Genetic analysis, breed assignment and conservation priorities of three native Danish horse breeds

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Summary

A genetic analysis was performed on three indigenous Danish horse breeds using 12 microsatellite markers from a standard kit for parental testing. These three breeds are all considered endangered based on their small population sizes. Genetic variation in these three breeds was comparable to other horse breeds in Europe, and they do not seem to be at immediate danger of extinction caused by genetic deterioration. The Knabstrupper breed had more genetic variation, as measured by expected heterozygosity and allelic richness, than the other two breeds (Frederiksborg and Jutland). F_{ST} statistics and population assignments confirmed population differentiation into three distinct breeds. The Frederiksborg and Knabstrupper breeds were closer to each other than to the Jutland breed. When establishing conservation priorities for the breeds, the priorities will depend on the conservation goals. Different methods for establishing conservation priorities are also discussed.

Keywords domestic breeds, genetic differentiation, inbreeding.

Introduction

Conservation of endangered animal species is often associated with wildlife management and reintroduction from captivity (Frankham *et al.* 2002), but domestic species are also of concern. During the United Nations Conference on Environment and Development in Rio de Janeiro in 1992, the Convention on Biological Diversity was signed. During this conference, it was also decided to conserve domestic animals. In Denmark, breeds of Danish origin were identified to be conserved in order to preserve biodiversity, cultural history and history of landscape. The committee on Conservation of Genetic Resources under The Danish Ministry of Food, Agriculture and Fisheries (Genressourceudvalget 2007) was asked to care for threatened races of domestic animals.

In Denmark, the number of horses has fallen dramatically since 1945. Because of mechanization, horses were almost eliminated from the farming industry. In 1945, the number of horses in Denmark was around 600 000, and in 1986 it was around 40 000 (Trock 1986). Among the Danish domestic horses, there are three breeds that are considered to be of Danish origin and are endangered: the Frederiksborg (FR), Knabstrupper (KN) and Jutland (JU) breeds.

The breeding of FR started in the 16th century at the royal stables. FR animals were bred for riding, driving and parades (Trock 1986), and efforts were directed towards obtaining horses with similar colours. A studbook was founded in 1861, and was primarily based on the original FR horses and on horses from other breeds such as the Thoroughbred, Arabian and German breeds. In the late 20th century, the studbook was closed, and only horses with at least 6/8 FR in the fifth generation were accepted.

The KN was originally a subpopulation of the FR. It was bred for the special spotted colour that is characteristic of KN (Lunn 1966). In 1970, a studbook for KN was founded. It has been kept as an open studbook, where mares with no previous studbook records and stallions from other breeds are accepted, and the characteristic coloured spotting pattern is the only requirement for inclusion in the studbook. It is the intention that the studbook will be closed in the near future and that only horses with at least 6/8 KN in the third generation will be accepted. In 2002, the KN Association and the Committee of Conservation of Genetic Resources cooperatively agreed to establish a breeding programme to multiply the number of pure Danish KN horses.

The JU was the horse of the farming industry in Jutland (Hedegaard 2003). It was based on heavy draught horses mainly of German origin. The studbook was founded in

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Table 1 Summary information for the three horse breeds.

Population	No. individuals sampled	No. individuals in population	N_e	Studbook maintained since	Studbook closed since	Inbreeding ($F \pm$ SE)
Frederiksborg	33	980	480	1861	1987	0.04 (0.0057)
Jutland	30	716	486	1861	1861	0.06 (0.0042)
Knabstrupper	34	781	420	1970	Open	0.03 (0.0099)

The effective population size (N_e) is based on a formula that takes into account differences in the number of animals of the two sexes. The level of inbreeding is calculated from pedigrees going seven generations back in time.

1861 and was a closed studbook, but accepted 5% Shire and Suffolk Punch breeds.

Because of their small population sizes, the three horse breeds are all classified as endangered (http://www.fao.org). Based on studbooks, there are currently 980 FR, 716 JU and 781 KN horses (Table 1).

In conservation genetics, one main objective is to preserve the genetic variability within populations, assuming there is a positive correlation between genetic variation and population viability. In the management of populations, the aim is to prevent loss of genetic variation because of inbreeding and/or drift and at the same time avoid migration among populations of different breeds. Microsatellites have been used in horses for parental testing (Bowling et al. 1997; Bowling 2001; Tozaki et al. 2001; Lee & Cho 2006), inferring genetic structures and genetic relationships (Cañon et al. 2000; Kelly et al. 2002; Bjørnstad et al. 2003; Aberle et al. 2004), assigning individuals to breeds (Bjørnstad & Røed 2001, 2002; Glowatzki-Mullis et al. 2005), inferring phylogenetic relationships (Tozaki et al. 2003) and determining the origins of lineages (Cunningham et al. 2001).

The potential for recognizing population-specific profiles for breed identification would be valuable for validating the quality and origin of livestock. In addition, the discrimination among populations is essential for effective and accurate management of both natural and livestock breeds (Bjørnstad & Røed 2001). The analysis of breed differentiation and the assignment of individuals to populations, subpopulations or breeds using microsatellite information and a Bayesian analysis approach have already proven to be powerful in resolving subtle conservation genetic issues in horses (Marletta et al. 2006; Druml et al. 2007).

If conservation plans are established, it might be necessary to prioritize among breeds or populations depending on the aim of the management plan (Fabuel et al. 2004). Three methods have been implemented in prioritizing conservation units. Caballero & Toro (2002) recently developed a method to estimate within- and between-population diversity on the basis of coancestry between all pairs of individuals, referring to identity-by-state instead of identity-by-descent. In contrast, the method of Weitzman (1992) estimates marginal loss on the basis of genetic distance between populations. The third method, by Petit et al.

(1998), calculates the contributions of different populations to total diversity and allelic richness (AR).

The mating of closely related individuals can lead to inbreeding depression. The frequency of homozygotes increases with higher inbreeding coefficients, and this leads to the expression of deleterious recessive alleles and inbreeding depression (Charlesworth & Charlesworth 1987; Hedrick & Kalinowski 2000). To avoid inbreeding depression, estimating relatedness based on molecular tools might be an advantage. But breeders do not have direct access to molecular tools and use studbooks to assess appropriate mating partners. If a correlation between individual relatedness based on molecular markers and individual inbreeding coefficient exists, an attempt to avoid inbreeding depression might be to not mate highly inbred individuals.

The general objective of this study was to determine the levels of genetic variability within and between the three indigenous horse breeds from Denmark, to infer the recent genetic relationship among the breeds and finally to assess the assignment of the analysed individuals to the different breeds. The study was based on microsatellite markers. A Bayesian approach was used to infer assignment to breeds and different approaches for evaluating the partitioning of genetic diversity were applied. The approaches of Weitzman (1992), Petit et al. (1998) and Caballero & Toro (2002) mentioned above as well as investigations of correlations between individual relatedness, individual inbreeding coefficient and individual heterozygosity were compared with respect to their utility for establishing conservation strategies for the breeds.

Materials and methods

Study population and sample collection

When individuals are chosen (graded) for studbooks, they are judged by a panel of experts on the basis of the appearance and movement (exterior judgement). Veterinary personnel took blood samples in EDTA tubes at the time of grading. Samples were kept at 4 °C or frozen at the Blood-type Laboratory, Nørlund Horse Hospital (Silkeborg), where they were used for parental testing (genotyping) in case of doubt. Ninety-seven individuals (33 FR, 30 JU and 34 KN) were chosen randomly from the database at the Blood-type

Laboratory (Table 1). All animals met the following criteria: they were living at the time of this study, they had no siblings or half siblings in this study and they had no parents/offspring in this study.

Microsatellite genotyping procedure

DNA extraction was performed with the standard CTAB procedure (Doyle & Doyle 1987). A total of 12 microsatellite markers from a standard kit used for genotyping and parental testing of horses (StockMarks®, Applied Biosystems) was used to genotype the DNA samples from the horses. The microsatellite markers (*VHL20*, *HTG4*, *AHT4*, *HMS7*, *HTG6*, *AHT5*, *HMS6*, *ASB2*, *HTG10*, *HTG7*, *HMS3* and *HMS2*) were multiplexed, with four and eight markers per reaction (see Table 2 for more details). The PCR reaction for the multiplex was carried out in a 14-µl reaction volume containing 2.5 µl StockMarks PCR Buffer (1.5 mM MgCl$_2$), 4.0 µl dNTP mix (200 µM of each dNTP), 0.5 µl *Taq* polymerase (AmpliTaq Gold, 5 U/µl), 1.0 µl template DNA, 0.5 µl each primer (20 ng/ml) and topped with deionized water. PCR conditions (using a 9700 GeneAmp) were an initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s. Cycling culminated

with 60 min of extension at 72 °C. The amplified loci were analysed on an ABI 310 Genetic Analyzer. The alleles were scored manually using the program GENOTYPER version 2.5.2 (Applied Biosystems). Trained personnel at the Blood-type Laboratory performed all the genotyping analyses.

Genetic variation

GENEPOP version 3.4 (Guo & Thompson 1992) was used to estimate gene frequencies, inbreeding coefficients (F_{IS}) and observed (H_O) and expected (H_E) heterozygosities (Weir & Cockerham 1984). H_O and H_E were estimated for each locus, for each breed and for the total population, pooling the three breeds. GENEPOP was also used to test for Hardy–Weinberg equilibrium (HWE) and genotypic linkage equilibrium (LE). Overall Bonferroni adjustments were used to correct for the effect of multiple tests (Rice 1989). Significance levels were $P < 0.05$ for each locus in each breed, $P < 0.00417$ for the breeds and $P < 0.00139$ for the total population. Because the KN breed was not in HWE at loci *ASB2* and *HTG10*, we tested to see if there was any evidence for the presence of null alleles using MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004), using Bonferroni adjustments implemented in this software.

Table 2 Details of the 12 microsatellite loci used for the analysis of Danish horses.

Locus	Size range (bp)	Chr.[1]	Dye label	Sequence	Reference
8-plex					
VHL20	85–105	30	FAM	F: 5′-CAAGTCCTCTTAGAAGACTAG-3′	Van Haeringen *et al.* (1994)
				R: 5′-AACTCAGGGAGAATCTTCCTCAG-3′	
HTG4	125–139	9	FAM	F: 5′-CTATCTCAGTCTTGATTGCAGGAC-3′	Ellegren *et al.* (1992)
				R: 5′-CTCCCTCCCTCCCTCTGTTCTC-3′	
AHT4	142–168	24	FAM	F: 5′-ACCCGCCTGAGCAAGGAAGT-5′	Binns *et al.* (1995)
				R: 5′-GCTCCCAGAGAGTTTACCCT-3′	
HMS7	168–188	1	FAM	F: 5′-CAGGAAACTCATGTTGATACCATC-3′	Guérin *et al.* (1994)
				R: 5′-TGTTGTTGAAACATACCTTGACTGT-3′	
HTG6	77–103	15	JOE	F: 5′-CCTGCTTGGAGGCTGTGATAAGAT-3′	Ellegren *et al.* (1992)
				R: 5′-CTCCATCTTGTGAAGTGTAACTCA-3′	
HMS6	150–170	4	JOE	F: 5′-CCTGAAGCAGAACATCCCTCCTTG-3′	Guérin *et al.* (1994)
				R: 5′-CTCCATCTTGTGAAGTGTAACTCA-3′	
HTG7	114–131	4	TAMRA	F: 5′-CCTGAAGCAGAACATCCCTCCTTG-3′	Marklund *et al.* (1994)
				R: 5′-ATAAAGTGTCTGGGCAGAGCTGCT-3′	
HMS3	146–176	9	TAMRA	F: 5′-CCAACTCTTTGTCACATAACAAGA-3′	Guérin *et al.* (1994)
				R: 5′-CCATCCTCACTTTTTCACTTTGTT-3′	
4-plex					
AHT5	124–139	8	JOE	F: 5′-ACGGACACAACCCTGCCTGC-3′	Binns *et al.* (1995)
				R: 5′-GCAGGCTAAGGGGGCTCAGC-3′	
ASB2	219–256	15	JOE	F: 5′-CCACTAAGTGTCGTTTCAGAAGG-3′	Breen *et al.* (1994)
				R: 5′-CACAACTGAGTTCTCTGATAGG-3′	
HTG10	83–111	21	TAMRA	F: 5′-CAATTCCCGCCCCACCCCCGCCA-3′	Marklund *et al.* (1994)
				R: 5′-TTTTTATTCTGATCTGTCACATTT-3′	
HMS2	215–236	10	TAMRA	F: 5′-CTTGCAGTCGAATGTGTATTAAATG-3′	Guérin *et al.* (1994)
				R: 5′-ACGGTGGCAACTGCCAAGGAAG-3′	

[1]Positions determined by Penedo *et al.* (2005).

Allelic richness, mean number of alleles per locus and the number of alleles sampled were calculated by FSTAT version 2.9.3 (Goudet 1995). Private alleles, effective number of alleles (N_e) – the number of alleles that would provide the same heterozygosity if they were all in equal frequency – and the number of alleles at each locus with frequencies <5% were calculated by GENALEX 6 (Peakall & Smouse 2006). The Kruskal–Wallis test and Mann–Whitney pairwise comparisons using Bonferroni correction with an indicative nominal level of 5% ($P < 0.0167$) were used to determine if there were significant differences in AR and H_E between breeds.

Breed differentiation

Genetic differentiation among breeds was characterized by estimating overall and pairwise F_{ST} values using FSTAT. The significance levels for the overall and pairwise F_{ST} values were determined after 10 000 permutations.

To infer the number of clusters in our samples ($n = 97$), STRUCTURE 2.2 was used (Pritchard et al. 2000). This program employs a Bayesian Markov chain Monte Carlo (MCMC) approach, uses multi-locus genotypes to identify the number of clusters (breeds or populations) and simultaneously assigns individuals to clusters without prior information on the origin of individuals. The Bayesian model assumes K (unknown) clusters that have different allelic frequencies at a set of independent loci. The likelihood of K is estimated from allelic frequencies. The highest likelihood value indicates the most likely number of clusters. Each individual is assigned probabilistically to a cluster or jointly to two or more clusters if its genotype indicates that it is admixed. The method assumes HWE and LE between loci within each population, and recent population admixture, migration or hybridization would probably produce departures from HWE and LE.

Posterior probability values for K [log likelihood, ln $P(D)$] were estimated by assigning priors from one to 10, with five independent runs of each. STRUCTURE was run with the 'admixture model', 10 000 burn-in steps and 100 000 MCMC replicates in all simulations. To compensate for the fact that likelihood maximization intrinsically favours partitions with more clusters, and because ln $P(D)$ did not provide an unequivocal number of clusters, the number of K clusters was also selected based on the log likelihood ratio test, using the formula $-2([\ln P(D)_k] - [\ln P(D)_{k-1}])$, with $df = df_k - df_{k-1}$ (Crawley 1993).

Identifying populations for conservation

Three approaches have been implemented for identifying priorities for conservation: a method based on coancestry (Caballero & Toro 2002), a method based on AR (Petit et al. 1998) and a method based on genetic distance (Weitzman 1992, 1993 & Thaon d'Arnoldi et al. 1998).

Caballero & Toro (2002) assumed a metapopulation consisting of n populations and i subpopulations with N_i breeding individuals. Mean coancestry within subpopulations (f), mean coancestry within metapopulation (f), inbreeding (F), self-coancestry (s) and mean distance between subpopulations (D) were calculated using MOLKIN V.2.0 (Gutiérrez et al. 2005). Total genetic diversity is $G_{DT} = 1 - f$, diversity between individuals is $GD_{BI} = s - f$ and diversity within individuals is $GD_{WI} = 1 - s$. GD_{BI} and GD_{WI} sum up to genetic diversity within subpopulations: $GD_{WS} = 1 - f$. Genetic diversity between subpopulations is $GD_{BS} = f - f$. The contribution of each breed to within- and between-breed diversity and the total contribution to diversity were calculated by running the analysis without the breed in question.

Petit et al. (1998) calculated the contribution of each subpopulation to total diversity. The total diversity can be partitioned into two components. The first is related to the level of diversity of the subpopulation and the second is related to its divergence from the other subpopulations. The authors suggested that AR should be the best indicator. It depends on effective population size and is therefore a better indicator of past demographic changes. Thus, AR is of interest in the context of conservation. A rarefaction method can be used to cope for unequal sample size. The approach according to Petit et al. (1998) was calculated in CONTRIB.

The Weitzman method is based on pairwise genetic distance between the subpopulations (Weitzman 1992, 1993). It is concerned with the distance between the units and the theory ignores diversity because of variation within units. The Weitzman approach has properties such that the removal of an element always decreases the variability and the addition of an element that is identical to another element does not increase variability (monotonicity in species). Moreover, the diversity in a set of subpopulations should increase if the distance between the subpopulations increases (monotonicity in distance). Nei's (1987) genetic distance was used to calculate the marginal loss of genetic diversity. The distance matrix was calculated in POPGENE 1.32 (Yeh & Boyle 1997).

Pearson's r-test was performed to infer correlation between the relatedness values and inbreeding coefficients of individuals. The objective was to examine if it is reasonable to use inbreeding coefficients calculated from pedigrees to avoid mating genetically related individuals. The relatedness matrix was calculated in GENALEX 6. Individual inbreeding coefficients were calculated from pedigrees going seven generations back in time, using a database created by the Danish Department of Horse Breeding. These are available on request from http://www.lr.dk/. It was assumed that individuals were unrelated seven generations ago. A correlation between individual heterozygosity and inbreeding coefficient was also tested. Individual heterozygosity was calculated as the number of loci for which the

horse was heterozygous, divided by the total number of loci in which the horse was scored.

Results

Genetic variation

The number of alleles detected at each locus varied between six (*HTG7*) and 10 (*VHL20*, *ASB2* and *HMS2*) (Table 3). There were significant differences in AR and H_E among breeds, and KN had a significantly higher AR and H_E than the two other breeds (Table 4). The numbers of private alleles in each breed were 3, 8 and 12 for FR, JU and KN respectively. Most of the private alleles were in very low frequencies and below 5%. Only one private allele at locus *ASB2* in the KN breed was in high frequency (25%). Two alleles at *HMS3* in the KN breed had frequencies of 10% and 8.3% respectively. Of all alleles detected in the JU population, 35.94% had a frequency of <5%, in the FR population, 31.75% had a frequency of <5% and in the KN population, 26.13% had a frequency of <5%. This was reflected in the mean number of effective alleles: 2.93, 2.80 and 4.44 for FR, JU and KN respectively. See Appendix S1 for allelic frequencies for each breed and at each locus.

Observed heterozygosity (H_O) values ranged from 0.115 (at *HMS3* in JU) to 0.882 (at *VHL20* in KN) (Table 3). All three breeds and the total population were in HWE, but KN had heterozygote deficit at loci *ASB2* and *HTG10*. Results indicated the presence of a null allele at *ASB2* ($P = 0.1289$) but not at *HTG10*. F_{IS} values across all loci were −0.015 (FR), 0.004 (JU) and 0.078 (KN). All loci were in LE (results not shown).

Table 4 Results of Mann–Whitney pairwise comparisons: above the diagonal are pairwise differences in H_E and below the diagonal are pairwise differences in allelic richness.

	FR	JU	KN
FR		2.187	0.006**
JU	2.385		0.015*
KN	0.006**	0.0033**	

FR, Frederiksborg; JU, Jutland; KN, Knabstrupper.
*$P < 0.05$, **$P < 0.01$.

Breed differentiation

The overall estimate of F_{ST} across all loci differed significantly from zero ($F_{ST} = 0.1032$, $P = 0.00113$). The pairwise F_{ST} values between populations ranged from 0.0577 (FR/KN) to 0.1406 (FR/JU), with the in-between value ($F_{ST} = 0.1225$) for KN/JU. When Bonferroni-corrected, there was significant differentiation between the breeds.

The Bayesian structure analysis did not return an unequivocal number of genetic clusters; ln $P(D)$ seemed to level out at four clusters and then started to fall. There seemed to be an optimum at three clusters. A log likelihood ratio test also indicated three clusters ($\chi^2_{K=2 \text{ vs. } K=3} = 124.08$, df = 88). Based on these findings, it was decided to set $K = 3$.

Results showed that the FR and JU samples were correctly assigned to a single population with probability $q \geq 0.8$, while KN individuals were assigned to more than one cluster (Table 5). In FR, seven individuals could not be assigned to the proper cluster (cluster 1) at the 0.80 level, and two of these were mis-assigned to cluster 2, and thereby

Table 3 Summary of genetic diversity: number of alleles (*n*) sampled in total and in each breed, allelic richness (AR) and observed (H_O) and expected (H_E) heterozygosity.

	All	Frederiksborg				Jutland				Knabstrupper			
	n	*n*	AR	H_O	H_E	*n*	AR	H_O	H_E	*n*	AR	H_O	H_E
VHL20	10	6	5.585	0.697	0.684	7	6.562	0.800	0.718	9	8.697	0.882	0.851
HTG4	8	6	5.594	0.733	0.657	7	6.400	0.733	0.697	6	5.703	0.676	0.717
AHT4	9	3	3.000	0.606	0.597	4	3.828	0.655	0.645	8	7.177	0.727	0.776
HMS7	7	5	4.963	0.767	0.734	4	4.000	0.538	0.538	7	6.454	0.818	0.778
HTG6	8	5	4.749	0.594	0.650	6	5.627	0.517	0.573	7	6.438	0.625	0.715
AHT5	9	6	5.927	0.844	0.696	4	3.794	0.467	0.572	9	8.190	0.844	0.824
HMS6	8	6	5.857	0.758	0.746	7	6.769	0.690	0.745	7	6.699	0.735	0.767
ASB2	10	4	4.000	0.583	0.601	5	4.989	0.667	0.731	9	8.524	0.600	0.826*
HTG10	8	7	6.535	0.393	0.490	6	5.919	0.846	0.758	8	7.725	0.710	0.834*
HTG7	6	4	3.999	0.606	0.555	5	4.956	0.733	0.688	5	4.455	0.454	0.607
HMS3	7	5	4.978	0.481	0.630	2	2.000	0.115	0.111	7	6.999	0.600	0.824
HMS2	10	6	5.800	0.833	0.779	7	6.362	0.533	0.540	6	5.824	0.862	0.762
All	100	63		0.663	0.653	64		0.611	0.613	88		0.712	0.772
Mean	8.33	5.25	5.082			5.33	5.101			7.33	6.907		

Bonferroni corrections were applied (Rice 1989). Significance levels were $P < 0.00139$ for the total population and $P < 0.00417$ for the individual breeds. Deviations from HWE are indicated by an asterisk.

Table 5 Proportion of assignment of each pre-defined population to each of the three clusters. The highest contributions are in boldface.

Given pop.	Inferred clusters			No. individuals
	1	2	3	
FR	**0.853**	0.093	0.054	33
JU	0.036	0.049	**0.915**	30
KN	0.229	**0.725**	0.046	34

FR, Frederiksborg; JU, Jutland; KN, Knabstrupper.
Cluster 1, FR; cluster 2, KN; cluster 3, JU.

to KN. Three individuals in JU could not be assigned to the proper cluster (cluster 3) at the 0.80 level, but none of these were mis-assigned. In KN, 14 individuals could not be assigned to the proper cluster (cluster 2) at the 0.80 level and six of these were mis-assigned to cluster 1, and thereby to FR. Five of the 14 individuals were admixed between cluster 1 and cluster 2 (see Fig. 1 for a bar plot of the STRUCTURE results).

Identifying populations for conservation

According to Caballero & Toro's (2002) method, the loss of the KN breed would provide the greatest loss caused by within-subpopulation diversity (GD_{WS}), but the loss of the JU breed would provide the greatest loss caused by between-subpopulation diversity (GD_{BS}) because of the distance of this breed from the other breeds. The loss of KN would provide the greatest loss to overall diversity (GD_T) (Table 6).

According to Petit et al.'s (1998) method, KN contributes the most to the total diversity (C_{DT}). It has a high within-breed diversity (C_{DS}) and divergence (C_{DR}). FR contributes to diversity but not to divergence. The opposite is the case for the JU breed. It is more divergent from the other breeds but has less within-breed diversity (Table 6).

According to the Weitzman (1992, 1993) approach, the loss of the JU breed would give the greatest loss to overall diversity. The KN breed would give a greater loss than the FR breed.

The individual relatedness and individual inbreeding coefficients were significantly correlated ($n = 97$, $r = 0.29$, $P = 0.0058$), but the individual heterozygosity and individual inbreeding coefficients were not ($r = 0.15$, $P = 0.129$).

Discussion

The level of AR, number of alleles sampled and heterozygosity found in the native Danish horses in this study were similar to those previously found in Lipizzan horses (Curik et al. 2003; Achmann et al. 2004), Spanish horses (Cañon et al. 2000; Solis et al. 2005; Marletta et al. 2006), German draught horses (Aberle et al. 2004), French horses (Glowatzki-Mullis et al. 2005) and Norwegian horses (Bjørnstad & Røed 2002). So, Danish horse breeds do not seem to be in immediate danger of extinction because of low genetic variation. The KN breed showed greater genetic variation than the other two breeds; this is probably because of the open studbook and is, therefore, probably a consequence of immigration. The FR and JU breeds have the same level of genetic variation, although they have different breeding policies: The JU studbook has been closed since 1861 whereas the FR studbook was closed more recently, in 1987. The JU breed might have lost some genetic variation because of drift, but drift might not have had enough time to work on the FR breed. The two breeds also have different breeding strategies. In the FR breed, a few stallions have almost monopolized the breeding market, and they seem to stay in the breeding pool until they are very old. The mean age of stallions is 12.62 ± 1.8 years. In the JU breed, the stallions are only in the breeding pool for a short time. The mean age of stallions in the JU breeding pool is 6.32 ± 0.16 years, and there is a much higher turnover rate in stallions. This might explain why the amount of genetic variation in the two breeds is similar despite the different breeding policies.

All breeds were in HWE, and there is no reason to assume deviations from random mating. The KN breed had heterozygote deficits at HTG10 and ASB2. Tests for the presence of null alleles indicated a null allele at ASB2 but not at HTG10. Recently, a base substitution in the sequence flanking the ABS2 marker has been described, the mutation being located in the L allele-priming site (Achmann et al. 2001). Thus, in this study, allele non-amplification can be the reason for the detected deviation from equilibrium in this marker. The remaining two breeds were in equilibrium for all 12 markers tested.

Both the assignment test and the F_{ST} statistics confirmed that there are three distinct breeds. The KN breed had previously been a part of the FR breed, but breeding strategies apparently have led to genetic differentiation.
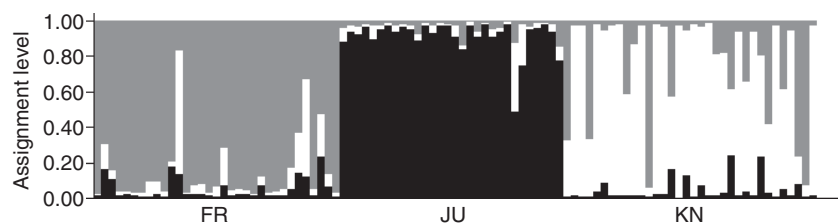
Figure 1 Bar plot from STRUCTURE. Each bar represents one individual. The shade of each bar indicates the cluster the individual is assigned to and the level of the assignment. Grey, FR; black, JU; white, KN.

	Loss of diversity[1,2] loss/gain		Contribution to richness[3,4] loss/gain		
	GD$_{WS}$/GD$_{BS}$	GD$_T$	C$_{DS}$/C$_{DR}$	C$_{DT}$	Marginal loss[2,5]
Total	0.812/0.034	0.846			
Frederiksborg	+0.009/−0.005	+0.005	0.5/−0.5	0.0	−32.58
Jutland	+0.015/−0.017	−0.002	−0.6/0.6	0.0	−67.42
Knabstrupper	−0.025/−0.004	−0.029	0.1/0.1	0.2	−37.86

Table 6 Loss of diversity, contribution to richness and marginal loss.

[1]Based on the method by Cabellero & Toro (2002).

[2]Positive values indicate gain of diversity and negative values indicate loss of diversity if the breed is lost.

[3]Based on the method by Petit *et al.* (1998).

[4]Positive values indicate that the breed contributes more than average to diversity or divergence and negative values indicate that the breed contributes less than average to diversity or divergence.

[5]Based on the method of Weitzman (1992).

Because the official KN studbook was founded in 1970 and remains open today, and because the FR studbook was closed only in 1987, there has not been enough time for drift to work on either of the breeds. Unwritten breeding rules and selective breeding might have formed the KN breed long before the official studbook was founded. The KN and FR are genetically very similar. In fact, at the 80% level, KN could not be assigned to its own cluster; it had a high percentage assigned to the FR breed. This finding could be because (i) the two breeds descended from the same breed, which split only recently, or (ii) both breeds have had high levels of immigration from the same breeds.

When deciding conservation priorities, several factors should be taken into account such as adaptation to specific environments or diseases and possession of special traits of cultural, scientific or future economic value (Ruane 1999). Genetic diversity within and between breeds can influence decisions affecting the breeds, or species, to be preserved. Choices should be based on objective criteria and computations. It is difficult to base priorities on subjective criteria such as beauty or interest in future or present generations (Thaon d'Arnoldi *et al.* 1998).

Conservation decisions will depend on future plans for the breeds in question. If the purpose is to use them in crossbreeding or introgression plans, the diversity between subpopulations should be prioritized. But on the other hand, if the purpose is to preserve a closed population capable to coping with future challenging environments or with diversified production conditions, the within-population diversity should be prioritized.

Results based on both Petit *et al.*'s (1998) and Caballero & Toro's (2002) methods indicate that if the KN breed is lost, it would be the greatest loss of within-population and overall diversity. The KN breed had significantly more genetic variation, as measured by H_E and AR, than the other two breeds. On the other hand, based on Weitzman's (1992) and Caballero & Toro's (2002) method, the greatest loss of between-population diversity would be because of the loss of the JU breed. This breed is more distinct from the other two breeds, as confirmed by F_{ST} statistics and STRUCTURE analysis. The Danish horse population, according to Caballero & Toro's method, will gain if the FR breed became extinct. This is because the method is based on a theoretical model in which subpopulations contribute to an infinite pool of genes (Fabuel *et al.* 2004). As a consequence of removing one subpopulation, gene frequencies would equalize in the remaining ones. This, in turn, would increase H_E. The variability of a population will increase if a group with the most related individuals is eliminated and substituted by randomly chosen individuals.

Several authors have criticized the Weitzman approach (Caballero & Toro 2002, Eding *et al.* 2002) because it ignores within-population diversity and because of the properties of monotonicity in species and diversity. This will favour inbred populations with extreme gene frequencies, whereas the coancestry approach will favour non-inbred populations with an even distribution of gene frequencies. On the other hand, an over-emphasis on within-breed variation will favour the largest breeds, those of current commercial value; they, therefore, are less endangered (Fabuel *et al.* 2004).

There was no correlation between individual heterozygosity and individual inbreeding coefficients, but a significant correlation between relatedness and individual inbreeding was found. Curik *et al.* (2003) did not find such correlations in Lipizzan horses while Cunningham *et al.* (2001) found close relatedness between relationship on the basis of allele sharing and relationship based on pedigree information in thoroughbred horses. Pemberton *et al.* (1999) found a correlation between relatedness and inbreeding in red deer (*Cervus elaphus*), but Coulson *et al.*

(1998) did not find such a relationship in the same population. Hedrick *et al.* (2001) found that the inbreeding coefficient explained a large amount of the variance in heterozygosity. Because the findings in previous studies are not unequivocal, it would not be advised to favour a breeding strategy excluding highly inbred animals from the breeding pool, based on the assumption that inbred animals are also genetically related to most other individuals.

In conclusion, the genetic diversity of the three native Danish horse breeds is comparable to other European horse breeds. The KN breed possesses more variation than either of the two other breeds. FR and JU possess similar levels of genetic variation. Even though two different breeding policies (open vs. closed studbooks) exist in the FR and JU breeds, different breeding strategies (monopolizing stallions vs. exchanging stallions) might have led to similar levels of genetic variation in these breeds.

Both F_{ST} statistics and assignment analysis confirmed the overall population to be differentiated into three distinct breeds, although FR and KN were genetically more similar and JU was more distant from the two other breeds. Coancestry and/or immigration from the same breed(s) might explain the similarities between FR and KN. If the aim of conservation is to preserve within-breed genetic diversity, KN possesses the most diversity and is more likely to cope with future challenges. If the aim is to preserve between-breed diversity, JU is more distant from the other two, and the loss of JU would mean the highest loss of total diversity. Even though individual heterozygosity and individual inbreeding are significantly correlated, it cannot be recommended to use a breeding strategy where highly inbred animals are excluded from the breeding pool.

Acknowledgements

References

Aberle K.S., Hamann H., Drögemüller C. & Distl O. (2004) Genetic diversity in German draught horse breeds compared with a group of primitive, riding and wild horses by means of microsatellite DNA markers. *Animal Genetics* **35**, 270–7.

Achmann R., Huber T., Wallner B., Dovc P., Müller M. & Brem G. (2001) Base substitutions in the sequences flanking microsatellite markers *HMS3* and *ASB2* interfere with parentage testing in the Lipizzan horse. *Animal Genetics* **32**, 52.

Achmann R., Curik I., Covc P., Kavar T., Bodo I., Habe F., Marti E., Sölkner J. & Brem G. (2004) Microsatellite diversity, population subdivision and gene flow in the Lipizzan horse. *Animal Genetics* **35**, 285–92.

Binns M.M., Holmes N.G., Holliman A. & Scott A.M. (1995) The identification of polymorphic microsatellite loci in the horse and their use in thoroughbred parentage testing. *British Veterinary Journals* **151**, 9–15.

Bjørnstad G. & Røed K.H. (2001) Breed demarcation and potential for breed allocation of horses assessed by microsatellite markers. *Animal Genetics* **32**, 59–65.

Bjørnstad G. & Røed K.H. (2002) Evaluation of factors affecting individual assignment precision using microsatellite data from horse breeds and simulated breed crosses. *Animal Genetics* **33**, 264–70.

Bjørnstad G., Nielsen N.Ø. & Røed K.H. (2003) Genetic relationship between Mongolian and Norwegian horses? *Animal Genetics* **34**, 55–8.

Bowling A.T. (2001) Historical development and application of molecular genetic tests for horse identification and parentage control. *Livestock Production Science* **72**, 111–6.

Bowling A.T., Eggleston-Stott M.L., Byrns G., Clark R.S., Dileanis S. & Wictum E. (1997) Validation of microsatellite markers for routine horse parentage testing. *Animal Genetics* **28**, 247–52.

Breen H., Downs P., Irwin Z. & Bell K. (1994) Intrageneric amplification of horse microsatellite markers with emphasis on the Przewalski's horse (*E. przewalskii*). *Animal Genetics* **25**, 401–5.

Caballero A. & Toro M.A. (2002) Analysis of genetic diversity for the management of conserved subdivided populations. *Conservation Genetics* **3**, 289–99.

Cañon J., Checa M.L., Carleos C., Vega-Pla J.L., Vallejo M. & Dunner S. (2000) The genetic structure of Spanish Celtic horse breeds inferred from microsatellite data. *Animal Genetics* **31**, 39–48.

Charlesworth D. & Charlesworth B. (1987) Inbreeding depression and its evolutionary consequences. *Annual Reviews* **18**, 237–68.

Coulson T.N., Pemberton J.M., Albon S.D., Beaumont M., Marshall T.C., Slate J., Guinness F.E. & Clutton-Brock T.H. (1998) Microsatellites reveal heterosis in red deer. *Proceedings of the Royal Society B, Biological Science* **265**, 489–95.

Crawley M.J. (1993) GLIM *for Ecologists*. Blackwell Scientific Publications, Oxford, UK.

Cunningham E.P., Dooley J.J., Splan R.K. & Bradley D.G. (2001) Microsatellite diversity, pedigree relatedness and contributions of founder lineages to thoroughbred horses. *Animal Genetics* **32**, 360–4.

Curik I., Zechner P., Sölkner J., Achmann R., Bodo I., Dovc P., Kavar T., Marti E. & Brem G. (2003) Inbreeding, microsatellite heterozygosity and morphological traits in Lipizzan horses. *Journal of Heredity* **94**, 125–32.

Doyle J.J. & Doyle J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–5.

Druml T., Curik I., Baumung R., Aberle K., Distl O. & Sölkner J. (2007) Individual-based assessment of population structure and admixture in Austrian, Croatian and German draught horses. *Heredity* **98**, 114–22.

Eding H., Crooijmans P.M.A., Groenne M.A.M. & Meuwissen T.H.E. (2002) Assessing the contribution of breeds to the genetic diversity in conservation schemes. *Genetics Selection Evolution* **34**, 613–33.

Ellegren H., Johannsson M., Sandberg K. & Andersson L. (1992) Cloning of highly polymorphic microsatellites in horses. *Animal Genetics* **23**, 133–42.

Fabuel E., Barragán C., Silió L., Rodriguez M.C. & Toro M.A. (2004) Analysis of genetic diversity and conservation priorities in Iberian pigs based on microsatellite markers. *Heredity* **93**, 104–13.

Frankham R., Ballou J.D. & Briscoe D.A. (2002) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, UK.

Genressourceudvalget (2007) *Arbejdsplan for Udvalget til Bevarelse af Benressourcer hos Danske Husdyr*. Available at: http://www.genres.dk/Arbejdsplaner/Arbejdsplan_2007.pdf) (last accessed on 3 August 2008).

Glowatzki-Mullis K.L., Muntwyler J., Pfister W., Marti E., Rieder S., Poncet P.A. & Gaillard C. (2005) Genetic diversity among horse populations with a special focus on the Franches-Montagnes breed. *Animal Genetics* **37**, 33–9.

Goudet J. (1995) FSTAT (Version 1.2): a computer program to calculate *F*-statistics. *Journal of Heredity* **86**, 485–6.

Guérin G., Bertaud M. & Amigues Y. (1994) Characterization of seven new horse microsatellites: *HMS1, HMS2, HMS3, HMS5, HMS6, HMS7* and *HMS8*. *Animal Genetics* **25**, 62.

Guo S.W. & Thompson E.A. (1992) Performing the exact test for Hardy–Weinberg proportions for multiple alleles. *Biometrics* **48**, 316–72.

Gutiérrez J.P., Royo L.J., Álvarez I. & Goyache F. (2005) MOLKIN v2.0: a computer program for genetic analysis of populations using molecular coancestry information. *Journal of Heredity* **96**, 718–21.

Hedegaard E. (2003) *Jyder – Heste og Folk. Den Jydske Hests Historie*. Arkona, Denmark.

Hedrick P.W. & Kalinowski S.T. (2000) Inbreeding depression in conservation biology. *Annual Review of Ecology and Systematics* **31**, 139–62.

Hedrick P., Fredrickson R. & Ellegreen H. (2001) Evaluation of d^2, a microsatellite measure of inbreeding and outbreeding, in wolves with a known pedigree. *Evolution* **55**, 1256–60.

Kelly L., Postiglioni A., De Andrés D.F., Vega-Pla J.L., Gagliardi R., Biagetti R. & Franco J. (2002) Genetic characterisation of the Uruguayan Creole horse and analysis of relationships among horse breeds. *Research in Veterinary Science* **72**, 69–73.

Lee S. & Cho G. (2006) Parentage testing of thoroughbred horses in Korea using microsatellite DNA typing. *Journal of Veterinary Science* **7**, 63–7.

Lunn N. (1966) Mere om Knabstrupperne. *Hipologisk Tidsskrift* **8**, 4–7.

Marklund S., Ellegren H., Eriksson S., Sandberg K. & Andersson L. (1994) Parental testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. *Animal Genetics* **25**, 19–23.

Marletta D., Tupsc-Yupanqui I., Bordonaro S., García D., Guastella A.M., Criscione A., Cañón J. & Dunner S. (2006) Analysis of genetic diversity and the determination of relationships among western Mediterranean horse breeds using microsatellites. *Journal of Animal Breeding and Genetics* **123**, 315–25.

Nei M. (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Peakall R. & Smouse P.E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288–95.

Pemberton J.M., Coltman D.W., Coulson T.N. & Slate J. (1999) Using microsatellites to measure the fitness consequences of inbreeding and outbreeding. In: *Microsatellites: Evolution and Application. World Animal Science A4* (Ed. by A.B. Chapman), pp. 151–64. Elsevier, Amsterdam.

Penedo M.C.T., Millon L.V., Bernoco D. *et al.* (2005) International equine gene mapping workshop report: a comprehensive linkage map constructed with data from new markers and by merging four mapping resources. *Cytogenetic and Genome Research* **111**, 5–15.

Petit R.J., El Mousadik A. & Ponds O. (1998) Identifying populations for conservation on the basis of genetic markers. *Conservation Biology* **12**, 844–55.

Pritchard J.K., Stephens M. & Donnelly P.J. (2000) Inferences of population structure using multilocus genotype data. *Genetics* **155**, 945–59.

Rice W.R. (1989) Analyzing tables of statistical tests. *Evolution* **43**, 223–5.

Ruane J. (1999) A critical review of the values of genetic distance studies in conservation of animal genetic resources. *Journal of Animal Breeding and Genetics* **159**, 699–713.

Solis A., Jugo B.M., Mériaux J.C., Iriondo M., Mazón L.I., Aguirre A.I., Vicario A. & Estomba A. (2005) Genetic diversity within and among four South European native horse breeds based on microsatellite DNA analysis: implications for conservation. *Journal of Heredity* **96**, 670–8.

Thaon d'Arnoldi C., Foulley J.L. & Ollivier L. (1998) An overview of the Weitzman approach to diversity. *Genetics Selection Evolution* **30**, 149–61.

Tozaki T., Kakoi H., Mashima S., Hirota K., Hasegawa T., Ishida N., Miura N., Choi-Miura N.-H. & Tomita M. (2001) Population study and validation of paternity testing for thoroughbred horses by 15 microsatellite loci. *Journal of Veterinary and Medical Science* **63**, 1191–7.

Tozaki T., Takezaki N., Hasegawa T., Ishida N., Kurosawa M., Tomita M., Saitou N. & Mukoyama H. (2003) Microsatellite variation in Japanese and Asian horses and their phylogenetic relationship using a European horse outgroup. *Journal of Heredity* **94**, 374–80.

Trock C. (1986) *Heste og Ponyavl*. Skarv, Denmark.

Van Haeringen H., Bowling A.T., Scott M.L., Lenstra J.A. & Zwaagstra K.A. (1994) A highly polymorphic horse microsatellite locus: *VHL20*. *Animal Genetics* **25**, 207.

Van Oosterhout C., Hutchinson W.F., Wills D.P.M. & Shipley P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535–8.

Weir B.S. & Cockerham C.C. (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358–70.

Weitzman M.L. (1992) On diversity. *The Quarterly Journal of Economics* **107**, 363–405.

Weitzman M.L. (1993) What to preserve? An application of diversity theory to crane conservation. *The Quarterly Journal of Economics* **108**, 157–83.

Yeh F.C. & Boyle T.J. (1997) Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**, 157.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Allelic frequencies for each breed and at each locus.

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