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Quantifying the effects of the mitochondrial genome on milk production traits in dairy cows: empirical results and modelling challenges

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ABSTRACT

Significant advances in livestock traits have been achieved primarily through selection strategies targeting variation in the nuclear genome, with little attention given to mitogenome variation. We analyzed the influence of the mitogenome on milk production traits of Holstein cattle in Croatia based on strategically generated next-generation sequencing data for 109 cows pedigree-linked to 7115 milk production records (milk, fat and protein yield) from 3006 cows (first 5 lactations). Since little is known about the biology of the relationship between mitogenome variation and production traits, our quantitative genetic modeling was complex. Thus, the proportion of total variance explained by mitogenome inheritance was estimated using 5 different models: (1) cytoplasmic model with maternal lineages (CYTO), (2) haplotypic model with mitogenome sequences (HAPLO), (3) amino acid model with unique amino acid sequences (AMINO), (4) evolutionary model based on a phylogenetic analysis using Bayesian Evolutionary Analysis Sampling Trees phylogenetic analysis (EVOL), and (5) mitogenome SNP model (SNPmt). The polygenic autosomal and X chromosome additive genetic effects based on pedigree were modeled, together with the effects of herd-year-season interaction, permanent environment, location, and age at first calving. The estimated proportions of phenotypic variance explained by mitogenome in 4 different models (CYTO, HAPLO, AMINO, and SNPmt) were found to be substantial given the size of mitogenome, ranging from 5% to 7% for all 3 milk traits. At the same time, a negligible proportion of the phenotypic variance was explained by mitogenome with the EVOL model. Similarly, in all models, no proportion of phenotypic variance was explained by the X chromosome. Although our results should be confirmed in other dairy cattle populations, including a large number of sequenced mitogenomes and nuclear genomes, the potential of utilizing mitogenome information in animal breeding is promising, especially as the acquisition of complete genome sequences becomes cost-effective.

Key words: Holstein cattle, milk production traits, complete mitogenome, Next Generation Sequencing, variance components

INTRODUCTION

Domestic cattle have profoundly influenced development of modern human societies, consolidating their status as the world's economically most important domestic animal. This central importance is particularly evident in the increasing demand for high-yielding breeds, with the emphasis on dairy cows. Over the last century, the milk yield per lactation has increased many times over (Britt et al., 2018, 2021), emphasizing the indispensable role of these animals in satisfying human needs and promoting agricultural progress.

Meeting the elevated production demands of high-producing dairy cows requires a significant amount of energy, which emphasizes the importance of bioenergetic homeostasis and lactogenesis in adapting to fluctuations in energy requirements and physiological processes during the lactation period (Cheng and Ristow, 2013; Weikard and Kuehn, 2018). The pivotal role in maintaining metabolic balance, essential for high milk production, lies with the mitochondria, the double-membrane-bound, semi-autonomous organelles in the cytoplasm of cells. The mitochondria are often referred to as the "powerhouse" of cells and make a significant contribution by generating around 90% of adenosine triphosphate (ATP)

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

through oxidative phosphorylation from carbohydrates and fatty acids (Wilson et al., 1985; Hadsell et al., 2011; Cheng and Ristow, 2013; Favorit et al., 2021). The importance of mitochondria is particularly evident when the high energy requirements for milk production compete for resources, potentially disrupting reproductive processes, resilience and overall health (Monzel et al., 2023). In addition, the role of mitochondria goes beyond energy provision and includes multifunctional tasks such as calcium signaling, regulation of membrane potentials, control of cell metabolism and involvement in apoptosis (Ballard and Melvin, 2010; Monzel et al., 2023).

Each cell contains several hundred to thousands of mitochondria, the inheritance of which in cattle, as in other mammals, is exclusively along the maternal lineage (Hutchison et al., 1974). The cattle mitogenome is a small circular molecule spanning 16,338 bp in length (Anderson et al., 1982), characterized by semi-conservative self-replication and exhibits the unique property of rapid evolution without recombination, as highlighted in many studies (Harrison, 1989; Javonillo et al., 2010; Prosdocimi et al., 2012; Castro Paz et al., 2014). It consists of 37 genes without introns, 13 of which encode respiratory chain proteins involved in energy metabolism, 2 ribosomal and 22 transfer RNAs essential for protein synthesis (Boore, 1999; Wallace et al., 1999), while a non-coding region is known as the control region or D-loop. Variation in the mitogenome is represented by unique sequences or haplotypes that have been shaped by mutations, drift and selection over a long period of time and passed on by maternal ancestors. According to their phylogenetic origin, unique cattle haplotypes are categorized into several highly divergent haplogroups (I, C, R, P, Q, T_1, T_2, T_3, T_4 and T_5), which are commonly used in domestication studies (Bradley et al., 1996; Achilli et al., 2008; Zhang et al., 2013; Verdugo et al., 2019) and diversity studies (Cubric-Curik et al., 2022; Dorji et al., 2022).

The effects of mitogenome variation on complex traits in humans are closely related to human health and have been well-investigated in many studies (Wallace, 2005, 2015; Gorman et al., 2016). In particular, various mitogenome mutations or haplotypes have been associated with several human diseases, e.g., cancer (Shen et al., 2011), diabetes (Liou et al., 2012), Alzheimer's disease (Ridge et al., 2012), Parkinson's disease (Ghezzi et al., 2005), and Leber hereditary optic neuropathy (Yu-Wai-Man et al., 2009).

In contrast, the effects of mitogenome variation in cattle have been studied in the context of production traits, while the first disease caused by a mutation in the mitogenome has only recently been reported (Novosel et al., 2022). However, most studies evaluating the effects of mitogenome variation on economically important traits such as milk production were conducted in the late 20th century. These studies were based on "cytoplasmic models," which assume that all observed maternal lineages in the pedigree have different mitogenome haplotypes (Bell et al., 1985; Kennedy, 1986; Schutz et al., 1992; Boettcher and Gibson, 1997; Albuquerque et al., 1998; Roughsedge et al., 1999). In these studies, the "cytoplasmic effects" explained from 0 to 10% of the phenotypic variability. In addition, Boettcher et al. (1996b) simulated the effects of maternal lineages from the normal distribution, analyzed the data with fixed and random models and concluded that random (cytoplasmic) models estimate the effects of the different maternal lineages more accurately. On the other hand, there are not many studies in which the effects of mitogenome polymorphism and milk production were estimated using genomic data because sequences data was available only for short regions such as D-loop, due to technical limitations in obtaining complete mitogenomes for large numbers of individuals (Brown et al., 1989; Schutz et al., 1994; Boettcher et al., 1996a; Qin et al., 2012). While nuclear genome information is now widely used to estimate breeding values (Boichard et al., 2015; Weigel et al., 2017; Cole and VanRaden, 2018), the role of the complete mitogenome in improving milk production and has not yet been fully explored. Recent technological advances, particularly the emergence of next-generation sequencing (NGS), have opened up the possibility of efficiently genotyping large numbers of complete mitogenomes at low cost. Moreover, informative single nucleotide polymorphisms (SNPs) of the mitogenome have been integrated into SNP arrays (Brajkovic et al., 2023) or might be extracted from whole genome sequences with low coverage (Sanglard et al., 2022b). These resources provide a solid foundation for further research on the utilization of complete mitogenome information in dairy cattle breeding.

The main objective of this study was to evaluate the effects of inherited mitochondria on milk production traits in cattle using the complete mitogenome sequence information. Analyses were performed on Croatian Holstein cows, with a focus on a comprehensive modeling of variation across the complete mitogenome. More specifically, our focus was on estimating the proportion of phenotypic variance explained by mitogenome variation (m^2) using 5 different models: (1) cytoplasmic model with maternal lineages (CYTO), (2) haplotypic model with mitogenome sequences (HAPLO), (3) amino acid model with unique amino acid sequences (AMINO), (4) evolutionary model based on a phylogenetic analysis using Bayesian Evolutionary Analysis Sampling Trees (BEAST) (EVOL), and (5) mitogenome SNP model (SNPmt). In assessing the relationship between inherited mitochondrial variation and milk production, we are unaware of a single study that has used similarly complex modeling and/or a study

Table 1. Descriptive statistics for milk production traits

Lactation	Variable (kg)	N	Mean	Standard deviation	Minimum	Maximum
1st	Milk	2390	6733	1582	1673	11980
	Fat	2389	258	65	83	589
	Protein	2388	220	52	82	386
2nd	Milk	1984	7440	1868	1537	11960
	Fat	2020	291	82	81	598
	Protein	2019	247	62	85	447
3rd	Milk	1336	7482	1916	2201	11982
	Fat	1360	293	84	89	586
	Protein	1359	246	64	91	458
4th	Milk	835	7344	2012	1770	11995
	Fat	850	288	87	94	581
	Protein	849	241	66	82	418
5th	Milk	484	7168	1968	2010	11962
	Fat	488	277	83	81	515
	Protein	486	232	62	83	428

that has used complete mitogenome information on a large scale. Furthermore, our decomposition of genetic variance into variance components within and between mitogenome regions is novel and opens new perspectives for analyzing the effects of non-recombining mitogenome SNP polymorphism on economically important production traits.

MATERIALS AND METHODS

Data and sampling strategy of maternal lineages

Pedigree and lactation data of Holstein cattle were provided by the Croatian Agency for Agriculture and Food, a national institution responsible for milk recording and estimation of genetic parameters. For pedigree verification, sampling strategy and maternal lineage imputation, MaGelLan 1.0 (Maternal Genealogy Lineage Analyzer) software (Ristov et al., 2016) was used to strategically select 109 Holstein cows from 20,973 lactating animals based on the 2016 report, with the aim that the resulting maternal lineage coverage is as diverse as possible. The 109 Holstein cows included in the sample thus represent 109 maternal pedigree lineages according to the pedigree data and comprise a total of 3,040 individuals with 7,576 records within the first 10 lactations, with each maternal pedigree lineage comprising 10 to 74 individuals. The pedigree for our 3,040 individuals comprised 6,336 individuals. The descriptive statistics for milk production traits over the first 5 lactations (305 d) used in the repeatability model, comprised 3,006 individuals and resulting in a total of 7,115 records, are presented in Table 1.

Sampling description

Milk, hair and tissue samples were collected from small (10 to 30 cows), medium (30 to 100 cows) and large (over 100 cows) farms registered with the Ministry of Agriculture. The samples were distributed across 7 counties and 40 farms in Croatia (Figure 1). A total of 109 samples were collected, including 86 milk samples, 22 hair samples, and one ear tissue sample. A strategy for the collection of milk samples as a non-invasive method, but taking into account the required amount of milk, storage temperature, liquid or pelletized form and storage time for the extraction of good quality DNA, is described in Brajkovic et al. (2018).

Molecular genetic analyses and mitogenome diversity

The molecular genetic analysis and software with information on i) DNA isolation, ii) mitogenome amplification by 3-step PCR, iii) DNA library preparation, iv) sequencing platform, v) the bioinformatic analysis of the Fastq sequence, vi) the calculation of the mitogenome depth and breadth of coverage, and vii) list of GenBank accession numbers are presented in our phylogenetic meta-analysis of the bovine mitogenome (Cubric-Curik et al., 2022) and in Table S1.

The diversity of the complete mitogenome and the diversity of 27 functional regions were summarized with the number of variable sites (S), the total number of mutations (Eta), the nucleotide diversity per site (π), the average number of nucleotide differences (k), the number of haplotypes (h) and the haplotype (gene) diversity (Hd). The summary of genetic parameters was calculated using DNAsp v6 (Rozas et al., 2017) and the software Arlequin v. 3.5.2.2. (Excoffier and Lischer, 2010).

Haplotype construction, classification, and phylogenetic analysis

To test the influence of mitogenome polymorphisms on phenotypic variance in milk traits (milk, fat, and

protein yield) of Holstein cattle, 3 types of haplotypes/haplogroups were used. First, mitogenome haplotypes were constructed based on all variable sites of the entire nucleotide sequences. Analyses were performed using Clustal Omega software (Sievers et al., 2011), MEGA7 software (Kumar et al., 2016) and DNAsp v6 software (Rozas et al., 2017), see also Table S1. Second, amino acid haplotypes were constructed based on a sequence of 3,828 amino acids translated from a nucleic acid sequence of 11,484 bp and comprising 13 protein-coding mitogenome regions with a total of 59 variable sites. Analyses were performed using MEGA7 software (Kumar et al., 2016) and SAS (SAS Institute, 2012), see also Table S1.

Third, evolutionary haplogroups of Holstein mitogenomes were formed based on an MCMC Bayesian evolutionary analysis performed using the BEAST v1.4.3 software package (Suchard et al., 2018) as part of a comprehensive phylogenetic meta-analysis of cattle (Figure 3) described in (Cubric-Curik et al., 2022). The 109 Holstein mitogenomes were grouped into 10 subclades representing evolutionary haplogroups, see Table S1 for more details.

To better understand the origin of mitogenome haplotypes and their estimated effect on milk production traits, we classified our mitogenomes into specific haplogroups using the MitoToolPy program (Peng et al., 2015) (Table S1, column "MTP"), which included 278 mitogenomes of the genus Bos as a reference base for the determination of haplogroups (266 for *Bos taurus*, 2 for *Bos primigenius* and 10 for *Bos indicus*). To comprehensively analyze our Holstein mitogenomes in a broader context, a median joining (MJ) network (Bandelt et al., 1999) was constructed using PopArt (Leigh and Bryant, 2015) to visualize the phylogenetic relationship with an additional 70 nucleotide sequences (see Table S2) from GenBank - NCBI (GenBank) (Clark et al., 2016), representing 62 haplotypes distributed across 8 distinct haplogroups (T_1, T_2, T_3, T_4, T_5, P, Q, R). Arleqin 3.5 software (Excoffier and Lischer, 2010) was used to create the haplotype frequency matrix for PopArt (Leigh and Bryant, 2015) input.

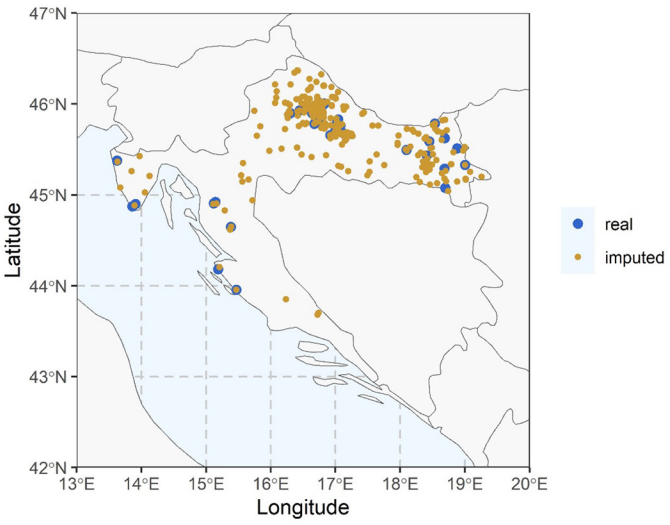

Figure 1. Geographical representation of the samples: The blue circles (real), represent the location of the sampled farms where milk/hair was collected covering 109 maternal lineages/mitogenomes, while the orange circles (imputed) represent the location of the farms for all cows and their milk records used in the analyses based on pedigree imputation of the previously collected 109 mitogenomes to all animals within the maternal lineages.

Quantitative genetic analyses

We employed 5 different models to estimate the magnitude of the association between mitogenomes and milk production traits. In each of the 5 models - CYTO, HAPLO, AMINO, EVOL, and SNPmt - we applied a Bayesian repeatability animal model that included the first 5 lactation records. This comprehensive analysis included 3 evaluated traits: milk, fat, and protein yield, resulting in a total of 15 assessments across 5 models. Our model can be described by:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_c\mathbf{c} + \mathbf{Z}_s\mathbf{s} + \mathbf{Z}_i(\mathbf{a} + \mathbf{x} + \mathbf{m} + \mathbf{p}) + \mathbf{e},$$

where $\boldsymbol{y}$ is $n_y \times 1$ vector of $n_y = 7{,}115$ milk, fat, and protein 305- yields (standardized to zero mean and unit variance); $\boldsymbol{X}$ is $n_y \times n_b$ design matrix for the $n_b = 12$ effects of the overall mean, the interaction between the number of calving and age at calving covariate and $\boldsymbol{b}$ is the corresponding vector of effects;; $\boldsymbol{Z}_c$ is $n_y \times n_c$ design matrix for $n_c = 2{,}654$ contemporary groups defined as herd-year-season effects $\boldsymbol{c} \sim N\left(\mathbf{0}, \mathbf{I}\sigma_c^2\right)$ where the calving seasons within a year were defined as: Spring (March to May), Summer (June to August), Autumn (September to November) and Winter (December to February); $\boldsymbol{Z}_s$ is $n_y \times n_s$ design matrix for $n_s = 807$ herd location (spatial) effects $\boldsymbol{s} \sim N\left[\mathbf{0}, \mathbf{S}\left(\sigma_a^2, \rho\right)\right]$ with $\boldsymbol{S}$ being Matérn covariance function based on Euclidean distances between the herd locations and parameterized with variance σ_s^2 and range ρ (see Selle et al., 2020 and references therein for further details); $\boldsymbol{Z}_i$ is $n_y \times n_i$ design matrix for $n_i = 6336$ individual animal effects with the following components: $\boldsymbol{a} \sim N\left(\mathbf{0}, \mathbf{A}\sigma_a^2\right)$ the additive genetic effect of autosomal DNA with pedigree-relationship matrix $\mathbf{A}$ (Henderson, 1976); $\boldsymbol{x} \sim N\left(\mathbf{0}, \mathbf{X}\sigma_x^2\right)$ the additive genetic effect of X chromosome DNA with pedigree-relationship matrix $\mathbf{X}$ (Grossman and Eisen, 1989; Fernando and Grossman, 1990); $\boldsymbol{m}$ the additive genetic effect of mitochondrial DNA modeled with different assumptions described below; $\boldsymbol{p} \sim N\left(\mathbf{0}, \mathbf{I}\sigma_p^2\right)$

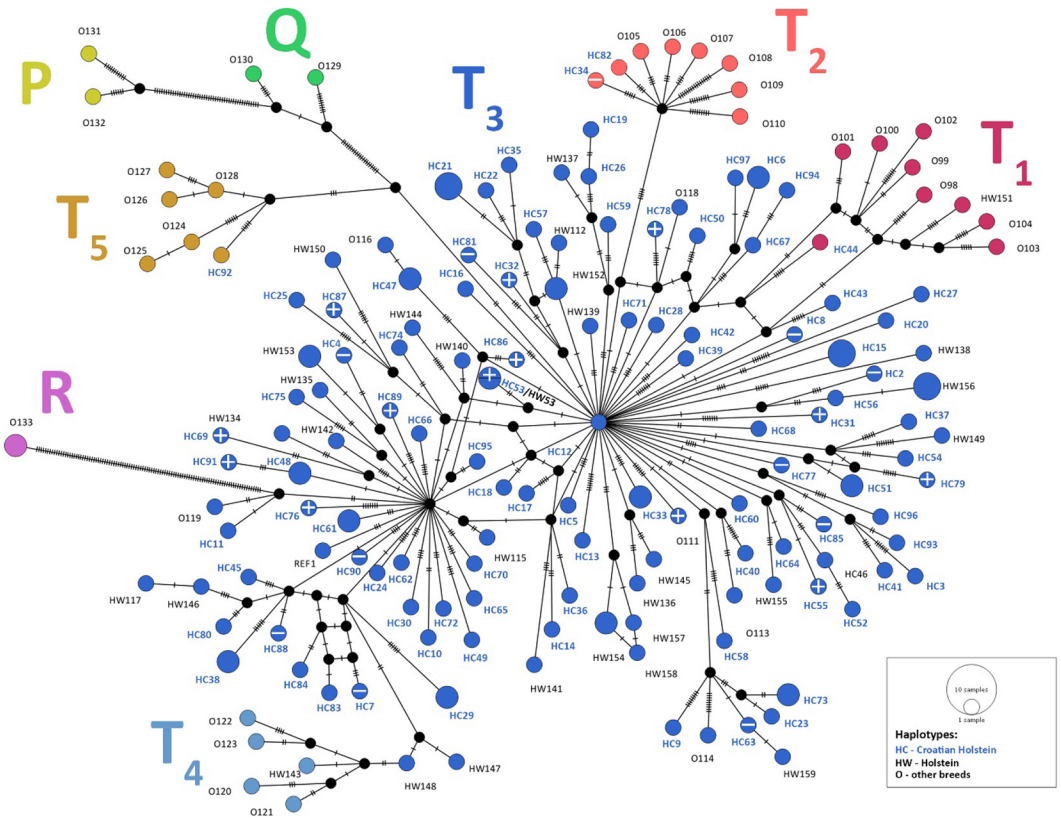

Figure 2. Median joining network representing the phylogenetic relationship (mutational differences) of all complete mitogenomes found in GeneBank and assigned to the Holstein breed (labeled with the letters HC if they were Croatian Holstein and HW if they were found in populations of other Holstein animals), together with several haplotypes representing cattle with other haplogroups (labeled with the letter O as representatives of other breeds). The plus sign within the haplotypes indicates the 10 percent of the best haplotypes with the largest random solution effects for milk, fat and protein, and the minus sign within the haplotypes indicates the 10 percent of the worst haplotypes with the smallest random solution effects.

the permanent environmental effect; and $\boldsymbol{e} \sim N\left(\mathbf{0}, \mathbf{I}\sigma_e^2\right)$ the residual; and $\mathbf{I}$'s are the identity matrices of corresponding dimensions.

The 5 models differed in their representation of mitogenome effects. Mitogenome is a circular haplotype, so we denote the effect of differently defined mitogenome haplotypes with $\boldsymbol{h}_m$ where subscript m denotes a model. In the CYTO model, the mitogenome effects were modeled by considering the effect of 109 maternal pedigree lineages h_c, which were assumed to be independent: $\boldsymbol{m} = \boldsymbol{Z}_c\boldsymbol{h}_c$, where $\boldsymbol{h}_c \sim N\left(\mathbf{0}, \mathbf{I}_{h_c}\sigma_{h_c}^2\right)$ and $\boldsymbol{Z}_c$ is mapping cows' mitochondrial effect to their maternal pedigree lineage effect. The HAPLO model fitted the effect of 96 unique complete mitogenome haplotype sequences $\boldsymbol{h}_h$: $\boldsymbol{m} = \boldsymbol{Z}_h\boldsymbol{h}_h$, where $\boldsymbol{h}_h \sim N\left(\mathbf{0}, \mathbf{I}_{h_h}\sigma_{h_h}^2\right)$ and $\boldsymbol{Z}_h$ is mapping cows' mitochondrial effect to their mitogenome haplotype effect, assuming that different nucleotide cbinations form different haplotypes that influence mitochondrial efficiency and consequently milk production. This is the same assumption as in the CYTO model, but more precise, since with many maternal pedigree lineages in the study it is to be expected that some have the same mitogenomes, but we do not observe that information for the CYTO model due to finite pedigrees. The AMINO model assumed that mutations at synonymous and non-protein coding nucleotides do not contribute to the differences in milk production which led to 48 amino acid sequences or different "AMINO haplotypes" $\boldsymbol{h}_a$: $\boldsymbol{m} = \boldsymbol{Z}_a\boldsymbol{h}_a$, where $\boldsymbol{h}_a \sim N\left(\mathbf{0}, \mathbf{I}_{h_a}\sigma_{h_a}^2\right)$ and $\boldsymbol{Z}_a$ is mapping cows' mitochondrial effect to their AMINO haplotype effect. This assumption implied that non-synonymous mutations lead to the synthesis of different amino acid sequences, which all jointly influence mitochondrial effect. The EVOL model fitted the effect of 10 phylogenetic haplogroups $\boldsymbol{h}_e$, suggesting that long-term selection or adaptations to "ancient" mutations and environments represents mitochondrial effects: $\boldsymbol{m} = \boldsymbol{Z}_e\boldsymbol{h}_e$, where $\boldsymbol{h}_e \sim N\left(\mathbf{0}, \mathbf{I}_{h_e}\sigma_{h_e}^2\right)$ and $\boldsymbol{Z}_e$ is mapping cows' mitochondrial effect to their phylogenetic haplogroup effect. Finally, the SNPmt model fitted the effect of 359 SNP mutations in mitogenome α on variation in milk production: $\boldsymbol{m} = \boldsymbol{W}\,\alpha$, where $\alpha \sim N\left(\mathbf{0}, \mathbf{I}_{h_a}\sigma_{h_a}^2\right)$

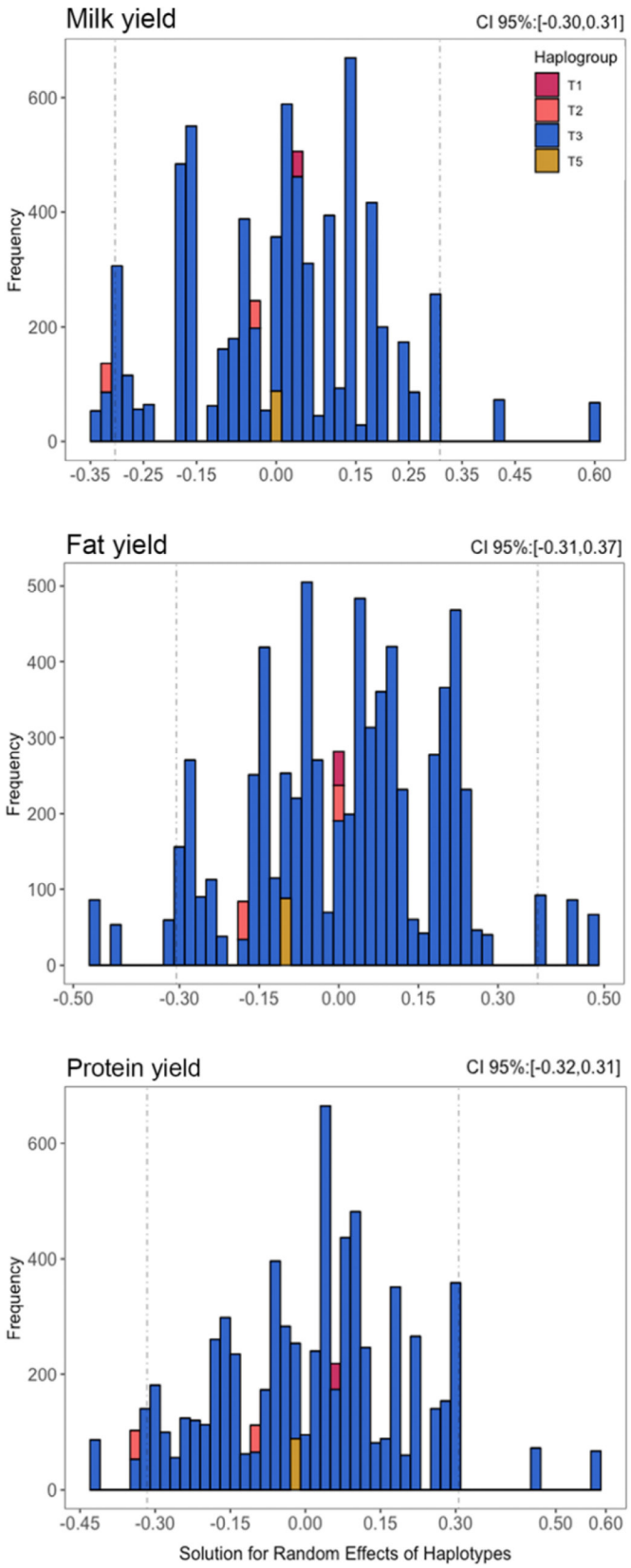

Figure 3. Distributions of haplotype effects in phenotypic standard deviations for milk production traits in the Croatian Holstein population.

and $\boldsymbol{W}$ is $n_i \times n_{snp}$ mitogenome allele matrix with elements equal to 0 for reference alleles and 1 for alternative alleles.

All models were fitted using tegrated Nested Laplace Approximation (INLA) as implemented in the R package R-INLA (v24.05.01–1; Rue et al., 2009) using R software (v4.4.0; R Core Team, 2021) and RStudio (v2024.4.0.735; RStudio Team, 2020). INLA, known as the Bayesian numerical approximation method, computes marginal posteriors for all model parameters. The main reason for using the R-INLA package was that it can model spatial effects through the stochastic partial differential equation (SPDE) approach of Lindgren et al. (2011). This approach can accommodate geographically referenced data, including areal and geostatistical data as well as spatial point process data (Lindgren and Rue, 2015). Use of this spatial modeling approach was deemed important to correct for spatial variation that could otherwise be captured by mitochondrial/maternal lineages in different regions of the country. The SPDE approach involved: i) construction of a mesh based on the locations of individual herds/farms, ii) delineation of spatial barriers given the specific shape of the country, iii) definition of a projection, iv) creation of a projector matrix, and v) configuration of the barrier model (Bakka et al., 2019). See Selle et al. (2020) for use of spatial modeling in quantitative genetics. Pedigree-based relationship matrices for autosomal and X chromosomes were constructed using R package nadiv (Wolak, 2012) and provided to the R-INLA call. All R code for data manipulation and model fitting including data will be available at GitHub and Zenodo (at the moment please use the Dropbox link: https://www.dropbox.com/scl/fo/l6ez8eykl1o03sw546z8a/ANYdV4BHjJ1H9ECO1iFTp2A?rlkey = csknoxhk27ifv0nhgf8oedtic&dl = 0).

Decomposition of genetic (co)variance components

We were particularly interested in estimating how much of the total phenotypic variance can be explained by variance between mitogenome effects $m^2 = \frac{\sigma^2}{\sigma_y^2}$ using different models. Specifically, we calculated the following parameters for each milk production trait: i) m^2_{CYTO}, the proportion of phenotypic variance explained by variance between maternal lineages $\sigma^2_{h_c}$, ii) m^2_{HAPLO} the proportion of phenotypic variance explained by variance between mitogenome haplotype sequences $\sigma^2_{h_h}$, iii) m^2_{AMINO}, the proportion of phenotypic variance explained by variance between AMINO haplotypes $\sigma^2_{h_a}$, iv) m^2_{EVOL} the proportion of phenotypic variance explained by variance between phylogenetic haplogroups $\sigma^2_{h_e}$, and v) m^2_{SNP}

the proportion of phenotypic variance explained by variance between mitogenome effects modeled with SNPs $\sigma^2_{h\_s}$. In the calculation of m^2_{SNP}, the variance between mitogenome effects $\sigma^2_{h\_s} = Var(m) = Var\left(\frac{W}{a}\right)$ included all genic/SNP locus variances as well as both intragenic covariances (between SNP loci within defined mitogenome genes/regions) and intergenic covariances (between SNP loci between defined mitogenome genes/regions). This innovative approach, inspired by the concept of Lara et al. (2022) for autosomal genomic analysis of genetic variance, was applied here for the first time on mitogenomes. This approach is important because of the lack of recombination in mitogenomes. Since the complete mitogenome comprises 37 coding genes/regions and one non-coding region, our analysis allowed us to estimate and compare the contribution of each gene/region to the total mitogenome variance $\sigma^2_{h\_s}$.

RESULTS AND DISCUSSION

Mitogenome diversity and classification

For a highly selected breed, the diversity of complete mitogenomes (16,344 bp long sequence) analyzed in 109 Holstein cows was unexpectedly high (Table 2).

A total of 96 different haplotypes (h) were observed, corresponding to a haplotype diversity (Hd) of 0.997, with 358 variable sites (S), a nucleotide diversity per site (π) of 0.00064 and an average number of nucleotide differences (k) of 10.509.

The observed diversity in the different functional regions was quite variable, with the highest diversity observed in the D-loop region (S = 74, π = 0.00376, k = 3.425, h = 65, Hd = 0.948), followed by ND5 (S = 43, k = 1.003, h = 33, Hd = 0.61) and ND4 (S = 35, k = 0.804, h = 32, Hd = 0.588), while the lowest diversity was observed in tRNA-Leu (S = 1, k = 0.018, h = 2, Hd = 0.018) and other tRNA regions. This agrees with the diversity observed in the global data set analyzed by Cubric-Curik

Table 2. Mitogenome diversity in 109 Holstein cows across different functional genes/regions

Functional gene/region	Length (bp)	S	Eta	π	k	h	Hd
12S	958	13	13	0.00034	0.328	14	0.303
16S	1571	18	18	0.00027	0.420	19	0.364
ATP6	681	12	12	0.00059	0.400	14	0.318
ATP8	201	6	6	0.00081	0.163	7	0.158
COX1	1545	25	25	0.00042	0.653	22	0.486
COX2	684	10	10	0.00037	0.255	10	0.192
COX3	804	16	16	0.00054	0.437	17	0.334
CYTB	1140	22	22	0.00042	0.476	22	0.407
D-loop	912	74	75	0.00376	3.425	65	0.948
D-loop beginning	364	12	12	0.00244	0.888	13	0.643
D-loop end	548	62	63	0.00464	2.538	55	0.888
**Inter CYTB tRNA-Thr*	3	1	1	0.00612	0.018	2	0.018
**Inter tRNA-Ser tRNA-Asp*	5	1	1	0.00367	0.018	2	0.018
ND1	957	21	21	0.00051	0.493	19	0.349
ND2	1044	22	22	0.00057	0.600	22	0.487
ND3	357	7	7	0.00041	0.146	7	0.125
ND4	1425	35	35	0.00056	0.804	32	0.588
ND4L	297	4	4	0.00043	0.127	5	0.124
ND5	1821	43	43	0.00055	1.003	33	0.610
ND6	528	16	16	0.00089	0.470	15	0.376
tRNA-Arg	69	1	1	0.00027	0.018	2	0.018
tRNA-Asn	73	1	1	0.00025	0.018	2	0.018
tRNA-Cys	67	1	1	0.00132	0.088	2	0.088
tRNA-Gln	72	1	1	0.00025	0.018	2	0.018
tRNA-Glu	69	1	1	0.00027	0.018	2	0.018
tRNA-Leu	75	1	1	0.00024	0.018	2	0.018
tRNA-Met	68	1	1	0.00027	0.018	2	0.018
tRNA-Ser	60	2	2	0.00091	0.055	3	0.054
tRNA-Thr	70	2	2	0.00052	0.037	3	0.037
tRNA-Val	67	1	1	0.00027	0.018	2	0.018
Mitogenome	16344	358	359	0.00064	10.509	96	0.997

S - Number of variable sites; Eta – the total number of mutations; π - Nucleotide diversity (per site); k - Average number of nucleotide differences; h - Number of Haplotypes; Hd - Haplotype (gene) diversity; The D-loop region is additionally subdivided into the D-loop beginning and the D-loop end (hypervariable regions 1 and 2) due to their specificity of connection and the inscription of entire mtDNA replication;**Inter CYTB tRNA-Thr* region according to the referent mitogenome (GenBank accession number V00654) does not belong either to the *CYTB or tRNA-Thr* and the same applies to *Inter tRNA-Ser tRNA-Asp region*. Other tRNA regions that did not show mutations are not included in the table.

et al. (2022), in which the D-loop was the most diverse mitogenome region, while the observed diversity of the NDH5 gene was among the highest.

The phylogenetic relationship (mutational differences) of all complete mitogenomes observed in the Holstein breed (haplotypes reported in GeneBank) together with several haplotypes representing all other existing haplogroups is shown in Figure 2.

Overall, most haplotypes of Holstein cattle (94%) not sampled in Croatia were classified as T_3, which was expected as T_3 is the predominant haplogroup characteristic of cattle of European origin (Figure 2), while only one T_1 (Italy) and one T_4 (Korea) haplotype were found (detailed description in Table S2). In the Croatian Holstein population, following the pattern observed for Holstein cattle, 91 haplotypes (95%) were assigned to the T_3 haplogroup, while we also identified 2 T_2 haplotypes, one T_1 haplotype and one T_5 haplotype. According to Brajkovic et al. (2022), the presence of T_1, T_2, and T_5 haplotypes is most likely the consequence of genetic upgrading of local Croatian breeds with Holstein bulls, as T_1, T_2, and T_5 haplotypes were observed in Istrian cattle (T_1 with 6.7%), Croatian Busha cattle (T_1 with 24% and T_2 with 32%), and Slavonian Syrmian Podolian cattle (T_5 with 25%)

Variance components and quantitative genetic parameters

The results of the quantitative genetic analysis of phenotypic variation for milk production traits in the Croatian Holstein breed are presented in Table 3 for different models analyzed (CYTO, HAPLO, AMINO, EVOL, and SNPmt). In addition to the estimated variance components, the contribution of mitochondrial variation was presented as a proportion of phenotypic variation alongside the additive contribution of autosomal chromosomes (h^2), the additive contribution of the X chromosome (x^2), and other random environmental effects presented as contemporary group and permanent environment effects. The estimated heritability (phenotypic variance explained by the additive autosomal component) was within the range found in less complex modeling of the same data set (Brajkovic, 2019). Specifically, the estimated heritability for milk yield was between 0.22 and 0.32 for all models (CYTO, HAPLO, AMINO, EVOL, and SNPmt), with estimated heritability for fat yield in a similar range, between 0.22 and 0.29, and for protein yield between 0.23 and 0.33. For all 3 milk traits, the highest heritability was observed in the EVOL and SNPmt model, while the CYTO and HAPLO models had the lowest heritability. This could be consistent with Van Vleck's recommendation: "Heritability (additive direct) can be overestimated from covariances between relatives with the same cytoplasm if cytoplasmic effects on the trait are real and if those effects are ignored." (Van Vleck, 1993).

The estimated proportion of phenotypic variance of milk yield, fat yield and protein yield captured by mitochondrial variation (m^2) was significant in all models except the EVOL model, where all estimates were zero or negligible and non-significant (Table 3).

These results suggest that grouping mitochondrial effect into main evolutionary haplogroups is missing variation within these groups. In all other models, the estimated m^2 for all 3 traits was significantly positive and ranged from 0.05 to 0.07. The highest estimates, either 0.06 or 0.07, were consistently obtained for all 3 traits for HAPLO model, while estimates obtained with CYTO and AMINO models were between 0.05 (fat yield) to 0.07 (protein yield). Slightly lower estimates (0.05) were obtained in SNPmt models for all 3 traits.

To our knowledge, this was the first time that mitochondrial and additive effects of the X chromosome were modeled together. This was important to avoid confounding between capturing variation due to the X chromosome and the mitogenome. For all 3 milk production traits, there was no significant proportion of phenotypic variance explained by X chromosome additive effects (x^2). However, null estimates are not biologically plausible, as it can be assumed that genes on the X chromosome contribute to small variations in milk production traits (Sanchez et al., 2023). It is noteworthy that in only one of our models (R-INLA version of 21.11.22) x^2 was between 0.01 and 0.04, but this did not affect the estimated m^2 values for any of the 3 milk production traits analyzed (see Supplementary Table S5). We attribute the instability of the X chromosome effects to the high correlation between the classical additive relationship matrix and the relationship matrix of sex (X chromosome), as evidenced by a Mantel test correlation of 0.955 ($P < 0.001$ after 100 permutations). To exclude possible confounding between X chromosome and mitogenome effects, we performed additional analyses excluding only the mitogenome effects. As we did not observe nonzero x^2 values, we concluded that our m^2 estimates were not influenced by confounding with X chromosome effects.

The random effects of the contemporary group and the permanent environment were stable in all different mitochondrial models.

SNPmt model reduced the estimate of variance and range between location effects indicating possible confounding between these 2 effects. The distributions of the estimated haplotype effects for the milk production traits (HAPLO model) are shown in Figure 3. The range of estimated haplotype effects was approximately between −0.5 and 0.5 phenotypic standard deviations, which is a large effect.

Table 3. Estimated variances and variance component ratios (±SE) for milk production traits in a repeatability animal model (first five lactations) in Croatian Holstein breed

Trait (kg)	Model	σ_a^2	σ_x^2	σ_h^2	σ_c^2	σ_p^2	σ_e^2	σ_s^2	ρ	h^2	x^2	m^2	c^2	p^2	e^2
Milk yield	CYTO	0.22	0.00	0.05	0.09	0.10	0.35	1.68	0.81	0.27 ± 0.03	0.00 ± 0.00	0.06 ± 0.02	0.11 ± 0.01	0.12 ± 0.02	0.43 ± 0.02
	HAPLO	0.22	0.00	0.05	0.09	0.09	0.35	1.75	0.81	0.28 ± 0.03	0.00 ± 0.00	0.06 ± 0.02	0.11 ± 0.01	0.12 ± 0.02	0.43 ± 0.02
	AMINO	0.26	0.00	0.05	0.09	0.08	0.35	1.93	0.83	0.32 ± 0.03	0.00 ± 0.00	0.06 ± 0.02	0.11 ± 0.01	0.10 ± 0.02	0.42 ± 0.02
	EVOL	0.30	0.00	0.00	0.09	0.07	0.34	1.47	0.84	0.37 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.01	0.08 ± 0.02	0.43 ± 0.02
	SNPmt	0.32	0.00	0.04	0.10	0.08	0.35	1.02	0.40	0.32 ± 0.03	0.00 ± 0.00	0.05 ± 0.01	0.10 ± 0.01	0.08 ± 0.02	0.36 ± 0.01
Fat yield	CYTO	0.22	0.00	0.05	0.10	0.10	0.36	1.82	0.79	0.26 ± 0.03	0.00 ± 0.00	0.05 ± 0.02	0.12 ± 0.01	0.13 ± 0.02	0.43 ± 0.02
	HAPLO	0.22	0.00	0.05	0.10	0.10	0.36	1.50	0.78	0.26 ± 0.03	0.00 ± 0.00	0.06 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.43 ± 0.02
	AMINO	0.25	0.00	0.04	0.10	0.10	0.36	2.04	0.84	0.29 ± 0.03	0.00 ± 0.00	0.05 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.43 ± 0.02
	EVOL	0.28	0.00	0.00	0.10	0.08	0.36	1.73	0.84	0.34 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.01	0.10 ± 0.02	0.44 ± 0.02
	SNPmt	0.29	0.00	0.05	0.11	0.09	0.37	0.82	0.40	0.30 ± 0.03	0.00 ± 0.00	0.05 ± 0.01	0.12 ± 0.01	0.09 ± 0.02	0.37 ± 0.01
Protein yield	CYTO	0.23	0.00	0.05	0.10	0.09	0.32	2.05	0.86	0.29 ± 0.03	0.00 ± 0.00	0.06 ± 0.02	0.13 ± 0.01	0.11 ± 0.02	0.40 ± 0.02
	HAPLO	0.23	0.00	0.05	0.10	0.09	0.32	3.23	0.91	0.29 ± 0.03	0.00 ± 0.00	0.07 ± 0.02	0.13 ± 0.01	0.11 ± 0.02	0.40 ± 0.02
	AMINO	0.27	0.00	0.06	0.10	0.08	0.32	2.32	0.88	0.32 ± 0.03	0.00 ± 0.00	0.07 ± 0.02	0.12 ± 0.01	0.09 ± 0.02	0.39 ± 0.02
	EVOL	0.31	0.00	0.00	0.10	0.06	0.32	1.52	0.87	0.39 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.01	0.08 ± 0.02	0.40 ± 0.02
	SNPmt	0.33	0.00	0.05	0.11	0.07	0.33	0.96	0.44	0.34 ± 0.03	0.00 ± 0.00	0.05 ± 0.01	0.12 ± 0.01	0.07 ± 0.02	0.34 ± 0.01

CYTO - cytoplasmic model with pedigree derived maternal lineages; HAPLO – haplotype model with mitogenome sequences; AMINO - amino acid model with unique amino acid sequences; EVOL – evolutionary model with phylogenetic haplogroups; SNPmt – model with SNP polymorphisms; $\sigma_a^2\sigma_c^2$ - additive autosomal variance; σ_x^2 - additive X chromosome variance; σ_h^2 - mitochondrial variance (between haplotypes); σ_c^2 – common herd-year-season variance; σ_p^2 – permanent environmental variance; σ_e^2 – residual variance; σ_s^2- spatial effect variance; ρ - spatial range parameter; Phenotypic variance proportion explained by additive effect of autosomal chromosomes (h^2), X chromosome component (x^2), mitochondrial component (m^2), contemporary group component (c^2); permanent environment (p^2).

For all traits analyzed, the best and worst haplotypes were those assigned to the T_3 haplogroup, the most common haplogroup in European cattle, while other non-T_3 haplotypes (T_1, T_2, and T_5) were mainly distributed within 50% of the worse haplotypes for milk production. The results suggest that if there is a difference between the haplogroups, their distribution of haplotype effects is likely to overlap. Unfortunately, we could not verify this statement due to the small number of non- T_3 haplotypes. High linear correlation between haplotype effects of all milk production traits ($r_{MILK\text{-}FAT}$ = 0.83; $r_{MILK\text{-}PROTEIN}$ = 0.98, and $r_{FAT\text{-}PROTEIN}$ = 0.85) were observed pointing to its "pleiotropic behavior" of non-recombining mitochondrial haplotypes considered as a "single gene."

Decomposition of mitogenome variance to gene regions

By applying the SNPmt model to estimate mitochondrial effects, we were able to decompose the contribution of functionally or positionally specific mitogenome regions to the total variance between mitogenome effects. For this analysis we used the approach of Lara et al. (2022) for autosomal genome. This approach is important because the mitogenome does not recombine, meaning that covariances between some functionally related SNPs can be an important component of variance between mitogenome effects. The results of the variance decomposition, separated by specific mitogenome region, are shown in Figure 4 and Table S3.

A very similar pattern of variance decomposition was observed for all 3 milk production traits, suggesting that the influence of the mitogenome on milk yield, fat yield and protein yield may occur through similar biological processes. For all 3 traits, the largest contribution to variance was observed for the D-loop end, followed by the ND5 and ND4, while the contribution of COX1, D-loop beginning, CYTB, 12S RNA, 16S RNA, ATP6, COX2, COX3, ND1, ND2, and ND6 was non-negligible.

At the same time, the estimated covariances were larger between SNPs located in different mitogenome regions and, with few exceptions, were predominantly negative (Figure 4). In contrast, the only substantial (negative) covariance within mitogenome regions was estimated between SNPs located in the D-loop end. We also analyzed variance of mitogenome regions as a function of the number of polymorphic sites using linear regressions, for more information see Figures S1 and S2.

Implications, limitations, and future work

The impact of mitogenome on milk production traits has been intensively studied at the end of 20th century using the cytoplasmic model (Bell et al., 1985; Kennedy,

1986; Schutz et al., 1994; Boettcher and Gibson, 1997; Albuquerque et al., 1998; Roughsedge et al., 1999). While estimated phenotypic variance explained by different maternal lineages (m^2 ranging from 0 to 10%) has pointed to the possible considerable effect of mitogenome, the observed results were never implemented in practical cattle dairy breeding. The lack of understanding why estimated cytoplasmic effects were zero in some populations and 10% in other populations is one potential explanation. Questioning how well maternal lineages used in the cytoplasmic models reflect the true variation present in cattle mitogenome, with high possibility that some maternal lineages are identical or at least phylogenetically connected, was another potential explanation. At the end, the lack of a breeding concept on how to utilize mitogenome variation was probably the final decisive explanation for ignoring cytoplasmic effects in practical cattle breeding. At the same time, simulations by Fortuna et al. (2024) have shown that the inclusion of mitochondrial DNA variation (mDNA) increases the accuracy in different animal categories by between +0.01 and +0.05, though with a considerable variation between replicates similar to large variation in past studies on phenotypic variance explained by different maternal lineages.

This study has been driven by recent advances in mitochondrial research, where the functional capabilities of mitochondria have implications for crucial biological processes within the cell that extend far beyond their fundamental role in oxidative phosphorylation, the Krebs cycle and fatty acid oxidation (Al-Kafaji and Golbahar, 2013; Picard et al., 2018; McGuire, 2019; Monzel et al., 2023; Murphy and O'Neill, 2024).

With this in mind, we would be surprised that variation in the mitogenome have no effect on highly intensive milk production, a stressful and energy-consuming biological process (Favorit et al., 2021). For example, mitochondrial protein gene expression and the oxidative phosphorylation pathway have been shown to be associated with feed efficiency and energy balance in dairy cows (Dorji et al., 2020, 2021). More recently, mitochondrial efficiency has been linked to mtDNA copy number and associated with production in beef (Sanglard et al., 2022b) and dairy (Laubenthal et al., 2016; Weikard and Kuehn, 2018) cattle.

We went beyond cytoplasmic modeling and showed, based on the complete mitogenome information, that substantial phenotypic variance in milk production traits (milk, fat, and protein yield), ranging from 5% to 7% across the 3 traits, was influenced by mitogenome. Our analyses were based on complex modeling and provided additional insights into the influence of the mitogenome on milk production traits. Thus, we were able to show that mitogenome diversity in Croatian Holsteins contributes significantly to considerable variation in milk production

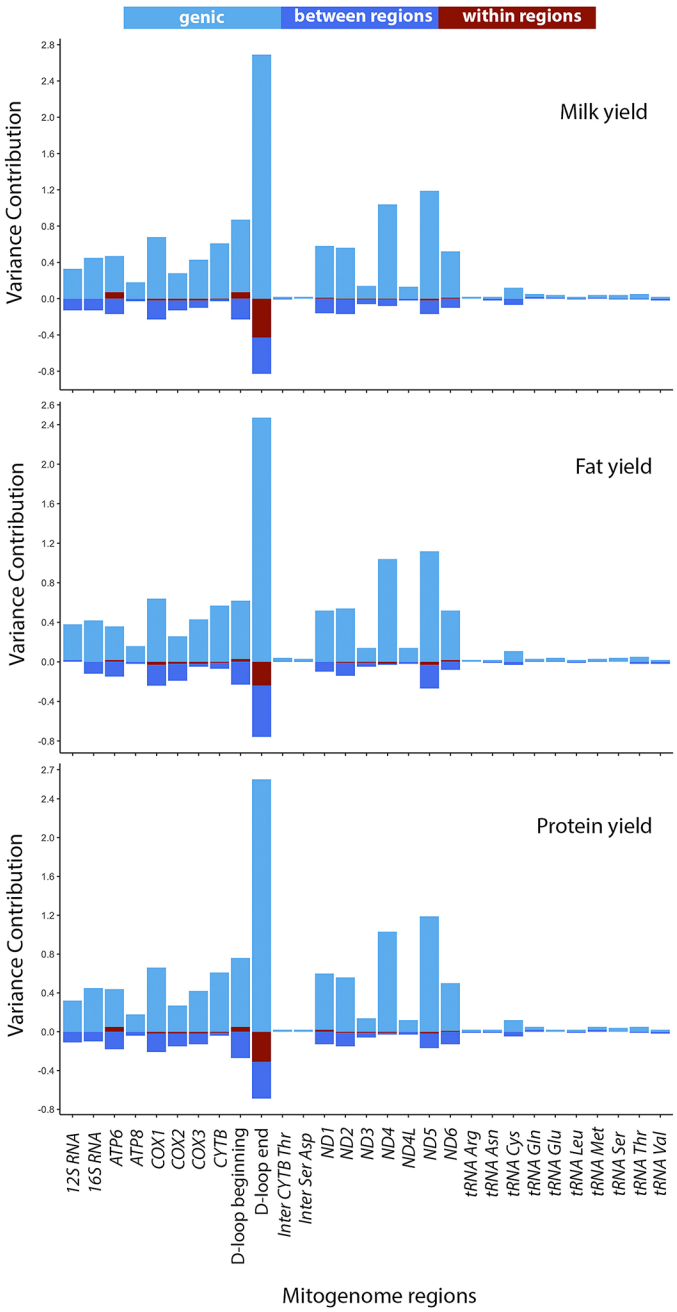

Figure 4. Mitogenome variance decomposition by specific mitogenome regions (variances and covariances between and within defined mitogenome regions) estimated for milk production traits in Holstein cows. A) milk yield, B) fat yield and C) protein yield.

traits between different haplotypes. We are aware that despite the large number of complete mitogenomes (109), the total number of lactating cows in the data set was relatively small compared with classical genetic analyses of quantitative traits in dairy cattle. For this reason, we expect that similar analyses will be performed in different dairy breeds based on a larger number of complete

mitogenomes and lactating cows. The routine use of low-coverage whole-genome sequences, which are already on the market, offers such an opportunity at no additional cost (Sanglard et al., 2022a). Alternatively, some commercial SNP arrays provide good coverage of complete mitogenome polymorphism (Brajkovic et al., 2023). We were not able to study the separation of the influence of the nuclear genome and the mitogenome because we did not have genotype information for the nuclear genome SNPs, though we did control for nuclear genome via expected autosomal and X chromosome relationships based on pedigrees. Observation that SNPmt model reduced the estimate of variance and range between location effects is puzzling and possibly indicates confounding between these 2 effects. This result is pointing toward a need for future research on modeling genetic and environmental/geographic effects with larger data sets.

Over 1,158 proteins are required for mitochondrial function in mammals, almost all of which are controlled by the nuclear genome, while interaction effects or incompatibility between nuclear and mitogenome SNPs have already been demonstrated (Wang et al., 2017; Dorji et al., 2020; Kwon et al., 2022; Ward et al., 2022). This indicates the need for further study of the separation of the influence of the nuclear genome and the mitogenome and possibly even their interaction. A nice example of such joint modeling of autosomal, nuclear mitochondrial (past mitogenome now part of nuclear genome), and mitogenome genetic variation for a complex trait in humans (neuroticism) was recently performed by Xia et al. (2023). In addition, we did not consider the effects of heteroplasmy (the occurrence of multiple mtDNA haplotypes within a single cell or organism), which is known to affect complex traits in humans (Ye et al., 2014).

Our study demonstrates a pleiotropic effect of mitogenomes with high correlations of the estimated haplotype effects between different milk production traits ($r > 0.83$), suggesting that selection of some haplotypes might be favorable for several traits. More drastically, this result opens the quest for superior mitogenomes that could be created by genetic engineering, especially since significant progress has recently been made in mitogenome editing in experimental mammals (Gammage et al., 2018; Rai et al., 2018; Klucnika and Ma, 2020; Barrera-Paez and Moraes, 2022). For the introduction of mitogenome gene editing in practical cattle breeding, either by introducing new variation or by enabling "recombination" between different haplotypes (simultaneous gene editing at several SNP positions), a much better understanding of how mitogenome genetic variation contributes to phenotypic differences without neglecting mito-nuclear interactions should obviously be studied. The separation of haplotype and single SNP effects in modeling the effects of the mitogenome on complex traits, together with comprehensive empirical evidence, is certainly the first step required.

CONCLUSION

In this pioneering study, we utilized complete mitogenome information to evaluate its influence on milk production traits in Croatian Holstein dairy cows. Our findings reveal substantial proportions of phenotypic variance explained by 4 different mitogenome models (CYTO, HAPLO, AMINO, and SNPmt), ranging from 5% to 7% across all 3 milk traits, while proportion of phenotypic variance explained by EVOL was negligible. Notably, the observed influence of the mitogenome on milk production appears to stem from the significant mitogenome diversity given its small physical size, a factor that may have been overlooked in previous cytoplasmic models. Furthermore, our study demonstrates that the integration of complete mitogenome information provides additional insights. For example, it allows the inference of haplotypes or SNPs that contribute to the estimated differences and reveals the pleiotropic effect of haplotypes, whether they are favorable or unfavorable for all 3 traits analyzed (milk, fat, and protein yield). Although these results need to be validated in other dairy cattle populations, especially with a larger number of sequenced mitogenomes and more phenotyped animals, the potential for leveraging mitogenome information in animal breeding is promising, especially as the cost-effectiveness of acquiring complete mitogenome sequences continues to improve.

NOTES

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Sampling was carried out during routine milking and was non-invasive, so no ethical approval was required. The authors have not stated any conflicts of interest. At the end, we thank two anonymous reviewers for their useful comments and helpful suggestions that improved this manuscript.

Data archiving statement Mitochondrial sequences of 109 Holstein cattle are deposited in GenBank (accession numbers from MZ901471 to MZ901579). The complete anonymized dataset used in this study will be available at Zenodo.

R code R code for data manipulation and model fitting will be available at GitHub (at the moment please use the Dropbox link: https://www.dropbox.com/scl/fo/l6ez8eykl1o03sw546z8a/ANYdV4BHjJ1H9ECO1iFTp2A?rlkey=csknoxhk27ifv0nhgf8oedtic&dl=0).

SUPPLEMENTAL MATERIAL Supplemental material is available in Dropbox link: https://www.dropbox.com/scl/fi/dab8urjtupykrcx1po8tz/Brajkovic_2024_JDS-mitogenome-milk-v43_supplemental_material.docx?rlkey=3gabe5xpr9zhye9pphmswt0nt&dl=0

Abbreviations used: AMINO = amino acid model with unique amino acid sequences; c^2 = phenotypic variance proportion explained by contemporary group component; CYTO = cytoplasmic model with maternal lineages; Eta = the total number of mutations; EVOL = evolutionary model based on BEAST phylogenetic analysis; h = number of haplotypes; h^2 = phenotypic variance proportion explained by additive effect of autosomal chromosomes; HAPLO = haplotypic model with mitogenome sequences; Hd = haplotype (gene) diversity; k = average number of nucleotide differences; m^2 = phenotypic variance proportion explained by mitochondrial component; MJ network = median joining network; NGS = next-generation sequencing; p^2 = phenotypic variance proportion explained by permanent environment; S = number of variable sites; SNPmt = mitogenome SNP model; x^2 = phenotypic variance proportion explained by X chromosome component; π = nucleotide diversity (per site); ρ = spatial range parameter;

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