Intra-line and inter-line genetic diversity in sire lines of the Old Kladruber horse based on microsatellite analysis of DNA

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ABSTRACT: The Old Kladruber horse is the oldest original Czech horse breed – gene resource, with pedigree records spanning three centuries. Because the population is closed, there is a concern about the loss of genetic variation. The genetic diversity within and amongst sire lines was evaluated using microsatellite markers and based on pedigree information. The DNA analysis, covering 16 microsatellite loci, revealed that the genetic diversity (observed heterozygosity = 0.637, genetic diversity = 0.678 and the mean number of alleles = 8.25) for the Old Kladruber horse is consistent with other populations of horses. Inter-line genetic difference in sire lines reached the mean value (the F_{ST} values ranged from 0.020 to 0.017). Differences amongst the sire lines were identified using genetic distances and principal component analysis. One gene cluster comprised black variation sire lines, while the second cluster included the sire lines of the grey variation. In the subjects monitored, the average inbreeding coefficient of 0.076 was estimated on the basis of pedigree information. The highest mean values of the coefficient of relationship were estimated within the colour variations. When comparing the genetic distance and the average coefficient of relationship, differences were found out. These results are useful for the development of breeding strategies which consider classical horse breeding as well as recent achievements of population and conservation genetics.

Keywords: genetic variability; genetic structure; conservation; microsatellite DNA; Kladruber horse; line

The Old Kladruber horse is the only original Czech horse breed. It is an important genetic resource with unique properties and high cultural and historic value. The Old Kladruber horse, which ranks amongst endangered breeds, with breeding mares numbering around 1000 individuals, can honestly claim a lineage in the Czech Republic going back more than four hundred years. The origin of this breed is linked to the foundation of a stud farm in Kladruby nad Labem in 1579 with the intention of producing high-quality Old Spanish blood-descendant horses for the needs of the Habsburg imperial court (Bílek, 1957). Initially, the permanent importing of Old Spanish horses from dependable European farms, in particular those of Italy, took place in order to refresh the genetic base of the stock. However, the Old Spanish horse began to lose popularity at the turn of the 18th and 19th centuries, and subsequently the stocks of such animals in Europe largely disappeared. Since then, the

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breeding of the horses of the Italo-Spanish type has remained exclusive to Kladruby, i.e. without introducing new blood from other breeds. Since this horse was the only significant carrier of the Italo-Spanish bloodline, other than the Lipizzaner, it has since been recognized as a separate breed called the Old Kladruber horse.

At present, it is a warm-blooded large-frame breed of a gala-carriage type, preserving to this day its typically baroque-era exterior. The breed is now kept by the National Stud in two colour varieties – the grey horse and the black horse.

Evaluating genetic diversity and relationship within and amongst populations of animals is a prerequisite for developing meaningful breeding programmes. Microsatellite DNA markers, thanks to their naturally high polymorphism, can be used to analyse genetic diversity among different breeds of livestock species (Børstand et al., 2000; Canon et al., 2000; Achmann et al., 2004; Aberle et al., 2004; Solis et al., 2005; Behl et al., 2007; Bao et al., 2009; Chen et al., 2009; Kusza et al., 2009; Zrůstová et al., 2009). Molecular genetic analyses of the Old Kladruber horse using DNA polymorphism and other polymorphic systems were conducted by Hořín et al. (1998). Some authors have demonstrated the use of microsatellites to show that individuals belong to a particular population or sub-population (Rannala and Mountain, 1997; Cornuet et al., 1999). Baumung and Sölkner (2003) verified that the analysis of microsatellite markers and analysis on the basis of lineage information can be compared in a simulated population.

The objective of this study is to determine the extent of intra-line and inter-line genetic variation and similarity in sire lines (hereinafter referred to as lines) of the Old Kladruber horse, based on microsatellite markers and pedigree information with regard to inbreeding coefficient. Another objective is to determine whether it is possible to determine the lineage connection of an individual based on the knowledge of that individual's microsatellite profile.

MATERIAL AND METHODS

Determining inter-line relationship using microsatellite markers

Blood samples were collected randomly from 324 Old Kladruber horses within a 10-year period (1990–2000), each time from about 10% of individuals from each line. Genomic DNA was isolated from whole blood using the NucleoSpin Blood Kit (Clontech Laboratories, Palo Alto, USA). Genotyping included 16 microsatellite loci (AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, LEX3 and VHL20) spread over at 16 chromosomes.

Microsatellite analysis

PCR amplifications were performed in multiplex reactions using 7.5 µl reaction volumes with 80 to 100 ng of template DNA, 1.25 µl of reaction buffer (10× Stockmarks Buffer), 2 µl of dNTP mix (1.25 mmol/l), 2 µl of Primer mix (20 nmol/l) and 1.25 IU of Taq Gold polymerase. These components were taken from the StockMarks for Horses Equine Genotyping Kit (Applied Biosystems, Foster City, USA). The reactions were carried out in the TGradient 96 thermal cycler (Whatman Biometra, Göttingen, Germany) using initial denaturation over 10 min at 95°C, followed by 31 cycles of 30 s at 95°C, 30 s at 60°C and 60 s at 72°C. The final exposure at 72°C was prolonged to 60 min. PCR products were diluted with 7.5 µl of water. The analysis of PCR products and allelic size computations were performed using ABI PRISM 310 (Applied Biosystems) and GeneScan analysis software.

Statistical analysis

The Old Kladruber horse lines, including the year of birth of the line founder, are described in Table 1.

Allele frequencies, observed heterozygosity, genetic diversity [expected heterozygosity assuming the Hardy-Weinberg equilibrium (HWE)], Hardy-Weinberg equilibrium test and genetic distances were estimated across the different loci and lines using the TFPGA 1.3 software package (Miller, 1997). This programme creates a standardised matrix of genetic distances corrected for a small number of animals (Nei, 1978). The cluster analysis, conducted using the UPGMA discriminatory method, was expressed as a dendrogram using bootstrap analysis. The dendrogram robustness was tested using 1000 bootstrap samples. The UPGMA algorithm was used due to its greater suitability, in comparison with the near-neighbour algorithm, in the presence of the migration of individuals amongst

Table 1. Name, colour variation, year of birth, breed and country of origin of the Old Kladruber horse line founder

Name	Colour	Born	Breed	Origin
Generale	grey	1787	Old Kladruber	Czech Republic
Generale-Generalissimus	grey	1938	Old Kladruber	Slovakia
Favory	grey	1779	Old Kladruber	Slovakia
Favory-Generalissimus	grey	1965	Old Kladruber	Czech Republic
Sacramoso	grey/black	1800	Italo-Spanish	Italy
Solo	black	1927	Old Kladruber	Czech Republic
Siglavi Pakra	black	1946	Lipizzaner	Slovenia
Romke	black	1966	Friesian	Netherlands
Rudolfo	grey	1968	Lusitanian	Portugal

the lines or families (Achmann et al., 2004). Intra-line and inter-line genetic differences in the lines studied were detected by fixation coefficients (F_{IS}, F_{ST}, and F_{IT}) estimated by the FSTAT programme (Gaudet, 2001) according to Weir and Cockerham (1984) as follows:

$$F_{IS} = \frac{H_s - H_I}{H_S} = \frac{\sigma_a^2 + \sigma_b^2}{\sigma_a^2 + \sigma_b^2 + \sigma_w^2}$$

$$F_{TS} = \frac{H_T - H_S}{H_T} = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_b^2 + \sigma_w^2}$$

$$F_{IT} = \frac{H_T - H_I}{H_T} = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_w^2}$$

where:

H_I = heterozygosity of the individual within the population

H_S = expected heterozygosity of the individual within the subpopulation (sire lines)

H_T = expected heterozygosity of the individual within the entire population

σ_a^2 = among sample variance component

σ_b^2 = between individual within the sample component

σ_w^2 = within individual component

Numbers of migrants per population (N_m) were estimated from the following relationship (Wright, 1969):

$$N_m = \frac{1 - F_{ST}}{4F_{ST}}$$

To be able to employ microsatellites for assigning individuals to the respective lines, an assignment test was used. For the test of assignment of individuals to the respective lines, the simulation method according to Rannala and Mountain (1997) was employed, which evaluates, through the Bayesian approach, the probability of correct assignment (P-value) of each individual to the line. The GENECLASS 2 programme (Piry, 2004) was used for the assignment test. Furthermore, principal component analysis (PCA) was used to allow for the assignment of individuals to the correct line. For principal component analysis, the GENETIX software package was used (Belkhir et al., 2001).

Determining the inter-line genetic relationship using pedigree information

The pedigree file for the 324 individuals included in the analysis comprised 5 generations of ancestors. Pedigree information was used to estimate the inbreeding coefficient (F_X) and coefficient of relationship (R_{XY}).

The completeness of the pedigree, which is of great importance for estimating the F_X and R_{XY} components, was evaluated according to Cassell et al. (2003):

$$cp = \frac{a_k}{\sum_{i=1}^{g} 2^i}$$

where:

cp = coefficient of pedigree completeness

a_k = number of known ancestors in g generations

Based on the pedigree, a relationship matrix was compiled, ranking from the oldest to the youngest subjects. An individual's inbreeding coefficient (F_X) was estimated as the coancestry coefficient (Falconer and Mackay, 1996).

$$F_X = f_{ZY} = 0.25\ (f_{AC} + f_{AD} + f_{BC} + f_{BD})$$

where:

f = coancestry coefficient between two individuals, such as X and Y, with A and B representing the parents of individual X, while C and D represent the parents of individual Y

Like the inbreeding coefficient (F_x), the coefficient of relationship (R_{XY}) between two individuals was estimated (Falconer and Mackay, 1996):

$$R_{XY} = 2f_{XY}$$

To estimate the coefficients of inbreeding and relationship, the INBREED procedure of the SAS software package was employed (SAS, 2005).

The rate of genetic similarity based on the pedigree information was evaluated through the cluster analysis method using the VARCLUS procedure of the SAS software package (SAS, 2005). Input data consisted of mean values of the inter-line coefficient of relationship.

RESULTS AND DISCUSSION

Determining the inter-line relationship using microsatellite markers

The total number of alleles detected at 16 microsatellite loci in the Old Kladruber horse was 132. The average number of alleles per microsatellite locus was 8.25 with a range of 4 to 14. The estimated average for the observed heterozygosity across microsatellite loci was 0.637, while the estimated mean value of genetic diversity was 0.678. The heterozygosity observed for each of the microsatellites ranged from 0.374 for the HTG6 microsatellite to 0.827 for the AHT4 microsatellite. As with heterozygosity, the lowest value of genetic diversity was found in the HTG6 microsatellite (0.406). However, the highest value of genetic diversity was achieved via the VHL20 microsatellite (0.835). General in-

Table 2. Characteristics and summary statistics for microsatellite loci analyzed in the population of the Old Kladruber horse

Locus	No. of alleles	Size range	Observed heterozygozity	Genetic diversity	Chromosomal location	Gene differentiation (G_{ST})
AHT4	10	143–160	0.827	0.821	24	0.076
AHT5	6	130–140	0.716	0.752	8	0.081
ASB2	14	236–255	0.821	0.846	15	0.060
ASB17	11	95–121	0.784	0.778	2	0.085
ASB23	12	178–207	0.534	0.703	3	0.073
CA425	6	234–244	0.651	0.622	28	0.029
HMS1	5	174–184	0.519	0.543	15	0.063
HMS2	12	217–238	0.725	0.734	15	0.044
HMS3	10	148–168	0.509	0.607	9	0.070
HMS6	10	155–172	0.685	0.713	4	0.075
HMS7	8	117–125	0.549	0.625	1	0.076
HTG4	6	127–137	0.682	0.700	9	0.106
HTG6	6	79–95	0.374	0.406	15	0.114
HTG7	4	117–125	0.556	0.600	4	0.162
LEX3	5	144–156	0.497	0.561	X	0.044
VHL20	7	85–105	0.765	0.835	30	0.115
Mean	8.25	–	0.637	0.678	–	0.081

formation about differences and aggregate statistics is shown in Table 2. A statistically conclusive deviation from the Hardy-Weinberg equilibrium was detected at ASB23 ($P < 0.01$), HMS3 ($P < 0.01$), HMS7 ($P < 0.01$), HTG7 ($P < 0.01$) and VHL20 ($P < 0.01$) loci. Similar values of observed heterozygosity and genetic diversity were also found in Spanish Celtic horses (Cañon et al., 2000), Lipizzaner horses (Achmann et al., 2004), German draught horses (Aberle et al., 2004) and Biłgorai horses (Ząbek et al., 2005). Conversely, Iwanczyk et al. (2006) reported that the values of heterozygosity and genetic diversity of Polish heavy horses were considerably lower.

Descriptive statistics of microsatellites throughout the lines: the number of individuals, the average number of alleles, observed heterozygosity and genetic diversity are presented in Table 3. The observed heterozygosity and genetic diversity showed similar values for all lines. The lowest value of heterozygosity was determined in the Generale line (0.569), while the highest value was found out in the Favory line (0.680). For genetic diversity, the Favory line showed the highest value as well (0.677), while the lowest genetic diversity was exhibited in the Rudolfo line (0.547), however, the value of genetic diversity in the latter line may be due to the inclusion of a small number of individuals of this line in the analysis. The values of observed heterozygosity and genetic diversity are influenced by the number of alleles. Table 3 clearly shows that with the exception of the Generale and Sacramoso lines, observed heterozygosity reached higher values than genetic diversity. This does not indicate a decrease in variability in the lines mentioned above. A decrease in effective population size (*Ne*) and reduced variability result in a loss of rare alleles from the gene pool, plus there is a reduction in genetic diversity below the observed heterozygosity, as rare alleles only slightly contribute to the value of heterozygosity (Cunningham et al., 2001). Higher observed heterozygosity values compared to those of genetic diversity were seen in endangered populations of Asturon ponies by Cañon et al. (2000), and the same was recorded within a small population of Biłgorai horses by Ząbek et al. (2005). Genetic variability in the Old Kladruber horse lines mentioned above is maintained by breeding schedules when parental pairs are set up from individuals with relationship as small as possible. Thanks to this, there was a reduction in the coefficient of inbreeding in the total population by 2.87%, from 7.75% in horses born in 1993 to the value of 4.88% in those born in 2003 (Jakubec et al., 2009).

The genetic differentiation values (G_{ST}) showed an overall 8.1% difference. This value is consistent with those published by Cañon et al. (2000) for the Spanish Celtic horse. However, it contrasts with the figures reported by Børnstad et al. (2000) for the Norwegian horse (12%) or, for example, the Spanish donkey breeds (3.6%) published by Aranguren-Méndez et al. (2001).

The values of the *F* statistics are listed in Table 4. The F_{ST} values are very similar, ranging from 0.024 to 0.127. These values correspond to the mean inter-line differences. Nonetheless, the total value of F_{ST} multiloci clearly indicates that about 7% of overall genetic variation is due to inter-line differences, while differences among individuals are responsible for the remaining 93%. On average, the proportion of heterozygosity in each line is reduced below 1%, while the same for the total population

Table 3. Characteristics and summary statistics for microsatellite loci analysed in Old Kladruber horse lines

Line name	*n*	Mean No. of alleles	Observed heterozygozity	Genetic diversity
Generale	40	4.38	0.569	0.575
Generale-Generalissimus	60	5.50	0.623	0.600
Favory	80	6.06	0.680	0.677
Favory-Generalissimus	68	5.69	0.656	0.622
Sacramoso	198	6.75	0.637	0.660
Solo	120	5.44	0.624	0.618
Siglavi Pakra	28	4.25	0.647	0.604
Romke	32	4.63	0.630	0.605
Rudolfo	22	3.94	0.659	0.547

Table 4. Estimated F_{IT}, F_{IS} of the entire population (F_{IS} total), intra-line F_{IT} and F_{IS}

Locus	F_{IT}	F_{IS}									F_{IS} total	F_{ST}
		Gene.	G.Gens.	Fav.	F.Gens.	Sac.	Sol.	SigP.	Rom.	Rud.		
AHT4	0.006	0.118	−0.289	−0.027	0.011	−0.022	−0.152	−0.199	−0.063	−0.061	−0.072	0.073
AHT5	0.059	0.093	−0.045	−0.018	−0.031	0.034	0.018	0.086	−0.108	−0.135	0.003	0.055
ASB2	0.039	0.102	−0.062	−0.088	−0.002	0.005	0.100	−0.319	−0.01	−0.124	−0.009	0.048
ASB17	0.009	−0.109	−0.085	−0.111	−0.235	0.007	−0.081	−0.059	−0.123	−0.358	−0.084	0.086
ASB23	0.249	0.61**	0.768**	0.213*	0.415**	0.105*	−0.060*	0.099	0.262	−0.538	0.209	0.051
CA425	−0.041	−0.092	−0.069	−0.073	−0.119	−0.007	−0.100	0.011	−0.038	−0.325	−.0665	0.024
HMS1	0.057	0.246	−0.364	0.06	0.086	−0.054	0.177	0.071	−0.25	−0.224	−.0062	0.062
HMS2	0.012	−0.067	0.052	−0.005	−0.088	−0.053	−0.002	0.071	0.176	−0.290	−0.022	0.041
HMS3	0.172	0.531**	−0.165*	0.227*	0.196	0.053	0.149	0.342	−0.148	−0.026	0.114	0.066
HMS6	0.049	−0.052	−0.184	−0.037	−0.222	0.092	0.076	−0.122	0.032	−0.139	−0.010	0.058
HMS7	0.140	−0.105	−0.134	0.124	−0.222	0.280**	−0.008	−0.149*	−0.047	−0.104	0.026	0.117
HTG4	0.041	−0.145	0.167	−0.154	−0.171	0.035	−0.106	−0.117	−0.125	−0.190	−0.052	0.089
HTG6	0.095	−0.094	0.109	0.117	−0.114	−0.029	0.175	0.041	−0.132	0.000	0.017	0.079
HTG7	0.095	−0.056	0.072	−0.081	−0.229	−0.009	0.017	−0.322	−0.154	0.474	−0.032	0.123
LEX3	0.120	0.00	0.020	0.026	0.320*	0.095	−0.034	0.108	0.460*	−0.026	0.096	0.027
VHL20	0.102	−0.251	0.016	−0.052	−0.177	0.139*	−0.047	−0.091	0.010	−0.163	−0.011	0.111
All	0.073	0.037	−0.021	0.008	−0.04**	0.039	−0.002	−0.035	−0.008	−0.158	0.003	0.070

Gene. = Generale, G.Gens. = Generale-Generalissimus, Fav. = Favory, F.Gens. = Favory-Generalissimus, Sac. = Sacramoso, Sol. = Solo, SigP. = Siglavi Pakra, Rom. = Romke, Rud. = Rudolfo

is reduced by 7.3%. Similar values were also found by Cañon et al. (2000) when comparing Spanish Celtic horses. The high F_{IS} values for the ABS23 and HMS3 loci correspond to the prevalence of homozygous individuals, which causes deviations from the Hardy-Weinberg equilibrium. The high F_{IS} alues of the ABS23 and HMS3 loci are caused by inbreeding as a result of reduced population size. The total F_{IT} value indicates the average rate of inbreeding coefficient within the population. This figure (7.3%) is consistent with the value estimated for the population of 1993 by Jakubec et al. (2009). Statistical deviations of the F_{IS} value from zero were found in the ABS23 locus in the Generale, General-Generalissimus, Favory, Favory-Generalissimus, Sacramoso and Solo lines, in the HMS3 locus of Favory, Generale and Generale-Generalissimus lines, in the HMS7 locus of Sacramoso and Siglavi Pakra lines, in the LEX3 locus of Favor-Generalisimus and Romke lines and in the VHL20 locus in the Sacramoso line. In the microsatellite loci and lines listed above there is a reduction in heterozygosity and this may result in inbreeding depression. Increased F_{IS} values, however, may be due not only to the increased inbreeding coefficient but also to the Wahlund effect, which causes decreased heterozygosity in the population as a result of the population splitting into sub-populations. In contrast, increased heterozygosity was found in the HMS3 and ASB23 microsatellite loci in the Generale-Generalisimus and Solo lines. Statistical deviations of the F_{IS} value from 0 in the ABS23, HMS3 and HMS7 loci correspond to deviations from the Hardy-Weinberg equilibrium of these loci. A significant deviation of F_{IS} from 0 for the average values was found only in the Favory-Generalisimus line. In other lines, no deviation from 0 has been detected, indicating the conservation of genetic variability. By contrast, the statistical decrease in the F_{IS} value in the Favory-Generalisimus line shows a desirable increase in genetic variability.

Standard inter-line genetic distances (D) according to Nei (1978) and differences using the pairwise coefficient F_{ST} are listed in Table 5. The lowest value of genetic distances according to Nei (1978) was found between the Sacramoso and Solo lines (0.049) and the highest value of genetic distance was estimated between the Romke and Rudolfo lines (0.429). Similar genetic diversity values were published by Ząbek et al. (2005) when comparing a small population of the Biłgorai horse with common horse breeds. Pairwise F_{ST} coefficients

Table 5. Genetic distance according to Nei (1978) (D) above the diagonal and F_{ST} with the number of effective migrants per population (in parentheses) below the diagonal

Line	Gene.	G.Gens.	Fav.	F.Gens.	Sac.	Sol.	SigP.	Rom.	Rud.
Gene.		0.197	0.144	0.204	0.212	0.356	0.390	0.367	0.214
G.Gens.	0.093 (2.5)		0.133	0.104	0.173	0.287	0.404	0.359	0.172
Fav.	0.059 (4.0)	0.054 (4.4)		0.119	0.112	0.183	0.267	0.219	0.161
F.Gens.	0.092 (2.5)	0.045 (5.4)	0.045 (5.3)		0.121	0.212	0.319	0.249	0.166
Sac.	0.091 (2.5)	0.075 (3.1)	0.042 (5.5)	0.051 (4.7)		0.049	0.191	0.093	0.176
Sol.	0.153 (1.4)	0.127(1.7)	0.076 (3.1)	0.095 (2.4)	0.020 (12.1)		0.171	0.061	0.253
SigP.	0.159 (1.3)	0.160 (1.3)	0.094 (2.4)	0.126 (1.7)	0.072 (3.2)	0.071 (3.3)		0.169	0.429
Rom.	0.152 (1.4)	0.147 (1.5)	0.079 (2.9)	0.102 (2.2)	0.031 (7.7)	0.017 (14.6)	0.062 (3.8)		0.316
Rud.	0.100 (2.3)	0.078 (3.0)	0.062 (3.8)	0.072 (3.2)	0.072 (3.2)	0.113 (2.0)	0.174 (1.1)	0.135 (1.6)	

Gene. = Generale, G.Gens. = Generale-Generalissimus, Fav. = Favory, F.Gens. = Favory-Generalissimus, Sac. = Sacramoso, Sol. = Solo, SigP. = Siglavi Pakra, Rom. = Romke, Rud. = Rudolfo

showed average values for most pairs of lines, high F_{ST} values were found only between the lines as follows: Generale vs. Solo, Generale vs. Siglavi Pakra, Generale vs. Romke, Generale-Generalissimus vs. Siglavi Pakra, Generale-Generalissimus vs. Romke and Siglavi Pakra vs. Rudolfo. The high F_{ST} values indicate higher genetic distances between the lines listed above. Nevertheless, lines with low estimated F_{ST} values involved Sacramoso vs. Solo and Sacramoso vs. Romke. The values of pairwise F_{ST} coefficient make clear that from 2% to 17.4%, the microsatellite variability in the Old Kladruber horse can be explained by splitting the population into the black stock and grey stock, which have long been kept separate, i.e. no exchange of breeding males or females has occurred. The F_{ST} values correspond to those of genetic distance (D). Estimation of gene flow is shown as the average number of migrants per population (N_m), see Table 5. The largest proportion of migrants was found between the Romke and Solo lines (14.6), while the smallest proportion was determined between the Siglavi Pakra and Rudolfo lines (1.1). Once again, this fact can be explained by the division of the breed into two more or less independent populations. Romke and Solo are black lines, between which there was an intensive exchange of breeding material from both sexes, while Siglavi Pakra represents a black and Rudolfo a grey line, meaning they were kept separate from each other. The number of migrants per population (N_m) is consistent with the values of genetic distance (D) and pairwise F_{ST} coefficient. The higher number of migrants is in line with the breeding objective, when the small population formed the basis for efforts to breed as few related individuals as possible, even between individual lines.

The UPGMA dendrogram (Figure 1) makes it obvious that the lines were divided into two main clusters. These represent the distribution of lines as per colour variation. The Solo, Romke, and Siglavi Pakra lines are the representatives of black horses, while Generale, Generale-Generalissimus, Favory, Favory-Generalissimus and Rudolfo represent grey horses. Members of the Sacramoso line are present in both colour variations.

Within the first cluster, the most similar lines are Sacramoso and Solo. These are the earliest black lines of Old Kladruber horses. The high similarity between these two lines is also due to the fact that one stallion within the Sacramoso line was renamed Solo as part of the black Old Kladruber horse restoration process and contributed decisively to the restoration process as a founder of a new line. In addition, the Romke line is closely related to these two lines. These three lines exhibited the highest similarity of microsatellite loci from the entire population. This corresponds to the average number of migrating individuals (N_m) among these three lines (Table 5). The Siglavi Pakra line showed a great genetic distance from the lines – Sacramoso, Solo and Romke (Table 5). However, this finding is not in line with generally known facts or breeding records. The founder of the Siglavi Pakra line was a Lipizzaner stallion, while this breed is the most closely related to the Old Kladruber, with a quite frequent exchange of breeding material occurring between the two in the past (Bílek, 1957). The Generale-Generalissimus and

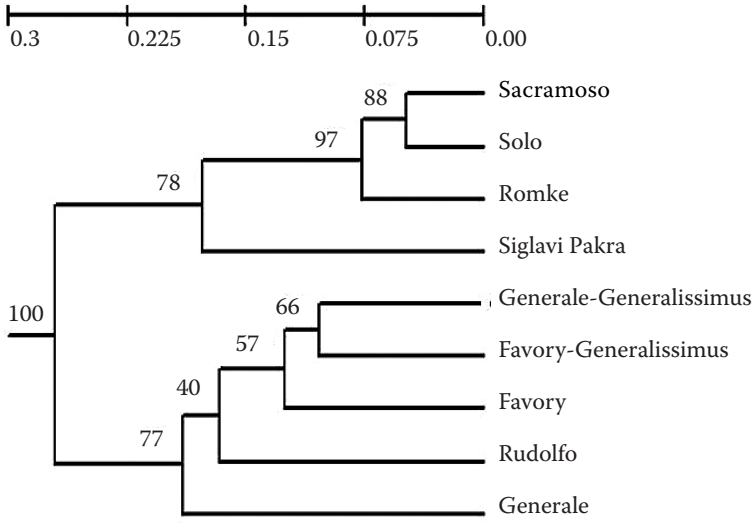

Figure 1. UPGMA dendrogram constructed based on genetic distances according to Nei (1978). The number for each outside branch is the bootstrap value for 1000 replications

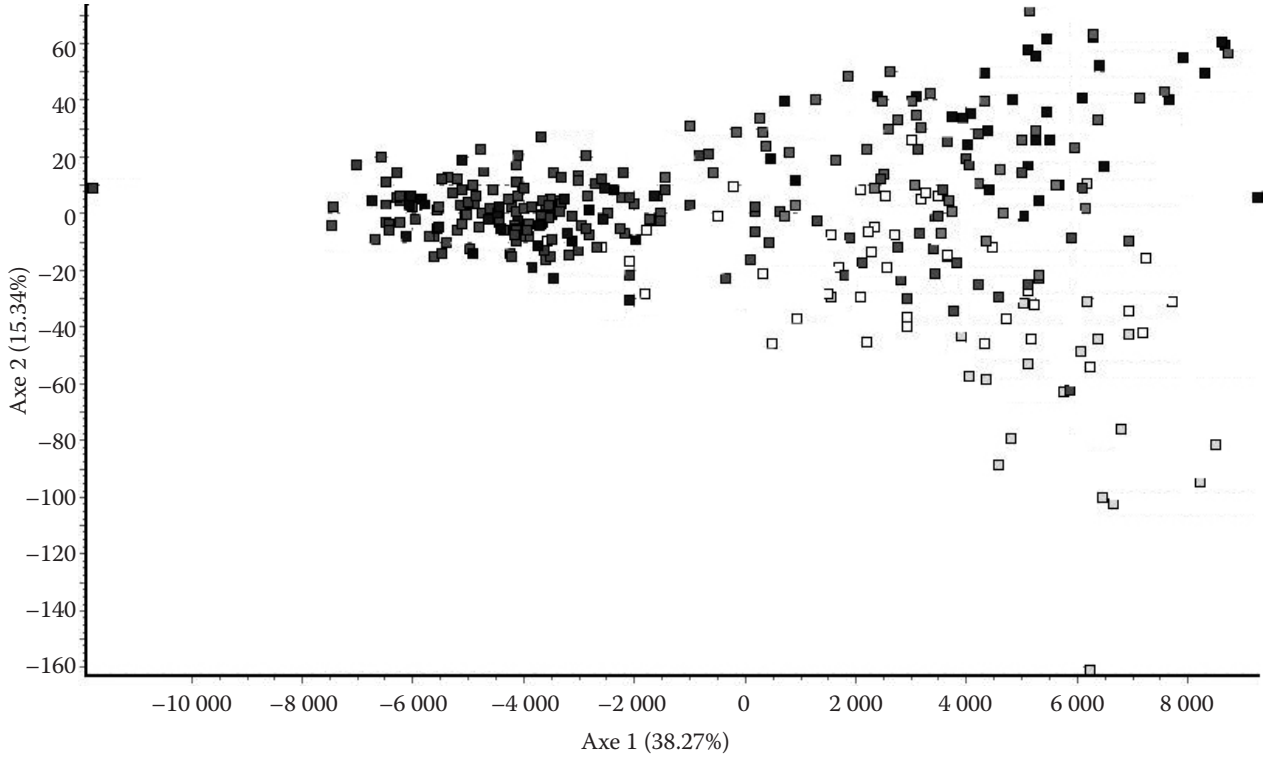

Figure 2. Scatter diagram of relative positions of 324 subjects defined by the method of principal components based on the correlation matrix of alleles at 16 microsatellite markers

Favory-Generalissimus lines showed the highest genetic similarity; however, as is clear from breeding documentation, the Generale-Generalissimus line is de facto the Generale line, while the Favory-Generalissimus is actually the Favory line. Therefore, it is not logical for the Generale-Generalissimus (i.e. Generale) line to be farther from the Generale line than the Favory-Generalissimus (i.e. Favory) line, or for the Generale-Generalissimus line to be closer to the Favory-Generalissimus line than the genuine Favory line. Despite being amongst the oldest lines and having been involved in restoring the Generalissimus line as well as the Favory line, Generale showed the largest distance to the three lines inside the other cluster. As mentioned above, such greater distance was affected by the number of migrants per population (*Nm* – Table 5) among those lines, as the biggest distance with respect to other lines should be displayed by Rudolfo, which comes from Spain according to the line's origin, while the original Old Kladruber Generale line and also the Lipizzaner Favory line should be closer to each other. In terms of the known origin of the lines, the Generale and Generale-Generalissimus line should, therefore, be placed within the same branch, while the neighbouring branch should include Favory and Favory-Generalissimus, and Rudolfo should be within the outermost branch.

Furthermore, Figure 1 implies that the second cluster is characterized by being less robust than the first one, as it has relatively lower bootstrapping values for the different branches. The bootstrapping values indicate the probability of correct inclusion of the individuals of each line in their respective lines based on molecular genetic analysis.

Figure 2 shows the results of PCA (principal component analysis) line analysis on the basis of 16 microsatellites. With the exception of a few individuals, axis 1 separates the individuals of black and grey variants. For the second axis, however, the differentiation was not so significant. The graphical representation also shows that the lines overlap each other, which mostly occurs within the right cluster, covering individuals of the black variant lines: Sacramoso, Solo, and Romke. Although the left individual clustering is not so distinctive, there is still some mixing of the lines throughout the range, due to the small size of the population and efforts to breed the individuals with the least kinship, as well as to breed lines using the alternating or rotating breeding method.

Table 6. Descriptive statistics of the inbreeding coefficient (F_X) per line

Line name	Average F_X	SD	Min	Max
Generale	0.096	0.022	0.062	0.139
Generale-Generalissimus	0.078	0.019	0.042	0.128
Favory	0.048	0.029	0.003	0.155
Favory-Generalissimus	0.049	0.030	0.005	0.096
Sacramoso	0.091	0.040	0.006	0.222
Solo	0.080	0.035	0.003	0.207
Siglavi Pakra	0.082	0.050	0.011	0.177
Romke	0.084	0.043	0.004	0.151
Rudolfo	0.051	0.025	0.011	0.085
All	0.076	0.038	0.003	0.222

Based on the results of the assignment test of subjects from the respective lines, only 53.7% of the subjects were included correctly, at the level of significance $P < 0.05$ and $P < 0.01$ (10 000 simulated individuals).

The results of the bootstrapping values (Figure 1), PCA method based on 16 microsatellites (Figure 2) and assignment test show that inter-line disparity is not obvious. For this reason, using solely microsatellite markers for the accurate classification of individuals into lines is not appropriate in the case of the Old Kladruber horse.

Determining inter-line genetic relationship using pedigree information

For all individuals tested, full pedigrees were determined up to generation 5 ($cp = 1$).

Descriptive statistics for the inbreeding coefficient of individuals in different lines are shown in Table 6, showing that for each line the average inbreeding coefficient ranged from 0.048 (Favory) to 0.096 (Generale). The degree of variability within the coefficient of inbreeding values was estimated to be the highest with the Siglavi Pakra line (SD = 0.050), while the lowest concerned the Generale-Generalissimus line (SD = 0.019). The average inbreeding coefficient throughout all lines totalled 0.076, with SD of 0.038. This estimated average inbreeding coefficient corresponds to the estimated value of F_{IT} in the molecular part of the analysis (Table 4, $F_{IT} = 0.073$), as well as the value published by Jakubec et al. (2009) in an analysis covering all individuals born in 1993 within the Old Kladruber population ($F_X = 0.0775$).

Estimated average intra-line and inter-line coefficients of relationship are shown in Table 7, exhibiting the fact that the intra-line average coefficient of relationship ranged from 0.179 (Favory and Sacramoso) to 0.304 (Generale-Generalissimus), while the average inter-line coefficient of relationship ranged from 0.039 (Generale vs. Sacramoso) to 0.214 (Generale vs. Generale-Generalissimus). The higher average coefficients of relationship indicate a higher affinity of subjects within the lines. Generally low inter-line values were found in colour variants (black horses vs. grey horses). Conversely, a higher average inter-line coefficient of relationship was found within the colour variant. This can be explained by the fact that when designing breeding plans, mating involves individuals within the colour variant, while breeding between colour variants occurs only to a small extent.

A cluster analysis chart (Figure 3) was compiled using the mean inter-line coefficients of relationship shown in Table 7. Following pedigree information, lines were divided into two basic clusters according to colour variations, as with the basis of microsatellite markers. There are slight changes within the two main clusters according to colour variations compared with those based on microsatellite markers. In the black variant, the highest relationship was shown by the Solo and Romke lines, with the Sacramoso line being another close relative of the two. However, even these results are not consistent with the relationship of the lines in terms of historical development as the Solo line began to exist by formally separating from

Table 7. The average intra-line (on the diagonal) and inter-line (above the diagonal) coefficient of relationship

Line	Gene.	G.Gens.	Fav.	F.Gens.	Sac.	Sol.	SigP.	Rom.	Rud.
Gene.	0.301	0.214	0.152	0.173	0.085	0.039	0.056	0.042	0.198
G.Gens.		0.304	0.153	0.176	0.087	0.040	0.045	0.043	0.176
Fav.			0.179	0.126	0.107	0.080	0.073	0.082	0.132
F.Gen.				0.231	0.111	0.086	0.090	0.099	0.146
Sac.					0.179	0.176	0.159	0.162	0.091
Sol.						0.257	0.195	0.212	0.052
SigP.							0.279	0.185	0.064
Rom.								0.215	0.065
Rud.									0.256

Gene. = Generale, G.Gens. = Generale-Generalissimus, Fav. = Favory, F.Gens. = Favory-Generalissimus, Sac. = Sacramoso, Sol. = Solo, SigP. = Siglavi Pakra, Rom. = Romke, Rud. = Rudolfo

the Sacramoso line, and thus they are actually identical. Therefore, from this perspective, the highest relationship should be exhibited by these two lines, with Siglavi Pakra as the next in line and the Romke line being the outermost.

For the grey colour lines, Generale and Rudolfo, and then Favory and Favory-Generalissimus were the closest relatives in determining genetic relationship using the method above. The rather narrow relationship between Favory and Favory-Generalissimus is due to the aforementioned restoration of Generalissimus using the Favory line. This method to determine the similarity among individuals of the Old Kladruber horse was previously used by Přibyl et al. (1997).

Comparing the estimated values based on microsatellite markers and pedigree information makes certain differences obvious concerning the formation of clusters within the colour variations. These differences and the estimated relative positions of lines, the results of principal component methods (Figure 2) and the assignment test make clear that including individuals in lines for the Old Kladruber horse breed only on the basis of the present microsatellite analysis is not reliable.

The results of the analysis show that inbreeding due to the low numbers of individuals in the Old Kladruber horse population, and efforts to reduce the impact thereof causes the inter-line genetic distances to reduce, with the reason being the high degree of inter-line breeding, in which the effect of the genetic founder sire is blurred, so from a genetic aspect, the line becomes more or less a formal unit.

The results also show that the use of current microsatellite analysis to estimate the inbreeding coefficient is not optimal. Creating parental pairs only on the basis of information on the inclusion of an individual in a line appears to be sufficient

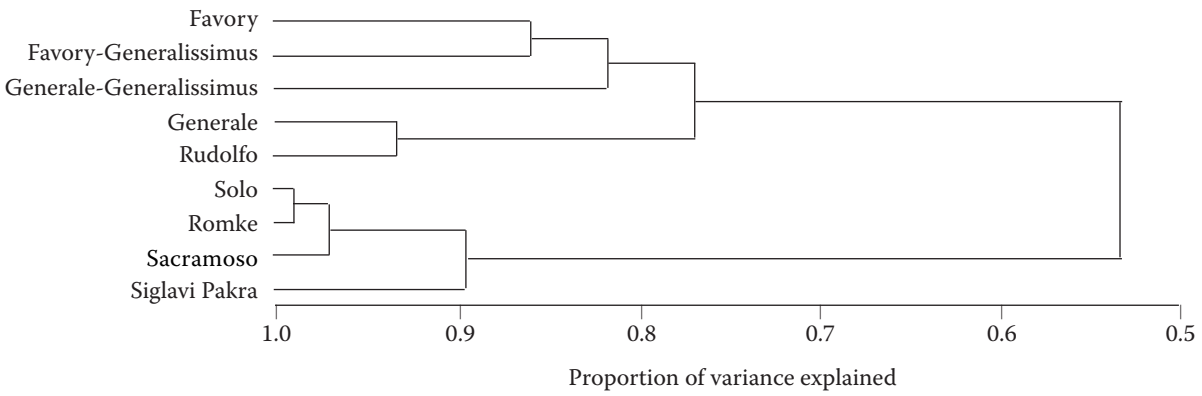

Figure 3. Cluster analysis constructed from the average inter-line coefficients of relationship

in an effort to reduce the coefficient of inbreeding. Similar conclusions were also drawn by Baumung and Sölkner (2003), stating in their work that even an incomplete lineage is more appropriate than microsatellite markers when estimating the inbreeding coefficient and other population parameters. These authors further report that comparing the significance of pedigree figures and microsatellite markers would need the monitoring of more than 100 (or better 200) markers in order to obtain matching informational values. Furthermore, the work implies that with such small populations, such as the Old Kladruber horse, using estimates of the coefficient of relationship is preferable for compiling parental combinations correctly. However, despite the differences observed between the two methods analysed, the information obtained from microsatellite analyses will be of future use in the Old Kladruber horse breeding process.

The results from this study shall be employed within the breeding process, which includes both conventional breeding procedures and methods combining molecular genetics and pedigree information.

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