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SHORT COMMUNICATION

Genetic characterization of the Bardigiano horse using microsatellite markers

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ABSTRACT

The study was aimed at investigating the genetic structure of the Bardigiano horse and its relationships with the Haflinger, Maremmano and Arabian breeds using 11 microsatellite markers. A total of 94 alleles were detected across the breeds, with a mean of 8.5 alleles per locus and a mean observed heterozygosity of 0.69. Compared to the other breeds, the Bardigiano horse showed quite a high genetic variability, as indicated by the mean number of alleles (7.0 *vs* 6.1÷7.6) and by the observed heterozygosity (0.72 *vs* 0.66÷0.71). Moreover, the genotype distributions in the Bardigiano groups of different sex and age were not significantly different. The overall F_{ST} value showed that the genetic differences among breeds accounted for 7.8% (P=0.001) of the total variation, and the pairwise F_{ST} values were all significant. The assignment test allocated between 96.8 and 98.9% of the individuals to the population they were collected from, with a mean probability of assignment of about 97% for all breeds, except for the Arabian, where it approached 100%.
The results have highlighted that the Bardigiano breed has a high within and between breed variability, which is considerably more than could be expected by looking at its evolution history. This justifies the need for the development of additional breeding strategies to preserve the existing genetic variability.

Key words: Horse, Bardigiano, Genetic characterization, Microsatellites.

RIASSUNTO

CARATTERIZZAZIONE GENETICA DEL CAVALLO BARDIGIANO MEDIANTE MARCATORI MICROSATELLITI

E' stata analizzata la struttura genetica del cavallo Bardigiano mediante 11 marcatori microsatelliti e ne è stata valutata la variabilità, anche in relazione alle razze Aveglinese, Maremmana e Araba. L'analisi ha evidenziato per la razza Bardigiana una soddisfacente variabilità, come indicato dai valori piuttosto elevati sia per numero di alleli (Na=7,0; Ne=3,6), che per grado di eterozigosi (Ho=0,72; He=0,71). Inoltre, nell'ambito della razza, non si sono rilevate differenze per questi parametri fra maschi e femmine, situazione che rappresenta un buon presupposto per il mantenimento della variabilità presente. Il confronto dei soggetti più giovani (nati dal 2003) con quelli più vecchi (nati entro il 1996) fa intravedere la tendenza, sia

pur non significativa, ad una riduzione di variabilità (Ho: 0,68 vs *0,73; He: 0,67* vs *0,69), il che suggerisce di porre particolare attenzione alla gestione genetica della razza. I valori di F_{ST} per le razze considerate a coppie sono risultati tutti significativi, evidenziando che il Bardigiano presenta una elevata originalità genetica, soprattutto rispetto all'Arabo, ma anche nei riguardi sia della razza Maremmana, di cui si riteneva fosse una "sotto-razza", sia dell'Aveglinese, utilizzata come razza incrociante nella prima metà del secolo scorso. La notevole differenziazione genetica ha permesso anche la corretta assegnazione di un'alta percentuale di individui alla razza di appartenenza (96,8%÷98,9%). La probabilità media di assegnazione è stata anch'essa molto elevata, collocandosi intorno al 97%, ad eccezione della razza Araba, per la quale si avvicina al 100%. Nel complesso, i risultati hanno messo in evidenza che la razza Bardigiana possiede una variabilità genetica entro e tra razze molto più elevata di quanto si potesse ritenere in base alla sua storia evolutiva, delineando una situazione favorevole per lo sviluppo di ulteriori programmi di miglioramento.*

Parole chiave: *Cavallo, Bardigiano, Caratterizzazione genetica, Microsatelliti.*

Introduction

The conservation of biodiversity still remains an important concern. Despite worldwide efforts following the Convention on Biological Diversity held in 1992, about one third of all livestock breeds are threatened by extinction (FAO, 2000). Horses are a component of global animal diversity and are suffering a progressive reduction of their genetic variability, just like other domestic species.

The Bardigiano horse (Cavallo Bardigiano, 2007) is one of the Italian autochthonous populations of limited size, classified in the subgroup of South European ponies and considered by some Authors as a variety of the Maremmana breed. In the early 20th century the Bardigiano breed underwent introgression, mainly from the Haflinger. By the end of the Second World War, the population size declined progressively to 5 stallions and 150 mares. In the 1970s a conservation programme was started with the establishment of the Studbook in 1977. At present the breed numbers about 3,240 head in Italy and a few dozen in Germany and Hungary, where national Associations were established in 2003. The selection is aimed at obtaining a taller and lighter horse, suitable for saddle and light draught. Since 1992 a grading-up programme with the Arabian horse has been implemented in order to improve the saddle purpose and the results are under evaluation.

Although the Bardigiano breed is no longer in danger of extinction, its limited population size suggests the need for adequate genetic programmes in order to avoid the risk of an excessive reduction of variability. The aim of this study is to describe the genetic structure of the Bardigiano horse and to compare it with breeds linked to its history, in order to evaluate its genetic differentiation and to provide tools for decision making.

Material and methods

The study was carried out on 103 purebred Bardigiano horses (48 females and 55 males), born in the period 1980-2005. Individuals from Arabian (155), Haflinger (104) and Maremmano (91) breeds were also included in the study. The animals of each breed had no parents in common.

DNA samples, extracted from blood or hair roots, were amplified in a single multiplex for eleven internationally recognized microsatellites (AHT4, AHT5, ASB2, HMS2, HMS3, HMS6, HMS7, HTG4, HTG7, HTG10, VHL20), as described in Blasi *et al.* (1999).

The Hardy-Weinberg equilibrium for each locus-breed combination and the linkage disequilibrium for loci pairs across

populations were tested with the FSTAT program (Goudet, 1995). Allele frequencies, as well as observed and expected heterozygosity were computed using the GENETIX package (Belkhir *et al.*, 1998). The observed and effective number of alleles per locus were estimated with the POPGENE software version 1.32 (Yeh *et al.*, 1999). The differences between breeds for the number of alleles and heterozygosity were evaluated using an F-test. The genotype distributions in the Bardigiano groups of different sex and age were compared using the GENEPOP software (Raymond and Rousset, 1995). F_{IS} statistics for each breed across loci were also computed with the FSTAT program (Goudet, 1995), testing the statistical significance with permutation tests. The between-breeds differentiation was estimated by the pairwise F_{ST}, using the GENETIX software (Belkhir *et al.*, 1998). In addition, both the Principal Component Analysis, with the PCA-GEN software (Goudet, 1995), applying 1000 randomization for testing, and the Factorial Correspondence Analysis, with the GENETIX package (Belkhir *et al.*, 1998), were performed. The GENECLASS2 software (Piry *et al.*, 2004) was employed for breed assignment, using the Bayesian option, which has been shown to perform better (Cornuet *et al.*, 1999).

Results and discussion

Microsatellite characteristics

Prior to other analyses, two basic assumptions were tested in order to verify the suitability of the considered markers for population studies: the independent assortment of the loci and their selective neutrality. For the first assumption, the markers included in the study were on different chromosomes, and therefore physically independent, except for HMS6 and HTG7, both on chromosome 4, and HMS3 and HTG4, both on chromosome 9. The test for linkage disequilibrium gave P values of 0.12 and 0.49 for the two pairs respectively, indicating that

Table 1. Descriptive statistics of the markers used.

Locus	Chr	Size range	Na	Ne	Ho	He
AHT4	24	148-168	10	5.1	0.79	0.80
AHT5	8	130-144	8	5.4	0.76	0.81
ASB2	15	222-256	14	5.5	0.68	0.82
HMS2	10	218-238	9	4.4	0.74	0.77
HMS3	9	152-170	8	4.4	0.70	0.77
HMS6	4	159-171	7	3.8	0.68	0.73
HMS7	1	173-185	7	4.8	0.67	0.79
HTG4	9	129-139	6	3.3	0.64	0.70
HTG7	4	120-130	5	2.1	0.50	0.52
HTG10	21	91-113	11	5.8	0.72	0.83
VHL20	30	89-107	9	7.8	0.75	0.87
Mean			8.5	4.7	0.69	0.77

Chr=chromosomal location; Na=number of observed alleles; Ne=number of effective alleles; Ho=observed heterozygosity; He=expected heterozygosity.

the concerned loci are not linked. For the selective neutrality assumption, the microsatellite loci in themselves are considered to be neutral, but, if linked to selected loci, might lead to biased estimates of the population structure. Selection can be seen by comparing the observed genotype distributions to the expected Hardy-Weinberg equilibrium. None of the deviations from the equilibrium, estimated for each locus-breed pair by using F_{IS} statistics, were significant, thereby suggesting that the 11 microsatellites were neutral markers. Based on these results, all the microsatellites were considered suitable for further analyses.

A total of 94 alleles were detected across breeds, with a mean of 8.5 alleles per locus (Table 1). The number of observed alleles at individual loci was highly variable, ranging from 5 (HTG7) to 14 (ASB2). Similarly, a high variability for the number of effective alleles and for its deviation from the number of observed alleles was found, reflecting differences in the allele distribution. The mean observed and expected heterozygosities were 0.69 (0.50÷0.79) and 0.77 (0.52÷0.87), respectively.

Breed structure

All the breeds were polymorphic for the analysed markers (allele frequencies available from the first Author) and exhibited a similar level of within-breed variability (Table 2). The only significant difference (P=0.036) was found in the observed number of alleles between the Haflinger and Maremmano. The F_{IS} values showed a significant deficiency of heterozygotes in the Arabian. This is not surprising, because, compared to other breeds, the Arabian horse has been subjected to a more intense selection, which has led to an increase in the inbreeding level. On the other hand, the low variability of the Arabian horse has already been shown in most studies, despite differences in type and number of markers used (Aberle *et al.*, 2004; Glowatzki-Mullis *et al.*, 2005; Marletta *et al*., 2006; Vega-Pla *et al.*, 2006).

The genetic structure of the Bardigiano breed was further investigated in relation to sex and age (Table 3). Females showed higher values for all the diversity parameters, but the differences were not significant, as confirmed by the analysis of the genotypic differentiation over loci (P=0.40). This result could depend on the fact that the number of males in the breed is not exceedingly low, representing about 7.8% of the breeding population (Cavallo Bardigiano, 2007). Considering the impact of males in determining the genetic structure of the following generations, this situation is a favourable condition for the maintenance of the existing variability. Similarly, the genotype distributions in animals of different age (born before 1996 and after 2003) did

Table 2. Within-breed variability.

Breed	Na	Ne	Ho	He	F_{IS}
Bardigiano	7.00 (1.41)	3.60 (0.90)	0.72 (0.08)	0.71 (0.08)	-0.017
Arabian	6.46 (1.81)	3.58 (0.96)	0.67 (0.11)	0.70 (0.11)	0.045*
Haflinger	6.09 (1.51)	3.64 (1.03)	0.69 (0.09)	0.71 (0.09)	0.027
Maremmano	7.64 (1.91)	3.98 (1.04)	0.72 (0.10)	0.73 (0.08)	0.027

Na=number of observed alleles; Ne=number of effective alleles; Ho=observed heterozygosity; He=expected heterozygosity. Standard deviation in brackets.
**: P<0.05.*

Table 3. Bardigiano variability according to sex and age.

Factor	Groups	Na	Ne	Ho	He
Sex	Males	6.4 (1.3)	3.5 (0.9)	0.69 (0.08)	0.70 (0.08)
	Females	6.5 (1.5)	3.7 (1.0)	0.75 (0.10)	0.71 (0.09)
Age	Born before 1996	5.6 (1.5)	3.6 (1.2)	0.73 (0.13)	0.69 (0.11)
	Born after 2003	5.8 (1.4)	3.3 (1.1)	0.68 (0.11)	0.67 (0.10)

Na=number of observed alleles; Ne=number of effective alleles; Ho=observed heterozygosity; He=expected heterozygosity. Standard deviation in brackets.

not show significant differences (P=0.21), but a trend was observed towards a reduction of variability, despite the short period considered. This finding is far from critical, but should be taken into consideration in future breeding plans, in the light of genealogical data results, which indicate a progressive increase of the inbreeding level in the breed (Sabbioni *et al.*, 2005).

Breed differentiation

The analysis of the genetic subdivision showed that the genetic differences between breeds accounted for about 8% of the total variation (F_{ST}=0.078; P=0.001). This estimate is identical to those reported by Cañón *et al.* (2000) and Marletta *et al.* (2006), who studied Spanish Celtic breeds and western Mediterranean breeds with a set of markers similar to that used in the present study, but lower than the F_{ST} values obtained with different markers in other studies on European breeds (Aberle *et al.*, 2004; Glowatzki-Mullis *et al.*, 2005; Vega-Pla *et al.*, 2006).

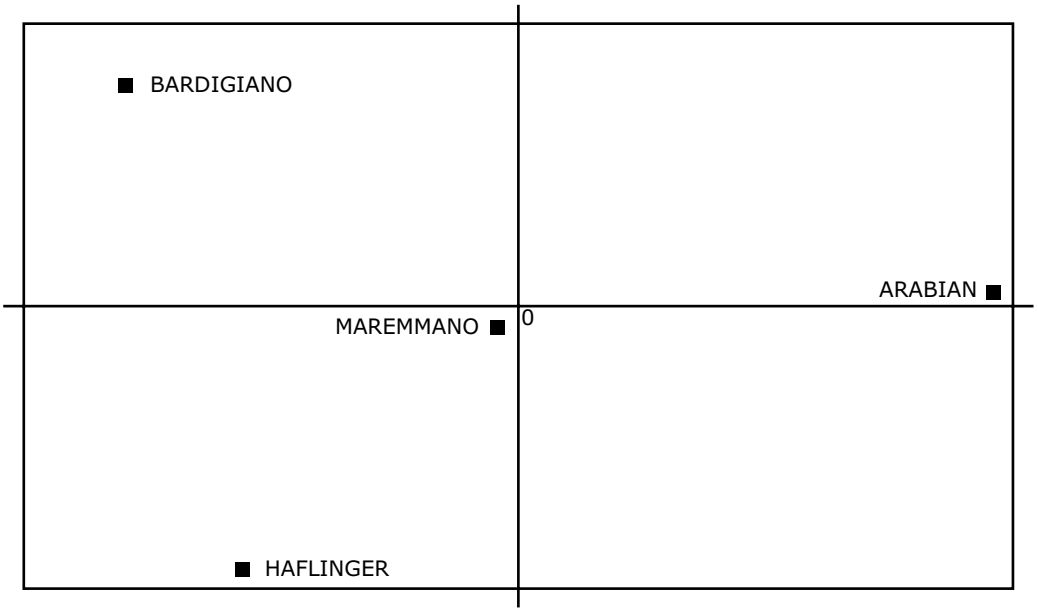
The pairwise F_{ST} values were significant for all the comparisons (P<0.01) and indicate that the Bardigiano breed has a high genetic originality, not only with respect to the Arabian breed (F_{ST}=0.1370), but also with respect to the Maremmano (F_{ST}=0.0748) and Haflinger (F_{ST}=0.0735) breeds. This result is rather unexpected, considering the relatively short evolution time, the small geographic scale and the historical relationships among these breeds. The establishment of the Herdbook, which limited the gene flow between the Bardigiano and the other breeds, is likely to have contributed to the present genetic differentiation.

The Principal Component Analysis (PCA), based on F_{ST} statistics, graphically illustrates the relationships among breeds, by condensing the information from a large number of alleles and loci into a few synthetic variables (Figure 1). The first two dimensions accounted for 61% and 23% of the genetic variance, respectively. The Bardigiano breed was significantly separated from the Arabian (P<0.01), which supports the hypothesis of their reproductive isolation; it was also differentiated from the Haflinger and Maremmano breeds, but to a lesser extent, suggesting a possible common origin or past genetic admixture. The results of the Factorial Correspondence Analysis (not reported), which shows the individuals as a cloud of points in a metric space, confirmed the results of the PCA and highlighted little overlapping of individuals belonging to different breeds.

Breed assignment

For each individual the probability of assignment to each sampled breed was calculated. Between 96.8 and 98.9% of the individuals were assigned to the population they were collected from, with a mean probability of assignment of about 97% for all breeds, except for Arabian, where it approa-

Figure 1. Principal Component Analysis.



ched 100% (Table 4). Probabilities of correct assignment of 62% to 97% were reported for different horse breeds tested with different sets of markers (Cañón *et al.*, 2000; Bjørnstad and Røed, 2001; Achmann *et al.*, 2004). The high precision obtained in the present study is likely to depend on the number of markers used, but even more so, on the high level of genetic differentiation among breeds. In fact, Bjørnstad and Røed (2002) demonstrated that the assignment precision is highly correlated with F_{ST}; also the number of loci correlates with the assignment performance, even though it becomes a less stringent factor when at least 10 loci are scored. The relatively high number of individuals sampled per breed might have contributed to the precision of assignment, but only to a certain extent, because the sample size was shown not to be a critical factor, as long as moderately large sample sizes (≥20 animals per population) are used (Bjørnstad and Røed, 2002).

It is interesting to highlight that the two Bardigiano individuals incorrectly assigned were attributed to the Haflinger and Ma-

Table 4. Breed assignment.

Breed	Individuals correctly assigned (%)	Mean probability of assignment (%)	Misassignment to
Bardigiano	98.1	97.2	Haflinger / Maremmano
Arabian	96.8	99.7	Maremmano
Haflinger	98.1	97.1	Bardigiano
Maremmano	98.9	97.5	Arabian

remmano, while two Haflinger individuals were attributed to the Bardigiano, consistent with historical information. Reciprocal incorrect assignments were obtained for Arabian and Maremmano.

The possibility to determine the breed of origin of an individual on the basis of its genotype is of practical interest in conservation genetics, where it can be more important to exclude a given population than to designate the most likely one, in order to avoid introgression of unwanted genes.

Conclusions

The results have revealed that the Bardigiano horse has quite a high genetic variability, as shown by the allele number and heterozygosity level. Moreover, the similar genotype distributions in males and females can positively contribute to the maintenance of the existing variability. On the other hand, the observed trend towards a reduction of variability suggests the need for a careful genetic management of the population in order to avoid the risk of an excessive increase in the inbreeding level. Unexpectedly, the Bardigiano breed exhibits a significant genetic differentiation from the Haflinger and Maremmano breeds, considered to be closely related.

The overall results highlight a favourable situation to implement further programmes of valorisation and genetic improvement of the Bardigiano horse, and provide tools for adopting adequate breeding strategies aimed at preserving its genetic variability.

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