



The Pentro horse: genetic characterization by microsatellite markers

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ABSTRACT

The Pentro horse population is an autochthonous breed from a breeding area characterized by climatic and geographic peculiarities. The horse population is interesting because of its rusticity, however, has run the risk of loosing its identity because of the introduction of exotic genetic material. This study presents data of the genetic characterization by means of microsatellite markers. A total of 12 microsatellite loci were used to score 147 individuals among those with the Pentro phenotype, as well as 16 individuals representing the variable part of the population. Allele frequencies were calculated for each locus, with the mean number of alleles ($N_{all} = 6.7$) and the expected heterozygosity ($H_e = 0.724$). The mean observed heterozygosity was 0.695. The obtained data were used to compare the genetic structure of the Pentro horse to six other Italian breeds. The N-J tree computed on individual genetic distances showed that 93% of Pentro horses clustered together.

Key words: Pentro horse, Microsatellite, Genetic biodiversity, Genetic distances.

RIASSUNTO

IL CAVALLO PENTRO: CARATTERIZZAZIONE GENETICA CON MARCATORI MICROSATELLITI

La popolazione equina denominata "cavallo Pentro" è un tipo genetico autoctono la cui area di allevamento mostra delle peculiarità climatiche e geografiche. Questa popolazione equina è interessante per la sua rusticità ma è stata a rischio di perdere la sua identità genetica a causa dell'introduzione di materiale genetico esotico. In questo lavoro vengono presentati i risultati della caratterizzazione genetica della popolazione utilizzando i marcatori molecolari microsatelliti. Per l'analisi sono stati utilizzati 12 loci microsatelliti e sono stati tipizzati 147 individui che presentavano morfologia "Pentro" più 16 individui a morfologia "non Pentro" che rappresentavano la parte variabile della popolazione. Sono state calcolate le frequenze alleliche ad ogni locus, il numero medio di alleli ($N_{all} = 6.7$) e l'eterozigotà attesa ($H_e = 0.724$). L'eterozigotà media osservata è di 0.695. I dati ottenuti sono stati utilizzati per confrontare il Pentro con altre sei razze Italiane. Il diagramma N-J costruito sulla base delle distanze genetiche individuali mostra che il 93% degli individui Pentro formano un unico gruppo.

Parole chiave: Cavallo Pentro, Microsatellite, Biodiversità genetica, Distanze genetiche.

Introduction

The remains of an autochthonous horse population called "Cavallo Pentro" is bred in the wild in the mountainous area of the Molise region in Italy.

The population today numbers about 250 heads among which only approximately 150 individuals show the morphological traits of the original Pentro horse (Miraglia *et al.*, 2001), well adapted to the difficult environment of the "Pantano della Zittola", its breeding area. This is a wide plain of about 2200 hectares, located along the border of the Abruzzo National Park in the mountainous territory between the regions of Abruzzo and Molise. Due to its climatic and geographic peculiarities, the Pantano is considered an ecological niche where some rare vegetal species can be found (Lucchese *et al.*, 1995).

Living conditions in this area are very difficult because of the harsh climate; the winter is cold with floods and abundant snow while the summer is very dry. Parasites are among the principal causes of death during this season. Moreover, the presence of predators, mainly wolves and bears from the nearby National Park, represent a constant danger for the livestock.

For a long time the population has been left completely free to graze, to mate and to defend itself from predators' attacks. It is only during the spring season that the breeders collect some of the colts to be sold for meat.

The origin of this population can be inferred relying only on hypothesis and indirect evidence. In any case, it is reasonable to attribute the first introduction to some Berber individuals. In recent decades, because of the disappearance of traditional breeding practices, the breeding of the Pentro horse has been abandoned.

During the 1920s and 30s stallions of different origin were introduced from breeds such as Murgese, Maremmano, TPR (Tiro Pesante Rapido), Appaloosa and others, partially altering the morphological and genetic characters of the Pentro population.

Because of its peculiar adaptive ability, the "Pentro horse" must be considered a genetic resource which needs to be preserved. Moreover,

the horse pasture is very important for the ecological equilibrium of the area.

Compared with other economically important livestock species, only a few papers have been published on horses regarding biological diversity and ecological research approach, and most of them used biochemical markers such as blood groups by Cothran *et al.* (2001). The genetic structure of Spanish Celtic horses was studied by means of microsatellite markers (Canon *et al.*, 2000), and the Italian breed Maremmano has been characterized by Ceriotti (1998).

In this paper a genetic characterization of the "Pentro horse" by means of microsatellite markers is presented. The aim of the work is the analysis of biodiversity and the relationship of "Pentro horse" versus other horse breeds.

Material and methods

Sample collection

During the summer season in the years 2000 and 2001, a census of the population was undertaken (Miraglia *et al.*, 2001). Morphological and genealogical data were collected and blood samples were taken from a total of 163 individuals, 147 of which belonging to the group "Pentro" and representing the total of the mating individuals in this group, and 16 belonging to the group "Other" as a sample of the morphologically variable part of the population. In addition, 6 Italian horse breeds (Maremmano - 40 individuals, TPR - 42 individuals, Murgese-16 individuals, Haflinger - 39 individuals, Trottatore - 40 individuals and Bardigiano - 41 individuals) were included as a comparison.

DNA samples were obtained from lymphocytes with the classical phenol-chloroform extraction protocol (Sambrook *et al.*, 1989).

Microsatellite analysis

Twelve microsatellite loci (HTG10, VHL20, HTG7, HTG4, AHT5, AHT4, HMS3, HMS6, HMS7, LEX003, HMS2, ASB2) were amplified with a protocol of multiplex PCR (Blasi *et al.*, 1999). For each locus the chromosomal location, the primer reference, number of alleles and size range in the studied populations are reported in Table 1. Genotypes were scored with an ABI 377 automatic sequencer,

and the GENESCAN and GENOTYPER computer packages.

Data analysis

Allelic frequencies and number of alleles for each locus were calculated for each population with the GENEPOP ver. 3.1d computer package (Raymond *et al.*, 1995). Hardy-Weinberg expectations were computed using the program ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000). Average observed and expected heterozygosities and number of alleles for each breed were comput-

ed with the Microsatellite toolkit (Park *et al.*, 2001). Statistical significance of differences among mean observed heterozygosities of breeds were calculated using the Student t procedure.

The locus LEX003 for its location on the X chromosome was not included in the calculation of average heterozygosities and number of alleles because it does not have paternal allele.

Genetic distance by Nei (1987) was used for the breed comparison, while distances among individuals were calculated as the proportion of shared alleles, (Dps=1-Ps) according to Bowcock *et*

Table 1. References, chromosomal location, number of alleles and size range for the 12 microsatellite loci.

| Locus | Reference | Chromosome | N. alleles | Size range (bp) |
|-------|-----------------------------------|------------|------------|-----------------|
| HTG10 | Marklund <i>et al.</i> , 1994 | 21 | 14 | 88-116 |
| VHL20 | Van Haringen <i>et al.</i> , 1992 | 30 | 11 | 87-107 |
| HTG7 | Marklund <i>et al.</i> , 1994 | 4 | 9 | 114-132 |
| HTG4 | Ellegen <i>et al.</i> , 1992 | 9 | 8 | 125-139 |
| AHT5 | Binns <i>et al.</i> , 1995 | 6 | 10 | 127-145 |
| AHT4 | Goddard <i>et al.</i> , 1998 | 24 | 10 | 146-164 |
| HMS3 | Guerin <i>et al.</i> , 1994 | 9 | 9 | 152-170 |
| HMS6 | Guerin <i>et al.</i> , 1994 | 4 | 8 | 157-171 |
| HMS7 | Guerin <i>et al.</i> , 1994 | 1 | 7 | 173-185 |
| LEX03 | Coogle <i>et al.</i> , 1996 | X | 12 | 195-217 |
| HMS2 | Guerin <i>et al.</i> , 1994 | 10 | 13 | 215-239 |
| ASB2 | Breen <i>et al.</i> , 1997 | 15 | 15 | 219-255 |

Table 2. Number of heterozygous observed and expected, p values and standard deviation for each locus in the two subsamples Pentro and Other.

| Locus | Pentro | | | | Other | | | |
|-------|---------|---------|---------|--------|---------|---------|-------|-------|
| | Obs. He | Exp. He | P | SD | Obs. He | Exp. He | P | SD |
| HTG10 | 0.584 | 0.736 | <0.001* | <0.001 | 0.625 | 0.833 | 0.044 | 0.001 |
| VHL20 | 0.698 | 0.842 | <0.001* | <0.001 | 0.750 | 0.855 | 0.290 | 0.002 |
| HTG7 | 0.635 | 0.732 | <0.001* | <0.001 | 0.625 | 0.690 | 0.426 | 0.005 |
| HTG4 | 0.584 | 0.591 | 0.094 | 0.002 | 0.750 | 0.665 | 0.926 | 0.001 |
| AHT5 | 0.886 | 0.804 | 0.001* | <0.001 | 0.688 | 0.889 | 0.051 | 0.001 |
| AHT4 | 0.594 | 0.696 | 0.012* | 0.001 | 0.875 | 0.744 | 0.487 | 0.004 |
| HMS3 | 0.667 | 0.724 | 0.071 | 0.003 | 0.750 | 0.738 | 0.660 | 0.003 |
| HMS6 | 0.742 | 0.721 | 0.037* | 0.002 | 0.688 | 0.847 | 0.148 | 0.003 |
| HMS7 | 0.710 | 0.757 | <0.001* | <0.001 | 0.875 | 0.738 | 0.822 | 0.004 |
| LEX03 | 0.726 | 0.768 | <0.001* | <0.001 | 0.750 | 0.762 | 0.345 | 0.003 |
| HMS2 | 0.683 | 0.786 | <0.001* | <0.001 | 0.750 | 0.802 | 0.383 | 0.003 |
| ASB2 | 0.782 | 0.805 | <0.001* | <0.001 | 0.688 | 0.861 | 0.105 | 0.001 |

* Significant P values.

al. (1994) using the MICROSAT program (Minch *et al.*, 1995). In the breed comparison the “Pentro” and the “Other” group were considered as a single population. Neighbour-joining diagrams (Saitou *et al.*, 1987) were constructed on genetic distances using the PHYLIP package ver. 3.5 c (Felsenstein, 1993). The program TreeView (Page, 1996) was used to visualize the diagrams.

Variability levels within and between breeds were estimated using the F statistics according Weir and Cockerman (1984) using the Fstat program (Goudet, 1995). The two groups composing the Pentro population were analyzed separately in order to check whether any difference in the genetic variability could be detected between the morphologically uniform part of the population and the group of heterogeneous individuals.

Deviation from Hardy-Weinberg equilibrium were tested across loci for the two groups.

The Fst index between the “Pentro” and the “Other” groups was also calculated in order to check an eventual subdivision of the population into the two subgroups.

Results and discussion

Genetic variability of the Pentro horse population

All the loci were well amplified and they were also polymorphic; the total number of observed alleles was 104 in the entire Pentro population; the average number of alleles per locus is 8.6. The lower number of alleles is 6 and is found for the

locus HMS7. The higher number of alleles is 11, and is found for the loci VHL20, HMS2 and ASB2.

The “Pentro” group resulted to be significantly ($P < 0.001$) in disequilibrium for 10 loci out of 12, while the “Other” had no significant disequilibrium. The number of heterozygous observed and expected and the P values are reported in Table 2.

The probability test for the heterozygous deficit confirmed these findings, for the “Pentro” group where a 5% significant value was observed in 10 out of the 12 loci.

The Fst value calculated between “Pentro” and “Other” was 0.0186.

Breed comparison

Average heterozygosity values and number of alleles are reported in Table 3 for each breed. Differences between the observed mean heterozygosities are not significant. This result indicates that the level of genetic homogeneity among individuals in the Pentro population is comparable with the levels observed for standardized breeds.

In order to test whether the “Pentro” group constitutes a separate unit in the population, genetic distances among single individuals were calculated as the proportion of shared alleles and used to build a Neighbour-Joining diagram. Data obtained with the same marker set for 6 Italian standardized breeds have been included in the analysis.

This method, proposed by Bowcock *et al.* (1994) for the study of human populations was recently used on horses as a statistical test for breed

Table 3. Number of individuals, mean heterozygosity values, mean number of alleles per locus and relative SD for the six horse breeds and for the Pentro population.

| Population | Sample size | N. alleles | SD | Average heterozygosity | | | |
|------------|-------------|------------|-----|------------------------|-------|----------|-------|
| | | | | expected | SD | observed | SD |
| Pentro | 132 | 8.6 | 1.9 | 0.754 | 0.019 | 0.693 | 0.012 |
| Maremmano | 40 | 7.4 | 2.3 | 0.729 | 0.022 | 0.727 | 0.021 |
| TPR | 42 | 6.3 | 1.4 | 0.727 | 0.021 | 0.755 | 0.020 |
| Haflinger | 39 | 6.5 | 1.5 | 0.681 | 0.034 | 0.661 | 0.023 |
| Bardigiano | 40 | 6.5 | 1.6 | 0.710 | 0.027 | 0.730 | 0.021 |
| Murgese | 16 | 5.8 | 1.3 | 0.748 | 0.020 | 0.716 | 0.034 |
| Trottatore | 40 | 5.6 | 1.1 | 0.717 | 0.031 | 0.684 | 0.022 |
| Average | | 6.7 | | 0.724 | | 0.709 | |

assignment (Bjornstad *et al.*, 2001). Bjornstad's study (2001) shows how 10 microsatellite markers are enough to give a result of 75% in terms of correct assignment while raising the number of markers up to 20 increases the successful assignment to 95%.

The tree shows a high degree of structuring of the breeds (Figure 1). On the total number of individuals included only 59 do not group exactly in the breed of origin. The Pentro horses group together in 93% of the cases, and no subgrouping of the individuals showing the morphological traits of the Pentro horse with respect to the group "Other" is observed. For the other breeds the percentage of individual clustering together are: TPR 90%, Trottatore and Bardigiano 85%, Haflinger 87%. Maremmano and Murgese are the less defined groups with only 47% and 56%, respectively.

A further investigation of the genetic structure of the population gave a value of population subdivision of 10.5% (F_{st} value is 0.105), repeating the analysis and excluding the Pentro population this value decreases to 9.6% (F_{st} value is 0,096).

The genetic correlation between the Pentro horse and three related breeds (Maremmano, Murgese and TPR) was analyzed and compared with more distant breeds (Haflinger, Bardigiano, and Trottatore). The NJ diagrams constructed on the Nei's genetic distance and using 500 bootstrap values, give a picture that is in accordance with historic records (Figure 2). The Pentro horse results to be closely related to the TPR and Murgese (bootstrap value 235/500), as expected if taking into account the recent introduction of individuals from those breeds in the population.

Discussion

The data reported in this work show how the Pentro horse population living in the "Pantano della Zittola" is the result of the adaptation to a difficult environment.

The condition of strong HW disequilibrium found for the majority of the loci analyzed (P value is statistically significant for 10 out of 12), is probably due to the small population size. The hypothesis of a Wahlund effect in order to justify the deviations from HW equilibrium seems not to be reliable by the fact that mating is free and not

planned by the breeders; obviously since the low number of individuals mating between relatives occurs very frequently. The F_{st} value calculated between "Pentro" and "Other" is 0.0186 indicating that there is no genetic differentiation between the two groups despite their morphological differences. The NJ diagram of single individuals genetic distances, where all the individuals assigned to the group "Other" are well dispersed the population, also confirms this finding.

Regarding the allele sharing distance among single individuals, all the breeds display a good level of correct clustering (Figure 1). The only exceptions are Maremmano and Murgese. In both the cases this is probably the effect of recent cross breeding with other breeds.

In the NJ diagram of populations, Nei's genetic distances display a pattern in accordance with historic records for each breed. The Pentro horse results closely related to the TPR, which were mainly used for introduction in the population. The Maremmano breed was also used in the last decades for cross breeding in the Pentro population, nevertheless it results to be located on the NJ diagram far away from the Pentro. This could be due to the weak uniformity of this breed which, in the same way, has had many different breeds (English pure-bred, Hackney and Arab) introduced (Gandini *et al.*, 1996). The Maremmano is also the breed showing the lowest level of correct assignment in the individuals NJ diagram (47%).

These results confirm the hypothesis of the Pentro horse being a genetic unit well differentiated from all the breeds analyzed in this work. This result is even more interesting if the introduction of genetic material from some of those breeds in the Pentro population is taken into account.

The Pentro horse, despite all the foreign genetic material which has been introduced, has conserved its uniqueness. This introduction did not cause a total fading of the morphological characteristics of the original population.

Conclusions

These results demonstrate that the Pentro horse population, despite the numerous influxes from others breeds, shows a similar degree of

Figure 1. N-J diagram of distances among single individuals constructed using the proportion of shared alleles.

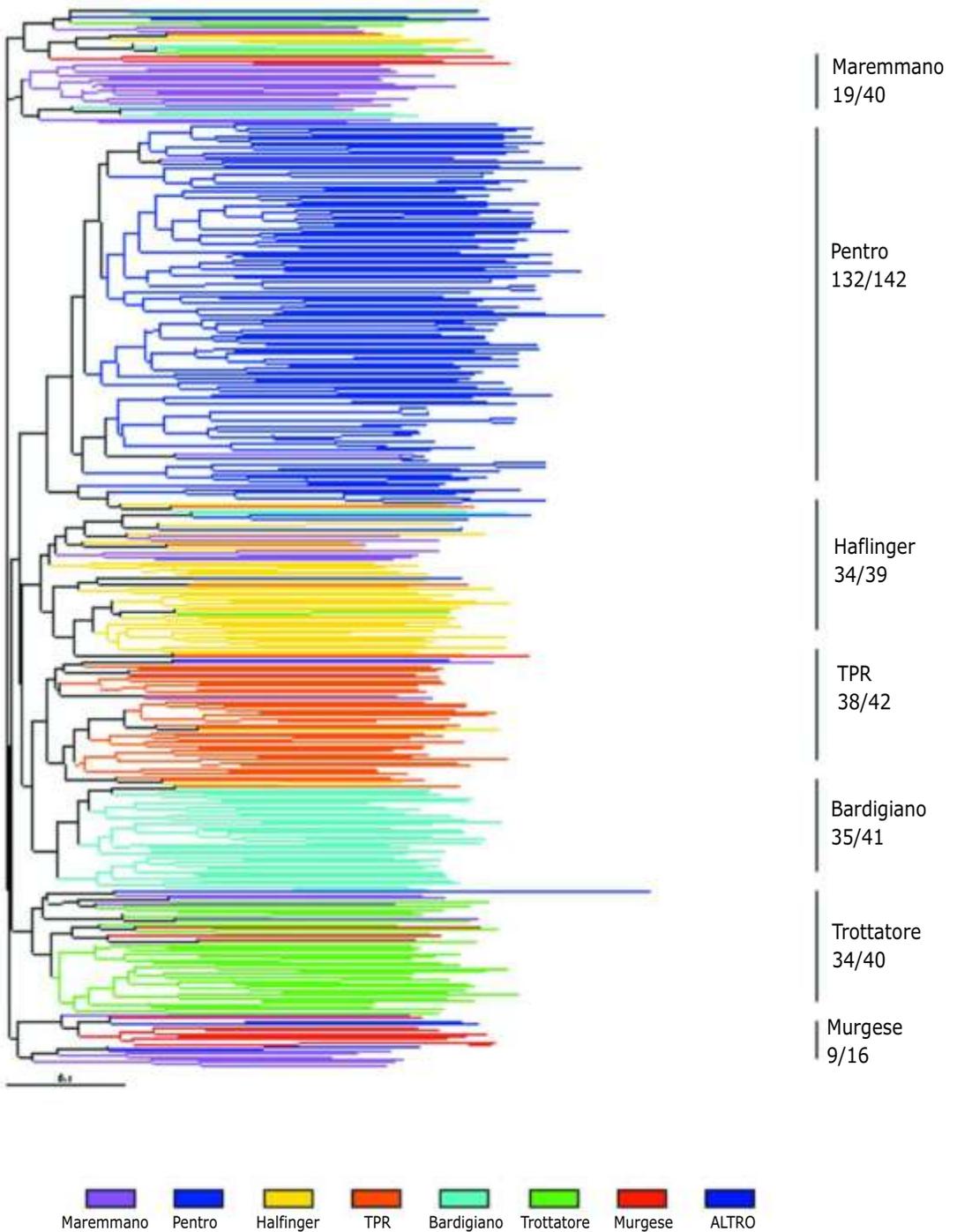
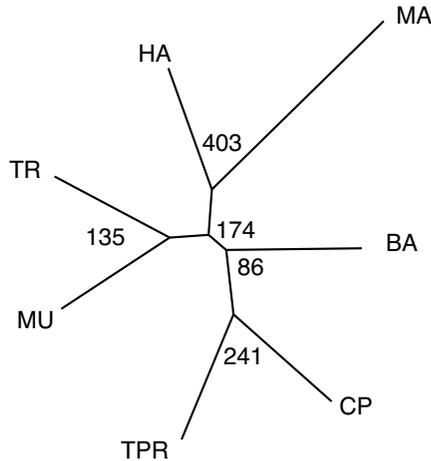


Figure 2. N-J tree between populations constructed using the Nei's distance. The bootstrap percentages are reported.



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HA= Haflinger; MA= Maremmano; BA=Bardigiano; CP=Cavallo Pentro; TPR=Tiro Pesante Rapido; MU=Murgese; TR=Trotatore.

genetic uniformity and identity compared to the other standardized breeds. This population constitutes a defined cluster and is differentiated from the other populations analyzed in this work. Interbreeding is a common practice between the breeders and it is used to modify the characteristics of breeds according to the needs and also to expand the size of a population when it is becoming too small. This practice is useful in a sense to preserve from excessive inbreeding, but it is also deleterious where small endangered populations are concerned. In this paper the case of the Pentro horse is presented, but other breeds seem to suffer from problems of this nature.

The uniqueness of the Pentro horse resides in its adaptive ability, which preserved it from complete "extinction" through mixing with other breeds. The preservation of not only a natural, but also a social and cultural environment, is essential for the conservation of livestock genetic resources.

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