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## Genomic diversity and population structure of the Czech Holstein cattle

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## HIGHLIGHTS

• The genomic diversity of the Czech and other Holstein subpopulations was investigated.

Inbreeding in the AI bulls was very high and far exceeded the inbreeding in the cows.

• Using of highly inbred AI bulls will lead to a high level of inbreeding in the Czech subpopulation.

• The differences in the Holstein subpopulations are consequence of different breeding programs.

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## ABSTRACT

Holstein-Friesian (HF) is a cosmopolitan breed distributed in more than 150 countries. It represents a large metapopulation with intensive gene flow, realised mainly through artificial insemination and the intensive use of the popular "star" bulls. The breed is known for its superiority in milk yield, production efficiency and black and white coat color. In contrast to the enormous size of the census population, which exceeds nine million animals in the U.S. alone, the genetic diversity of this highly commercialised breed is surprisingly low, necessitating genetic monitoring, especially of subpopulations in individual countries. Our main objective in this study was to analyze genomic diversity (estimated by genomic inbreeding and effective population size) and population structure (relationship to other subpopulations) of the subpopulation from the Czech Republic and, based on highthroughput SNP array genotypes. We analysed 2178 animal samples (32,865 autosomal SNP) from 12 subpopulations and the Simental cattle breed (98 animals), which represents an outlier population. Czech bulls showed high genomic inbreeding ( $F_{ROH>2Mb}=0.133$ ), well above the inbreeding level of Czech cows (F<sub>ROH>2Mb</sub>=0.091), with particularly high recent inbreeding (ROH>8Mb). Unexpectedly, the estimated effective population size (NeLD) was relatively high, ranging from 202 (GONE) to 283 (NeEstimator v2), depending on the estimation algorithm. Our phylogenetic analyses showed that the Czech HF belonged to the "core metapopulation HF ", together with Belgian, British, Canadian, Dutch, French, German, and USA subpopulations, which was separated from the Swiss, Irish, and Croatian subpopulations. We also showed that Czech AI bulls differed slightly from cows, especially in genes affecting meat and carcass. Our results have defined the population structure of the Czech HF and indicate the potential problems of increased inbreeding due to selection of AI bulls.

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## 1. Introduction

Management of genetic diversity in intensively selected cattle populations is an essential component to ensure successful long-term increase in selection response to production and adaptation traits that provide optimal genetic improvement (Boichard et al., 2016; Goddard, 2009; Hill, 2000). Maintaining sufficient genetic variability is also necessary to meet production requirements in different environments, to enable sustained genetic improvement and to adapt to changing breeding objectives (de Jong and Bijma, 2002; Strandén et al., 2019). Genetic diversity also represents a key factor to avoid negative consequences due to inbreeding (Doekes et al., 2018; Ferenčaković et al., 2017; Howard et al., 2017) and/or increased prevalence of genetic defects (Cole, 2015; VanRaden et al., 2011). However, the genetic diversity of dairy breeds has been greatly reduced, jeopardizing successful long-term genetic improvement. For example, despite large numbers of animals (large census), the genetic diversity of commercial breeds is often relatively low because only a few sires are used to reproduce with large numbers of offspring (Hodges, 2006).

Holstein-Friesian (HF) is a trans-border breed spread in more than 150 countries and represents a large metapopulation. It is known for its superiority in milk yield, production efficiency and black and white piebald coat color, and is now the most widespread dairy breed in the world. In the Czech Republic, the Holstein subpopulation (CZE) has long been bred for high milk yield. (www.holstein.cz). Similar to other national subpopulations, the CZE has been developed over the past fifty years from domestic breeding material and imported semen and embryos from international bulls and cows with high milk yields. More specifically, more than 1 million AI semen and several thousand embryos have been imported, along with 200 live bulls and 25,000 heifers imported mainly from France, Germany, the Netherlands, and Denmark.

The genomic diversity of subpopulations of HF is relatively low worldwide, and for example, the effective population size (Ne) for Holstein populations in Australia, Canada, Denmark, Spain, Ireland, and the United States of America ranges from 49 to 127 (Doekes et al., 2018). The main reason for reduced genomic diversity is the overuse of a small number of superior bulls. This is most evident in the variability of the Y chromosome, where it was shown that 220,872 Holstein bulls, all from the Interbull database, descended from only five founders, three of which came from North America (Yue et al., 2015). This example also shows that intensive use of a small number of popular bulls known for their superior milk production results in high gene flow and connectivity between subpopulations, but this further reduces the genetic diversity of the overall metapopulation. Management of genomic diversity and inbreeding has therefore become an important issue in many national HF breeding programs (Ablondi et al., 2022; Doekes et al., 2018; Forutan et al., 2018; Rodríguez-Ramilo et al., 2015). The CZE subpopulation is no exception, as current breeding objectives focus on high milk yield and intensive use of a small number of mainly U.S. elite bulls is substantial, while selection (especially genomic) is known to increase inbreeding.

The main objective of this study was to estimate the genomic diversity status (genetic diversity, genomic inbreeding, and effective population size) of the CZE and compare it with other HF subpopulations. Genomic relatedness, population structure, and admixture of HF subpopulations, especially those affecting breeding of CZE, were estimated. We made additional efforts to analyze the influence of imported bulls on current CZE (cows).

## 2. Material and method

## 2.1. Analysed animals and quality control

The genome-wide data of 2178 animals of Holstein cattle from twelve countries; Belgium - 43 bulls (BEL<sub>B</sub>), Canada - 98 bulls (CAN<sub>B</sub>), Croatia - 84 cows (HRV<sub>C</sub>), Czech Republic - 301 bulls (CZE<sub>B</sub>), 298 Czech

Republic - cows (CZE<sub>C</sub>), France - 141 bulls (FRA<sub>B</sub>), Netherlands - 290 bulls (NLD<sub>B</sub>), Ireland - 129 bulls (IRL<sub>B</sub>), Great Britain - 42 bulls (GBR<sub>B</sub>), Germany - 343 bulls (DEU<sub>B</sub>), Switzerland - 177 bulls (CHE<sub>B</sub>), USA - 232 bulls (USA<sub>B</sub>), were analysed. Animals were genotyped using Illumina BovineSNP50 BeadChip v1, v2 and v3 (n = 2094) and GGP100K Neogen Ltd (n = 84). In addition, genome-wide data from 98 animals, originally Czech Simental cattle (SIM<sub>C</sub>), genotyped with the Illumina BovineSNP50 BeadChip v3 were used as an outlier population. Only SNPs co-occurring in these three panels were selected for analysis. The merged data set included 32 865 successfully reassigned SNPs occurring in all panels used. Quality control of genotype data was performed using PLINK 1.9 software (Chang et al., 2015). Data were trimmed using the following parameters: only autosomal SNPs with known chromosomal positions to exclude bias between males and females, individual call rate > 0.9, and SNP call rate > 0.9. This process ultimately resulted in the use of 29,502 SNPs.

## 2.2. Genetic diversity and runs of homozygosity inbreeding

Genomic diversity, represented as observed  $(H_0)$  and expected  $(H_F)$ heterozygosity (gene diversity), and Wright's inbreeding coefficient ( $F_{IS}$ ) were calculated in PLINK 1.9. Genome-wide  $F_{IS}$  values indicate deviation from Hardy-Weinberg heterozygosity equilibrium, with negative values usually the result of avoidance of mating between close relatives, while positive values indicate inbreeding or mating between closely related individuals (Wright, 1965). Genome-wide diversity was quantified using a segment-based approach. Runs of homozygosity (ROH) segments were determined using detectRUNS (Biscarini et al., 2019). ROH segments were determined according to the following criteria (Ferenčaković et al., 2013): the minimum number of SNPs included in the ROH was set at 15; zero missing calls per window were allowed for ROHs > 2 Mb, one for ROHs from 4 to 8 Mb, and two for ROHs > 8 Mb categories, while no heterozygous SNPs were allowed. The maximum distance between the two SNPs was set at 1 Mb. The ROH-based inbreeding coefficient (FROH) was defined as the proportion of the genome in ROH relative to the total autosomal genome covered by SNPs on the chip. The F<sub>ROH</sub> was estimated as the proportion of the autosome in ROH covering 29 chromosomes (F<sub>ROH</sub>=L<sub>ROH</sub>/L<sub>AUTOSOME</sub>). With respect to ROH length, three inbreeding coefficients were estimated, indicating recent ( $F_{ROH>8Mb}$ ), intermediate ( $F_{ROH4-8Mb}$ ), and distant (F<sub>ROH2-4Mb</sub>) inbreeding.

## 2.3. Effective population size

We calculated the contemporary population effective size for each subpopulation using two different approaches. To estimate only the contemporary effective population size, we used the approach described and implemented in NeEstimator v.2 software (Do et al., 2014), which uses the Jack-Knife method to estimate 95% confidence intervals and removes SNPs with frequencies below 5%. We also applied a recently developed approach implemented in the software GONE that provides both historical and contemporary estimates (Saura et al., 2021). The estimation approach developed in GONE is based on the functional relationship between gamete and/or linkage disequilibrium patterns and effective population size (Hill, 1981), but is calculated using a complex modeling approach. A genetic algorithm is implemented in the software GONE to derive the historical set of effective population size series that best minimises the sum of squared differences between the observed d<sup>2</sup> values (averaged squared correlations between two loci allele frequencies weighted by their variance) of the bins and those predicted at corresponding different demographic trajectories (Saura et al., 2021). In this study, we have referred to our estimates as NeLD to indicate that the estimates are based on gametic and/or linkage disequilibrium. NeLD is the size of an idealised population, often considered a Wright-Fisher population, that exhibits the same degree of genetic drift-change in gametic or linkage disequilibrium-as the

population under consideration. For more information on NeLD and potential biases, see Ryman et al. (2019), Waples (2021) and Waples et al. (2014).

## 2.4. Genetic relationship and population structure

The phylogenetic relationship between the 12 subpopulations of HF and the outlying Simental cattle population was represented by the Neighbor-Net network inferred from Nei's pairwise genetic distance (Nei, 1972). Nei's genetic distances were calculated using the StAMPP package (Pembleton et al., 2013). The phylogenetic network (Neighbor-Net) was created and drawn using SitsTree5 software (Huson and Bryant, 2006). Discriminant analysis of principal components (DAPC), implemented in the R package Adegenet (Jombart and Ahmed, 2011), was used to determine genetic structure and differentiation among Holstein subpopulations. The optimal number of principal components (PCs) reflecting the highest proportion of variance in the database was tested based on the a-score (Jombart and Ahmed, 2011). In addition, DAPC was used to assign individuals and obtain the affiliation probability, which represents the total genetic background of an individual. The population structure was further evaluated using a Bayesian clustering approach implemented in the software STRUCTURE (Pritchard et al., 2000). Before analysing genetic differentiation within and between populations, data sets were pruned based on the threshold for linkage disequilibrium between SNPs (0.05) with a window size of 50 and a step size of five SNPs. After this cleaning of the data, only 2223 SNPs remained for subsequent analyses. The analysis was performed with an admixture and correlated allele frequency model, using 106 iterations with a burn-in period of 105. Runs were repeated 20 times for each assumed K (1-13). The most likely K value in the data set was determined according to (Evanno et al., 2005) using STRUCTURE HARVESTER (Earl and vonHoldt, 2012). Visualisations of population structure were performed using the web-based tool CLUMPAK (Kopelman et al., 2015).

# 2.5. The genetic influence of imported bulls on the Czech Holstein subpopulation

The Czech Holstein population is open, with significant import of genetic material from abroad. The term Czech bulls refers to bulls born in the Czech Republic. These are bulls from imported embryos (mainly from the USA) and sons of heifers born from these embryos. The sires of the Czech bulls are the best bulls of the world population of Holstein cattle (mainly from the USA). The Czech Holstein cow entered the herd in the nineties of the last century by crossing with foreign bulls (from different countries, mainly from Europe). Most cows in the Czech Republic are inseminated by foreign bulls (from different countries). Insemination doses of Czech bulls make up only a small part of the insemination doses used in the Czech Republic. As mentioned above, most of the insemination doses used for production cows come from abroad (Europe, USA). Therefore, the Czech Holstein cows (CZE<sub>C</sub>) have a different genetic background than the studied Czech Holstein bulls (CZE<sub>B</sub>). Therefore, to measure the influence of imported bulls, we calculated the genetic differentiation between female and male Czech Holsteins (CZE<sub>C</sub> versus CZE<sub>B</sub>).

Thus, genome-wide  $F_{ST}$  (coefficient of population differentiation) estimates were calculated for each SNP (Weir and Cockerham, 1984) using the SNP & Variation Suite (SVS) v8.7.0 software package (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). Subsequently,  $F_{ST}$  estimates were normalised by mean frequency, while transformed values were represented as -log(P). As outliers, 0.1% SNPs with the highest values were selected (30 SNPs), which corresponded to  $F_{ST}$  value of 0.074 and a -log(P) value of 9.058. In addition, candidate genes and QTLs were annotated within 0.4 Mb wide genomic regions around each outlier (0.2 Mb from each side). While genes were annotated using information from the SNP & Variation Suite (SVS) v8.7.0 software package

(Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com), QTLs were annotated using the GALLO R package (Fonseca et al., 2020) to query the Animal QTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/ index, accessed 04/29/2022) for previously identified QTLs in the regions of interest. Trait enrichment analysis was performed for the annotated QTLs, and enriched classes and traits per chromosome with an FDR-adjusted P value of less than 0.05 were considered significant and represented as -log(P).

## 3. Results and discussion

## 3.1. Genetic diversity and runs of homozygosity inbreeding

Our analyses showed that inbreeding levels were higher in all subpopulations of HF compared to SIM<sub>C</sub> (Table 1 and Fig. 1). For example, the estimated mean  $F_{ROH>2Mb}$  in SIM<sub>C</sub> (0.045) was more than twice as small as in the twelve HF subpopulations (see below for more details on population structure), where the  $F_{ROH>2Mb}$  ranged from 0.091 to 0.133. At the same time, the estimated  $F_{ROH>2Mb}$  was lower in tree subpopulations (CHE<sub>B</sub>, HRV<sub>C</sub>, and IRL<sub>B</sub>) outside the "core" HF, ranging from 0.077 to 0.088 (Table 1). The similar trend was observed for other indicators of genetic diversity (H<sub>O</sub>, H<sub>E</sub>, and F<sub>IS</sub>), although the observed differences for H<sub>O</sub> and H<sub>E</sub> were not significant. Interestingly, with the exception of HRV<sub>C</sub>, CHE<sub>B</sub>, IRL<sub>B</sub>, but also CZE<sub>C</sub>, and GBR<sub>B</sub> subpopulations, all the remaining subpopulation had significantly positive  $F_{IS}$  values. The  $F_{IS}$  negative values usually indicating of avoidance of mating between close relatives, while positive values indicate inbreeding or mating between closely related individuals.

In contrast,  $F_{IS}$  values were significantly negative in  $CHE_B,\ HRV_C,$  and  $IRL_B,\ indicating$  avoidance of inbreeding, while a negative  $F_{IS}$  value was also observed in the  $SIM_C$  breed. Quite large differences, higher  $F_{IS}$  and  $F_{ROH>2Mb}$  values, were observed in CZE\_B than in CZE\_C, indicating that the reduction in diversity and extreme increase in inbreeding is due to imported semen. Of particular concern is the very high recent inbreeding ( $F_{ROH>8Mb}$ ) observed in CZE\_B, which exceeded the levels of

#### Table 1

Genetic diversity indicators ( $H_o$ ,  $H_E$  and  $F_{IS}$ ) and runs of homozygosity based inbreeding level in 12 HF subpopulations and Czech Simental cows.

	Animals	Ho	H <sub>E</sub>	F <sub>IS</sub>	F <sub>ROH&gt;2Mb</sub>
BELB	43	0.365	0.358	$0.012{\pm}0.006$	0.112
-		$\pm 0.024$	$\pm 0.022$		$\pm 0.006$
CANB	98	0.363	0.357	$0.017{\pm}0.004$	0.114
		$\pm 0.015$	$\pm 0.015$		$\pm 0.004$
CZE <sub>C</sub>	298	0.372	0.371	-0.007	0.091
		$\pm 0.008$	$\pm 0.008$	$\pm 0.002$	$\pm 0.002$
CZEB	301	0.356	0.358	$0.036 {\pm} 0.003$	0.133
		$\pm 0.008$	$\pm 0.008$		$\pm 0.003$
DEUB	343	0.363	0.363	$0.017{\pm}0.002$	0.113
		$\pm 0.008$	$\pm 0.007$		$\pm 0.002$
FRAB	141	0.366	0.360	$0.010 {\pm} 0.003$	0.108
		$\pm 0.012$	$\pm 0.012$		$\pm 0.003$
GBR <sub>B</sub>	42	0.368	0.359	$0.005 {\pm} 0.005$	0.102
		$\pm 0.025$	$\pm 0.022$		$\pm 0.005$
HRV <sub>C</sub>	84	0.373	0.368	-0.010	0.088
		$\pm 0.016$	$\pm 0.015$	$\pm 0.003$	$\pm 0.003$
CHEB	177	0.373	0.368	-0.010	0.084
		$\pm 0.011$	$\pm 0.010$	$\pm 0.002$	$\pm 0.002$
IRLB	129	0.374	0.375	-0.013	0.077
		$\pm 0.012$	$\pm 0.011$	$\pm 0.003$	$\pm 0.003$
NLD <sub>B</sub>	290	0.365	0.365	$0.012{\pm}0.002$	0.108
		$\pm 0.008$	$\pm 0.008$		$\pm 0.002$
USAB	232	0.364	0.358	$0.016 {\pm} 0.003$	0.113
		$\pm 0.010$	$\pm 0.009$		$\pm 0.002$
SIM <sub>C</sub>	90	0.376	0.373	-0.016	0.045
		$\pm 0.015$	$\pm 0.014$	$\pm 0.004$	$\pm 0.004$

 $\rm H_O$  is observed heterozygosity,  $\rm H_E$  is expected heterozygosity,  $\rm F_{IS}$  is Wright's inbreeding coefficient and  $\rm F_{ROH>2~MB}$  is ROH based genomic inbreeding coefficient.



**Fig. 1.** Distribution of individual genomic inbreeding in 12 HF subpopulations [Belgium - BEL<sub>B</sub>, Canada - CAN<sub>B</sub>, Croatia - HRV<sub>C</sub>, Czech Republic - CZE<sub>B</sub> (bulls), Czech Republic - CZE<sub>C</sub> (cows), France - FRA<sub>B</sub>, Netherlands - NLD<sub>B</sub>, Ireland - IRL<sub>B</sub>, Great Britain - GBR<sub>B</sub>, Germany - DEU<sub>B</sub>, Switzerland - CHE<sub>B</sub>, USA - USA<sub>B</sub>)] and Czech Simmental breed - SIM<sub>C</sub> (cows). (a) Boxplot representation of genomic inbreeding ( $F_{ROH>2Mb}$ ); (b) stacked bar representation of the partitioning of genomic inbreeding to remote ( $F_{ROH2-4Mb}$ ), intermediate ( $F_{ROH2-8Mb}$ ) and recent ( $F_{ROH>8Mb}$ ) origins of autozygosity.

all other (sub)populations analysed (Fig. 1B).

In general, very high recent inbreeding was observed in all HF subpopulations (F<sub>ROH>8Mb</sub>), reflecting the strong intensity of selection in bulls. This explains why genetic diversity was low in all bull populations, while inbreeding was high. However, the observed differences in inbreeding levels, e.g., HF bulls sampled subpopulations versus SIM<sub>C</sub>, were too large to affect the observed trends. For example, a very high level of inbreeding (F<sub>ROH>2Mb</sub>) was observed in the Italian HF population (mainly cows), where the observed level of inbreeding ranged from 0.09 for animals born between 2002 and 2005 to 0.16 for animals born between 2016 and 2020 (Ablondi et al., 2022). However, the estimated  $F_{ROH>2Mb}$  in Ablondi et al. (2022) is based on a denser SNP array ( $\approx$ 70 K) and is not fully comparable, although the difference is not expected to be significant. The estimated level of inbreeding of 0.12 ( $F_{ROH>2Mb}$ ) reported by Lozada-Soto et al. (2022) in the USA HF is consistent with our estimates. A similarly high degree of inbreeding (F<sub>ROH>2Mb</sub> equal to 0.111) was also estimated by Szmatoła et al. (2019) in the Polish HF (bulls and cows).

## 3.2. Effective population size

Contemporary estimates of linkage (gametic) disequilibrium effective population size obtained using algorithms from NeEstimator v2 (Do et al., 2014) and GONE (Santiago et al., 2020) software are shown in Table 2. There were often no significant differences between the estimates obtained with two different approaches because the 95% confidence intervals (95% CI) largely overlapped, although some exceptions were also observed (CZE<sub>C</sub>, CZE pooled,  $HRV_C$ , and  $IRL_B$ ).

Unfortunately, in some subpopulations ( $HRV_C$ ,  $BEL_B$ , and  $GBR_B$ ) the observed 95% CIs were quite wide and provided less informative estimates (Table 2).

In addition to the estimates presented separately for the CZE<sub>C</sub> and CZEB samples, we also estimated NeLDO for the pooled CZE sample (bulls and cows). Compared with the other HF subpopulations, estimated Ne<sub>LD0</sub> was among the highest in the pooled CZE sample (Table 2). However, we should be aware that the estimates based on the bull samples are much lower, as expected and commented by Ablondi et al. (2022). Although our subpopulation samples consisted mainly of bulls, much higher estimates than expected (Table 2) were observed, mostly above 100, which is higher than the critical value of 50 recommended by (FAO, 1998). Estimates of effective population size (Ne<sub>LD</sub>) obtained from genome-wide data in other studies ranged from about 80 in the North American (Canada and USA) HF population (Sargolzaei et al., 2008) to about 150 in the Australian HF cattle (Hayes et al., 2003). Our estimates for the NLD<sub>B</sub> were much higher than those of (Doekes et al., 2018), which ranged from 69 to 102 for the period from 1995 to 2015, although these estimates related to inbreeding effective population size were reported without confidence intervals. For example, Ablondi et al. (2022) observed an inbreeding effective population size of 55 in the Italian population HF, estimated from the pedigree, whereas a much higher

#### Table 2

Contemporary and historical linkage (gametic) disequilibrium effective population size estimated by two different approaches implemented in the NeEstimator v2 (Do et al., 2014) and GONE (Santiago et al., 2020) software in HF subpopulations.

HF subpopulation (Origin)	Ne <sub>LD0</sub> - NeEstimator v2	Ne <sub>LD0</sub> - GONE	Ne <sub>LD10</sub> - GONE
BEL <sub>B</sub> (Belgium)	135 (89–255)	<b>120</b> (90–161)	<b>95</b> (73–126)
CAN <sub>B</sub> (Canada)	107 (85–140)	<b>97</b> (83–114)	102 (87–120)
CHE <sub>B</sub> (Switzerland)	102 (88–118)	110 (99–123)	118
			(106 - 132)
CZE <sub>C</sub> (Czech Republic)	359 (329-395)	181	117
		(167–197)	(109–127)
CZE <sub>B</sub> (Czech Republic)	147 (132–164)	154	86 (81–93)
		(143–167)	
CZE pooled (Czech	261 (240-283)	202	109
Republic)		(193–213)	(105–114)
$DEU_B$ (Germany)	196 (181–212)	188	<b>94</b> (89–101)
		(175–203)	
FRA <sub>B</sub> (France)	146 (123–176)	155	117
		(136–178)	(103–134)
GBR <sub>B</sub> (Great Britain)	145 (107–219)	108 (81–145)	124 (91–168)
HRV <sub>C</sub> (Croatia)	130 (105–166)	206	<b>55</b> (48–65)
		(167–255)	
IRL <sub>B</sub> (Ireland)	<b>61</b> (51–72)	105 (92–121)	200
			(172–234)
NLD <sub>B</sub> (Netherlands)	207 (190-226)	179	109
		(165–195)	(102–118)
USA <sub>B</sub> (USA)	158 (144–175)	140	<b>99</b> (91–108)
		(128–154)	

 $Ne_{LD0}$  is the estimated linkage (gametic) effective population size in the contemporary population, whereas  $Ne_{LD10}$  refers to historical estimates of the same populations 10 generations back.

estimate (120) was based only on SNPs placed on the same chromosomes (Ne<sub>LD</sub>). Surprisingly, historical estimates (Ne<sub>LD10</sub>) were mostly lower than contemporary estimates (Ne<sub>LD0</sub>), suggesting that Ne<sub>LD</sub> has increased over the past 10 generations, whereas a significant decrease was observed only in the IRL<sub>B</sub>. The increase in inbreeding effective population size from 65 (animals born between 1960 and 1979) to 101 (animals born between 2000 and 2013) was also observed in the Spanish HF (Rodríguez-Ramilo et al., 2015).

We would like to emphasize that the observed NeLD estimates should be used with caution for several reasons that may have influenced our estimates. For example, estimating effective population size is very complex when populations are subdivided, especially when there is a high unbalanced migration rate between subpopulations, which is the case in this study where HF can be considered as a large metapopulation (Ryman et al., 2019). Thus, the presence of admixture or migration from other HF subpopulations or even breeds (Simmental cattle), as observed in this study (see below), may lead to biased estimates of  $Ne_{LD}$ . At the same time, estimates of historical NeLD are even more unreliable when there is a possible bias due to the presence of admixed individuals. The subpopulations analysed had overlapping generations, which is another cause of the potential bias in estimating effective population size in this study (Waples et al., 2014). Finally, estimates of effective population size can differ drastically between inbreeding and linkage (gametic) disequilibrium in populations undergoing migration (Ryman et al., 2019), which is to be expected in HF subpopulations, with additional complications arising from the presence of selection and non-random mating.

## 3.3. Genetic relationship and population structure

The population structure of the HF metapopulation was analysed using different approaches. For example, Fig. 2 shows the relationships between 12 HF subpopulations and SIM<sub>C</sub> resulting from DAPC and Neighbor-Net analysis. The first four discriminant functions of DAPC explained 97% of the total variation, whereas each discriminant

function contributed to the separation of SIM<sub>C</sub>, HRV<sub>C</sub>, IRL<sub>B</sub>, and CHE<sub>B</sub> from the "core" HF metapopulation (Fig. 1A, B, C). The NeighborNet network derived from the pairwise genetic distances of Nei is consistent with the DAPC analysis and shows clear differentiation of the HRV<sub>C</sub>, IRL<sub>B</sub>, and CHE<sub>C</sub> subpopulations toward SIM<sub>C</sub> apart from the other HF subpopulations (Fig. 2D). The results support the hypothesis that the formation of the HF subpopulations in some EU countries is due to the crossing of imported HF semen with local Simmental cows. Our hypothetically subdivided Czech HF subpopulations of bulls and cows (CZE<sub>B</sub> and CZE<sub>C</sub>), although slightly separated from each other, were placed in the "core" HF metapopulation with subpopulations BEL<sub>B</sub>, CAN<sub>B</sub>, DEU<sub>B</sub>, FRA<sub>B</sub>, GBR<sub>B</sub>, NLD<sub>B</sub> and USA<sub>B</sub>. This result is logical because semen from popular and highly productive North American bulls was intensively imported into the Czech Republic and other European countries.

This also explains the neighbor position of CZE<sub>B</sub> near USA<sub>B</sub>, whereas CZE<sub>C</sub> is on the other side of the Neighbor-Net network toward HRV<sub>C</sub> (Fig. 2D). The visual illustration of the relationship between the subpopulations of HF is further quantified by the pairwise genome-wide  $F_{ST}$  estimates (Table 3). The three slightly separated subpopulations (HRV<sub>C</sub>, IRL<sub>B</sub>, and CHE<sub>B</sub>) had high estimated mean  $F_{ST}$  (MF<sub>ST</sub>) that ranged from 0.019 to 0.027, whereas the MF<sub>ST</sub> for the main HF "gene pool" ranged from 0.010 to 0.014.

For unsupervised identification of population structure and estimation of admixture level, we used the algorithm implemented in the software STRUCTURE because it can reveal "hidden structure" and quantify admixture without determining a priori membership in individual clusters. We ran STRUCTURE from K = 1 to K = 13, assuming that the highest number of potential clusters is 13, i.e., one breed plus 12 subpopulations, each representing a single cluster. The Ln Pr(G|K) value increased slightly and steadily, with only a significant drop at K = 6caused by lower Ln Pr(G|K) values in some runs (Fig. 3A). At the same time, the highest rate of change of Ln Pr(G|K) between successive K values was observed at K = 5 (Fig. 3B). Thus, following the recommendations of Pritchard et al. (2000), Falush et al. (2007), and Evanno et al. (2005), it is very likely that K = 5 applies to the analysed data set. While SIM<sub>C</sub> clearly stood out as a different "yellow" cluster (in this case representing "SIM<sub>C</sub> cluster") from the HF metapopulations, the other four clusters were distributed across all HF subpopulations, confirming the extensive gene flow known to occur through semen importation (Fig. 3C).

This result suggests that any differences in the subpopulations of HF are due to the composition of the estimated five clusters, probably because of their different breed development histories, selection intensities, and breeding goals. Interestingly, the main cluster defining  $SIM_C$  with the mean individual membership equal to 0.777 (presented by yellow color in the Fig. 3C) was present in all HF subpopulations, ranging from 0.089 to 0141 in the "core" HF metapopulation. Much higher proportion of the "SIM<sub>C</sub> cluster" (yellow) was estimated in the HRV<sub>C</sub> (0.356), CHE<sub>B</sub> (0.296) and IRL<sub>B</sub> (0.214) subpopulations further explaining its slight differentiation from the "core" HF metapopulation presented in Fig. 2. In CZE<sub>B</sub>, we found some degree of clustering with a considerable presence of "red" clusters, which was also noticeable in DEU<sub>B</sub>, NLD<sub>B</sub>, and USA<sub>B</sub> (Fig. 3C).

# 3.4. The genetic influence of imported bulls on the Czech Holstein subpopulation

Our phylogenetic network (Neighbor-Net) and unsupervised STRUCTURE analyses revealed slight genetic differences between samples from the cows ( $CZE_C$ ) and samples from the imported AI bulls (Figs. 2 and 3). To predict future genetic changes expected from high use of AI semen in the Czech Republic HF, we identified genomic regions where genetic differences (estimated  $F_{ST}$  values) between  $CZE_C$  and  $CZE_B$  were most pronounced, and their genomic positions are shown in Fig. 4.

The genes located in these regions were identified and are presented



**Fig. 2.** visualization of the genetic relationship of 12 HF subpopulations [Belgium - BEL<sub>B</sub>, Canada - CAN<sub>B</sub>, Croatia - HRV<sub>C</sub>, Czech Republic - CZE<sub>B</sub> (bulls), Czech Republic - CZE<sub>C</sub> (cow), France - FRA<sub>B</sub>, Netherlands - NLD<sub>B</sub>, Ireland - IRL<sub>B</sub>, Great Britain - GBR<sub>B</sub>, Germany - DEU<sub>B</sub>, Switzerland - CHE<sub>B</sub>, USA - USA<sub>B</sub>)] and Czech Simmental breed – SIM<sub>C</sub> (cows). A) Variation of the first two discriminant function of the DAPC. B) Variation of the third discriminant function of the DAPC. C) Variation of the fourth discriminant function of the DAPC. D) Neighbor-Net inferred from pairwise Nei's genetic distances.

## Table 3

Population differentiation among 12 HF subpopulations [Belgium - BEL<sub>B</sub>, Canada -CAN<sub>B</sub>, Croatia - HRV<sub>C</sub>, Czech Republic - CZE<sub>B</sub> (bulls), Czech Republic - CZE<sub>C</sub> (cow), France - FRA<sub>B</sub>, Netherlands - NLD<sub>B</sub>, Ireland - IRL<sub>B</sub>, Great Britain - GBR<sub>B</sub>, Germany - DEU<sub>B</sub>, Switzerland - CHE<sub>B</sub>, USA - USA<sub>B</sub>)] and Czech Simmental breed – SIM<sub>C</sub> (cows) based on genome-wide F<sub>ST</sub> estimates.

	BELB	CANB	CHEB	CZEB	CZE <sub>C</sub>	DEUB	FRA <sub>B</sub>	GBR <sub>B</sub>	HRV <sub>C</sub>	IRL <sub>B</sub>	NLD <sub>B</sub>	USA <sub>B</sub>	MF <sub>ST</sub>
BELB													0.011
CANB	0.003												0.014
CHEB	0.013	0.013											0.019
CZEB	0.004	0.008	0.019										0.014
CZE <sub>C</sub>	0.005	0.008	0.013	0.007									0.011
DEUB	0.001	0.004	0.012	0.002	0.004								0.010
FRAB	0.005	0.008	0.017	0.005	0.003	0.003							0.012
GBR <sub>B</sub>	0.003	0.003	0.013	0.005	0.004	0.002	0.003						0.011
HRV <sub>B</sub>	0.025	0.028	0.028	0.027	0.021	0.023	0.023	0.023					0.027
IRLB	0.017	0.022	0.019	0.020	0.011	0.016	0.015	0.016	0.028				0.020
NLD <sub>B</sub>	0.002	0.007	0.014	0.004	0.003	0.002	0.003	0.003	0.022	0.013			0.011
USAB	0.003	0.006	0.018	0.002	0.007	0.002	0.005	0.004	0.027	0.021	0.004		0.013
$SIM_{C}$	0.056	0.059	0.049	0.060	0.051	0.055	0.056	0.057	0.057	0.047	0.054	0.059	0.055

in the Supplement along with their physical location, estimated  $F_{ST}$  values, and significance (Supplemental Table 1). The identified genes are located on chromosomes 2 (PLEKHA3, FKBP7, DFNB59, PRKRA, OSBPL6, NFE2L2, HNRNPA3, MALRD1, PLXDC2, SPAG16, RHBDD1, COL4A3), 3 (NOTCH2, REG4, HMGCS2, PHGDH, DDX18, CCDC93,

HDAC4), 4 (SFRP4, EPDR1, STARD3NL, TRGC6, BBS9, RP9, NT5C3A, FKBP9, KBTBD2, AVL9, CREB5, JAZF1), 5 (MYRFL, RAB3IP, BEST3, LRRC10, CCT2, FRS2), 6 (LDB2), 7 (FNIP1, RAPGEF6, CGC42SE2), 8 (MSRA, KIF13B), 9 (OLIG3, UTRN, SASH1, UST), 11 (HMCN2, ASS1, FUBP3, PRDM12, EXOSC2, ABL1), 13 (MACROD2, TAF3, ATP5C1, KIN,



**Fig. 3.** Results of unsupervised population structure and admixture analysis using the algorithm STRUCTURE for 2178 individuals HF from 12 subpopulations HF (Belgium - BEL<sub>B</sub>, Canada – CAN<sub>B</sub>, Croatia - HRV<sub>C</sub>, Czech Republic bulls - CZE<sub>B</sub>, Czech Republic cows - CZE<sub>C</sub>, France - FRA<sub>B</sub>, Netherlands - NLD<sub>B</sub>, Ireland - IRL<sub>B</sub>, Great Britain - GBR<sub>B</sub>, Germany - DEU<sub>B</sub>, Switzerland - CHE<sub>B</sub>, USA - USA<sub>B</sub>) and for 98 Czech Simmental cows (SIM<sub>C</sub>). A) Plot of Ln Pr(G|K) values as a function of the number of clusters (K). B) Plot of K values for each K based on the second order rate of change of the likelihood function as a function of K. C) Graphical representation of the selection of results from STRUCTURE at K = 4, K = 5, and K = 6, where each individual is represented by a vertical line divided into K coloured segments whose length is proportional to the estimated membership of the inferred cluster.

ITIH2. ITIH5, PTPN1, FAM65C, PARD6B, BCAS4, ADNP, DPM1), 19 (PRPSAP1, QRICH2, RNF157, FOXJ1, EXOC7, ZACN, GALR2, SRP68, EVPL, CDK3, TEN1, ACOX1, FBF1, MRPL38, TRIM65, TRIM47) and 29 (PAG3, PAG6, PAG11, FGF19, FGF4, FGF3, ANO1, MRPL21, IGHMBP2, MRGPRF, TPCN2). Based on QTL annotation and their enrichment analyses (Fonseca et al., 2020), it was recognised that the majority of QTLs annotated in these regions mainly affect meat and carcass traits (Supplementary Figure 1). This result informs breeders, as the main decision makers, that the intensive use of current AI bulls influences meat and carcass traits in addition to increasing milk production, which is defined by the breeding objective of the Czech HF.

## 4. Conclusion

In this study, we have shown that the effective population size (NE<sub>LD</sub>) in the Czech HF population was relatively high, ranging from 202 (GONE) to 283 (NeEstimator v2). Compared to other HF subpopulations, this should not be a problem for breeding. However, the inbreeding observed in the AI bull sample was very high ( $F_{ROH>2Mb}=0.133$ ) and far exceeded the inbreeding observed in the cow sample ( $F_{ROH>2Mb}=0.091$ ). Of particular concern was the very high recent inbreeding

 $(F_{ROH>8Mb}=0.08)$  of the CZE<sub>B</sub>, indicating intentional mating of close relatives, as further evidenced by the high and significant  $F_{IS}$  (0.036  $\pm$ 0.003). It is very likely that the future use of highly inbred AI bulls will lead to a high level of inbreeding in the entire Czech HF subpopulation. Our phylogenetic analyses showed that the Czech HF belonged to the "core HF metapopulation", together with Belgian, British, Canadian, Dutch, French, German, and USA subpopulations, which was slightly separated from the Swiss, Irish, and Croatian subpopulations. Finally, to predict future genetic changes expected from the high use of AI bull semen in the Czech Republic HF, we identified genomic regions where genetic differences (estimated FST values) between CZEC and CZEB were most pronounced. Our further enrichment analyses of SNPs with the 0.1% highest FST values revealed that genes located in the most differentiating genomic regions primarily influence meat and carcass traits. This information is of importance to breeders, as they might expect continued use of current AI bulls to result in changes in meat and carcass performance in addition to increased milk production.

## CRediT authorship contribution statement

Lubos Vostry: Writing - original draft, Methodology, Visualization,



**Fig. 4.** Manhattan plot showing genomic distribution of  $F_{ST}$  values distinguishing Czech HF cows (CZE<sub>C</sub>) and bulls (CZE<sub>B</sub>). The horizontal yellow line represents the significance threshold ( $F_{ST} = 0.074$ ) for the SNPs with the highest  $F_{ST}$  differentiation (0.1%).

Formal analysis, Data curation, Writing – review & editing. Hana Vostra-Vydrova: Writing – original draft, Methodology, Visualization, Formal analysis, Data curation, Writing – review & editing. Nina Moravcikova: Formal analysis, Methodology. Radovan Kasarda: Methodology. Vlatka Cubric-Curik: Writing – original draft, Methodology. Michaela Brzakova: Formal analysis. Johan Solkner: Methodology. Mario Shihabi: Formal analysis, Visualization. Jorge Angel Hidalgo Moreno: Data curation. Maria Spehar: Data curation. Ino Curik: Writing – original draft, Methodology, Visualization, Writing – review & editing.

## **Declaration of Competing Interest**

The authors declare that they have no conflict of interest.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2023.105261.

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