Impacts of genetic drift and restricted gene flow in indigenous cattle breeds: evidence from the Jutland breed

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Summary

Indigenous cattle breeds represent a unique genetic resource, and understanding their variability, population structure and breeding units is important for their sustainable conservation. The endangered Jutland breed was widespread in Denmark in the eighteenth century, but decreased in population size following the introduction of modern farming. We investigated the impact of recent anthropogenic fragmentation of the breed by analysing 737-bp mitochondrial DNA and 23 microsatellites in 207 individuals. The results revealed the Jutland breed as a unique genetic entity with high levels of genetic diversity, and only limited introgression from other black-pied breeds. The data reflected the impacts of fragmentation and restricted gene flow in breeds with small segregated herds, and revealed the rapid differentiation of herds resulting from genetic drift. The application of a management strategy that conserves diversity and minimizes increase in inbreeding is important for the future conservation of the Jutland breed and other indigenous cattle breeds.

Keywords: *fragmentation, genetic diversity, indigenous cattle breeds, introgression*

Résumé

Les races de bétails indigènes representent une ressource génétique unique. Comprendre la variabilité génétique, la structure de la population et unités d'amelioration génétique est essentiel. La race Jutland, menacée aujourd'hui, était très répandue au Danemark durant le 18eme siècle avant de voir sa population décroitre suite a l'avènement des méthodes modernes d'agriculture. Nous avons etudiés l'impact de la fragmentation anthropogénique de cette race en analysant l'ADN mitochondrial (737-bp) et 23 microsatellites dans 207 individus. Les résultats dévoilent La race Jutland comme une entité génétique unique présentant une grande diversité, et montrent seulement une introgression limité d'autres races tachetés noires. Les données reflètent les impacts de la fragmentation et un flux de genes limite au sein des espèces avec de petits troupeaux issus d'une ségrégation et revèlent une differentiation rapide des troupeaux résultant de la dérive génétique. La mise en place d'une stratégie de management qui conserve la diversité et qui empêche les croisements est importante pour la conservation future de la race Jutland et des autres races de bétails indigènes.

Mots-clés: *la fragmentation, la diversité génétique, les races de bétail Indigènes, introgression*

Resumen

Las razas indígenas del ganado representan un fuente única de recurso genético, la comprensión de su variabilidad, estructura de población, y de sus unidades de cría son importantes factores para sus conservación sostenible. La raza de ganado en peligro de extinción de Jutlandia se encontraba muy dispersa en Dinamarca en el siglo XVIII, pero disminuyó e tamaño tras la introducción de la agricultura moderna. En este estudio, se investigó el impacto antropogénico en la fragmentación de la raza usando el análisis de 737 pares de bases del ADN mitocondrial y 23 microsatélites en 207 animales. Los resultados muestran que la raza en Jutlandia contiene una entidad genética única con altos niveles de diversidad genética, y limitada solamente por la introgresión de otras razas comunes de ganado. Los datos reflejan el impacto de la fragmentación y el flujo genético en razas restringidas y separadas en pequeños rebaños; y pone en manifiesto la rápida diferenciación de los rebaños como resultado de la deriva genética. La aplicación de una estrategia de gestión que conserve la diversidad y evite la mezcla de razas es importante para la futura conservación de la raza de Jutlandia y otras razas de ganado indígenas.

Palabras clave: *fragmentación, diversidad genética, razas de ganado Indígenas, introgression*

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Introduction

The diversity of domestic cattle reflects millennia of artificial breeding, selection and geographic separation, which have led to a broad spectrum of phenotypes (Hanotte *et al.*, 2002). Since the introduction of modern farming in the eighteenth century, an erosion of genetic diversity has taken place in livestock (Tapio *et al.*, 2006). A few high-performance cattle breeds have been utilized in the industrial farming system, where selective breeding and assisted reproduction have resulted in an overall loss of genetic variability (Kantanen *et al.*, 2000; Taberlet *et al.*, 2008).

The indigenous breeds are – in contrast to the highly selected commercial breeds – exposed to lower selection pressures. They have been subjected to local use and traditional husbandry management, and have – as a group – generally maintained a large pool of genetic variability, despite their small population sizes (Tapio *et al.*, 2006). As a consequence, there are high levels of phenotypic variation among indigenous breeds, a variety of adaptations to local environmental conditions and high fitness under natural conditions (Tapio *et al.*, 2006; Dalvit *et al.*, 2008). They represent a unique genetic resource, and can potentially contribute genetic diversity beneficial for meeting present and future breeding objectives (Sørensen, Sørensen and Berg, 2005; Toro, Fernández and Caballero, 2009). At present, a high number of indigenous breeds have low effective population sizes (N_e) and are listed as at risk by the Food and Agriculture Organization of the United Nations (FAO, 2007). Understanding the variability, genetic structure and breeding units of indigenous breeds is important for conserving them in the most sustainable way (Taberlet *et al.*, 2008; Toro, Fernández and Caballero, 2009).

In Denmark, the Danish livestock industry has focused on the commercial Holstein and Jersey breeds (Sørensen, Sørensen and Berg, 2005; Taberlet *et al.*, 2008). As a consequence, indigenous breeds such as the Red Danish, Agersoe Cattle and the Jutland breed have been neglected. These breeds have suffered significant declines in population sizes, prompting concern about their long-term genetic viability (Danish Ministry of Food, Fisheries and Agriculture, 2008).

One indigenous breed that has been maintained through the efforts of private collectors is the Jutland breed. It presumably originated from black- and grey-pied cattle and was widespread in Denmark in the eighteenth century, mainly on the Jutland peninsula (Kantanen *et al.*, 2000; Negrini *et al.*, 2007; Danish Ministry of Food, Fisheries and Agriculture, 2010; Withen *et al.*, 2011). Since then it has experienced a drastic decline in population size and is now classified as endangered (FAO, 2007). Estimated from unequal sex ratios (Hedrick, 2002, equation 6.6a), the effective population size of the Jutland breed was estimated at 40 out of a census size of ∼350 individuals in 2007 (http://dad.fao.org). This is similar to the effective population size of commercial Danish breeds: Danish Holstein ($N_e = 49$), Danish Jersey ($N_e = 53$) and Danish Red ($N_e = 47$) (Sørensen, Sørensen and Berg, 2005). As these breeds have considerably larger population sizes of 560 000 to 3 700 000 individuals (Sørensen, Sørensen and Berg, 2005), they are not classified as endangered (http://iuncredlist.org).

Before the introduction of modern farming, the Jutland breed was used in both dairy and beef production under traditional usbandry management. In the eighteenth–twentieth century, there was a large export, of mainly steers, to the North German market (Danish Ministry of Food, Fisheries and Agriculture, 2010). In 1881, the first herd book was published and in 1950 part of the breed was crossed with black-pied cattle from Holland and Germany to form the Danish Black-Pied. A few private collectors continued to breed the original Jutland breed, and the breed currently consists of four main herds and a number of herds of smaller size, which have been separated for at least the last five decades without admixture from other breeds (S. Benzon, personal communication). A management strategy for the breed is currently being planned on the basis of the present survey.

We used 737-bp of the mitochondrial DNA (mtDNA) control region and 23 microsatellites to estimate genetic diversity and structuring within the Jutland breed. To elucidate the genetic relationship between the Jutland breed and breeds which may have been admixed with it, we included microsatellite data from the following six breeds: the German Black-Pied ($N_e = 172$) (H. Simianer, personal communication), Dutch Belted ($N_e = 136$), Dutch Friesian ($N_e = 225$), Groningen White-Head ($N_e = 149$), Meuse–Rhine–Yssel ($N_e = 689$) and the Danish Black-Pied ($N_e = 4$). Effective population sizes were estimated from unequal sex ratios (Hedrick, 2002, equation 6.6a) based on data from FAO's Domestic Animal Diversity Information System (http://dad.fao.org).

Materials and methods

Samples

Blood and tissue samples were collected between 1997 and 2007 from 207 registered Jutland breed animals. Four main herds were sampled: Kortegaard ($n = 21$), Vesterboelle ($n = 28$) and Westergaard ($n = 22$) all from Northern Jutland, and Oregaard from Funen ($n = 82$), in addition to 54 animals from smaller herds consisting of one to four animals, mainly from Jutland. The animals were selected to be as unrelated as possible (avoiding siblings and parent/offspring) according to the Danish Register for Livestock. Due to difficulties in amplifying mtDNA and microsatellites for a number of samples, not all animals were represented in every analysis.

Dr H. Lenstra from the European Cattle Genetic Consortium (ECGC) kindly provided microsatellite data

from the Danish Black-Pied ($n = 44$), the German Black-Pied ($n = 20$) and four Dutch cattle breeds: Dutch Belted ($n = 24$), Dutch Friesian ($n = 34$), Groningen White-Head ($n = 25$) and the Meuse–Rhine–Yssel ($n = 39$). The ECGC dataset is based on 30 microsatellite loci, which are recommended by the International Society for Animal Genetics (ISAG) and FAO. The loci are unlinked and neutral, and widely used in genetic studies of cattle. All samples were collected in 1999. Unfortunately mitochondrial data were not available for these breeds.

In the following, we use *herds* to refer to the analysis of the four main herds. The term *breeds* is used in reference to the comparative analysis between the Jutland breed and the six black-pied breeds.

DNA extraction

DNA was extracted and purified from 100 µl blood or 20 mg tissue samples according to the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol and was stored at $-20\,°C$ in the laboratory.

MtDNA

The control region (d-loop) of the mtDNA was amplified in PCR using two flanking primers 5′-CTGCAGTCTCACCA TCAACC-3′ (Loftus *et al.*, 1994) and 5′-AGAGTTAACA GGAAGGCTGG-3′ (Kim *et al.*, 2003). The amplification was performed in a 25 µl volume PCR containing 1 µl purified DNA, $1 \times$ PCR buffer, 1.5 mM MgCl$_2$, 1 µM of each primer, 2 µM of each dNTP and 1 unit *Taq* polymerase (Thermo Scientific). The following cycling conditions were used: 2 min at 94 °C; 35 cycles of 1 min at 94 °C; 1 min at 57 °C; 1 min at 72 °C and a final extension for 10 min at 72 °C. PCR blanks were always included and remained negative throughout. Purification of PCR products was performed with *Invisorb Vacuum Manifold kit* (Invisorb) following the manufacturer's protocol. The PCR product was run on a 2 percent agarose-gel and verified afterwards.

Sequencing was performed using ABI Prism®BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) in a 10 µl volume containing 1 µl DNA template and 10 µl HiFi. Two primer pairs were used in the sequencing 5′-CTGCAGTCTCACCATCAACC-3′ (Loftus *et al.*, 1994), 5′-CGAGATGTCTTATTTAAGAGG-3′ (Cymbron *et al.*, 1999), 5′-CGCTCCGGGCCCATAAACC G-3′ and 5′-GCCTGCGTTTATATATTGAC-3′ (Withen *et al.*, 2011). The fragments of 597 and 588-bp, respectively, overlapped resulting in a 737-bp sequence. Cycle sequencing products were purified prior to separation on a 5 percent denaturing polyacrylamide gel in an ABI Prism®377 DNA sequencer (Applied Biosystems) using the DyeEx 2.0 Spin kit (Qiagen).

The two mtDNA sequence fragments were assembled and edited in SEQUENCHER version 4.8 (Gene Code Cooperation) and for each individual a consensus sequence was composed using the bovine mtDNA reference sequence (Anderson *et al.*, 1982; accession no. NC_00156, GenBank). The sequences were aligned in SE-AL version 2.0a11 (Rambaut, 1996) and formatted in CLUSTALX version 2.0.7 (Larkin *et al.*, 2007).

Microsatellites

Thirty unlinked microsatellite loci were genotyped for each sample as recommended by the ISAG and FAO. StockMarks® Horse and Cattle Paternity PCR Typing Kit (Applied Biosystems) were used for 11 of the markers: *TGLA227* (18), *BM2113* (2), *TGLA53* (16), *ETH10* (5), *SPS115* (15), *TGLA126* (20), *TGLA122* (21), *INRA23* (12), *ETH3* (19), *ETH225* (9) and *BM1824* (1), numbers in parentheses indicate chromosome location (http://www. thearkdb.org/arkdb). The PCR was run, using 1 µl DNA template, according to the manufacturer's protocol with all 11 markers amplified in one PCR reaction (Supplementary Table S1). Sequencing was done on an ABI Prism®BigDye terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) in a 13 µl volume containing 1 µl DNA, 11.5 µl HiDi-formamid and 0.5 µl Rox500.

The remaining nineteen markers: *INRA063* (18), *INRA005* (12), *ILST005* (10), *HEL5* (21), *HEL1* (15), *INRA035* (16), *ETH152* (5), *HEL9* (8), *CSSM66* (14), *INRA032* (11), *HEL13* (11), *INRA037* (10), *BM1818* (23), *ILST006* (7), *MM12* (9), *CSRM60* (10), *ETH185* (17), *HAUT24* (22) and *HAUT27* (26) were amplified with QIAGEN® Multiplex PCR Kit (Qiagen) in six reactions with unique PCR conditions following the manufacturer's protocol using 1 µl DNA as the template. The reactions were combined in three groups and took place in 96-well microtitre plates with 1 µl DNA in 13 µl volume (Supplementary Table S1). All loci were sequenced on an ABI Prism®BigDye terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) in a 10 µl volume containing 2 µl DNA template, 7.5 µl HiDi-formamid and 0.5 µl Rox500. To be able to compare our data with the ECGC dataset, we standardized our microsatellite data to the ECGC data by including ECGC reference DNA in our sequencing and scoring our samples accordingly.

Statistical methods

Validation of microsatellite data

The microsatellite loci were checked for possible allelic stuttering, allele drop out and null alleles in MICRO–CHECKER version 2.2 (Van Oosterhout *et al.*, 2004), which resulted in elimination of the following seven loci: *TGLA227, TGLA122, INRA32, BM1818, INRA35, BM2113* and *ETH185*, yielding a total of 23 loci.

Genetic diversity

Quantification of mtDNA genetic variation was estimated in DNASP version 5.0 (Rozas *et al.*, 2003) as haplotype

(H) and nucleotide (π) diversity according to Nei (1987) along with Tajima (1989) to test for selection. Haplogroup relationship (T, T1, T2 and T3) for each sequence was determined according to Troy *et al.* (2001). JMODELTEST version 0.1.1 (Posada, 2008) was used to find the substitution model that best fitted the observed data based on the Akaike Information Criterion constrained to compute likelihood scores only for models available in ARLEQUIN version 3.11 (Excoffier, Laval and Schneider, 2005). A median-joining network (Bandelt, Foster and Röhl, 1999) was constructed in NETWORK version 4.5.1.6 (http://www.fluxus-engineering.com) using the default settings.

Basic microsatellite diversity indices; mean number of observed alleles (A_O), observed (H_O) and unbiased expected (UH_E) heterozygosities and numbers of private alleles (A_P), were estimated in GENALEX version 6.3 (Peakall and Smouse, 2006). Fixation indexes (f) and significant deviation from Hardy Weinberg Equilibrium (HWE) were estimated in GENODIVE version 2.0b17 (Meirmans and Van Tienderen, 2004), each tested for significance by 999 permutations. In GENEPOP version 3.4 (Raymond and Rousset, 1995) significant deviations from linkage equilibrium (LE), was estimated for each locus pair using Fishers Exact Test based on 10^4 batches with 10^5 de-memorizations and 10^5 permutations per batch resulting in standard error <0.03 followed by sequential Bonferroni correction to reduce type 1 errors.

Genetic differentiation

Haplotype differentiation, defined as Φ_{ST} (Weir and Cockerham, 1984), was estimated in ARLEQUIN applying Tamura and Nei's substitution model (Tamura and Nei, 1993). Genetic differentiation, defined as F_{ST} (Weir and Cockerham, 1984), based on microsatellite data was estimated in GENODIVE version 2.0b17, and tested for significance with 10^3 permutations followed by Bonferroni correction. Reynold's genetic distance (D_R) was estimated using ARLEQUIN with 10^3 permutations to test for significance. The results were visualized as a NeighborNet in SPLITSTREE version 4.10 (Huson and Bryant, 2006).

Genetic structure

Structuring was estimated in STRUCTURE version 2.2 (Pritchard, Stephens and Donnelly, 2000) based on an admixture model with no prior information. A burn-in of 10^4 iterations followed by an Markov Chain Monte Carlo (MCMC) of 10^5 iterations for each value of K ($K = 1–12$) was applied, with five repetitions for each K. The most likely number of clusters was determined following the delta-K method (Evanno, Regnaut and Goudet, 2005) in STRUCTURE HARVESTER version 0.56.3 (Earl, 2009). CLUMPP (Jakobsson and Rosenberg, 2007) was applied to permute membership matrices across

replicates and generate a mean permuted matrix for a given value of K. The results were displayed using DISTRUCT (Rosenberg, 2004).

Assignment test was performed in GENALEX version 6.3 based on the "leave one out" option. The individual of interest was removed from the dataset prior to estimating the population genotype frequencies. The smaller herds were treated as unknown samples when assigned to populations and therefore not influencing the population genotype frequency estimates.

Gene flow

Pairwise gene flow rates were estimated in BAYESASS+ version 1.3 (Wilson and Rannala, 2003). The programme uses an MCMC algorithm to estimate recent gene flow assuming low levels of migration (<1/3 of the population total in each generation) and linkage equilibrium among loci. The method infers recent migration rates from the observed disequilibrium among genotypes in the population, generated by novel multi-locus genotypes introduced by migrants or individuals recently descended from migrants. BAYESASS+ may be applied to non-stationary populations that are far from HWE. We used a burn-in of 10^6 iterations followed by an MCMC of 4×10^6 iterations with a sampling frequency of 2×10^3. Delta values were set to 15 for allele frequency, migration rate and inbreeding level. To test for convergence, the programme was run with two different "seed" values (10 and 10^3). The results were examined in R ver. 2.12.2 (R Development Core Team, 2011) using the BOA package (Smith, 2007). Convergence was assumed when no significant difference was found between the mean of the first 10 percent of the chain and the mean of the last 50 percent (Geweke, 1992).
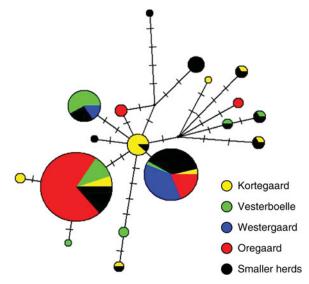
Results

Genetic diversity

The mtDNA analysis yielded 207 sequences of 737-bp (accession no. JQ234675–JQ234881). All haplotypes belong to the T3 haplogroup. There were 29 variable sites, resulting in 18 haplotypes. No sign of selection was detected (Table 1), which is consistent with expectations for this region (Anderson *et al.*, 1982; Tajima, 1989). Half of the haplotypes were shared among several herds with the most frequent found in 46 percent of the individuals (Figure 1). The overall haplotype diversity (H) was 0.72 and ranged from 0.17 in Westergaard to 0.81 in Kortegaard. The nucleotide diversity (π) was 0.0035 and varied from 0.0007 in Westergaard to 0.0043 in Vesterboelle (Table 1a).

The 23 microsatellite loci had 149 alleles in total, ranging from two alleles (*ILST005*) to 12 alleles (*TGLA53*) per locus. The observed heterozygosity (H_O) was 0.555–0.656 and mean number of alleles (A_O) was 4.6–4.8 per locus in the four herds, each having seven to eight unique

Table 1. Summary statistics of (a) the four main herds within the Jutland breed based on mtDNA and microsatellites and (b) the black-pied breeds based on microsatellites.

	mtDNA						Microsatellites					
	n	H	H_P	h	Π	D	A_O	A_P	H_O	UH_E	f	LE
(a)												
Kortegaard	21	8	2	0.810 ± 0.065	0.0038 ± 0.0007	−1.52	4.8	7	0.656 ± 0.031	0.636 ± 0.026	−0.054	2*
Vesterboelle	28	7	2	0.704 ± 0.059	0.0043 ± 0.0006	−0.39	4.6	7	0.555 ± 0.036	0.525 ± 0.032	−0.083*	3*
Westergaard	22	2	—	0.173 ± 0.010	0.0007 ± 0.0004	−0.95	4.6	7	0.598 ± 0.041	0.568 ± 0.035	−0.036	1*
Oregaard	82	4	2	0.323 ± 0.063	0.0014 ± 0.0003	−0.62	4.7	8	0.605 ± 0.025	0.624 ± 0.025	0.018	6*
(b)												
Jutland breed	207	18	—	0.717 ± 0.025	0.0035 ± 0.0002	−1.44	6.5	8	0.611 ± 0.022	0.664 ± 0.022	0.055*	60*
Danish Black-Pied	44	—	—	—	—	—	5.7	1	0.608 ± 0.032	0.661 ± 0.029	0.060*	5*
German Black-Pied	20	—	—	—	—	—	5.6	3	0.722 ± 0.030	0.676 ± 0.027	−0.060	0
Dutch Belted	24	—	—	—	—	—	5.0	7	0.622 ± 0.039	0.631 ± 0.035	0.003	2*
Dutch Friesian	34	—	—	—	—	—	5.1	1	0.619 ± 0.034	0.649 ± 0.030	0.042	1*
Groningen White-Head	25	—	—	—	—	—	4.1	—	0.565 ± 0.038	0.562 ± 0.027	0.013	1*
Meuse–Rhine–Yssel	39	—	—	—	—	—	5.5	7	0.685 ± 0.027	0.683 ± 0.025	0.003	2*

MtDNA information based on 737 bp includes number of haplotypes (H), number of private haplotypes (H_P), haplotype diversity (h), nucleotide diversity (π) and Tajima's D (D). Information from the 23 microsatellite loci includes mean number of alleles (A_O), number of private alleles (A_P), observed heterozygosity (H_O), unbiased expected heterozygosity (UH_E), inbreeding coefficient (f) and number of significant pairwise linkage disequilibria (LE). *Indicates $p < 0.05$.

Figure 1. Median-joining network of the 18 mitochondrial haplotypes found in the Jutland breed. Each circle corresponds to a haplotype and the relative size of the circle represents the haplotype frequency. Substitutions are indicated with bars on the branches.

alleles (A_P). Vesterboelle showed a significant excess of heterozygotes compared to HWE (Table 1a). Non-random association among loci (LE) was found in all herds (Table 1a). The smaller herds had $LE = 48$, which resulted in $LE = 60$ in the Jutland breed (Table 1b).

The observed heterozygosity (H_O) was 0.611 for the Jutland breed and 0.565–0.722 for the other breeds with a mean number of alleles (A_O) of 6.5 and 4.1–5.7, respectively (Table 1b). Unique alleles ($A_P = 1$–8) were found in all breeds except for Groningen White-Head. Deviations from HWE were found in the Jutland breed and the Danish Black-Pied, which both showed an excess of homozygotes (Table 1b). Linkage disequilibrium ($LE = 1$–5) was found among pairs of loci in the Danish Black-Pied and all Dutch breeds. The Jutland breed had the highest amount ($LE = 60$) (Table 1b).

Genetic differentiation

All pairwise mtDNA Φ_{ST} comparisons among the herds revealed significant differentiation (Table 2a). Kortegaard and Vesterboelle were found to be the least differentiated, and Oregaard and Westergaard the most highly differentiated herds based on mtDNA. All pairwise microsatellite F_{ST} comparisons were also significant, but in contrast to the mtDNA data, the microsatellite data showed Kortegaard and Oregaard as the least differentiated herds, and Kortegaard and Vesterboelle as the most differentiated.

The genetic differentiation (F_{ST}) and genetic distance estimates (D_R) revealed significant genetic differentiation between herds and breeds (Table 2b and Figure 2a). All four herds were more closely related to the Danish and German Black-Pied than they were to each other. Overall, the Jutland breed was closely related to the Danish- and German Black-Pied breeds (Table 2b and Figure 2b).

Table 2. Pairwise estimates of genetic differentiation among (*a*) the four herds and (*b*) the Jutland breed and the black-pied breeds.

(*a*) Herds	Herds	Kortegaard	Vesterboelle	Westergaard	Oregaard	
	Kortegaard	–	0.162*	0.124*	0.079*	
	Vesterboelle	0.119*	–	0.160*	0.151*	
	Westergaard	0.338*	0.352*	–	0.116*	
	Oregaard	0.280*	0.337*	0.657*	–	
(*b*) Breeds	Herds/breed	Kortegaard	Vesterboelle	Westergaard	Oregaard	Jutland breed
	Danish Black-Pied	0.046*	0.100*	0.057*	0.053*	0.017*
	German Black-Pied	0.080*	0.134*	0.114*	0.086*	0.052*
	Dutch Belted	0.127*	0.183*	0.147*	0.114*	0.086*
	Dutch Friesian	0.108*	0.175*	0.136*	0.100*	0.080*
	Groningen White-Head	0.172*	0.230*	0.230*	0.162*	0.137*
	Meuse–Rhine–Yssel	0.102*	0.144*	0.107*	0.093*	0.066*

Values based on (*a*) pairwise comparisons of 737 bp mtDNA (Φ_{ST}) under the diagonal and 23 microsatellite loci (F_{ST}) above the diagonal and (*b*) 23 microsatellite loci (F_{ST}). All values from the microsatellite analysis are based on 10^3 permutations. *Indicates $p < 0.05$.

Genetic structure

Clear population structure was found among herds and among breeds (Figure 3). The distinction of the Jutland breed and the German and Dutch breeds was found throughout. The herds showed structure for lower values of *K* than the breeds. The most likely number of clusters was three ($\Delta K = 455$), which divided the samples into three groups: (1) Kortegaard, Oregaard and the smaller herds, (2) Vesterboelle and Westergaard and (3) the German and Dutch breeds. The Danish Black-Pied showed membership coefficients in all three groups (Figure 3).

The assignment test showed that the majority of individuals from the four main herds were assigned to their herd of origin (Table 3*a*). The smaller herds assigned primarily to Kortegaard, Vesterboelle and Oregaard (Table 3*a*). The majority of individuals from the breeds were assigned to their breed of origin, except for the Danish Black-Pied, where half of the individuals were assigned to the Jutland breed (Table 3*b*).

Gene flow

Gene flow among the herds (Table 4*a*) was high ($m > 0.10$) from Kortegaard to the smaller herds, from Vesterboelle to Oregaard, and from the smaller herds to Vesterboelle. All other gene flow rates were low ($m < 0.10$) among the herds.

Two gene flow rates were high ($m > 0.10$) among the breeds (Table 4*b*), from the Jutland breed to the Danish Black-Pied and from the Dutch Friesian to the German Black-Pied. Low gene flow rates ($m < 0.10$) were found among the rest of the breeds (Table 4*b*).

Discussion

Genetic diversity of the Jutland breed

This study reveals the Jutland breed as a highly structured breed that is significantly differentiated from other related breeds, which make it a valuable reserve of genetic variation for both the indigenous and commercial breeds.
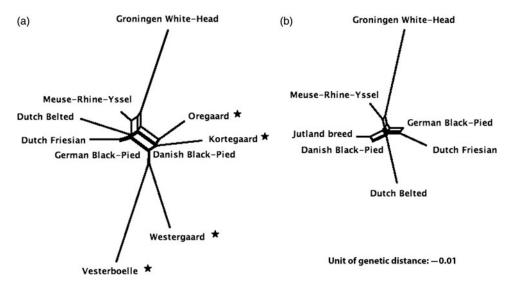

Figure 2. NeighborNets based on genetic distances (D_R) among (*a*) the four main Jutland breed herds (marked with a star) and the black-pied breeds, and (*b*) the entire Jutland breed and the black-pied breeds. The NeighborNets are based on 23 microsatellite loci.
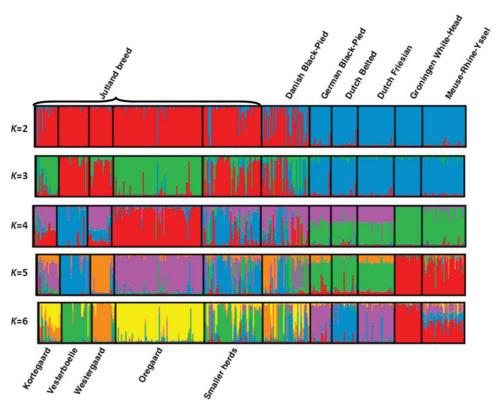
Figure 3. Estimated population structure among the Jutland breed herds and the black-pied breeds based on 23 microsatellite loci. Black vertical lines divide individuals into breeds, as indicated above the figure, and herds as indicated below the figure.

The Jutland breed has high genetic diversity (Table 1). All haplotypes belong to the common T3 haplogroup and most are shared among multiple herds (Figure 1). T3 is the most common haplogroup in Europe, and we found no evidence of introgression of breeds of the T, T1 or T2 haplogroups, which are more prevalent in the Middle East, Anatolia and Africa (Troy *et al.*, 2001).

The haplotype diversity is higher than that observed in other indigenous Danish breeds (Withen *et al.*, 2011). This corresponds well with their history of being widely used for local husbandry and not commercial selected breeding (Tapio *et al.*, 2006; Dalvit *et al.*, 2008). The level of genetic variation (H_O) is similar to that found in the Northern European black-pied breeds (Table 1); despite the Jutland breeds considerably lower effective population size as compared with the German and Dutch breeds. Different microsatellite loci have been applied to studies of genetic diversity in other cattle breeds, which makes direct comparisons of results complicated. However, based on data sets that have analysed a large number of same loci, the Jutland breed shows a level of genetic diversity comparable with the observed heterozygosity and mean allele number reported in other indigenous European cattle breeds (Kantanen *et al.*, 2000; Tapio *et al.*, 2006; Dalvit *et al.*, 2008; Withen *et al.*, 2011).

The small size of each herd – during at least the last five decades – is expected to lead to a reduction in genetic variation and an increase in levels of inbreeding (Reed and

Frankham, 2003). Historically the herd sizes are expected to have been larger when the Jutland breed was widespread (Kantanen *et al.*, 2000; Negrini *et al.*, 2007; Withen *et al.*, 2011). We did not observe significant levels of inbreeding in Kortegaard, Westergaard and Oregaard. Unfortunately, we do not have historic data to address whether a change in inbreeding and genetic diversity has taken place. As of now, these three herds can be seen as stable.

Vesterboelle shows signs of out-breeding (Table 1a). This might in part be ascribed to introgression from a Dutch-born grey-pied bull of unknown origin, which has been used extensively in the breeding programme of this herd (Dalsgaard, 2001). Vesterboelle's small population size and the extensive introgression are expected to lead to deviations from HWE (Hedrick, 2002). The introgression has most likely led to the distinctness of the herd, based on microsatellite markers (Table 2a and b). Unfortunately, we did not have genetic samples from the grey-pied bull, so we can only speculate that the introgression might have led to the observed pattern. The bull's origin must be of a breed, which was not included in this analysis, as Vesterboelle is distinct from the analysed breeds (Table 2b; Figures 2a and 3).

The high haplotype diversity found in Kortegaard and Vesterboelle may be due to high diversity of the founder females. In contrast, the lower haplotype diversity observed in Westergaard and Oregaard suggests high relatedness among the founder females. This is most

Table 3. Assignment of individuals to (*a*) herds and (*b*) breeds. Rows represent the herd/breed of origin. Columns represent the herd/breed to which an individual is assigned.

(*a*)	Kortegaard	Vesterboelle	Westergaard	Oregaard			
Kortegaard	19	0	1	1			
Vesterboelle	1	26	0	1			
Westergaard	1	2	18	1			
Oregaard	2	0	0	80			
Smaller herds	13	27	3	11			
(*b*)	Jutland breed	Danish Black-Pied	German Black-Pied	Dutch Belted	Dutch Friesian	Groningen White-Head	Meuse–Rhine–Yssel
Jutland breed	148	54	3	1	1	0	0
Danish Black-Pied	19	21	1	2	1	0	0
German Black-Pied	0	3	11	0	4	0	2
Dutch Belted	0	0	0	22	1	0	1
Dutch Friesian	0	3	1	1	29	0	0
Groningen White-Head	0	0	0	0	0	23	2
Meuse–Rhine–Yssel	1	1	0	2	0	0	35

Assignment test are based on the "leave one out" option, where the individual of interest is removed from the dataset prior to estimating the population genotype frequencies.

pronounced in Westergaard, which showed very low levels of haplotype variation (Table 1*a*). The high number of haplotypes of low frequency was probably a remnant of a historically larger genetic diversity from a time when the breed was more abundant (Figure 1).

Impacts of restricted breeding

All results suggest the four main herds represent distinct units (Tables 1*a*, 2*a* and 4*a*; Figures 2*a* and 3). The contrast of low levels of linkage disequilibrium in each herd (0–2 percent) and high linkage disequilibrium in the Jutland breed (24 percent), with the smaller herds being the main contributor (16 percent), suggests the Wahlund effect (Wahlund, 1928) when grouping the herds (Table 1*b*). This is supported by the genetic distances among herds (Table 2*a*; Figure 2*a*), assignment of individuals almost exclusively to the herd of origin (Table 3*a*) and the clustering patterns (Figure 3). These findings correspond with the history of the breed, and the presumed separation of the four herds for at least the past 50 years, with only low levels of admixture (Table 3*a*).

The limited gene flow – among most of the herds – has resulted in genetic drift and resulting high level of differentiation among them (Table 4*a*) (Fernández, Toro and Caballero, 2008; Toro, Fernández and Caballero, 2009). This is well known from other small breeds (Kantanen *et al.*, 2000; Withen *et al.*, 2011). The high level of differentiation among herds (Figure 2*a*) may be due to a separation time longer than five decades (S. Benzon, personal communication).

The joint grouping of (1) Kortegaard and Oregaard and (2) Vesterboelle, Westergaard and a large number of individuals from the smaller herds could reflect the hierarchical structuring of the Jutland breed (Figure 3; *K* = 3). As the number of clusters increases it becomes evident that Vesterboelle, Westergaard and Oregaard are genetically distinct, whereas Kortegaard share genotypes with each

of them (Figure 1; *K* = 4, 5, 6). In accordance with their history of originating from the main herds, the smaller herds represent the genetic diversity found in the four herds (Table 3*a*; Figures 1 and 3). Their resemblance to the German and Dutch breeds (Figure 3) and the unique haplotypes are most likely the result of minor introgression from these (Figure 1).

Genetic relationship among the black-pied breeds

Overall, the breeds are less differentiated than the herds (Table 2*b*) and have a higher level of gene flow (Table 4*b*), this is expected when accounting for genetic drift and differences in population size (Reed and Frankham, 2003). The Dutch breeds – and to a lesser extent the German Black-Pied – are distinct from the Jutland breed (Tables 2*b* and 4*c*; Figures 2*b* and 3*b*). Information on historic exchange of animals among the countries is limited; our results indicate that there has been limited exchange of animals between the breeds (Table 4*b*).

The putative origin of the Danish Black-Pied as a mix between the Jutland breed and black-pied cattle from Holland and Germany (Danish Ministry of Food, Fisheries and Agriculture, 2010) is supported by several of our analyses (Tables 3*b* and 4*b*; Figure 3), with the Jutland breed being by far the largest contributor (Tables 3*b* and 4*b*; Figure 3). All four herds have contributed almost equally with genetic material to the Danish Black-Pied breed (Table 2*b*; Figure 3), which is likely to have contributed to the observed Wahlund effect within this breed (Table 1*b*).

Management of a subdivided breed

The management of a subdivided breed, such as the Jutland breed, implies a compromise of two main factors: (1) maintenance of the highest possible levels of genetic

Table 4. Estimates of gene flow rates (*m*) among (*a*) the Jutland breed herds and (*b*) the black-pied breeds.

(*a*)

	Kortegaard	Vesterboelle	Westergaard	Oregaard	Smaller herds
Kortegaard	*0.97 (0.02)*	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)
Vesterboelle	0.01 (0.01)	*0.68 (0.01)*	0.01 (0.01)	0.01 (0.01)	*0.31 (0.02)*
Westergaard	0.06 (0.02)	0.01 (0.01)	*0.88 (0.03)*	0.01 (0.01)	0.05 (0.02)
Oregaard	0.01 (0.01)	*0.20 (0.02)*	0.00 (0.00)	*0.79 (0.02)*	0.00 (0.00)
Smaller herds	*0.22 (0.02)*	0.00 (0.01)	0.00 (0.00)	0.01 (0.01)	*0.76 (0.02)*

(*b*)

	Jutland breed	Danish Black-Pied	German Black-Pied	Dutch Belted	Dutch Friesian	Groningen White-Head	Meuse–Rhine–Yssel
Jutland breed	*0.95 (0.01)*	0.03 (0.01)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)
Danish Black-Pied	*0.19 (0.03)*	*0.72 (0.02)*	0.00 (0.01)	0.01 (0.02)	0.08 (0.02)	0.00 (0.01)	0.00 (0.01)
German Black-Pied	0.01 (0.01)	0.01 (0.01)	*0.68 (0.02)*	0.01 (0.02)	*0.27 (0.03)*	0.02 (0.02)	0.01 (0.01)
Dutch Belted	0.00 (0.01)	0.00 (0.01)	0.00 (0.01)	*0.98 (0.01)*	0.00 (0.01)	0.00 (0.01)	0.00 (0.01)
Dutch Friesian	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	*0.99 (0.01)*	0.00 (0.00)	0.00 (0.00)
Groningen White-Head	0.00 (0.01)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)	*0.98 (0.02)*	0.01 (0.01)
Meuse–Rhine–Yssel	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.01)	0.00 (0.01)	*0.98 (0.02)*

Migration rates (*m*) into each population are shown with standard errors (SE) given in parentheses. Rows represent the population from which each individual was sampled, and columns represent the populations from which they migrated. Diagonal values represent the proportions of individuals derived from the source populations in each generation. Migration rates ≥0.10 are in italics.

diversity and (2) conservation of unique herd characteristics (Toro and Caballero, 2005). The two factors can be achieved if the four main herds are kept completely isolated. However, this will result in an increase in herd inbreeding levels, which can compromise their short-term survival (Reed and Frankham, 2003; Taberlet *et al.*, 2008). Alternatively, the herds could be united as one large breeding unit. The trade-off would be loss of genetic diversity, washing out singularity of the herds and a long-term decrease in effective population size (Toro and Caballero, 2005).

We propose a management strategy where the herds are managed separately, but with an increased amount of gene flow among them, to avoid further genetic drift. A minimum of one migrant per herd per generation has been shown to counteract the detrimental effects of genetic drift (Fernández, Toro and Caballero, 2008; Toro, Fernández and Caballero, 2009). To conserve the uniqueness of the Jutland breed, introgression from the German and Dutch Black-Pied breeds should be avoided.

Conclusion

Losses of indigenous cattle breeds are happening at a high rate worldwide, and FAO has recommended immediate action should be taken to conserve local cattle breeds and possible valuable genetic variation (FAO, 2007b). Our characterization of the indigenous Danish cattle breed; the Jutland breed, shows it to be a valuable reservoir of unique genetic variation. Our results further demonstrate the rapid diversification of the Jutland breed herds due to limited gene flow and genetic drift. The Jutland breed has experienced limited introgression from related Northern European black-pied breeds. We propose conserving the Jutland breed herds as separate breeding entities with moderate gene flow among the herds. This will assure conservation of the highest possible amount of genetic variation and fitness for future management of the Jutland breed.

Supplementary material

Supplementary online material is available at http://cambridge.journals.org/AGR.

Acknowledgements

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