GENETIC DIVERSITY IN MILK PROTEINS AMONG ESTONIAN DAIRY CATTLE

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Summary. The aim of the study was to determine the main polymorphisms in casein beta (*CSN2*), casein kappa (*CSN3*) and lactoglobulin beta (*LGB*) genes in Estonian dairy cattle and compare these among breeds. The study was based on 7 single nucleotide polymorphisms among 122 individuals from three cattle breeds. Allele and genotype frequencies were calculated, and Hardy-Weinberg equilibrium and genotypic disequilibrium tests were performed.

Genotypic differentiation was statistically significant between Estonian Red and Estonian Native cattle and between Estonian Red and Estonian Holstein cattle. Regarding *CSN3*, Estonian Holstein were characterized by a high genotype AA frequency in comparison with the other common AB genotype and higher A-allele and AB genotype frequency for *LGB*. The proportion of uncommon *CSN3* B-allele (BB and BE genotypes) in the studied breeds was higher in the Estonian Red. For *CSN2*, the A2 allele occurred more frequently in Holstein and Estonian Native cattle than in Estonian Red.

The studied genetic variants of milk proteins influence milk yield, milk composition and may have a range of implications for human health. Utilization of natural genetic resources represented by local breeds is helpful in cattle breeding, and in sustaining the breeds.

Key words: dairy breeds, casein beta, casein kappa, lactoglobulin beta, SNP, ASO-PCR.

GENETINĖ ESTIJOS PIENINIŲ GALVIJŲ PIENO BALTYMŲ ĮVAIROVĖ

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Santrauka. Tyrimų tikslas buvo nustatyti Estijos pieninių galvijų beta kazeino (*CSN2),* kapa kazeino (*CSN3*) ir beta laktoglobulino (*LGB*) genų polimorfizmą ir atlikti tarpveislinį palyginimą. Tyrimas paremtas 7 vieno nukleotido polimorfizmais 122 individų, priklausančių trims galvijų veislėms. Buvo apskaičiuotas alelių ir genotipų dažnis, atlikti Hardi–Vainbergo pusiausvyros ir genotipų pusiausvyros nebuvimo testai.

Genotipinis skirtumas buvo statistiškai reikšmingas tarp Estijos žalųjų ir Estijos vietinių galvijų veislių bei Estijos žalųjų ir Estijos holšteino veislių. Ryšium su *CSN3,* Estijos holšteinai turėjo didesnį AA genotipo dažnį palyginti su kitu įprastu AB genotipu ir didesnį *LGB* A alelio ir AB genotipo dažnį. Neįprasto *CSN3* B alelio (BB ir BE genotipų) dažnis buvo aukščiausias tarp Estijos žalųjų palyginti su kitomis tirtomis veislėmis. *CSN2* A2 alelis tarp holšteinų ir Estijos vietinių galvijų buvo sutinkamas dažniau, nei tarp Estijos žalųjų galvijų.

Tirti pieno baltymų genetiniai variantai veikia pieno kiekį, pieno sudėtį ir gali turėti įvairiapusį poveikį žmogaus sveikatai. Natūralių genetinių išteklių, kuriems atstovauja vietinės veislės, panaudojimas yra svarbus galvijų veisimui ir tausojamajam veislių naudojimui.

Raktažodžiai: pieninės veislės, beta kazeinas, kapa kazeinas, beta laktoglobulinas, SNP, ASO-PGR.

Introduction. Breeding of dairy cattle has made a substantial contribution to meeting the demands for food production. In Estonia, where milk production represents more than a third of total agricultural production revenues, breeding of dairy cattle is the most important branch of animal husbandry. Currently the development of the dairy industry is characterized by orientation towards higher added-value products, such as cheese, fermented milk products and ice cream, the production of which has increased over recent years (Agriculture and Rural Life …).

The effects of milk protein gene variants, *LGB* and *CSN3*, on milk yield, milk composition and cheese making ability, were studied extensively (Jakob and Puhan 1992; Velmala *et al*. 1995; Lundén *et al*. 1997; Freyer *et al*. 1999; Boettcher *et al.* 2004; Tsiaras *et al*. 2005; Kübarsepp *et al*. 2006; Pečiulaitiene *et al*. 2006; Hallén *et al.* 2007). Breed characterization and analysis of relationships among breeds were carried out based on genetic variation in milk proteins (A. Lundén *et al*. 1997; Lien *et al*. 1999; Beja-Pereira *et al*. 2002; Lirón *et al.* 2002, Jann *et al*. 2004; Chessa *et al*. 2007). Studying several cattle breeds on several continents it was established that there exists a geographical association in distribution of casein locus haplotypes (Beja-Pereira *et al*. 2002; Jann *et al.* 2004).

Findings on the association between human health and milk casein (reviewed by Kaminski *et al.* 2007) have increased interest in the genetic status of dairy cattle breeds, especially with respect to *CSN2*. Casein beta gene variants (A1 and A2) are believed to be associated with allergies to milk protein, diabetes, neurological disorders

such as autism and schizophrenia and also with resistance to heart diseases. Bioactive milk peptides may function as health care products, representing therapeutic value through both treatment of infection and disease prevention (Clare and Swaisgood 2000). Milk opioid-derived peptides may thus be involved in defense against noxious agents and could have dietary and health applications (Trompette *et al.* 2003). However, Truswell (2005) indicated unconvincing evidence related to A1 and A2 beta-casein milk effects in humans. Bell *et al.* (2006) concluded that definite health benefits deriving from A2 beta-casein variants were unproven and required further investigation. The difference between *CSN2* A1 and A2 milk is a changed nucleotide (C→A), resulting in a different amino acid at position 67 of the peptide chain (histidine in A1 and proline in A2 variants, respectively).

There are approximately 100 000 dairy cows in Estonia (census size 104 100 in 2007) for which there are milk records for more than 90%. About 75% of cows are Holstein, 25% Estonian Red and less than 1% are Estonian Native. Although the Estonian Native breed has a similar genetic background to other native Nordic breeds, the influence of the Jersey breed is apparent and there are genetic links with Brown Swiss and Ayrshire. In the early 1990s the Red Holstein was crossed with Estonian Native to improve milk yield, but current conservation legislation does not allow the use of foreign genetic material in breeding programs.

In the early 1970s the Estonian cattle breeds were characterized by milk protein polymorphisms to establish the possibilities for using the markers in selection (Toome 1972). Previous studies established differences in milk properties of Estonian breeds and populations, and revealed an effect of casein kappa and lactoglobulin beta genetic variants (Kübarsepp et al. 2006). The Estonian Native breed was analyzed for casein (alpha, beta and kappa) and lactoglobulin beta variants using isoelectric separation to determine genotypic effects on milk rennet properties (Jõudu *et al*. 2007). Among the sampled cows, 16 different aggregate genotypes were found and a significant overall effect on rennet coagulation parameters was indicated.

However, there is little precise knowledge of the distribution of genetic markers for lactoproteins among Estonian dairy cattle. The aim of this study was to determine the principal polymorphisms for casein beta (*CSN2*), casein kappa (*CSN3*) and lactoglobulin beta (*LGB*) genes in Estonian dairy cattle breeds.

Material and Methods. A total of 122 cattle from Estonian Red (ER), Estonian Holstein (EHF) and Estonian Native (EN) breeds were included in the analyses. Blood samples were collected from unrelated individuals following the recommendations spread by ISAG/FAO advisory group on animal genetic diversity (FAO, 1998). The sampled Estonian Red cattle (40) and Estonian Holsteins (42) strictly excluded upgraded animals (to avoid introduced genetic material). The Estonian Native sample (40) comprised some upgraded individuals because of the small population size and previous genetic improvement through introduction of Red Holstein and Jersey genes.

Blood samples were collected in tubes containing K_3EDTA. DNA was extracted from whole blood according to Miller *et al*. (1988) or by using a commercial Puregene Gentra Blood kit (Minneapolis, USA).

Allele specific oligonucleotide (ASO) primers were designed for detecting polymorphisms in the *CSN2* gene in the BTA6 exon VII at nucleotide positions 8101, 8219 and 8267. Single nucleotide polymorphisms (SNPs) at these positions determine A1 and A2, A3, and B alleles, respectively. SNPs C8101A, C8219A and C8267G were discriminated by using six different oligos forming primer pairs, as given in Table 1.

Table 1. **Single nucleotide polymorphism sites** (SNP)**, allelic determination for the *CSN2* gene and primer sequences used for ASO-PCR**

SNP/Allele	Forward primer	Reverse primer
8101/A1	5'-TCCCTTCCCTGGGCCCATCC**A**-3'	5'-TCAGTGAGAGTCAGGCTCTGG-3'
8101/A2	5'-TCCCTTCCCTGGGCCCATCC**C**-3'	5'-TCAGTGAGAGTCAGGCTCTGG-3'
8219/A3	5'-GATGAACTCCAGGATAAAAT-3'	5'-AGGGAAGGGCATTTCTTT**T**-3'
8267/B	5'-TCCCTTCCCTGGGCCCATCC**A**-3'	5'-TCAGTGAGAGTCAGGCTCTG**C**-3'

The primers were designed according to the bovine genomic sequence GenBank Accession No. X14711. PCR was performed in a total volume of 10 μL, consisting 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P40; NAXO, Estonia), 2.5 mM $MgCl_2$ (NAXO, Estonia), 0.25 mM of each dNTP (Fermentas, Lithuania), 0.4 μM of each primer (TAG Copenhagen A/S, the Netherlands), and 0.5 U of Taq-Smart DNA polymerase (NAXO, Estonia). There was approximately 40 ng of template DNA. AB GeneAmp PCR System 9700 and AB 2720 thermal cyclers (Applied Biosystems, USA) were used. Cycling conditions were: 95 ºC incubation for 10 min followed by 35 cycles of denaturation at 95 ºC for 30 s, 64 ºC (A1-, A2- and B-allele) or 51 ºC (A3-specific allele reaction) annealing for 30 s, 72 ºC extension for 30 s and an additional extension at 72 ºC for 7 min at the end of cycle 35. The reaction products were analyzed using electrophoresis on 2% agarose gel in 0.5 X TBE (45mM Tris-borate; 1mM EDTA-Na_2) buffer. EtBr was added to gels to visualize the analysis results under UV light. Electrophoresis was carried out applying the BIO-RAD Sub-Cell® GT System (USA).

CSN3 genotyping included amplification of a 345-bp sequence of casein kappa (*CSN3*) gene at exon IV located at BTA6. Primer sequences were taken from the literature (Velmala *et al.* 1993) and were the following: forward 5'-CATTTATGGCCATTCCACCAAAG-3' and reverse 5'-CATTTCGCCTTCTCTGTAACAG-3'. PCR was carried

out from approximately 100 ng of genomic DNA in a total volume of 25 µL containing 1X PCR buffer (NAXO, Estonia), 1 mM $MgCl_2$ (NAXO, Estonia), 100 µM of each dNTP (Fermentas, Lithuania), 1.0 µM of each primer (TAG Copenhagen A/S, the Netherlands) and 0.15 U of Taq DNA polymerase (NAXO, Estonia) per sample. An initial incubation at 94 ºC for 3 min was followed by 34 cycles of 94 ºC for 30 s, 48 ºC for 30 s and 72 ºC for 30 s and final 72 ºC for 5 min.

SNPs at positions 13104 and 13124 were examined to determine the nucleotide changes (A→C and A→G, respectively) by restriction analysis. These polymorphisms determine the A, B and E allele of *CSN3*. The digestions were performed using endonuclease *Hinf*I to detect the presence of A or B alleles and *Bsu*RI to detect the presence of the E allele. Restriction digests were carried out in a final volume of 14 µL, containing 12 µL of PCR product, 1X reaction buffer (NAXO, Estonia) and 1 U of restriction enzyme (Fermentas, Lithuania). The digested fragments were separated by electrophoresis on a 3% agarose gel. According to allelic status of the sample, fragments 132, 131, and 82 bp (referring to A-allele) or 263 and 82 bp (B-allele) were found after digestion with *Hinf*I.

*Bsu*RI digested fragments are 191 and 145 bp (and a nonvisible 9 bp fragment) in the case of a nucleotide change at A13124G (E-allele), but 336 bp if no mutation has occurred ("non-E" genotypes). Electrophoresis results, showing the three bands with *Bsu*RI, were interpreted as being heterozygous, AE or BE, depending on restriction results with *Hinf*I: two bands segregating, one 82 bp and another, containing fragments 132 bp and 131 bp, is genotype AE, whereas samples showing an uncut band of 263 bp and a 82 bp band were genotyped as BE.

A 247 bp fragment of the *LGB* gene, covering SNP site 5261 of exon IV (BTA11), was amplified using forward 5'-TGTGCTGGACACCGACTACAAAAAG-3' and reverse primer 5'-GCTCCCGGTATATGACCACCCTCT-3' (Medrano and Aquilar-Cordova 1990). Restriction digestion with *Bsu*RI (Fermentas, Lithuania) identifies SNP T5261C of the *LGB* gene, detecting two alleles, A and B. PCR was carried out in a volume of 25 µL, containing 1X PCR Buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, Lithuania), 1.0 µM of each primer (DNA Technology A/S, Denmark) and 0.12 U of Taq DNA Polymerase (Fermentas, Lithuania) per sample. The amount of template DNA was about 100 ng. According to the polymorphism, 148 and 99 bp fragments of digested PCR product refer to the A-allele; the B-allele specific polymorphism creates an additional digestion site for endonuclease *Bsu*RI in the middle of the 148 bp fragment, so that a 99 bp and two 74 bp fragments result. Thus, the heterozygous genotype has three detectable bands after electrophoretic separation of 148, 99 and 74 bp. The incubation for restriction digestion of the *LGB* PCR products lasted overnight and the reaction products were separated on 4% agarose gel.

Samples with various genotypes of the casein loci, typed by ASO PCR and PCR-RFLP, were selected for sequencing to verify the results. Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA).

Statistical analyses were carried out with Fstat293 (Goudet 2001) and Genepop 3.4 (updated version of Raymond and Rousset, 1995) packages. Allele frequencies, gene diversity and Nei's heterozygosities were analyzed. A Hardy-Weinberg equilibrium exact test was also carried out and the heterozygosity excess and deficiency were examined. Genotypic differentiation of the studied breeds was analyzed on the basis of the F_{ST} index (Fstat). Pair-wise gene and genotypic differentiation was computed to evaluate the genetic differences between breeds. Genotypic disequilibrium was studied by each lactoprotein gene pair and by each SNP pairs within caseins. Markov chain parameters were set to 10000 dememorisations, 100 batches and 5000 iterations per batch.

Results and Discussion. We detected three allelic variants of both *CSN2* and *CSN3* genes and two variants of the *LGB* gene in each breed. No private alleles were found among Estonian dairy cattle breeds. *CSN2* occurs at equal frequencies for A1 and A2 alleles, but the occurrence of these alleles differed according to breed, being at a higher frequency for the A1 allele in ER cattle, but at a lower frequency in EN and EHF breeds. Allelic differentiation was highly significant for A1 and A2 alleles (SNP 8101) between ER and EN breeds (P=0.0004). The B-allele at *CSN2* was similarly rare in all breeds (Table 2). The A3 variant was not detected at *CSN2*. The results of sequencing confirmed the accuracy of the allele-specific method for genotyping *CSN2*. Additionally, we identified a nucleotide change (C→T) at position 8261 compared with the reference X14711. The I-allele, detectable as a A→C change at position 8132 was not identified in our samples sequenced in this study. Further analyses indicated the occasional presence of an I-allele specific mutation (A→C) in the Estonian dairy cattle population. According to Jann *et al.* (2002), the distribution of the I-allele range is from 0.00 to 0.12 among European cattle breeds.

Sequencing *CSN3* revealed all polymorphic sites in the analyzed DNA fragment; A, B and E allelic variants and additional SNPs that were not detected using restriction analysis - C13068T and A13165G, defining the B-allele were recorded, and C13065T, defining the rare H-allele, was recorded (allelic variant not found). *CSN3* shows prevalence of the A-allele at a frequency ranging from 0.600 in ER to 0.869 in EHF. The B-allele of *CSN3* ranged from 0.095 in EHF to 0.375 in ER. Differences between the A and B allele (SNP 13104) were highly significant (P=0.0001) for ER and EHF breeds. The mean occurrence of the E-allele was 0.033, showing only slight variance among the breeds (Table 2). At the *LGB* locus the B allele was predominant over the A-allele in all breeds (Table 2).

Table 2. **Comparison of allele frequencies in Estonian cattle breeds by *LGB*, *CSN2*, and *CSN3* and prominent genotypes by breeds**

	Estonian Native	Estonian Red	Estonian Holstein	All
Sample size	40	40	42	122
LGB A	0.188	0.138	0.319	0.211
LGB B	0.813	0.863	0.681	0.789
CSN2 A1	0.375[a]	0.663[a]	0.429	0.488
CSN2 A2	0.600[a]	0.313[a]	0.548	0.488
CSN2 B	0.025	0.025	0.024	0.025
CSN3 A	0.725	0.600[b]	0.869[b]	0.734
CSN3 B	0.238	0.375[b]	0.095[b]	0.234
CSN3 E	0.038	0.025	0.036	0.033
Prevalent *CSN2*-*CSN3* genotype:	*A2A2-AA*	*A1A1-AA*	*A1A2-AA*	*A1A2-AA*
Prevalent *LGB* genotype:	*BB*	*BB*	*AB*	*BB*

[a] $P<0.001$ allelic differentiation between EN and EHF computed by SNP 8101; [b] $P<0.001$ allelic differentiation between ER and EHF computed by SNP 13104

In general the heterozygous genotype A1A2 prevailed at *CSN2* (38.5%) in the EHF breed, but not in ER and EN, where the homozygous A1A1 and A2A2 genotypes were most frequent. The majority of the sampled individuals were homozygotic AA at *CSN3* (56.6% overall) except for ER, where heterozygous AB individuals occurred at the same frequency as AA individuals. At *LGB* 60.3% were BB individuals and heterozygotes were only prevalent for EHF.

The most frequent combinations of casein genotypes were double homozygous genotypes *CSN2A1A1-CSN3AA* in ER (35% within breed), *CSN2A2A2-CSN3AA* in EN (25%) and in EHF (23%). *CSN2A1A2-CSN3AA* in EN and in EHF breeds were rarer (2% and 4%). A favorable genetic marker for protein yield and cheese production, *CSN3 B*, was rare in the homozygous state and the genotype *CSN3BB* combined with *CSN2A1A2* was found in ER only (10% within ER, 3.3% all over breeds). Heterozygous *CSN3AB* combined with *CSN2A1A2* was found in 20% of ER. Derived from genotype combination data, haplotype *CSN2A1-CSN3A* was typical to ER, while *CSN2A2-CSN3A* was commonly found in EHF and in EN.

The overall heterozygosity was 0.425. Analyses showed breed-wide differences in gene diversities at *LGB* and at *CSN3* (Table 3). The observed heterozygosity ranged from 0.275 in ER to 0.528 in EHF at *LGB* and from 0.238 in EHF to 0.450 in ER at *CSN3*. Based on the exact probability test (GENEPOP), all breeds were in Hardy-Weinberg equilibrium. Regardless of the extent of divergence of the F_{IS}-values from zero at some protein loci (0.210 and 0.202 at *CSN2* in ER and in EN; -0.200 at *LGB* in EHF) neither heterozygote deficiency nor excess was established in the populations (P=0.482 and P=0.525, respectively). Comparing the present results of the genetic structure of EN with those of Jõudu *et al.* (2007), *CSN3* was less polymorphic (the E-allele was not detected), in their study, but diversities among *CSN3* with *LGB* were similar. However, Jõudu *et al*. (2007) established a significant heterozygosity excess (P=0.005) in *CSN3*. We suppose that using a different sampling procedure could have affected the result: the rare allele was not exposed because some pedigrees were not represented in that study whereas we sampled across the whole breed.

Table 3. **The breed- and gene-wise estimates of expected heterozygosity (H_E) and F_{IS} values**

Breed	*LGB*		*CSN2*		*CSN3*	
	H_E	F_{IS}	H_E	F_{IS}	H_E	F_{IS}
EN	0.308	-0.054	0.506	0.210	0.422	-0.008
ER	0.240	-0.147	0.470	0.202	0.506	0.110
EHF	0.440	-0.200	0.522	-0.003	0.237	-0.004
Total	0.339	-0.139	0.526	0.131	0.410	0.044

In EHF all lactoprotein genes had negative F_{IS}-values, probably caused by ongoing gene flow from the global Holstein population. Estonian Holstein was the most heterogeneous breed regarding *LGB* and *CSN2* genes, but showing lower heterozygosity for *CSN3*. The low diversity estimates (*CSN2*, *LGB*) for Estonian Red, to some extent, could be influenced by sampling. Upgraded animals were carefully avoided, but decreasing population size might be a reason for diversity loss. Since the 1980s, the proportion of ER cattle has decreased from 60% to 25% in the Estonian national dairy herd.

Due to the physical linkage of *CSN2* and *CSN3* gene loci at BTA6, genetic linkage disequilibrium should be observed between genotypes carrying these genes. Our analysis showed only significant disequilibrium between *CSN2* and *CSN3* in ER (P=0.0003), for EHF it bordered

on statistical significance (P=0.05) and was weak for the EN breed (P=0.228). This result could indicate the existence of intrabreed subpopulations of EN that vary according to *CSN2*-*CSN3* allelic linkages. It could also explain statistically hidden genotypic disequilibrium as well as imbalance of F_{IS}-values (+0.210 and -0.008, at *CSN2* and *CSN3*, respectively).

According to our observations, genotypes showed statistically non-significant linkage in EN and EHF breeds (P=0.287 and P=0.222, respectively) at *LGB* and *CSN2*. When analyzing the data of the five single SNP loci at casein genes, the highest P-values were obtained between polymorphic sites 8101 and 13104 (P=0.00009), showing associations between *CSN2 A1/A2* and *CSN3 A/B* allelic variants in ER. Also, some weak genotypic disequilibrium (P=0.151 and 0.195) were demonstrated between polymorphisms at 8267 and 13124 for *CSN2 B* and *CSN3 E* in EN and EHF breeds.

Pair-wise differentiation of breeds was computed for milk protein polymorphisms. The most distant breeds were ER and EHF (F_{ST} 0.110). There was less deviation between ER and EN breeds (0.066). The differentiation was negligible between EN and EHF breeds (0.021) (Table 4). The difference was markedly high at *CSN3* between ER and EHF (0.161, P= 0.0002), and also between EN and ER (0.134, P-value 0.002) at *CSN2*.

Table 4. **Pair-wise genotypic differentiation of breeds** (F_{ST} index below the diagonal) **and *P*-values for statistical significance** (above the diagonal)

	Estonian Native	Estonian Red	Estonian Holstein
Estonian Native		0.009	0.073
Estonian Red	0.066		0.000
Estonian Holstein	0.021	0.110	

The F_{ST}, which measures genetic differentiation, was 0.066 over all milk protein loci. This estimate, interpretable as population subdivision, did not exceed the 95% confidence interval of the F_{ST} value obtained from selectively neutral DNA microsatellite data (95% *CI* 0.045 0.078) (unpublished data). A high level of between-individual variation (93.4% from the total variation) and low between-breed variation likely signifies similar breeding goals as well as similar natural selection among the studied breeds within a relatively contained geographic location.

Conclusions. The occurrence of milk protein gene variants in Estonian dairy cattle, including Estonian Native cattle, was generally similar to that for common European dairy cattle. However, the distribution of genotypes, mostly with regard to casein genes, differentiated the breeds.

Our findings indicated the genetic advantage of small breeds for special traits, especially the Estonian Native cattle, which are able to produce wholesome *CSN2 A2* milk that might naturally lower risks associated with allergies, diabetes and heart diseases. If the health-conferring properties of this milk are confirmed it represents support for the important status of the native cattle. The best possibilities for meeting the needs for cheese manufacturing companies seemed to favor the older type of Estonian Red and Estonian Native breed, which were demonstrated to produce milk with better milk protein composition. Despite the decreasing effective population size of Holstein cattle at a global scale, the EHF displayed the highest heterozygosity of *LGB* and *CSN2* genes.

Utilization of natural genetic resources represented by local breeds might be helpful in cattle breeding programs and in sustaining the breeds. However, due to different genetic background of Estonian dairy breeds the marker effects should be assessed for marker-assisted selection before the implementation. Maintaining the existing within-breed trait variation is of great importance, but the obvious risk of minor breeds being threatened by inbreeding must be considered, especially if pedigrees with particular genetic advantages are intensively exploited.

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