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Bottleneck Study and Genetic Structure of Iranian Caspian Horse Population Using Microsatellites

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Abstract: Genetic diversity within the Iranian Caspian horse was evaluated using 8 different microsatellite pairs on 45 Caspian horse blood samples. This molecular characterisation was undertaken to evaluate the problem of genetic bottlenecks, if any, in this breed. The number of alleles per locus varied from 3 to 5 with mean value of 4.125. All markers have relatively high PIC value (>0.6), observed heterozygosity; 0.9433, expected Levene's heterozygosity 0.6856 and expected Nei's heterozygosity equal to 0.6762. This study indicated the existence of substantial genetic diversity in the Caspian horse. No significant genotypic linkage disequilibrium was detected across the population, suggesting no evidence of linkage between loci. A mode-shifted distribution, significant heterozygote excess on the basis of different mutation models, as revealed from Sign, Standardized differences and Wilcoxon rank tests suggested that there was recent bottleneck in the existing Caspian horse. Urgent conservational strategies on this population, is recommended.

Key words: Iran, Caspian horse, bottleneck, genetic structure, microsatellites

INTRODUCTION

The Caspian horse is a beautiful creature with a wonderful temperament. Thought to be extinct for 1300 years, Caspians once graced the royal seal of King Darius-550 B.C. Caspians were often depicted in ancient Persian statuettes, friezes and writings going back to 3000 B.C. The Caspian Breed was rediscovered in 1965 (www.kristull.com/caspian.htm).

To avoid further loss of potential unique genes and to preserve the genetic diversity within the breed, an objective breed classification based on genetic uniqueness is of priority (May, 1990; Hall and Bradley, 1995). Characterisation at the morphological and genetic levels is the first step towards formulating breeding policies and prioritising the breeds for conservation in an effective and meaningful way.

Recently an array of DNA based markers has been developed to carry out studies of genetic variation (Bradley *et al*., 1996; Canon *et al*., 2000). The term microsatellites, also Short Tandem Repeats (STRs), refers to a class of codominant DNA markers which are inherited in a Mendelian fashion. Microsatellites are highly polymorphic and abundant sequences dispersed throughout most eukaryotic nuclear genomes (Litt and Luty, 1989). In recent years, they have been extensively used in parentage testing, linkage analyses, population genetics and genetic studies (Goldstein and Pollock, 1997). Many microsatellites are informative due to their high polymorphisms and they are useful in evaluating breeds for genetic diversity. Several studies on establishing genetic relationships and differentiation based on microsatellite markers have been reported in livestock breeds, including horses (Arranz *et al*., 2001; Gupta *et al*., 2005; Bjornstad and Roed 2001; Fan *et al*., 2002; Ivankovic *et al*., 2002; Kantanen *et al*., 2000).

The present study involved molecular characterization based on eight microsatellite markers to detect historical population bottlenecks, in Caspian horse. This is critical as the overall population of these equines has gone down rapidly during the last few decades.

MATERIALS AND METHODS

Whole blood samples were collected from 45 Caspian horses from the jugular aseptically. Genomic DNAs were extracted using salting-out method with some modifications (Miller *et al*., 1988). Eight microsatellite markers were selected for this study (Table 1). These microsatellite markers have been reported by the horse applied genetics committee of ISAG for study of genetic diversity and parentage verification (www.user.gwdg.de/FAO/horses.html). PCR mix (15 μL) including 1X PCR buffer; 5 mM $MgCl_2$; 0.25 μM

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Table 1: Characteristics of 8 microsatellite loci used in this study

Locus	Chromosome	Primer sequence	Accession numbers	Allele range
		GAAGCTGCCAGTATTCAACCATTG		
HMS6	4	CTCCATCTTGTGAAGTGTAACTCA	X74635	153-169
		CCAACTCTTTGTCACATAACAAGA		
HMS3	9	CCATCCTCACTTTTTCACTTTGTT	X74632	148-186
		AACCGCCTGAGCAAGGAAGT		
AHT04	21	CCCAGAGAGTTTACCCT	None	139-171
		CCTGCTTGGAGGCTGTGATAAGAT		
HTG6	15	GTTCACTGAATGTCAAATTCTGCT	None	85-112
		CCTGAAGCAGAACATCCCTCCTTG		
HTG7	4	ATAAAGTGTCTGGGCAGAGCTGCT	None	120-130
		TTTAATCAAAGGATTCAGTTG		
LEX33	4	TTTCTCTTCAGGTGTCCTC	AF075635	203-217
		CAAGTCCTCTTACTTGAAGACTAG		
VHL20	30	AACTCAGGGAGAATCTTCCTCAG	None	89-107
		GAGGTTTGTAATTGGAATG		
ASB23	3	GAGAAGTCATTTTTAACACCT	X93537	128-154

primers; dNTPs 200 μM; 1 unit of Taq polymerase and genomic DNA 150 ng/reaction were under amplified. PCR conditions for all loci were included initial denaturation at 95°C for 2.5 min; followed by 36 cycles of 95°C for 30 sec; annealing 60°C for 30 sec; extension 72°C for 45 sec and final extention of 72°C for 5 min. The PCR products were electrophoresed on 8% denaturing polyacrylamide gels visualized by rapid silver staining (Sanguinetti *et al*., 1994).

Genotypes at the different polymorphic loci were individually recorded. Allele and genotype frequencies were estimated by direct counting.

Computation and statistical analysis: Heterozygosity (Nei, 1978) and other genetic diversity variables were calculated using POPGENE computer package (Yeh *et al*., 1999). Polymorphism Information Content (PIC) values were calculated using the method described by Buchanan and Thue (1998).

Bottleneck events in the population were tested by two methods. The first method consisted of three excess heterozygosity tests developed by Cornuet and Luikart (1996): (I) Sign test, (ii) Standardized differences test and (iii) a Wilcoxon sign-rank test. The probability distribution was established using 1000 simulations under three models: Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and Two-Phase Model of mutation (TPM). The second method was the graphical representation of the mode-shift indicator originally proposed by Luikart *et al*. (1998). Loss of rare alleles in bottlenecked populations is detected when one or more of the common allele classes have a higher number of alleles than the rare allele class (Luikart *et al*., 1998). This test was re-scaled so that frequency distribution of the allele frequency classes in each population would be based on equal 0.05 increments. These two methods were conducted using Bottleneck v1.2.02 (http://www.ensam.inra.fr/URLB).

RESULTS AND DISCUSSION

Genetic characterization of this breed was attempted using known polymorphic microsatellites for studying the genetic variability within the population. Microsatellites were polymorphic in Caspian horses and the values of diversity measures at each locus are presented in Table 2. A total of 33 alleles were observed at all the loci ranging from 3 to 5 with a mean of 4.1250 alleles per locus. The mean effective number of alleles in the Caspiani horse population was 3.183. The mean expected Levene's and Nei's heterozygosities were 0.686 and 0.676, respectively. This basic information indicated the existence of high genetic variability within the Caspiani horse population. PIC values ranged from 0.53 to 0.681 with a mean value 0.6 in terms of their suitability for genetic diversity studies and the remaining loci were reasonably informative (Table 2). Heterozygote deficiency analysis revealed significant deviations from HWE at all loci ($p<0.05$). An exact test for genotypic linkage disequilibrium yielded no significant P values across the population and therefore independent assortment was assumed. This high genetic variability can be exploited by horse breeders for planning breeding strategies and prioritizing the breed for its conservation.

In a population at mutation-drift equilibrium (i.e., the effective size of which has remained constant in the recent past), there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit. Populations that have experienced a recent reduction of their effective population size exhibit a correlative reduction of the allele numbers and gene diversity (H_e or Hary-Weinberg heterozygosity) at polymorphic loci. But the allele numbers is reduced faster than the gene diversity. Thus, in a recently bottlenecked population, the observed gene diversity is higher than the expected equilibrium gene diversity which is computed

Table 2: Observed (no) and effective (ne) number of alleles, allele size range, observed heterozygosity (Ho), expected heterozygosity (He), polymorphism information content (PIC) in different locus

	No. of alleles				He		
Locus	no	ne	Allele size range (bp)	Ho	Levene	Nei	PIC
HMS6	5	3.55	153-169	0.97	0.73	0.72	0.681
HMS3	3	3.60	148-186	1.00	0.62	0.62	0.596
AHT04	4	2.69	139-171	0.86	0.63	0.63	0.651
HTG6	4	3.77	85-112	0.98	0.74	0.73	0.555
HTG7	4	3.57	120-130	1.00	0.73	0.72	0.588
VHL20	5	3.93	89-107	1.00	0.75	0.74	0.63
ASB23	4	2.47	128-154	0.74	0.61	0.60	0.62
LX33	4	2.91	203-217	1.00	0.66	0.65	0.53
Mean	4.125	3.18	-	0.94	0.69	0.68	0.606

Table 3: Test for null hypothesis under three microsatellite evolution models

Test/Model	IAM	TPM	SMM
Sign test: number of loci with heterozygosity excess(probability)	Expected = 4.61(0.01201)*	4.74(0.01503)*	4.7(0.09407)
Standard differences test: Ti values(probability)	3.359(0.00039)*	2.696(0.00351)*	1.932(9.02668)*
Wilcoxon rank test (probability of heterozygosity excess)	0.00195*	0.00195*	0.00586*

*Rejection of null hypothesis (bottleneck)

from the observed number of alleles, under the assumption of a constant-size (equilibrium) population (Luikart *et al*., 1998). Strictly speaking, gene diversity excess has been demonstrated only for loci evolving under the Infinite Allele Model (IAM). If the locus evolves under the strict Stepwise Mutation Model (SMM), there can be situations where this gene diversity excess is not observed (Cornuet and Luikart, 1996). However, few loci follow the strict SMM and as soon as they depart slightly from this mutation model towards the IAM, they will exhibit an gene diversity excess as a consequence of a genetic bottleneck. Because few microsatellite loci follow the strict (one-step) SMM, it is recommend using the Two-Phased Model of mutation (TPM) with our Bottleneck test. The TPM is intermediate to the SMM and IAM. Most microsatellite data sets better fit the TPM than the SMM or IAM. The TPM we recommend for microsatellites consists of mostly one-step mutations, but a small percentage (5-10%) of multi-step changes (Luikart *et al*., 1998). Three tests have proposed to determine whether a population exhibits a significant number of loci with gene diversity excess. The first test namely sign test suffers from low statistical power. Standardized differences test (Cornuet and Luikart, 1996) in not very useful since it requires at least 20 polymorphic loci. The Wilcoxon test (Luikart and Cornuet, 1997) provides relatively high power and it can be used with as few as four polymorphic loci and any number of individuals (15-40 individuals and 10-15 polymorphic loci is recommend to achieve high power). It has also proposed a qualitative descriptor of the allele frequency distribution (mode-shift indicator) which discriminates bottlenecked populations from stable populations (Luikart and Cornuet, 1997).

Since the population of Caspian horses true to their breed has gone down drastically and only a few thousands are available, it is possible that demographic bottlenecks might have occurred. Because bottlenecks influence the distribution of genetic variation within and among populations, the genetic effects of reductions in population size require evaluation. To characterize this, Sign, Standardized differences and Wilcoxon sign rank tests were utilized. The values of average Heterozygosity (He) and their probabilities (H>He) in the Sign test, under three models of microsatellite evolution (IAM, SMM and TPM) were calculated and used to measure the expected number of loci with heterozygosity excess. In the present study, evidence for a bottleneck was detected with all methods. The expected number of loci with heterozygosity excess were 4.61 and 4.74 for IAM and TPM under null hypothesis, respectively (Table 3). The probabilites value in these cases were 0.012 and 0.015, respectively and thus reject the null hypothesis indicating bottleneck under these models. However the expected number of loci with heterozygosity excess was 4.7 in SMM with probability 0.094. Therefore, null hypothesis was not accepted when using the Sign test maening that Caspian horse population has undergone a recent genetic bottleneck. The standardized difference test provided the T2 (probability) statistics equal to 3.359 (0.0004), 2.696 (0.00354) and 1.932 (0.0267) for the IAM, TPM and SMM models, respectively. The probability values were less than 0.05 for IAM, TPM and SMM, thus hypothesis of mutation-drift equilibrium was not accepted under each three models. Using the Wilcoxon rank test (a non-parametric test) the probability values were 0.0019 (IAM), 0.0019 (TPM) and 0.0058 (SMM), thus rejects the null hypothesis indicating bottleneck under all models. Taking results from all the three tests together, it is clear that serious demographic bottlenecks have most probably occurred in this breed.

The Mode-shift indicator test was also utilized as a second method to detect potential bottlenecks, as the nonbottleneck populations that are near mutation-drift

equilibrium are expected to have a large proportion of alleles with low frequency. This test discriminates many bottlenecked populations from stable populations (Luikart, 1997). A graphical representation utilizing allelic class and proportion of alleles showed a clear deviation from normal L shaped distribution. This distribution clearly verified that the studied population has experienced a recent bottleneck.

CONCLUSIONS

The present research contributes to the knowledge of population structure and assessment of existing genetic diversity in the Iranian Caspian horse population. Further genetic analysis of other Iranian horse breeds and their comparisons need to be carried out to determine the phylogenic evolutionary relationships and genetic distances among the indigenous equine populations. The strong inference that the Caspian breed has undergone major bottlenecks is also important for equine conservationists and it is necessary to do urgent conservational strategies on this population.

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