Frequencies of milk protein variants and haplotypes estimated from genotypes of more than 1 million bulls and cows of 12 French cattle breeds

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ABSTRACT

Due to their major effects on milk composition and cheese-making properties and their putative effects on human health, there is a great deal of interest in bovine milk protein variants. The objectives of this study were to estimate frequencies of milk protein variants and haplotypes in 12 cattle breeds as well as their trends over time to assess the effect of selection on milk traits. Milk protein variants and haplotypes were identified from SNP genotype data from more than 1 million animals from 12 dairy, beef, or dual-purpose cattle breeds that had been genotyped for genomic selection. We examined a total of 15 loci in the genes that encode β -lactoglobulin (β -LG) and 3 caseins (α_{S1} -CN, β -CN, and κ -CN); genotypes were directly called from customized SNP chips (50.6%) or imputed (49.4%). Variants *A* and *B* of β -LG were frequent in the 12 breeds. For the caseins, we found 3 variants for α_{S1} -CN (*B*, *C*, and *D*), 6 for β -CN (*A1*, *A2*, *A3*, *B*, *C*, and *I*), and 5 for κ -CN (*A*, *B*, *C*, *D*, and *E*). For α_{S1} -CN, the *B* variant was the most frequent in all breeds except Jersey. For β -CN, the *A2* variant was the most abundant in all breeds except Tarentaise, although in Normande animals, the *I* variant (30.9%) was almost as common as *A2* (39.7%). The *C* variant was very rare except in the Tarentaise sample (4.8%). The most frequent variant for κ -CN was *A* in 5 breeds (including Holstein), and *B* in the 7 other breeds. The *B* variant was present at a particularly high frequency in Jersey (82.6%) and Normande (85.5%) animals. The *C* and *E* variants of κ -CN appeared to be particularly frequent in the Tarentaise (12.7%) and Holstein (9%) breeds, respectively. We found 20 haplotype combinations of α_{S1} - β - κ CN that were present at a frequency >0.1% in at least

one breed; however, only 6 to 9 haplotypes were found in any given breed, demonstrating a strong degree of linkage disequilibrium. The most frequent haplotypes were *B-A1-A*, *B-A2-A*, *B-A2-B*, *B-I-B*, *C-A2-A*, and *C-A2-B*. Some alleles were predominantly found in only one haplotype, such as the *E* and *C* variants of κ -CN and the *I* variant of β -CN, which were mainly found in the *B-A1-E*, *B-A1-C*, and *B-I-B* haplotypes, respectively. We observed changes in the frequency of certain variants over time in several breeds, such as an increase in the frequency of variants *A* of β -LG, *I* of β -CN, and *B* of κ -CN. With these results, we update and complete frequency data that were first estimated 30 to 50 yr ago, and, for the first time in these breeds, we assess the effect of selection on milk protein variants.

Key words: milk protein, variant and haplotype frequencies, genotype, French cattle breeds

INTRODUCTION

Cow milk contains 6 major proteins: α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN (representing about 80% of the total protein content) and the whey proteins α -LA and β -LG. Long before the availability of DNA molecular markers, some of the genetic polymorphisms of milk proteins could be directly identified by their protein phenotypes using electrophoresis (Aschaffenburg and Drewry, 1957). With that technique, it is possible to distinguish forms or variants of the same protein that differ in charge or size. In bovines, the main variants in milk proteins are typically caused by variations in the sequence of amino acids and, more precisely, from missense variations in the exons of genes (Farrell et al., 2004). The main variations in milk protein genes that induce cow protein variants are known (Martin et al., 2002). The α_{S1} - (*CSN1S1*), β - (*CSN2*), α_{S2} - (*CSN1S2*), and κ - (*CSN3*) CN genes are closely linked in a 248-kb region on chromosome 6 (between 85.4 and 85.7 Mb on the ARS-UCD1.2 genome assembly), which comprises the casein locus or haplotype. The genes that encode

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the α -LA and β -LG whey proteins are located on chromosomes 5 (*LALBA*, 31.2 Mb) and 11 (*PAEP*, 103.2 Mb), respectively. To date, few variants have been identified in the *LALBA* and *CSN1S2* genes in cattle breeds, particularly in French breeds, whereas many polymorphisms have been described in the other 4 genes: *CSN1S1*, *CSN2*, *CSN3*, and *PAEP* (Grosclaude, 1988; Caroli et al., 2009).

There is a great deal of scientific interest in milk protein variants because they have major effects on milk composition and cheese-making properties (Ng-Kwai-Hang et al., 1984; Bovenhuis et al., 1992) and they may have effects on human health (He et al., 2017). We can assume that intensive selection to increase yields of milk, protein, and fat in dairy cattle has resulted in changes in the frequency of these variants. It is therefore important to review the current situation in cattle breeds. With modern methods of molecular analysis, it is now much easier to determine genetic variants in DNA than in protein. Sequencing is the method of choice because it can detect all variants, although sample sizes are still limited (Meier et al., 2019). Genotyping is cheap and can be multiplexed with many genetic markers on SNP chips. Since the development of the low-density SNP BeadChip from Illumina Inc. (San Diego, CA; Boichard et al., 2012), bovine SNP chips can be customized to include additional variants. Because of their effects on milk production traits, known SNPs in *CSN1S1*, *CSN2*, *CSN3*, and *PAEP* were added in 2013 to the Eurogenomics BeadChip, which is routinely used for genomic selection in Europe (Boichard et al., 2018). Fifteen of these SNPs (3 to 7 per gene) are missense variants that affect the structure of the resulting protein, and their inclusion on the bead chip has enabled the genotyping of both male and female cattle for the main protein variants of these genes. In recent years, a dramatic expansion of genomic selection programs has created new possibilities for the characterization of these protein variants in all French cattle breeds for which a selection program has been developed. In November 2019, the SIGENO database hosted by INRAE (Institut national de recherche pour l'agriculture, l'alimentation et l'environnement, Paris, France) on behalf of French breeders included more than 1 million genotypes from 12 dairy, beef, or dual-purpose cattle breeds.

Milk protein variants are of special interest because they affect fat and protein milk composition, as well as technological properties of milk. Some of them are also thought to affect human health. In addition, casein genes are grouped in haplotypes that are transmitted almost without recombination. Therefore, frequencies of casein variants do not vary independently of each other, potentially leading to unexpected or undesirable changes. To update and build upon the results of

Grosclaude (1988), published more than 30 yr ago, the goal of this study was to present recent estimates of the frequencies of protein variants and casein haplotypes in 12 French cattle breeds, and to describe how those frequencies have evolved over time for assessing the effect of selection on milk production and composition traits, based on a large number of animals genotyped mainly for the purpose of genomic selection.

MATERIALS AND METHODS

Animals, Genotyping, and Imputation

More than 1 million animals representing 12 French dairy (Abondance, **ABO**; Jersey, **JER**; Brown Swiss, **BSW**; Holstein, **HOL**; Montbéliarde, **MON**; Tarentaise, **TAR**), dual-purpose (Normande, **NOR**; Simmental, **SIM**; Vosgienne, **VOS**), or beef (Blonde d'Aquitaine, **BLA**; Charolaise, **CHA**; Limousine, **LIM**) cattle breeds of Alpine (**CHA**, **ABO**, **BSW**, **MON**, **TAR**, **SIM**, and **VOS**), Northwest (Oceanic; **HOL**, **JER**, and **NOR**), or Southern (Iberic; **BLA** and **LIM**) origins (Grosclaude et al., 1990; Jann et al., 2002; Gautier et al., 2010) were genotyped, mainly for the purpose of genomic selection (Table 1). All genotypes obtained from international exchanges were excluded. The number of genotyped animals differed greatly depending on the breed. With 543,897, 289,875, and 96,558 genotyped animals, respectively, the 3 main French dairy breeds (**HOL**, **MON**, and **NOR**) alone accounted for nearly 88% of the total number of animals with genotypes. These were followed by the beef breeds **CHA**, **BLA**, and **LIM** (37,633, 14,878, and 14,439, respectively). In the other breeds, which are of regional importance in France, fewer animals had genotypes available: 9,979 for **ABO**, 8,628 for **BSW**, 5,573 for **TAR**, 3,731 for **VOS**, 3,312 for **SIM**, and 1,401 for **JER**.

In all breeds, most animals were candidates for genomic selection and were born in the last 10 yr. In dairy breeds, 80 to 94% of these young animals were females, whereas in beef breeds, less than 50% were females. Older animals (about 20,000) were progeny-tested bulls and constituted the initial reference populations for genomic evaluation. This sample is a nearly complete representation of the French AI bull population born from 1990 to 2008. Before 1990, the sample was more limited and included mainly elite bulls. The year of birth of the oldest genotyped bulls of each breed ranged from the early 1950s (**HOL** and **CHA**) to 2004 (**JER**) but, in most of the breeds, the oldest genotyped bulls were born in the 1960s and 1970s.

All animals were genotyped between 2008 and 2019, and thus different versions of Illumina chips were used (Table 2), with or without customization. High-, me-

Table 1. Description of the 12 cattle breeds, with information on the genotyped bulls and cows

Breed abbreviation	Breed name	Bulls (no.)	Cows (no.)	Total animals (no.)	Birth year of oldest bulls	Birth year of oldest cows	Production	Origin
BLA	Blonde d'Aquitaine	8,648	6,230	14,878	1962	1987	Beef	Iberic
CHA	Charolaise	21,076	16,557	37,633	1951	1989	Beef	Alpine
LIM	Limousine	10,800	3,639	14,439	1961	1996	Beef	Iberic
ABO	Abondance	1,097	8,882	9,979	1970	1981	Dairy	Alpine
BSW	Brown Swiss	1,545	7,220	8,765	1981	1979	Dairy	Alpine
HOL	Holstein	110,330	433,567	543,897	1950	1950	Dairy	Oceanic
JER	Jersey	97	1,304	1,401	2004	2010	Dairy	Oceanic
MON	Montbéliarde	30,167	259,708	289,875	1969	1983	Dairy	Alpine
TAR	Tarentaise	907	4,666	5,573	1964	1999	Dairy	Alpine
NOR	Normande	15,549	81,009	96,558	1970	1980	Dual-purpose	Oceanic
SIM	Simmental	1,483	1,829	3,312	1973	2003	Dual-purpose	Alpine
VOS	Vosgienne	238	3,493	3,731	1968	1997	Dual-purpose	Alpine

dium-, or low-density chips, which contained, respectively, around 777k (**HD**), 50k (**MD**), and 7 to 20k (**LD**) SNPs, were used in each breed (Table 2). Only a small proportion of the animals in each breed were genotyped with the HD chip, from 0.1% in HOL to 7.1% in SIM, and none in JER. Most of the 4,361 HD genotypes (from 182 to 817 per breed, average of 396) originated from research projects. The percentage of animals genotyped with an MD chip varied depending on the breed, from 18% in MON to 92.6% in SIM. Likewise, the percentage of animals in each breed that were genotyped with an LD chip was variable, from 0.2% (SIM) to 81.8% (MON). To obtain high imputation accuracy, it was recommended in each breed to accumulate MD genotypes before switching to LD, and this explains the higher proportion of MD genotypes when the total number of genotypes was limited. Since 2013, in addition to the standard content of generic SNPs, it has been possible to use specific versions of MD and LD chips that include additional SNPs, such as the missense variants in the milk protein genes. For

11 of the 12 breeds, a relatively large proportion of the animals had been genotyped with these customized chips (63.6 to 91.8%), whereas they were used for only 30.1% of the LIM animals (Table 2). Repeatability of genotyping results across chip versions is a crucial aspect. For that purpose, we set up a high-level quality control procedure. For each new chip and before its use in large-scale routine production, the same batch of 288 animals selected to cover a large genetic variability was re-genotyped and concordance between their genotypes was checked across chips. When some genotypes differed, SNP chip clusters were inspected and, when necessary, adjusted. Finally, markers that remained not interpretable were removed, but this never occurred for milk protein variants. In addition, these variants were always present (at least) twice on the same chip and all tests were required to be concordant. Because of this validation effort, we considered that genotyping results did not depend on the chip version.

The raw genotypes were then processed with the quality control procedure used in the French national

Table 2. Percentage of animals among the 12 French cattle breeds¹ that were genotyped using generic or customized (with missense SNPs in protein-coding genes) chips of various densities

Chip ²	BLA	CHA	LIM	ABO	BSW	HOL	JER	MON	TAR	NOR	SIM	VOS
HD generic	2.0	1.5	2.6	1.9	1.0	0.1	0.0	0.2	3.0	0.5	7.1	4.5
MD generic	18.1	27.6	61.7	14.0	16.1	22.0	8.2	11.6	19.8	18.0	29.3	24.3
MD customized	47.9	14.6	4.4	16.9	20.9	8.9	18.7	6.4	12.8	7.8	63.4	11.6
LD generic	0.1	0.0	5.6	0.0	5.2	5.5	0.0	2.9	0.0	5.2	0.0	0.0
LD customized	31.9	56.3	25.7	67.1	56.8	63.4	73.2	78.9	64.3	68.5	0.2	59.7
Total generic	20.2	29.2	69.9	16.0	22.3	27.6	8.2	14.7	22.8	23.7	36.4	28.7
Total customized ³	79.8	70.8	30.1	84.0	77.7	72.4	91.8	85.3	77.2	76.3	63.6	71.3
Total HD	2.0	1.5	2.6	1.9	1.0	0.1	0.0	0.2	3.0	0.5	7.1	4.5
Total MD	66.0	42.2	66.2	31.0	37.0	30.9	26.8	18.0	32.6	25.8	92.6	35.9
Total LD	32.0	56.3	31.2	67.1	62.0	68.9	73.2	81.8	64.3	73.7	0.2	59.7

¹Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Jersey (JER), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

²High density (HD; ≈ 777 k SNP), medium density (MD; ≈ 50 k SNP), or low density (LD; ≈ 10 – 20 k SNP).

³Milk protein variants were present on all customized chips and imputed for the generic chip.

evaluation system (Boichard et al., 2012): (1) only animals with an individual call rate higher than 95% were kept, (2) SNPs with a call rate lower than 90% were deleted, (3) for generic markers (but not for custom markers), a minor allele frequency >1% in at least one major French dairy cattle breed (HOL, MON, or NOR) was required, and (4) SNPs with genotype frequencies in Hardy-Weinberg equilibrium ($P > 10^{-4}$) were kept. Following this procedure, 53,469 SNPs were retained, which included 15 missense variants in milk protein genes.

To recover as complete information as possible, genotypes of all animals were imputed within breed to these 53,469 SNP, all present on the most recent version of the customized MD chip by Eurogenomics (EuroGMD BeadChip, Illumina Inc.). Imputation was performed with FImpute software (Sargolzaei et al., 2014). Because imputation involves phasing (i.e., determination of the parental origin of alleles), casein haplotypes were obtained automatically and simultaneously. Imputation accuracy was high in all breeds [$<0.5\%$ genotype error rate in the large dairy breeds, 1.1 to 1.5% in the beef breeds (Saintilan et al., 2014), and $<1\%$ in the regional dairy breeds (Sanchez et al., 2016)]. All real or imputed milk protein genotypes were then included in further analysis and frequencies were obtained by allele counting.

Correspondence Between Protein Variants and SNP Genotypes

After imputation, 15 missense variants located in the exons of the casein and *PAEP* genes were characterized for all genotyped animals. For each gene, between 2 and 6 SNPs from the chip were used to call 3 to 7 different variants of the resulting protein (*CSN1S1*: 2 SNPs, 3 variants; *CSN2*: 6 SNPs, 7 variants; *CSN3*: 3 SNPs, 5 variants; *PAEP*: 4 SNPs, 5 variants). The correspondence between DNA variants and protein variants is given in Figure 1. For *CSN3*, a combination of 2 mutations is required to distinguish alleles *C* and *D*. In *PAEP*, alleles *A* and *B* differ in 2 variants in complete linkage disequilibrium. Among these 15 SNPs in the 4 milk protein genes, it was therefore possible to distinguish variants *B*, *C*, and *D* of α_{S1} -CN; *A1*, *A2*, *A3*, *B*, *C*, *F*, and *I* of β -CN; *A*, *B*, *C*, *D*, and *E* of κ -CN; and *A*, *B*, *C*, *D*, and *W* of β -LG. In addition to allelic frequencies for the 4 proteins, we also calculated the frequencies of casein haplotypes (*CSN1*–*CSN2*–*CSN3*) among the 12 breeds. In each breed, we first calculated whole-population frequencies and then estimated the trend of frequencies over time. The frequencies were computed based on animal birth year for males and

females separately, and results were only retained if the year in question contained at least 50 genotyped bulls or cows.

To highlight patterns of similarity in the milk proteins between breeds, we applied a hierarchical clustering analysis, based on casein haplotypes, using the “hclust” function of the R package (Müllner, 2013) “fastcluster.”

RESULTS

Frequencies of Milk Protein Variants

Frequencies of protein variants are presented for each breed in Table 3. Among the 12 breeds, variants *A* and *B* were the main variants of β -LG. The *A* variant was the most frequent in BLA (71.6%), LIM (61.8%), CHA (60.5%), SIM (59.2%), HOL (58.5%), JER (55%), and MON (54.3%), whereas *B* was most frequent in BSW (68.8%), VOS (68.8%), TAR (68.2%), NOR (57%), and ABO (53.5%). A rare variant (*D*) was found in SIM (2.3%) and ABO (0.3%), and at a very low frequency ($<0.1\%$) in the HOL, MON, NOR, and BLA samples. We did not identify the *C* and *W* variants in these 12 breeds.

For caseins, 3 variants were found for α_{S1} -CN (*B*, *C*, and *D*), 6 for β -CN (*A1*, *A2*, *A3*, *B*, *C*, and *I*) and 5 for κ -CN (*A*, *B*, *C*, *D*, and *E*). Therefore, with the exception of the κ -CN *F* variant, we detected all possible casein variants that can currently be distinguished using SNP analysis. The most frequent casein variant in each breed was *B* or *C* for α_{S1} -CN, *A1* or *A2* for β -CN, and *A* or *B* for κ -CN.

For α_{S1} -CN, the *B* variant was found to be the most frequent in 11 of the 12 breeds and was highly predominant in 8 breeds (HOL, 99.6%; MON, 97.5%; VOS, 94.1%; SIM, 94.1%; BLA, 93.7%; BSW, 88.9%; LIM, 86.8%; and CHA, 86.2%). Jersey was the only breed in which the *C* variant was most frequent (59.1%, compared with 40.9% for the *B* variant). The *D* variant was found in the BLA, CHA, LIM, and HOL samples, but at a frequency $<0.1\%$ in each case.

We identified a larger number of polymorphisms for β -CN, but the *A2* variant was the most abundant in all breeds (from 39.7% in NOR to 82.8% in ABO) except TAR, in which the *A1* variant was slightly more frequent (49.7% vs. 45.8% for the *A2* variant). In NOR, the *I* variant (30.9%) was almost as common as the *A2* variant (39.7%); it was also found at a relatively high frequency in MON (16.7%), VOS (12.4%), BLA (10.5%), and HOL (8.4%). One of the characteristics of the MON breed was a high frequency of the *B* variant (29.1%), which also appeared at a relatively high frequency in NOR (18.6%), BSW (14.6%), and CHA

* The κ-CN-D variant, observed in Tarentaise, results from the association between the A allele at position 85,656,619 (the C variant) and the A allele at position 85,656,772 (the A variant) and likely results from a unique recombination event.

Figure 1. Relationships between missense SNPs and protein variants for the genes that encode the bovine milk proteins α_{s1}-CN (*CSN1S1*), β-CN (*CSN2*), κ-CN (*CSN3*), and β-LG (*PAEP*). REF and ALT indicate reference and alternate alleles on the ARS-UCD1.2 assembly.

(13.4%). The *C* variant was rarely found, except in TAR (4.8%) and to lesser extent in CHA (1.1%).

The most frequent variant for κ-CN was *A* in BLA, HOL, ABO, SIM, and VOS, and *B* in the 7 other breeds. The *B* variant was found at a particularly high frequency in JER (82.6%) and NOR (85.5%), while the frequencies of *A* and *B* variants were more balanced in all other breeds. Three other variants appeared to be breed-specific: the *C* and *D* variants were found exclusively in TAR (12.7% and 0.12%, respectively) whereas

the *E* variant was present in HOL samples (9.5%) and at frequencies <1% in JER and MON.

Casein Haplotype Frequencies

In the entire sample, 20 of the possible 90 ($3 \times 6 \times 5$) α_{s1}-β-κ-casein haplotype combinations were found at a frequency >0.1% in at least one breed (Table 4). One haplotype was always predominant in each breed, with a frequency above 0.6, 0.4, and 0.3 in 2, 5 and 11

Table 3. Percentages of β -LG, α_{S1} -CN, β -CN, and κ -CN variants in 12 French cattle breeds¹

Protein	Variant ²	BLA	CHA	LIM	ABO	BSW	HOL	JER	MON	TAR	NOR	SIM	VOS
β -LG	<i>A</i>	71.6	60.5	61.8	46.2	31.2	58.5	55.0	54.3	31.8	43.0	59.2	31.2
	<i>B</i>	28.4	39.5	38.2	53.5	68.8	41.5	45.0	45.7	68.2	57.0	38.4	68.8
	<i>D</i>	<0.1	0	0	0.31	0	<0.1	0	<0.1	0	<0.1	2.3	0
α_{S1} -CN	<i>B</i>	93.7	86.2	86.8	55.9	88.9	99.7	40.9	97.5	66.7	73.4	94.1	94.1
	<i>C</i>	6.2	13.8	12.5	44.1	11.1	0.34	59.1	2.5	33.3	26.6	5.9	5.9
	<i>D</i>	<0.1	<0.1	0.69	0	0	<0.1	0	0	0	0	0	0
β -CN	<i>A1</i>	13.9	19.7	22.3	15.7	6.5	30.0	4.4	7.7	49.7	9.1	35.8	38.4
	<i>A2</i>	68.4	63.2	66.4	82.8	78.7	59.2	76.6	46.4	45.5	39.7	55.6	48.5
	<i>A3</i>	0	<0.1	<0.1	<0.1	<0.1	0.26	0	<0.1	0	1.64	<0.1	0
	<i>B</i>	7.04	13.43	8.17	<0.1	14.62	2.09	12.99	29.10	0	18.59	3.80	0.20
	<i>C</i>	0.21	1.10	0.39	0.31	<0.1	<0.1	0	<0.1	4.75	<0.1	0	0.48
	<i>I</i>	10.5	2.4	2.8	1.1	0.16	8.4	6.0	16.7	0	30.9	4.7	12.4
κ -CN	<i>A</i>	53.8	34.4	46.9	64.8	25.6	56.9	16.9	38.2	30.5	14.5	71.8	65.5
	<i>B</i>	46.2	65.6	53.1	35.2	74.4	33.6	82.6	61.6	56.7	85.5	28.2	34.5
	<i>C</i>	0	0	0	0	0	0	0	<0.1	12.7	0	0	0
	<i>D</i>	0	0	0	0	0	0	0	0	0.12	0	0	0
	<i>E</i>	0	<0.1	0	<0.1	<0.1	9.50	0.50	0.19	0	<0.1	0	0

¹Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Jersey (JER), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

²Only variants found with a frequency >0.1% in at least one breed were included.

breeds, respectively. Among breeds, the most frequent casein haplotype varied: *B-A1-A* (32.2% in SIM and 32.4% in VOS), *B-A2-A* (43.4% in BLA, 40.1% in LIM, and 43.7% in HOL), *B-A2-B* (28.5% in CHA, 61.6% in BSW, and 40.1% in MON), *B-I-B* (30.3% in NOR), *C-A2-A* (43.8% in ABO), and *C-A2-B* (58.7% in JER and 32.8% in TAR). This reflected a high degree of diversity across breeds. We were able to distinguish 5

other casein haplotypes with a frequency >9% in some breeds: *B-A1-B* in BLA (12.2%), CHA (15.2%), LIM (17.8%), ABO (14.6%) and TAR (23.4%); *B-A1-C* in TAR (12.5%); *B-A1-E* in HOL (9.3%); *B-B-A* in MON (28.7%); and *B-B-B* in JER (12.9%) and NOR (18.5%). Finally, the *B-C-A*, *B-C-B*, and *C-A3-A* haplotypes were present at lower frequencies, in TAR (4.6%), CHA (1.1%), and NOR (1.5%), respectively. The 6 other

Table 4. Percentages of casein haplotypes in 12 French cattle breeds^{1,2}

α_{S1} - β - κ haplotype ³	BLA	CHA	LIM	ABO	BSW	HOL	JER	MON	TAR	NOR	SIM	VOS
<i>B-A1-A</i>	1.7	4.5	4.3	1.0	5.9	12.4	0.39	3.0	13.7	0.59	32.2 ²	32.4 ²
<i>B-A1-B</i>	12.2	15.2	17.8	14.6	0.5	8.3	3.5	4.5	23.4	8.5	3.6	6.0
<i>B-A1-C</i>	0	0	0	0	0	0	0	0	12.5	0	0	0
<i>B-A1-E</i>	0	0	0	<0.1	0	9.3	0.50	0.18	0	<0.1	0	0
<i>B-A2-A</i>	43.4 ²	21.0	40.1 ²	19.8	6.3	43.7 ²	16.0	4.0	11.9	11.3	31.3	27.2
<i>B-A2-B</i>	18.9	28.5 ²	13.7	19.1	61.6 ²	15.2	1.6	40.1 ²	0.36	3.7	18.5	15.5
<i>B-A2-E</i>	0	0	0	0	0	0.14	0	0	0	<0.1	0	0
<i>B-B-A</i>	5.4	8.4	0.63	<0.1	8.9	<0.1	0	28.7	0	<0.1	2.9	0
<i>B-B-B</i>	1.6	5.0	7.4	0.02	5.6	2.0	12.9	0.32	0	18.5	0.91	0.20
<i>B-C-A</i>	0	<0.1	0	<0.1	0	0	0	0	4.6	0	0	<0.1
<i>B-C-B</i>	0.20	1.1	0.39	0.23	0	0	0	0	0.13	0	0	0.47
<i>B-I-A</i>	0.41	<0.1	0.29	0	0	0.35	0.32	0.12	0	0.30	0.30	<0.1
<i>B-I-B</i>	10.0	2.4	2.2	1.1	0.15	8.1	5.6	16.6	0	30.3 ²	4.4	12.4
<i>C-A2-A</i>	2.9	0.45	1.3	43.8 ²	4.4	<0.1	0.14	2.2	0.31	0.65	5.1	5.9
<i>C-A2-B</i>	3.2	13.2	10.5	0.18	6.5	<0.1	58.7 ²	0.10	32.8 ²	24.0	0.69	<0.1
<i>C-A2-C</i>	0	0	0	0	0	0	0	0	0.14	0	0	0
<i>C-A3-A</i>	0	0	0	0	0	0.24	0	0	0	1.5	<0.1	0
<i>C-B-A</i>	<0.1	<0.1	0.15	0	0.12	0	0	<0.1	0	0	0	0
<i>C-I-B</i>	<0.1	0	0.31	0	0	0	0.11	<0.1	0	0.25	<0.1	0
<i>D-A2-B</i>	<0.1	<0.1	0.64	0	0	0	0	0	0	0	0	0

¹Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Jersey (JER), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

²The most frequent haplotype for each breed.

³Only haplotypes found with a frequency >0.1% in at least one breed were included.

observed casein haplotypes had a frequency <1% in all breeds.

Trends of Protein Variant and Casein Haplotype Frequencies

We estimated the trends of variant and casein haplotype frequencies in bulls and cows. In each breed, we calculated allele frequencies for every year in which at least 50 genotyped bulls or cows were born. For bulls, it was possible to follow the trend of frequencies since 1990 in the major breeds (HOL, MON, NOR, CHA, BLA, LIM, and SIM). In contrast, this analysis was much more limited for breeds of regional importance such as ABO (data available only since the 2000s), and TAR and VOS (2010s). With only 97 Jersey bulls genotyped, it was not possible to estimate any frequency trend in this breed (Appendix Figures A1, A2, A5, and A6). In all dairy breeds, the number of genotyped males was much lower than the number of genotyped females, and from 1990 to 2008, only progeny-tested bulls were genotyped. These limited numbers explain why frequencies were less stable for males than for females. As the birth years of genotyped cows spanned a more recent period, we were able to estimate frequencies only since the 2000s for all breeds, except JER (from 2015; Appendix Figures A3, A4, A5, and A6). In a given year, evolutionary patterns of protein variant and casein haplotype frequencies were similar between bulls and cows. A closer look at the HOL, MON, and NOR breeds, in which we observed the largest changes, is shown in Figure 2.

For β -LG, we observed different profiles among breeds. For the 3 beef breeds (BLA, CHA, and LIM), frequencies were relatively stable over time. In the other breeds for which we had enough information—HOL, MON, NOR, and SIM—the *B* variant was more frequent than the *A* variant until the mid-to-late 1990s, and then the frequencies either balanced out (NOR) or inverted (HOL, MON, and SIM).

For the past several decades, variant *B* of α_{S1} -CN has been largely predominant in all breeds (except in JER and to a lesser extent in ABO and TAR), and variant frequencies have been relatively stable over time.

The situation is more complex for β -CN because of the higher number of variants. The A2 variant has been predominant for years in beef breeds (BLA, CHA, and LIM) as well as in the dairy breeds ABO and BSW. In other breeds, instead, variants other than A2 have been relatively common for decades: variant A1 in HOL, TAR, SIM, and VOS and variants *B* and *I* in MON and NOR. In both MON and NOR, it is noteworthy that, starting around 2010, variant *B* has tended to become less frequent in favor of variant *I*.

For κ -CN, the situation is similar to that of β -LG: 2 main variants, *A* and *B*, coexist in varying frequencies depending on the breed. Frequencies have been stable over time in most breeds with the exception of HOL and MON. In HOL, the *A* variant was much more frequent than the *B* variant in the 1980s, but the frequencies have gradually balanced out over time; today, *A* and *B* are present at similar frequencies. In contrast, in the MON breed, the *B* variant is currently more frequent than *A*, but frequencies were more balanced before the 2000s.

Casein haplotype frequencies were relatively stable over time (Appendix Figures A5 and A6). However, we did observe an increase over the last 10 yr in the frequencies of some casein haplotypes in certain breeds: *B-A2-B* in HOL and MON, *B-I-B* in NOR and MON, and *C-A2-A* in ABO.

Breed Classification Based on Casein Haplotypes

Figure 3 presents a classification of the breeds based on casein haplotype frequencies. The Oceanic group (JER and NOR) was clearly differentiated from the other breeds. The Hispanic group was also easily identified (LIM, BLA) and separated from the other breeds of Alpine and Central Europe origins, with CHA being intermediate. Among them, SIM and VOS and, separately, MON and BSW were very close to each other. However, based on this single locus, HOL and TAR positions were not in agreement with their geographical origins.

DISCUSSION

Here, we estimated the frequencies of milk protein variants and casein haplotypes in a sample of more than 1 million bulls and cows of 12 milk, dual-purpose, or beef French cattle breeds of Alpine, Northwest (Oceanic), or Southern (Iberic) origins. This large-scale study updated and built upon similar estimates that were obtained more than 30 yr ago in French breeds on proteins but with much smaller sample sizes (Grosclaude, 1988). To our knowledge, this study is the first of this magnitude, and it enables a long-term perspective of the trend of variant frequencies over the last few decades and over a large number of cattle breeds, which is crucial to appreciate the effects of selection. Beyond dairy breeds, this study also provides results in 3 beef breeds. Although these results are of limited practical importance, they help us interpret the effect of the geographical origins of the breeds and illustrate that beef breeds have greater genetic variability than dairy breeds.

Figure 2. Trends of the frequencies of β -LG, α s1-CN, β -CN, and κ -CN variants in Holstein (HOL), Montbéliarde (MON), and Normande (NOR) bulls.

Figure 3. Cluster dendrogram based on all casein haplotypes in 12 French breeds.

The variant frequencies we estimated in the 12 breeds are consistent with those published in the review of Caroli et al. (2009), in which the variants β -LG-A, β -LG-B, α_{S1} -CN-B, β -CN-A2, κ -CN-A, and κ -CN-B were described as the most common in *Bos taurus* based on multiple studies published between 1962 and 2002.

However, we also detected additional variants in β -CN (*I*) and κ -CN (*C* and *E*) that were not identified in the study of Grosclaude (1988), although they probably already existed at that time.

The κ -CN-*C* variant, which was specific to the Alpine TAR breed in our study, was first identified more than 40 yr ago in Grey Alpine cattle (Di Stasio and Merlin, 1979). This variant was also later described in TAR cows, at a frequency of 12% (Delacroix-Buchet et al., 1993), very close to the frequency we estimated here in the population of genotyped TAR animals (5,573 animals, 12.7%). Because the genotyping in TAR was performed recently, it was not possible to estimate the trend of frequencies before 2006. Since 2006, however, the κ -CN-*C* variant has been present in TAR at a relatively stable frequency; TAR was also the only breed in which the *D* variant of κ -CN was present. This variant is not caused by a specific mutation but instead results from the combination of the 2 mutations responsible for the *C* and *A* variants. The *D* variant is rare and very likely originated from a recombination event between the 2 loci responsible for the *B* and *C* alleles, which are 153 bp apart. The κ -CN-*E* variant was identified in the late 1980s in different breeds (Erhardt, 1989) but was almost exclusively found in the HOL breed in this study, with a stable frequency of nearly 10% since the 1980s. Its presence at low frequency in the MON breed likely originates from the limited HOL introgression in

this breed. The β -CN-*I* variant, which is derived from A2, was discovered in the early 2000s in the Italian Red Pied (Jann et al., 2002) and here was found in almost all breeds. Its frequency has greatly increased in recent decades in MON (from 10 to 20%), NOR (from 20 to 40%), and BLA (from 4 to 15%), but has remained relatively constant in the other breeds, including HOL ($\approx 8\%$). The β -CN-*C* variant, which is derived from A1, was found at a frequency close to 5% in the TAR breed, whereas it was rare in the other breeds.

In addition to the emergence of some variants, we noted changes in the frequency of the most common variants. These changes were most marked in some dairy (HOL and MON) and dual-purpose (NOR and SIM) breeds; few changes were observed in beef breeds (BLA, CHA, and LIM). For the remaining dairy or dual-purpose breeds, it was more difficult to draw conclusions because we could not estimate variant frequencies before 2006 (ABO) or even 2015 (TAR, VOS, and JER) reliably.

Validity of conclusions about frequency trends depends on the representativeness of the genotyped animals over years. Old males were progeny-tested bulls who contributed to the initial reference population for genomic selection. For males born from 1990 to 2008, they included nearly all French progeny-tested bulls. Before 1990, the samples were smaller and often limited to elite bulls. Younger males born after 2008 were all candidates for genomic selection. Therefore, both old and recent males born after 1990 can be considered representative of their breed, in spite of their pedigree-based selection. For cows, the situation differed across breeds. In the common dairy breeds, only a small proportion of females were genotyped in the early years

but many of them originated from research projects. Since 2013, genotyping for selection purposes has become much more common and generally on an entire herd. Therefore, the representativeness of the female sample is best in recent years but is good over the whole period. In beef breeds, the situation is similar, but with a 5-yr gap, as genomic selection started later in beef than in dairy breeds. Samples from males are likely to be representative, but this is more doubtful for females. In regional dairy breeds, the number of males in the sample pool is limited, whereas females with phenotypes were genotyped to build the reference population. Because of this, they can be considered representative of the entire breed.

Protein variants affect the concentrations of β -LG or caseins in milk, and some also modify the chemical properties of the proteins. Consequently, they have effects on the cheese-making abilities of milk, particularly on coagulation properties (time and firmness) and cheese yields (Ng-Kwai-Hang et al., 1986; Bovenhuis et al., 1992; Delacroix-Buchet et al., 1993; Ikonen et al., 1999; Caroli et al., 2000; Bonfatti et al., 2010). The β -LG-*B*, α_{S1} -CN-*C*, β -CN-*B*, β -CN-*I*, and κ -CN-*B* variants were found to be associated with increased fat and casein contents in milk, and therefore, with better cheese-making traits (Ng-Kwai-Hang et al., 1986; Bovenhuis et al., 1992; Delacroix-Buchet et al., 1993; Bonfatti et al., 2010). In contrast, the β -LG-*A*, α_{S1} -CN-*B*, κ -CN-*A*, and κ -CN-*E* variants have positive effects on milk yield but adverse effects on milk composition and cheese-making traits. The β -CN-*C* variant is very detrimental for cheese-making properties (Delacroix-Buchet and Marie, 1994). The effects of the variants may therefore explain some of the changes in frequencies over time that we observed. For example, we found increases in the frequencies of the favorable variants β -CN-*I* and κ -CN-*B* mainly in breeds that have been selected for milk composition. In contrast, the frequency of variant β -LG-*B* decreases over time in the HOL, MON, and NOR dairy breeds in favor of variant *A*, which is responsible for milk with poorer cheese-making ability. Previous studies have shown that an increase in β -LG content (as a percentage of total protein content in milk), which is caused by variant *A*, is counterbalanced by a relative decrease in caseins, particularly α_{S1} -CN and β -CN, which are the most abundant caseins in bovine milk (Ng-Kwai-Hang et al., 1987; Heck et al., 2009). This decrease in casein content likely explains the adverse effect of variant *A* on the cheese-making properties of milk. Other studies found that genotype *AA* had favorable effects on milk and protein yields (Bovenhuis et al., 1992; Ikonen et al., 1999), which could explain why variant *A* of β -LG has been selected in some major dairy breeds.

As the imputation process involves phasing, construction of haplotypes was straightforward. This procedure is very accurate, especially with the strong family structure of cattle breeds and with the very low rate of recombination among the 15 loci in the casein region that were studied here. Although we observed a large diversity of protein variants and casein haplotypes across breeds, we found only 20 casein haplotypes out of 90 possible combinations. Of these, only 14 were found at a frequency $>1\%$ in at least one breed, and only 6 to 9 were found in each breed. Of the haplotype combinations that were present in each breed, 5 were the most frequent haplotypes in at least one breed (*B-A2-A*, *B-A2-B*, *B-I-B*, *C-A2-A*, and *C-A2-B*). The low number of haplotypes and the strong linkage disequilibrium between casein variants is mainly due to the vicinity of casein genes to each other on the genome, which results in the co-transmission of casein gene variants across generations and in a low rate of recombination between them. Grosclaude (1988) estimated casein haplotype frequencies in HOL, MON, NOR, and TAR cattle; in that study, *B-A2-A* was the most frequent haplotype in HOL (37%), MON (31%), and TAR (30%), whereas *B-B-B* was predominant in NOR (45%). Our analysis revealed very different results for the MON, NOR, and TAR breeds: the most frequent casein haplotypes from the 1980s are no longer the main haplotypes, which are now *B-A2-B*, *B-I-B*, and *C-A2-B*, respectively. However, the *B-A2-A* haplotype is still the most frequent in HOL (44%). It therefore seems that there is now a greater diversity of major casein haplotypes among breeds than in the past, partly due to the emergence of some new casein variants. The recent discovery of certain variants (such as β -CN-*I*) also partly explains this finding.

The κ -CN-*E* variant, which was found at its highest frequency in HOL, is mainly associated with the α_{S1} -CN-*B* and β -CN-*A1* variants, which together form the *B-A1-E* haplotype. The frequency of the *E* variant has been quite stable since the 90s, which is likely linked to the fact that in those years the goal of selection was changed (lower weight given to milk production in favor of improvements to milk composition). Similarly, the κ -CN-*C* variant, which is found almost exclusively in TAR, is mainly associated with the α_{S1} -CN-*C* and β -CN-*A2* variants to form the *C-A2-C* haplotype. Likewise, variant *I* of β -CN is almost completely associated with the *B* variants of the other 2 casein genes to form the *B-I-B* casein haplotype. The *C* variant of β -CN is always associated with the *B* variant of α_{S1} -CN and, in most breeds, with variant *B* of κ -CN to form the *B-C-B* haplotype, with the exception of the TAR breed, in which the *B-C-A* haplotype is much more frequent.

One locus, even a very polymorphic one, is not sufficient for estimating genetic distances among breeds.

However, some patterns of similarity that we detected in the milk proteins (Figure 3) are consistent with the known origins of the breeds (Grosclaude et al., 1990; Jann et al., 2002). For instance, both the JER and NOR, breeds that originate in the English Channel area, have a high proportion of the *C-A2-B* haplotype. Both BLA and LIM, which have Iberic origins and are known to be closely related, have a high frequency of *B-A2-A*. The SIM and VOS breeds, from Central Europe, share similar haplotype profiles, with high frequencies of *B-A1-A*, *B-A2-A*, and *B-A2-B*. The MON and BSW breeds, of Alpine origin, have the same 2 predominant haplotypes, *B-A2-B* and *B-B-A*. Nevertheless, we did find exceptions, as shown by the large degree of genetic diversity in the 6 Central European and Alpine breeds. The HOL breed appears to be unique in harboring the *E* variant of κ -CN and shares some similarities with all groups of breeds, especially in having a high frequency of *B-A2-A*, like the Iberic breeds. This classification based on only one locus can be compared with that of Gautier et al. (2010) based on 44,706 SNPs spanning the whole genome. Although the classifications agree quite well, results were highly different for 2 breeds, HOL and TAR. In Gautier et al. (2010), TAR is grouped with Alpine breeds, whereas HOL belongs to another group not studied here.

Some studies have suggested that certain milk protein variants have effects on human health. In particular, it has been claimed that the β -CN-A2 allele is better tolerated in some parts of the human population (He et al., 2017). Although the relationship between A1 bovine milk consumption and human disease incidence has not been confirmed, some recommendations have appeared worldwide to increase the frequency of variant A2. A similar health effect could also be obtained with any variant of the A2 family; that is, those with a proline at position 67 (variants A2, A3, *E*, *D*, *I*, *J*, *H*). Because *I* is frequent in some breeds (HOL, MON, and especially NOR), should A2 be selected, including *I* allele in selection decision would provide faster results without wasting genetic variability. However, due to the strong linkage disequilibrium between variants of caseins, it is important to note that an increase in the frequency of variant A2 of β -CN will also induce increases in the frequencies of certain variants of α_{S1} -CN and κ -CN. Our results indicate that selecting for the A2 variant would also increase the frequency of the *C* variant of α_{S1} -CN in ABO, JER, TAR, and NOR, and of the *A* variant of κ -CN in HOL and ABO, both of which have unfavorable effects on the cheese-making properties of milk. These results illustrate the “hitchhiking” phenomenon: when directly selecting for some alleles, the response to selection modifies the neighboring region due to the

haplotypic structure of the genome and linkage disequilibrium (Smith and Haigh, 1974). Generally, geneticists do not recommend direct selection on individual genes because they frequently explain a limited part of the variability of the major traits. Should selection be based on individual genes, it would be preferable to include this information in a total merit index (Segelke et al., 2016). If breeders want to select for some casein variants, we strongly recommend defining the objective in term of haplotypes instead of individual variants. Even if these variants are not directly selected, detailed and continuous monitoring is of interest to make breeders aware of the frequency changes induced by selection and the resultant consequences on milk properties.

CONCLUSIONS

The expansion of genomic selection and large-scale genotyping provides us with new opportunities to revisit milk protein variant frequencies that can now be studied at a much larger scale. The use of custom SNP chips enables the study of targeted variants of interest and, with the imputation and phasing of genotypes now being routine, genotypes and haplotypes of these variants can be recovered for any genotyped animal. The approach used here can be applied to any variant or haplotype as soon as it is included in the SNP genotyping chips. Here we provide frequencies of milk protein variants and casein haplotypes from large populations of a wide range of French cattle breeds raised for different objectives (milk, dual-purpose, or even beef) and of different origins (Oceanic, Alpine and Iberic). With these results, we updated and built upon values estimated more than 30 yr ago and, for the first time in these breeds, we can assess the effect of indirect selection on milk protein variants. In the 2 major dairy breeds (HOL and MON), we observed a clear increasing trend in frequency of the *A* allele of β -LG and the *B* allele of the κ -CN. If the first is likely due to the selection for milk yield, the latter was not expected and may have different origins, including selection for milk protein content or direct selection on this allele. The *I* allele of the β -CN also showed increasing frequency in MON and NOR. These changes in frequencies may have major effects on milk properties. From haplotype information, we were able to predict the effect of selection for some alleles on other variants. In particular, we noted a clear risk of reducing κ -CN-*B* frequency (favorable to cheese-making properties) when selecting for β -CN-A2 in Holstein. Finally, detailed and continuous monitoring of milk protein genes is important to make breeders aware of the frequency changes induced by selection, with their consequences on milk properties.

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APPENDIX

Figure A1. Trends of the frequencies (%) of β -LG and α s1-CN variant alleles in bulls. Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

Figure A2. Trends of the frequencies (%) of β -CN and κ -CN variant alleles in bulls. Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

Figure A3. Trends of the frequencies (%) of β -LG and α s1-CN variant alleles in cows. Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Jersey (JER), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

Figure A4. Trends of the frequencies (%) of β -CN and κ -CN variant alleles in cows. Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Jersey (JER), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

Figure A5. Trends of casein haplotype frequencies (%) in bulls. Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).

Figure A6. Trends of casein haplotype frequencies (%) in cows. Blonde d'Aquitaine (BLA), Charolaise (CHA), Limousine (LIM), Abondance (ABO), Brown Swiss (BSW), Holstein (HOL), Jersey (JER), Montbéliarde (MON), Tarentaise (TAR), Normande (NOR), Simmental (SIM), and Vosgienne (VOS).