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ORIGINAL ARTICLE

The Agersoe cattle: the last remnants of the Danish island cattle (*Bos taurus*)?

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Summary

A phenotypically interesting strain of cattle existed on the small island of Agersoe, on the west coast of Zealand, Denmark, in the beginning of the last decade. The cattle share a great resemblance to the extinct Danish breed, the Island cattle. The objective of this study was to genetically characterize the Agersoe cattle, using microsatellites, amplified fragment length polymorphism (AFLP) and mtDNA markers, and analyse the genetic variability within the breed and the genetic relationship to 14 European breeds with focus on the Red Danish and Jutland breed. The results show diversity in nuclear markers comparable to that of modern breeds and that the Agersoe cattle are separable from the two native breeds. The absence of inbreeding and the degree of genetic diversity are taken as a sign of recent admixture. The Agersoe cattle did not exhibit a consistent association with any of the European breeds. Several arguments based on this survey have been put forward in favour of characterizing the Agersoe cattle as being the last remnants of the Danish Island Cattle.

Introduction

A variety of cattle breeds have been founded throughout the history of modern agriculture; each with a genetic composition uniquely shaped by the natural and artificial selection enforced upon them in time and space. Many of these local breeds were later abandoned because of low productivity skills in an ever-evolving and highly productive farming system. The discarded breeds were destined to decimation, if not extinction. Although selection for better profitability appears reasonable from a farmers' point of view, it is quite the opposite when seen in a broader conservation context, and arguments have been made about the importance of preserving the present day diversity (Ruane 2000).

That this matter is by no means trivial can be realized by the fact that the Food and Agriculture

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Organization of the United Nations (FAO) has registered 171 European cattle breeds that are now extinct and further proclaims that only approximately half of the 482 European breeds have healthy populations (FAO 2000). The awareness of the ongoing genetic erosion has, within the recent years, caused a change in view, and indigenous breeds are now conceptualized as potential genetic resources necessary for the agriculture of the future. One of these breeds that might turn up as a valuable genetic resource is the Danish Agersoe cattle.

A private collector of indigenous domestic animals came across a very interesting strain of cattle when he visited the island of Agersoe, on the west coast of Zealand, in the early 1990s. The owner was well aware of the history of his livestock and could in great detail account for the origin of the founders which, for some, went as far back in time as the 18th century (S. Benzon, personal communication). The phenotype of the Agersoe cattle apparently bears many resemblances with those of the supposed extinct local type, the Island Cattle. This, combined with the history of the livestock, has convinced many that the Agersoe cattle are indeed the last remnants of the Danish Island cattle.

This local breed was indigenous to the Danish islands, hence the name, and it was kept by the vast majority of farmers up till somewhere in the late 19th century. In the middle of 19th century, the need for a more productive dairy breed arose and, primarily, the Angler was introduced and crossed with the Island cattle, the outcome was the Red Danish. This development was very critical for the Island cattle and their population size steadily decreased as farmers replaced them with the more productive Red Danish.

The objectives of this study were to analyse the genetic composition of the Agersoe cattle and the two contemporary breeds, Red Danish and Jutland breed, using microsatellites, AFLP and mtDNA markers to (i) estimate the level of genetic variability present in each breed; (ii) assess the degree of differentiation between the three breeds; (iii) estimate the levels of admixture between the three Danish breeds; and (iv) evaluate their genetic relationship with other European cattle breeds.

Materials and methods

Sampling and DNA extraction

Blood samples were collected from 123 animals representing the three native breeds: the Agersoe cattle (n = 42), Red Danish (n = 33) and Jutland breed (n = 48). Each of the sampled animals is registered in the national register for farm animals named 'Centrale Husdyrsbrugs Register' (CHR). The consanguinity between the sampled animals could not be determined because of the lack of herd books.

Microsatellite allele frequency data from six additional breeds (29 markers) were obtained from the European Resgen project, an inventory of European farm animal genetic resources concerning characterization and conservation of livestock. The Bohemian Red (n = 25), Polish Red (n = 48), Angler (n = 50), Red Holstein dual purpose (n = 25), Dutch Friesian (n = 34) and German Black-Pied (n = 20) were used. An additional number of subpopulations of two Danish breeds were included: Red Danish (n = 36) and Jutland breed (n = 44) both from the French International Institute for Agricultural Research (INRA). Furthermore, six Nordic breeds, each typed for 20 of the 28 markers, were obtained from the Nordic genetic diversity project (http://neurocad.lva.lt). These were the Dolafe (n = 35), Western Fjord (n = 41), Telemark (n = 46), Western Red Polled (n = 36), the Swedish Red Polled (n = 33) and the Danish Black-Pied (n = 27).

Genomic DNA was extracted and purified from whole blood using a *DNeasy Blood & Tissue kit* (Qiage006E) following the manufacturer's protocol and stored at −20°C.

MtDNA

The mitochondrial control region (D-loop) was amplified using the polymerase chain reaction (PCR) with the flanking primers 5′-CTG CAG TCT CAC CAT CAA CC-3′ (Loftus *et al.* 1994) and 5′-AGA GTT AAC AGG AAG GCT GG-3′ (Kim *et al.* 2003). The PCR was performed in 50 μl volumes containing 1 μl DNA, 1 μM of each primer, 1× PCR buffer, 1.5 mM $MgCl_2$, 2 μM of each dNTP and 1 U *Taq* polymerase (Thermo Scientific). The amplification was carried out using the following cycling conditions: 10 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 57°C, 1 min at 72°C and final extension at 72°C for 5 min The PCR product was purified using *Qiaquick PCR purification kit* (Qiagen, Copehagen, Denmark) according to the manufacturer's protocol.

Sequencing was performed using *ABI-Prism®BigDye Terminator v3.1 Cycle Sequencing Kit* (Applied Biosystems, Carlsbad, CA, US) in a 20 μl volume containing recommended concentrations. The cycling conditions were 96°C, 30 s; 50°C, 15 s; and 60°C, 4 min for 25 cycles (ramp rate, 1°C/s). Sequencing

was carried out in two overlapping fragments created using the primer combinations 5′-CTG CAG TCT CAC CAT CAA CC-3′ (Loftus *et al.* 1994), 5′-CGA GAT GTC TTA TTT AAG AGG-3′ (Cymbron *et al.* 1999) and 5′-CGC TCC GGG CCC ATA AAC CG -3′, 5′- GCC TGC GTT TAT ATA TTG AC-3′ (the latter two primers were designed for this study). The fragment sizes were 597 and 588 base pairs, respectively. Cycle sequencing products were purified prior to separation on a 5% denaturing polyacrylamide gel in an ABI PRISM®377 DNA sequencer (Applied Biosystems) using the *DyeEx 2.0 Spin kit* (Qiagen). Consensus sequences were created in SEQUENCHER® version 3.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned using CLUSTALW (Thompson *et al.* 1994) in BIOEDIT 5.0.9 (Hall 1999). Variation in the obtained sequences was described by comparing it with the bovine mtDNA reference sequence (Anderson *et al.* 1982) downloaded from GenBank (accession no. NC_00156). Sites containing gaps in the aligned sequences were excluded from subsequent analyses.

AFLP

The *Eco*RI/*Taq*I AFLP procedure was applied using the primer combinations E35/T32, E39/T33 and E45/T32 (Ajmone-Marsan *et al.* 1997). The genotyping was performed at *Keygene Genetics*, Wageningen (The Netherlands) following their standard protocol. The bi-allelic markers are dominant and were scored generating a matrix of binary data (0/1), absence and presence of bands, respectively.

Microsatellites

The cattle were genotyped for 30 microsatellite markers as recommended by ISAG/FAO. The markers were multiplexed as proposed by Dr Katayoun Moazami-Goudarzi, INRA, Jouy-en-Josas (http://dad.fao.org/en/refer/library/guidelin/marker.pdf) with slight modifications regarding choice of dye and multiplexing. The *StockMarks®Cattle Paternity PCR Typing kit* (Applied Biosystems) was used for amplification of 11 of the markers in one PCR, and *Qiagen®Multiplex PCR kit* (Qiagen) was used for the remaining 19 markers, both following the manufacturer's standard protocol. Multiplexes, fluorescence-labels and PCR cycling conditions are depicted in Table S1. The obtained fragments were separated on a 5% denaturing polyacrylamide gel in an ABI PRISM® 377 DNA Sequencer (Applied Biosystems), and the data were analysed using the software GENESCAN 3.1® and GENOTYPER 2.0® (Perkin, Carlsbad, CA, US). Scoring of the allele sizes was standardized using reference DNA (European Cattle Genetic Diversity Consortium 2006).

Statistical analysis

Validation of microsatellite and AFLP data

The microsatellite loci were tested for the presence of null alleles, short allele dominance, scoring of stutter peaks and typographic errors in MICRO-CHECKER version 2.2 (Van Oosterhout *et al.* 2004). Each population-locus combination was tested for significant deviation from Hardy–Weinberg equilibrium (HWE) and locus-by-locus deviations from linkage disequilibrium by an exact test in GENEPOP version 3.4 (Raymond & Rousset 2003) followed by sequential Bonferroni correction (Holm 1979) to reduce type I errors.

AFLP data were tested for outlier loci, which were removed prior to further analysis, to assure neutrality (Beaumont & Balding 2004).

Genetic variability

Variability of the mtDNA was estimated for each breed as unbiased haplotype diversity (h) and nucleotide diversity (π) (Nei 1987) using DNASP version 4.0 (Rozas *et al.* 2003). For the AFLP fragments, unbiased expected heterozygosity (H_E) (Nei 1987) within each population was estimated (Lynch & Milligan 1994). The allelic frequencies at each locus were estimated using a Bayesian method based on 10^6 permutations with non-uniform prior distribution of allelic frequencies (Zhivotovsky 1999) as implemented in AFLP-SURV version 1.0 (Vekemans *et al.* 2002) assuming HWE.

Variability for microsatellite markers was evaluated in each breed through the observed (H_O), unbiased expected (H_E) heterozygosity (Nei 1987) and mean number of alleles (A) as implemented in EXCEL MICROSATELLITE TOOLKIT (Park 2001), and the number of private alleles (A_P) was identified by manual inspection. Mean estimates and standard deviations of the F-statistics were calculated by jack-knifing over loci, and the significance was obtained by the bootstrapping procedure implemented in FSTAT version 2.9 (Goudet 1995). The heterozygote deficiency (f) and locus-by-locus deviations from linkage disequilibrium (LE) were estimated for each of the three breeds by the Markov chain method in GENEPOP.

Genetic structure and relationships

The degree of differentiation between the three breeds was quantified using Wright (1921)'s F_{ST}.

Overall and population pairwise estimates were obtained for all marker types. For the mtDNA data, the Φ_{ST} was estimated in ARLEQUIN version 3.11 (Schneider *et al.* 2000). Lynch & Milligan (1994)'s estimate of F_{ST} based on the AFLP data was calculated using AFLP-SURV. The variance-based method devised by Weir & Cockerham (1984) implemented in FSTAT version 2.9 was used to estimate θ_{ST} for the microsatellite data. The statistical significance of estimate was tested against a null distribution based on 10^4 permutations of genotypes among populations. An analysis of the molecular variance, AMOVA (Excoffier *et al.* 1992), as implemented in ARLEQUIN, was conducted using the microsatellite data.

A PCO was performed based on the Jaccard similarity index (Jaccard 1908) between individual AFLP genotypes using MVSP version 3.13 (Kovach Computing Services, Anglesea, UK). The genetic structure was further assessed using the microsatellite data in STRUCTURE version 2.1 (Pritchard *et al.* 2000), using the admixture model and the correlated allele frequency option. A burn-in of 10^5 iterations followed by a Markov chain Monte Carlo (MCMC) of 10^6 iterations was applied. It was assured at the end of each burn-in period that the key parameters had converged before starting the collecting data procedure. Likelihoods for $K = 1$ to $K = 14$ were estimated each with five repetitions. The most likely K was found according to Rosenberg *et al.* (2001). Results were visualized in DISTRUCT version 1.1 (Rosenberg 2004).

The genetic relationship of the mtDNA haplotypes between the Danish breeds was analysed as a median-joining (MJ) network and a neighbour-joining (NJ) tree constructed in SPLITSTREE version 4.0 (Huson & Bryant 2006). The MJ network was based on the NeighborNet algorithm by Bandelt *et al.* (1995), and the NJ tree on Kimura's 2-parameter model (1980) using the Anderson *et al.* (1982) sequence as the outgroup, and a bootstrap test of 10^3 permutations to test the reliability of the interior branches (Felsenstein 1985).

Furthermore, genetic relationship among Agersoe cattle, the two Danish breeds sampled here and the 12 European breeds was evaluated by constructing a NeighborNet based on Reynolds genetic distance (D_R) (Reynolds *et al.* 1983) between the breeds. This was based on only 20 microsatellite markers because of the lack of data for the entire data set. The network and distances were calculated using SPLITSTREE and MICROSAT version 2 (http://hpgl.stanford.edu/projects/microsat/).

Admixture and breed integrity

The level of admixture, among the three Danish breeds, was quantified using STRUCTURE. Three pairwise comparisons ($K = 2$) were performed to obtain membership coefficients (q) for each individual, with a burn-in of 10^5 steps followed by $2*10^5$ MCMC iterations. Each comparison was performed independently twice, and the obtained probabilities were compared and found to be virtually identical.

The breed integrity was calculated as the probability that two randomly chosen individuals within the same breed are more alike than individuals randomly sampled across breeds (Wiener *et al.* 2004). The measure used for similarity was the proportion of shared alleles between individuals (*psa*) (Bowcock *et al.* 1994) and was defined as:

Breed integrity = 1 − probability ($psa_{\text{same-breed}} < psa_{\text{random individual}}$)

The overall probability for each breed was calculated as the average of individual probabilities based on 10^4 independent calculations of $psa_{\text{same-breed}}$ and $psa_{\text{random individual}}$.

Results

Validation of assumptions prior to the analysis of the microsatellite data

The microsatellite locus *INRA035* was excluded from further analysis as it deviated significantly from HWE in the population-locus comparisons. None of the AFLP fragments showed significant deviation from neutrality.

The genetic variability

The 123 D-loop sequences (accession no. HM625981-HM626103) all belonged to the T3 haplogroup and contained 24 polymorphic sites and one insertion. The polymorphisms gave rise to 14 distinct haplotypes of which 10 were not shared amongst the three populations (Table 1; Figure 1). The genetic diversity in the Danish Red was considerably higher than in the Agersoe and Jutland breeds (Table 1; Figure 1a,b).

The AFLP procedure gave rise to 100 polymorphic loci (E35/T32: 31 loci, E39/T33: 34 loci and E45/T32: 35 loci), and under the assumption that all loci are bi-allelic and in Hardy–Weinberg proportions, the genetic diversity within populations was described as the expected heterozygosity ($H_{E(AFLP)}$). The results are given in Table 1 and depict comparable, but not

Table 1 Diversity measures and summary statistics for the Agersoe Cattle, Red Danish and Jutland Breed based on 785- bp mtDNA, 100 AFLP fragments and 29 microsatellite loci. Sample size (n), number of haplotypes (H), number of private haplotypes (H_P), haplotype diversity (h), nucleotide diversity (π), expected heterozygosity for AFLP [$H_{E\ (AFLP)}$], mean number of alleles (A), number of private alleles (A_P), expected heterozygosity for microsatellites [$H_{E\ (microsat)}$], observed heterozygosity for microsatellites [$H_{O\ (microsat)}$], deviation from Hardy–Weinberg equilibrium (f) and deviation from linkage equilibrium (LE)

Breed	n	H	H_P	h (%)	π (%)	$H_{E\ (AFLP)}$	A	A_P	$H_{E\ (microsat)}$	$H_{O\ (microsat)}$	f	LE
Agersoe Cattle	42	4	–	18.2 (7.9)	0.064 (0.028)	0.306 (0.017)	4.7 (1.3)	4	0.595 (0.024)	0.584 (0.014)	−0.009[a]	3*
Red Danish	33	10	9	83.1 (4.2)	0.562 (0.037)	0.270 (0.017)	4.7 (1.1)	9	0.611 (0.022)	0.578 (0.016)	0.028[a]	2*
Jutland Breed	48	4	1	36.2 (8.3)	0.155 (0.037)	0.334 (0.015)	4.6 (1.4)	23	0.625 (0.023)	0.599 (0.013)	0.029[a]	1*
Total	123	14	–	78.0 (2.4)	0.354 (0.022)	–	4.7 (1.5)	–	–	–	0.010[a]	4*

*$p < 0.05$.
[a]Not significant.

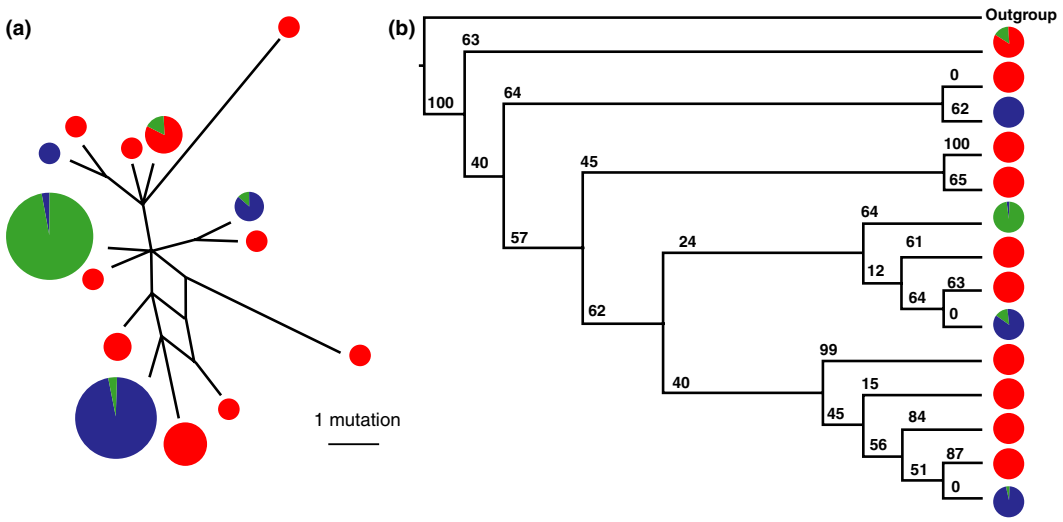

Figure 1 Median-joining network (a) and Neighbour-joining tree (b) of the mtDNA haplotypes found in the three Danish breeds; Agersoe cattle (green), Jutland breed (blue) and Red Danish (red). Circles corresponding to the haplotype frequencies are drawn to scale for the MJ network but not for the NJ tree. Bootstrap values are indicated on the branches of the NJ tree.

identical, variability levels within populations (two-tailed z-test, $p < 0.05$).

A total of 181 alleles were found for the 29 microsatellite loci. The number of alleles at each locus ranged from three (*INRA005* and *HEL13*) to ten (*TGLA22*) with an overall average of 4.7. Summary statistics for each breed are given in Table 1 as observed number of alleles (A), number of private alleles (A_P), expected heterozygosity [$H_{E(microsat)}$], observed heterozygosity [$H_{O(microsat)}$]. The variability levels within populations were comparable but not identical (two-tailed z-test, $p < 0.05$).

None of the Danish breeds showed significant deviations from HWE (Table 1). Significant associations between pair of locus were found in all three populations. The Agersoe cattle had three loci pairs in linkage disequilibrium; *CSRM60-INRA037* (10 and 10), *CSSM66-ILSTS005* (14 and 10) and *INRA063-INRA032* (18 and 11). The Red Danish had two; *CSRM60-INRA037* (10 and 10) and *ETH185-BM1824* (17 and 1), and the Jutland breed had one; *CSRM60-INRA037* (10 and 10). The numbers in parenthesis indicate the chromosome of origin.

The two autosomal markers both show highest variability in the Jutland breed, especially regarding the number of private alleles (A_P) (Table 1). The ranking as the second most diverse breed depends on the marker. The AFLP results depict the Agersoe cattle, whereas the microsatellite data indicate the Red Danish. The mitochondrial marker interestingly depicts the Red Danish as having substantially higher within-breed diversity than the other two populations (Table 1).

Table 2 Population pairwise differentiation (F_{ST}) among the three breeds based on 789 bp of mtDNA (a), 100 AFLP fragments (b) and 29 microsatellite loci (c), respectively

	Agersoe cattle	Jutland breed
(a)		
Jutland breed	0.68*	
Red danish	0.39*	0.25*
(b)		
Jutland breed	0.133*	
Red danish	0.159*	0.151*
(c)		
Jutland Breed	0.139*	
Red Danish	0.147*	0.172*

*$p < 0.05$.

The genetic structure and relationship among the breeds

All population pairwise estimates were statistically significant (Table 2). It is evident that some discrepancies exist between the results, but the autosomal markers both identified the Agersoe cattle and the Jutland breed as being the least differentiated pair, whereas they were the most differentiated based on the mtDNA (Table 2; Figure 1a,b).

The degree of differentiation between breeds was 0.15 for the microsatellite and AFLP markers, whereas the estimate based on the mtDNA was substantially higher ($\Phi_{ST} = 0.44$, $p < 0.01$). Analysis of the microsatellite data showed that the mean within-population estimate of inbreeding (F_{IS}) was 3.7% but not significant ($p > 0.05$). The mean reduction in an individual's heterozygosity relative to that expected at the panmictic metapopulation level (F_{IT}) was 18.5% ($p < 0.05$), whereas the mean differentiation between populations (F_{ST}) was 15% ($p < 0.05$).

The genetic distances between the 17 breeds, based on 20 microsatellite markers (Figure 2), indicate a star-shaped pattern, which is common in cattle phylogenies because of a common ancestry. The breeds are divided into three distinct clusters; the Lowland Pied (blue), the Baltic Red (red) and the Nordic cluster (purple). The Danish Red and the Jutland breed cluster within the expected groups (Baltic Red and Lowland Pied, respectively), whereas the Agersoe cattle clustered solely between these two groups (Figure 2).

The principal coordinate analysis (PCO) between AFLP genotypes (Figure 3) cluster the animals in three groups according to the breed of origin. The Agersoe and Jutland breeds are separated by component 1, whereas the Red Danish is separated from the Agersoe and most of the Jutland breed by component 2. This appears to reflect the high F_{ST}-values and indicates differentiation among the breeds. The analysis further suggests that some animals from the Jutland breed and the Agersoe cattle seem to share some genetic resemblance. This is indicated by their close proximity and intermediate placement between the two clusters. No breed substructure was readily identifiable in the plot.

Partitioning the Danish breeds into five clusters was found to be the most likely (Figure 4). The

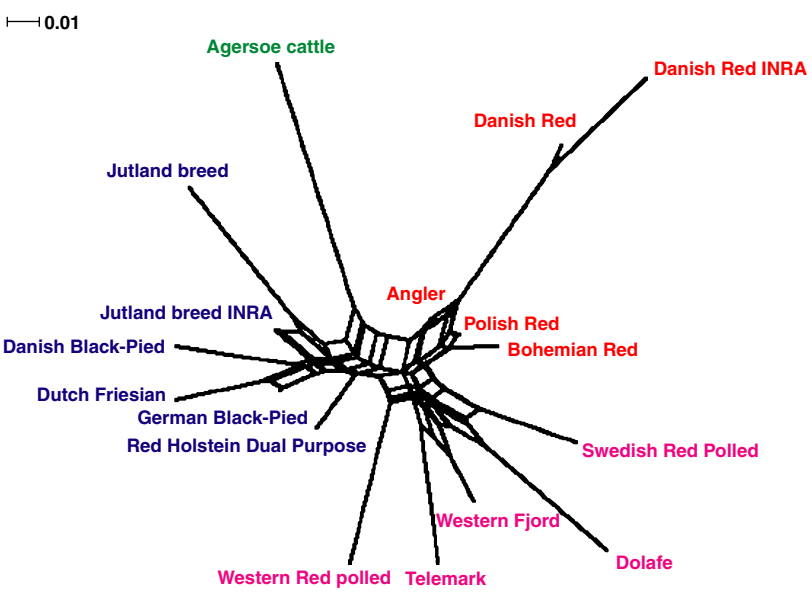

Figure 2 NeighborNet showing the relationship among the 15 European cattle breeds based on 20 microsatellite loci. Colours correspond to Lowland Pied (blue), Nordic breeds (purple), Baltic Red (red) and Agersoe Cattle (green).

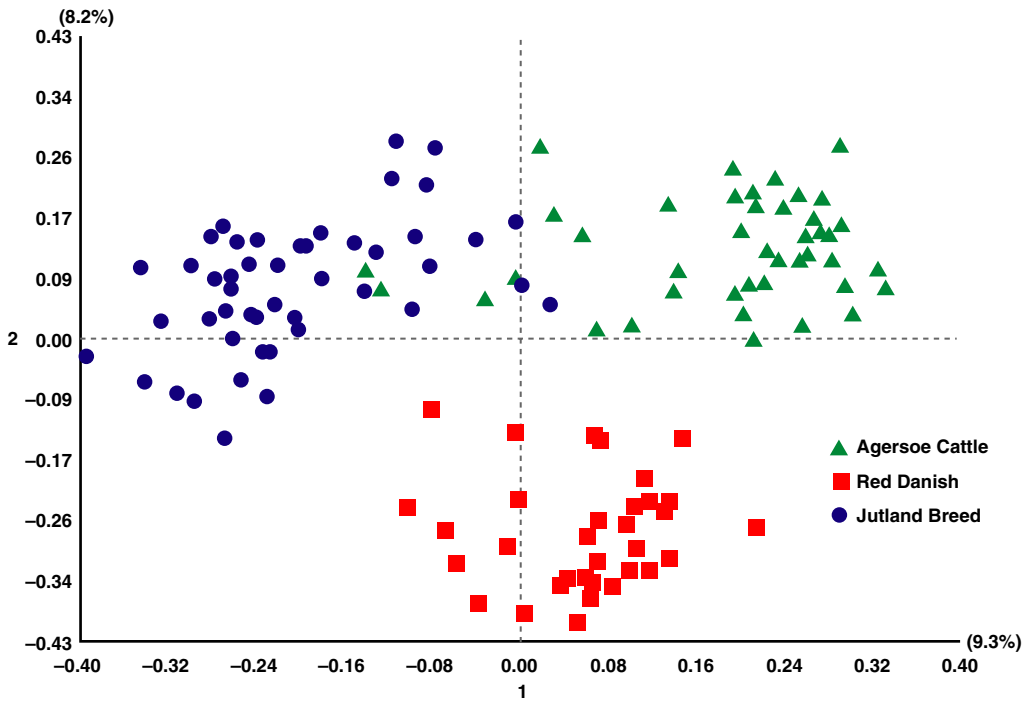

Figure 3 Principal coordinate analysis (PCO) of the Jaccards similarity measure between individuals of the three breeds based on 100 AFLP fragments.

Agersoe cattle all fell in the same cluster, as did the two Danish Red populations, whereas substructure was revealed in the two Jutland breed populations (Figure 4).

Admixture and breed integrity

In the pairwise comparison between the Agersoe cattle and the two Danish breeds (Figure 5), it became evident that some animals did not cluster distinctly according to their breed of origin. In the comparison with the Red Danish and the Jutland breed, 2 and 10 animals, respectively, did not have ($q = 0$ and $q = 1$, respectively) within their probability limits (Figure 5c). Furthermore, it was revealed that four and two individuals from these breeds, respectively, had a statistically significant affinity for the Agersoe cattle ($q \neq 0$, $p < 0.05$) (Figure 5a,b).

The calculation of the breed integrity showed similar levels of integrity and ranked the breeds in the following order from least to most cohesive: Jutland breed (0.843), Red Danish (0.868) and Agersoe cattle (0.888).

Discussion

A recent survey performed by Baumung *et al.* (2004) indicates that most diversity studies involving cattle breeds use microsatellite loci (90%), whereas the application of mtDNA and AFLP markers is less common (29% and 8%, respectively). In this study, these three genetic markers were applied to characterize three Danish cattle breeds. The reason for

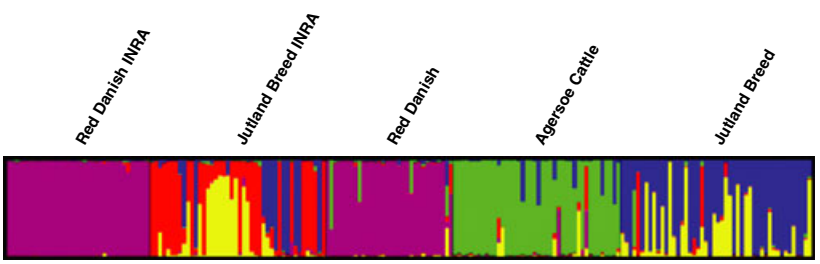

Figure 4 Estimated population structure among five Danish cattle breeds based on 29 microsatellite loci. A vertical line, divided into membership fractions in five clusters, represents each individual. Black vertical lines divide individuals into the populations as indicated above the figure.

applying the AFLP procedure was its ability to detect genetic structure (Ajmone-Marsan *et al.* 2002), as well as the fact that it is based on a different set of mutations, making it complimentary to the information derived from the microsatellites. The mitochondrial marker was used in the survey to evaluate the female-mediated characteristics of each breed, thereby avoiding the masking effects of a possible male admixture between them.

Validation of assumptions prior to the analysis of the microsatellite data

Deviations from HWE measured by the inbreeding coefficient (*f*) can be ascribed to a variety of causes. Outbreeding or over-dominant selection may lead to heterozygote excess, whereas inbreeding or gathering of subpopulations (Wahlund effect) results in heterozygote deficiency (Hartl & Clark 1997). None

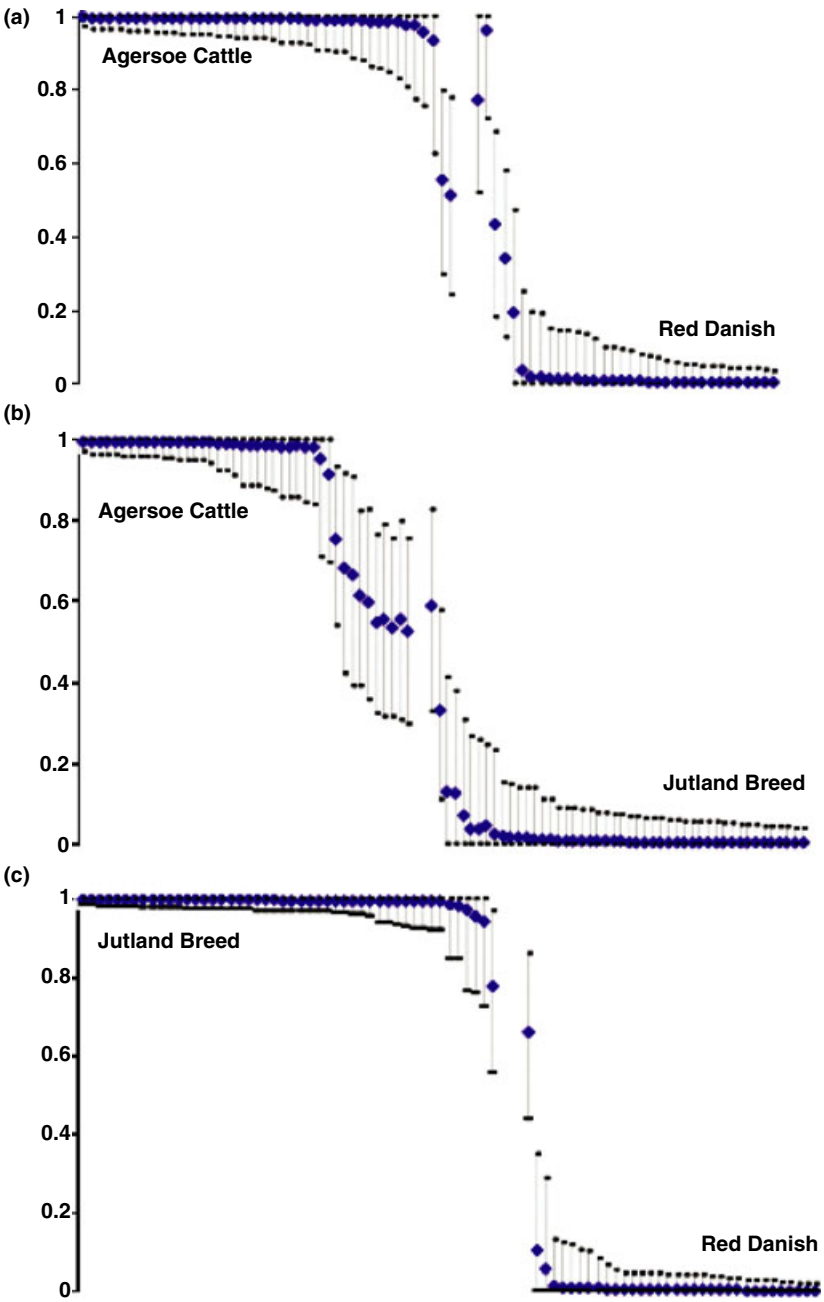

Figure 5 Posterior distribution of admixture coefficients (*q*) with 90% confidence intervals (CI) for the pairwise comparisons of between Agersoe Cattle, Red Danish and Jutland Breed. Individuals are represented by ♦, and vertical bars indicate CI's.

of the Danish breeds showed significant deviations from Hardy–Weinberg proportions. Nonetheless, population structure is evident from the STRUCTURE analysis where two genetic clusters were identified within the Jutland breed (Figure 4). The most likely explanation is the Wahlund effect because of the fact that the samples were from two different subpopulations (Jutland breed 1950 and Jutland breed).

The *a priori* assumption for the 30 microsatellites, as for the mtDNA and AFLP markers, is that they are invisible to selection. The results from the *U*-test showed one to three pair of loci with significant deviations from linkage equilibrium (*LE*) in each breed. Given the physical placement of the loci, it is evident that the observed disequilibrium can only be as a result of physical linkage for *INRA037* and *CSRM60* as they are both located on chromosome 10, but as we did not find them to deviate significantly from *LE* in the prior validation, we included them in the further analyses. However, several other factors can lead to the observed deviations, including selection, sampling of family members and admixed populations (Conrey & Mills 2003). In this study, the latter two possibilities appear likely.

The populations of the Red Danish and the Jutland breeds have each originally been purchased from 3 to 4 farms (S. Benzon, personal communication), which might have resulted in the partition of the haplotypes found in the Jutland breed into two groups, and the Red Danish in several haplogroups (Figure 1b). As linkage equilibrium is obtained only gradually over generations, dependent on the physical distance (Hartl & Clark 1997), this could explain the linkage disequilibrium observed in these two breeds. With regard to the Agersoe cattle, the likely cause of the observed deviations is the sampling of family members as all animals were derived from one herd.

The genetic diversity

The genetic variability found in the mtDNA was much higher in the Red Danish than in the two other breeds (Table 1; Figure 1a,b), which is expected because of their mixed origin. When comparing the obtained nucleotide diversity with the average of selected European breeds (Mannen *et al.* 1998) only the Red Danish reach the same level of variability (0.34–0.47%), while the Jutland breed and the Agersoe cattle exhibit lower polymorphism (Table 1). The low degree of genetic variability can be attributed to the almost complete fixation of one haplotype in the Agersoe cattle (90.5%) and the Jutland breed (79.2%) (Figure 1a). Fixation of a haplotype because of genetic drift is inevitable in a finite population, but the fixation time is dependent on effective population size and the number of generations in genetic isolation. The observed monomorphisms in the two breeds could very likely be the consequence of these two factors.

The estimate of the variability within the three breeds based on the AFLP and microsatellite markers was not very different but still significant (Table 1). When comparing diversity estimates between breeds, it is important that the studies are based on the same set of markers. Wiener *et al.* (2004) and Schmid *et al.* (1999) found expected and observed heterozygosities of 0.56–0.69 and 0.56–0.67, respectively. The Danish breeds' variability was within this interval. It was not possible to compare the results from the AFLP procedure to other breeds because of the lack of published studies using present primer combinations.

The genetic structure

The average degree of differentiation between the three breeds based on the AFLP (F_{ST}) and microsatellite markers (θ_{ST}) showed that 15% of the total variation was because of difference between breeds, while the rest could be ascribed to differences between animals within breeds. The findings for the microsatellites are high compared to other studies, indicating that the sampled breeds are more differentiated compared with modern established breeds (Martin-Burriel *et al.* 2007; Wiener *et al.* 2004).

A greater part of the variation in the mtDNA was as a result of differences between breeds, whereas only 57% could be assigned to differences between individuals within breeds ($1 - \Phi_{ST}$). This is expected when considering the observed distribution of haplotypes between breeds (Figure 1a,b). This distribution is very likely the consequence of genetic drift and the lack of female-mediated geneflow between populations. The low differentiation between the Jutland breed and Agersoe cattle is surprising, as crossing of Angler and Island cattle resulted in the Red Danish breed, and therefore a lower differentiation between the Agersoe and Red Danish is expected (Table 2; Figure 1a,b).

The genetic structure of the breeds (Figures 3 and 4) showed that the animals clustered according to their breed of origin. In the PCO (Figure 3), no substructuring was readily identifiable, whereas the STRUCTURE analysis (Figure 4), which included the Red Danish INRA and Jutland breed INRA, showed the presence of substructure within the Jutland breed populations, where two distinct genetic clus-

ters were identified. This is most likely because of the compound nature of the livestock.

The genetic relationship

The MJ network revealed a low degree of hybridization among the Danish breeds (Figure 1a), as indicated by the presence of boxes, whereas the absence indicates that the relations between taxonomic units are substantially tree like. The NJ tree revealed a mixed clustering of breeds among the five haplotypes clades (Figure 1b). Low bootstrap probabilities (<50) were found for 37% of the branches and most likely represent signs of hybridization or admixture between breeds (Schmid *et al.* 1999; Wiener *et al.* 2004).

The Agersoe and Jutland breed each had four haplotypes of which one was prevailing (Figure 1a); this can be as a result of a decrease in population size and thereby a low number of founders. The high diversity in Red Danish can be subscribed to the admixed origin from the crossing of Island cattle with Angler. The low number of shared haplotypes among the breeds is consistent with the assumed separation and thereby low levels of female-mediated geneflow.

The genetic relationships among the 17 European breeds are based on the 'drift-only' distance (D_R), which is accurate in describing the relationship between the closely related European cattle breeds (Laval *et al.* 2002) (Figure 2). The NeighborNet is star shaped, as expected, because of hybridization or recent common ancestry. The Agersoe cattle are placed between the two major clusters, Lowland Pied and the Baltic Red each containing the Jutland breed and Red Danish, respectively. This solo grouping of the two breeds might be evidence of genetic drift because of a small population size for several generations or a unique genetic history.

Admixture and breed integrity

Previous studies, of membership coefficients (q) in pairwise comparisons between breeds, have revealed that animals tend to cluster distinctly into groups according to their breed of origin with non-overlapping probability limits (Koskinen 2003). Deviations from this pattern are indicative of genetic admixture between the involved populations (Pritchard *et al.* 2000). In this study, both admixture and immigration were detected between the sampled populations (Figures 2 and 5). This was expected based on the history of the breeds.

The Agersoe cattle have allegedly been kept in almost reproductive isolation, with only confined admixture mediated by Red Danish bulls (S. Benzon, personal communication). Our results paint a different picture, where the Jutland breed seems to have been the main source of admixture in the Agersoe cattle (Figures 3 and 5b). This admixture could account for the relatively high diversity in the autosome, the relatively low diversity found in the mtDNA and for the deviation of unity for the calculated breed integrity.

There was no indication of admixed animals as the result of hybridization between the Red Danish and Jutland breed. The candidate depicted in Figure 5c is most likely a hybrid between Jutland breed and the Agersoe cattle. The overall low population admixture indicates a deliberate breeding strategy for the Agersoe cattle where the Jutland breed is primarily being used. The breed integrity indicates a greater breed definition than e.g. Friesian, Dexter and Ayrshire (Wiener *et al.* 2004). Overall, within-breed diversity, differentiation and integrity estimates for the autosome markers appear to be similar for the three breeds. Limiting the geneflow among the breeds will assure future 'pure' breeds.

Are the Agersoe cattle remnants of the Island Cattle?

The following arguments based on the present genetic survey can be put forward in support of this. First, the low diversity in the mtDNA in the Agersoe cattle corresponds very well with the alleged breed history (Table 1; Figure 1). Second, it constitutes animals that are genetically distinct from the Red Danish and the Jutland breed as evident from the F_{ST} estimates (Table 2), breed integrity calculation and the analysis of the genetic structure (Figures 3, 4 and 5). This lack of association could be interpreted as an indication of a long separation time. Another interesting aspect is the long branches observed for the three Danish breeds in the NeighborNet (Figure 2). The length of each terminal branch corresponds to the effect of genetic drift and it seems that the Agersoe, Jutland and Red Danish have experienced a great deal of drift or low levels of artificial selection comparable to that of other indigenous breeds.

As all three Danish breeds stand out in their well-defined differentiation from each other and the 12 European breeds, they constitute a unique genetic resource and therefore conservation measures should be assured to preserve them. A management

strategy, where the highest possible levels of genetic diversity within each breed are maintained, the genetic differentiation among them is preserved, and inbreeding is avoided, is preferable.

As there is no way of inferring allele frequencies or genetic diversity extant in the Island cattle, we cannot conclude that the Agersoe breed is in fact the Island cattle. However, from this study, it is clear that the Agersoe cattle represent an old breed and their history taken into account, they might be the last remnants of the Island Cattle.

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Supporting information

Additional Supporting Information may be found in the online version of this article.

Table S1 Multiplexes, fluorescence-labels and PCR cycling conditions.

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