171	173	175	177	179	181					H (O)	H (E)
HMS7 TH	0,1915	-	0,1277	0,4362	0,0957	0,1489					0,787	0,733
AR	0,2245	0,2653	0,2755	-	0,1429	0,0918					0,837	0,782
	153	160	161	162	164	166	168	172			H (O)	H (E)
HMS3 TH	0,5000	-	0,0106	0,0851	0,0851	0,0745	0,2128	0,0319			0,787	0,691
AR	0,2755	0,0306	-	0,2347	0,3776	0,0306	0,0408	0,0102			0,755	0,730
	157	159	162	166	168						H (O)	H (E)
HMS6 TH	0,1277	0,0106	0,4681	-	0,3936						0,681	0,616
AR	0,1224	0,2653	0,0204	0,0612	0,5306						0,653	0,635
	219	225	227	229	234	238					H (O)	H (E)
HMS2 TH	0,0426	0,3723	0,5319	0,0532	-	-					0,532	0,580
AR	0,0102	-	0,2347	0,2347	0,1224	0,3061					0,816	0,779

HTG10	TH	91	95	97	99	103	108	110				H (O)	H (E)
		0,2340	0,0426	-	0,3830	0,2234	0,1170	-				0,766	0,741
	AR	-	0,2143	0,1633	-	0,3776	0,1224	0,1224				0,714	0,763
HTG4	TH	127	129	131	137							H (O)	H (E)
		0,5213	0,0213	0,4255	0,0319							0,596	0,552
	AR	0,3776	0,1224	0,5000	-							0,653	0,599
HTG6	TH	79	85	91	95	101						H (O)	H (E)
		0,4362	0,4574	0,0106	0,0745	0,0213						0,511	0,601
	AR	0,5714	0,2551	-	0,1735	-						0,571	0,584
HTG7	TH	121	127	129								H (O)	H (E)
		0,1809	0,3191	0,5000								0,638	0,622
	AR	0,1224	0,1939	0,6837								0,571	0,485
VHL20	TH	85	86	92	93	94	95	97	101	105	H (O)	H (E)	
		-	0,2660	-	0,1064	-	0,3191	0,3085	-	-		0,681	0,729
	AR	0,3878	-	0,1224	-	0,0714	-	0,0102	0,0714	0,3367		0,755	0,718
Average heterozygosity	TH											0,672	0,678
	AR											0,695	0,665

Table 2: Summary statistics for genetic variation at 12 microsatellite loci surveyed in populations of Thoroughbred (TH) and Arabian (AR) horses from Romania. *H* (O) - Observed heterozygosity. *H* (E) - Expected heterozygosity.