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Original article

Haplotype diversity of the *myostatin* gene among beef cattle breeds

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Abstract – A total of 678 individuals from 28 European bovine breeds were both phenotyped and analysed at the *myostatin* locus by the Single Strand Conformation Polymorphism (SSCP) method. Seven new mutations were identified which contribute to the high polymorphism (1 SNP every 100 bp) present in this small gene; twenty haplotypes were described and a genotyping method was set up using the Oligonucleotide Ligation Assay (OLA) method. Some haplotypes appeared to be exclusive to a particular breed; this was the case for 5 in the Charolaise (involving mutation Q204X) and 7 in the Maine-Anjou (involving mutation E226X). The relationships between the different haplotypes were studied, thus allowing to test the earlier hypothesis on the origin of muscular hypertrophy in Europe: muscular hypertrophy (namely nt821(del11)) was mainly spread in different waves from northern Europe milk purpose populations in most breeds; however, other mutations (mostly disruptive) arose in a single breed, were highly selected and have since scarcely evolved to other populations.

muscular hypertrophy / *myostatin* gene / haplotype diversity / beef cattle breeds

1. INTRODUCTION

The bovine muscular hypertrophy (***mh***) syndrome has been widely documented since it was first described [1,17,18,25]. It occurs at different frequencies in many European cattle breeds. It was first believed that the

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syndrome had the same origin and mutation [8,11] until recently. When the gene underlying the trait was identified as *myostatin* [14,21,24], it was found to have a surprisingly high number of polymorphisms [15]. Some changes at the nucleotide level truncate the protein product resulting in a lack of its expression and in the known muscular hypertrophy phenotype.

In the many cattle breeds in which it appears, the ***mh*** phenotype shows differences in intensity probably due to differences in selection pressure maintained during generations on related traits that vary depending on market necessities as well as on management requirements. In particular, many breeders have tried to limit the occurrence of dystocia, which decreases the economical value of the ***mh*** phenotype. As a consequence, some breeds which are well adapted to the binary local management and muscular hypertrophy (*e.g.* Asturiana de los Valles, Parthenaise or Blanc Bleu Belge), show a high frequency of the mutated gene in their population while others have a very low frequency of any mutation (Pirenaica or Bazadaise). Since a large pleiotropic effect is observed, genotype identification of the ***mh*** gene is important to avoid difficulties when selection on this gene is practised, whether fixation, elimination or introgression is desired.

Six disruptive mutations were described earlier [4,15,21] in different breeds and explain the most extreme phenotypes. However, some individuals in different breeds do show a phenotype not corresponding to their genotype at the known polymorphic sites.

To analyse all these cases and search all other possible mutations that could explain the different phenotypes, we screened the entire coding region and splice sites for mutations in the gene in a large number of breeds by using the Single Strand Conformation Polymorphism (SSCP) technique. This method makes the detection of previously described mutations easy and enables unknown mutations to be identified in new individuals. All the mutations identified were defined in haplotypes and were later grouped according to their possible link with particular phenotypes. An easy and fast method of genotyping based on an oligonucleotide ligation assay (OLA, [16]) which describes the complete *myostatin* genotype was used to screen the samples, allowing population analysis at the gene level. The greater the knowledge of this gene, the better the management will be, allowing other genes with smaller effects on muscular development to be identified. This study also used distance information estimated from the nucleotide differences between haplotypes to reconstruct the evolutionary history of the bovine populations, allowing the earlier hypothesis on the origin of muscular hypertrophy in Europe to be tested.

2. MATERIALS AND METHODS

2.1. Animals

A total of 678 animals from 28 breeds were used in this study; 4 Spanish breeds: Asturiana de los Valles (AV), Asturiana de la Montaña (AM), Rubia

Gallega (RG) and Pirenaica (PI); 12 French breeds: Aubrac (AU), Bazadaise (BZ), Blonde d'Aquitaine (BA), Bretonne Pie Noire (BR), Charolaise (CH), Gasconne (GA), Inra95 sire line (IN95), Limousine (LI), Maine-Anjou (MA), Normande (NO), Parthenaise (PA) and Salers (SA); 2 Belgian breeds represented by the Blanc Bleu Belge Mixte (BBB mixte) and by the Blanc Bleu Belge culard (BBB); 9 British breeds: Aberdeen Angus (AA), Ayrshire (AY), British Shorthorn (BS), Devon (DE), Galloway (GL), Hereford (HE), Traditional Hereford (HT), Longhorn (LH) and South Devon (SD) and 1 Italian: Marchigiana (MG). Amongst these individuals, a total of 505 individuals belonging to AV, AM, RG, PI, BZ, CH, GA, IN95, MA, and PA were previously phenotyped according to the Euromh (BIO4-CT98–0421) scoring system (Ménissier, unpublished results). Briefly, all animals were scored for three phenotypic traits: (1) *general body conformation* (animal general classification, muscling depth, back width); (2) *muscle expression* (shoulder muscle depth, *longissimus dorsi* and great dorsal hypertrophy, thigh circumference, croup slope, inter-muscular definition) and (3) *hypertrophy associated traits* (bone size, tail position, posture and gait, abdomen shape, skin type). Each class was scored from 0 (no hypertrophic signs) to 2 (high double muscling) and a general mean note called the Phenotypic score (PS) was produced. Although phenotypes often differ dramatically from one breed to another, this phenotype scoring allowed different sets of animals that belonged to four different categories (0–0.5; 0.5–1; 1–1.5; > 1.5) to be chosen for each breed.

2.2. SSCP

All 678 individuals were analysed with the SSCP technique, aimed at detecting all mutations present and not described, for posterior sequencing. DNA was extracted according to standard methods [19,32]. The SSCP (single stranded conformation polymorphism) [29,30] technique was used to allow sequence variants to be detected from migration shifts in PCR amplified fragments of the gene. PCR primers were designed to produce eight partially overlapping fragments of different lengths (238 to 419 bp) (Fig. 1) based on previous information ([12,13,15]; M. Georges unpublished information). The eight fragments were generated from four different duplex PCR combinations of primers (Tab. I) in a 50 μL total reaction volume containing 200 μM dNTPs, 2 mM Cl_2Mg, 1 U Taq (Biotools), 5 to 20 pmol of each primer and 100 ng DNA with the following PCR conditions: 94 °C 1′, 55 °C 1′ and 72 °C 1′ during 30 cycles initiated with 5′ denaturing (94 °C) and 5′ final extension at 72 °C. Three microliters of each PCR sample were mixed with an equal volume of denaturing loading buffer (0.05% xylene-cyanole, 0.05% bromophenol blue, 5.5 mM EDTA, pH 8.0, in deionised formamide), were heat-denatured at 95 °C for 5 min, and were snap-chilled on ice. The samples were then loaded

Table I. Primers used in multiplex for the amplification of the different fragments of the *myostatin* gene. Numbering of primers refer to fragment number (shown in Fig. 1). Below, mutated primers used in the PASA technique to define the haplotypes.

	Forward Primers	**Reverse Primers**
Multiplex A	2.1for 5′-GATTGATATGGAGGTGTTCG-3′	2.1rev 5′-CAACATTTGGGTTTTCCTTC-3′
	2.4for 5′-GAGCATTGATGTGAAGACAGTGTTG-3′	2.4rev 5′-ATAAGCACAGGAAACTGGTAGTTATT-3′
Multiplex B	2.2for 5′-ATTTATAGCTGATCTTCTAACG-3′	2.2rev 5′-AGGATTTGCACAAACACTGT-3′
	2.3for 5′-CTAGTAAAGGCCCAACTGTG-3′	2.3rev 5′-GCCATTCTCATCTAAAGCTT-3′
Multiplex C	1.1for 5′-ATTCACTGGTGTGGCAAGTTGTCTCTCAGA-3′	1.1rev 5′-TTGAGGATGTAGTGTTTTCC-3′
	3.1for 5′-GAAATGTGACATAAGCAAAATGATTAG-3′	3.1rev 5′-AGCAGGGGCCGGCTGAACCTCTGGG-3′
Multiplex D	1.2for 5′-GAACAGCGAGCAGAAGGAAAATGTGG-3′	1.2rev 5′-CCCTCCTCCTTACATACAAGCCAGCAG-3′
	3.2for 5′-TTGTATTTTTGCAAAAGTATCCTCA-3′	3.2rev 5′-ATACTCTAGGCTTATAGCCTGTGGT-3′
	Mutated Primers	**Common Primers**
F94L.mut	5′-CTCCTGGAACTGATTGATCAGTTA-3′	**2.4rev**
nt419.mutrev	5′-GTATTGTATCTTAGAGCTATTG-3′	**2.1for**
nt821.mutrev	5′-GCATCGAGATTCTGTCACAA-3′	**2.1for**
nt267.mut	5′-CCAAGGCTCCTCCACTCCTGGAG-3′	**2.4rev**
nt324.mut	5′-GCAGTGACGGCTCCTTGGAAGAT-3′	**2.4rev**
S105.mut	5′-AGAGATGCCAGCAGTGACGGCTG-3′	**2.4rev**

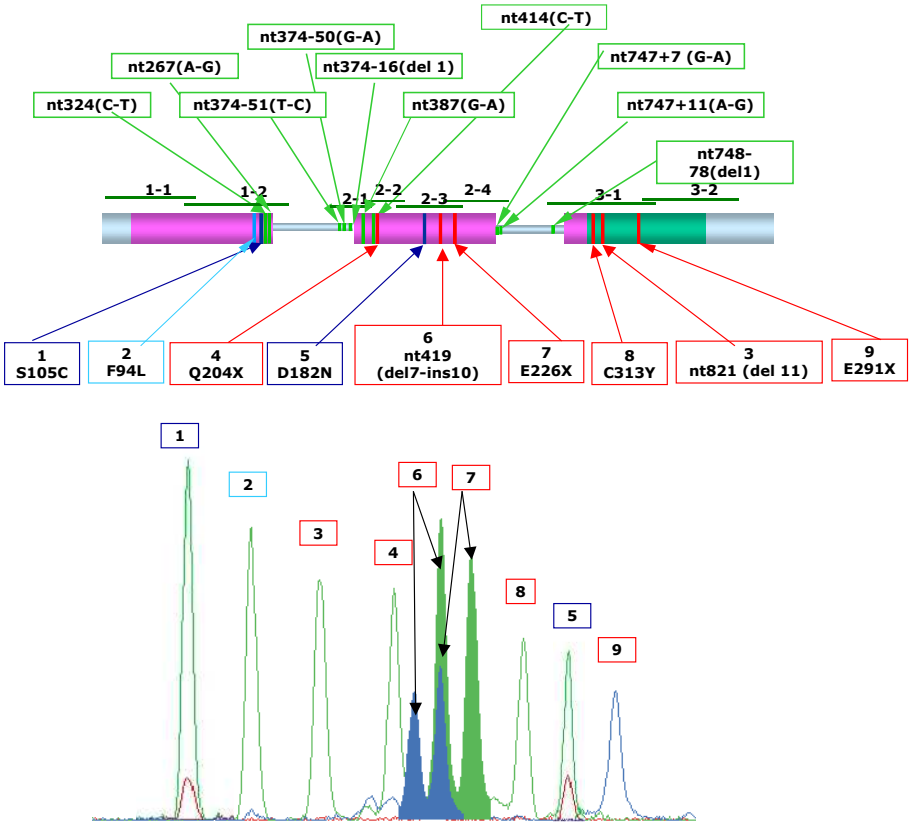


Figure 1. Myostatin gene. The top of the figure shows the three exons of the gene with the different mutations (silent are green, missense but not disruptive are blue and disruptive are red). The bottom of the figure shows the OLA image of an individual obtained either in a ABI PRISM 310 or 3700 automatic sequencer. Green peaks refer to wild type, and blue peaks refer to mutated alleles in the ligand. Red peaks belong to the TAMRA size standard. In this example, the individual is wild type for all mutations but also shows the mutated alleles for nt419(del7-ins10) and E226X, so it will be considered as double heterozygote for these two mutations, but wild homozygote for the rest. The numbers on the peaks refer to the mutation involved, indicated in the squares.

onto non-denaturing polyacrylamide gels using the Penguin Dual-Gel Water Cooled Electrophoresis System (OWL Scientific, Woburn, MA). A cooling device (Cooling Plus 8–30e, Heto-Holten A/S, 3450 Allerød, Denmark) kept the apparatus at the constant chosen temperature during the electrophoresis. Several variables were tested in order to achieve an optimum separation of the alleles: acrylamide concentrations (from 6 to 16%), acrylamide *bis* ratio (19:1, 29:1, 100:1), glycerol level (0%, 5% or 10%), electrophoresis temperature

(from 6 to 26 °C) and buffer conditions (0.5X or 0.6X). The bands were visualised by silver-staining according to the method of Bassam *et al.* [2] with minor modifications [3]. Silver-stained allele-specific SSCP bands were excised from the gel, placed in 100 μL of distilled water and subjected to 95 °C for 15 min, two freezing (−70 °C) and thawing cycles and then centrifuged at 10 000 × *g* for 2 min. Five microliters of the resulting solution were PCR re-amplified in 50 μL reactions using the corresponding primers of the excised fragments. The PCR re-amplified allele-specific SSCP bands were purified and sequenced in an ABI 310 DNA Sequencer using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer BioSystems) according to the manufacturer's instructions and were compared against known alleles for the bovine *myostatin* gene (GenBank Accession n° AF019620 and AF019761, M. Georges, unpublished results).

2.3. OLA reactions

The nine mutations which were chosen in this study (Fig. 1) changed an amino-acid and were previously screened or identified in this study [4, 15, 21]. For this purpose, the primers were designed to allow strand ligation to occur when the two probes matched exactly and thus producing a differentiated result which was easy to interpret. The test was based on the simultaneous amplification of the three *myostatin* exons using prior primers and conditions detailed elsewhere [28].

2.4. Haplotype definition

PCR amplification of specific alleles (PASA, [36]) was performed to generate fragments covering two exons (up to 2 kb). Six different allele specific primers (Tab. I) were necessary to solve the combinations of variants on a single chromosomal segment. Sequencing of the PCR fragments enabled the different haplotypes, found among the European breeds studied, to be defined. The evolutionary history between the different haplotypes was then analysed using the Kimura distance [23] and their graphic representation through the Neighbour Joining method.

3. RESULTS

Optimal SSCP electrophoresis conditions were obtained using a 12% 100:1 acrylamide-*bis* acrylamide ratio (in all cases but for multiplex D, where 16% gave the best results), 10 °C constant temperature (24 °C for multiplex D), 5% glycerol and 200 V constant voltage (4 W constant power for multiplex D). Electrophoresis was performed on both multiplex A and B for 7 h and on multiplex C and D overnight (17 h). These conditions optimised the discrimination

between the control alleles, allowing clear patterns corresponding to all but one (nt748–78(del1)) polymorphism described to date (figures not shown) [26,27]. This method allowed the identification of seven new mutations (Fig. 1), five being within the coding region. Amongst these mutations, two were missense mutations: the first mutation referred to as ***S105C*** resulted from a C → G transversion at coding nucleotide 314 in exon 1 and changed a Serine for a Cysteine found in the PA breed; the second mutation found in the MA breed and referred to as ***D182N*** resulted in a G → A transition at coding nucleotide 544 producing an amino acid change from Aspartic acid to Asparagine. Three other mutations were silent, two were identified in Exon 1 and referred to as ***nt267*** (A → G transition) and ***nt324*** (C → T transition) respectively, and the third (G → A) called ***nt387*** was found in Exon 2. All were found in the different French breeds: AU, BZ, SA for the first mutation, SA, AU, CH, IN95, MA for the second; the third mutation was found in different French and British breeds (AY, MA, SA, GL).

The other two new mutations located in the second intron and not affecting any coding sequence, were named ***nt747 + 7(G-A)*** and ***nt747 + 11(A-G)*** respectively, and were both found in British breeds. Most mutations described were transitions, so the average transition to transversion in the whole coding gene was 11:4. Transitions from G to A were more frequent (63%) than from C to T (36%). The nucleotide differences rate along the 1764 nt of the coding region including the splice sites was 10^{-2}.

A total of twenty distinct haplotypes were unambiguously defined by using a standard PASA technique and are shown in Table II. Figure 1 shows the typical image of the simultaneous OLA screening for nine mutations involved in the most frequent haplotypes and which have been classified as being important to explain the different phenotypes. Since each primer used in this genotyping method was specifically labelled, the interpretation of the haplotype was very simple, each mutation being differentiated both by range and by colour as shown in Figure 1. Once the animals were genotyped using the SSCP analysis, all those with available phenotypic scores (a total of 505) were classified according to the four categories shown in Table III. In some breeds, *e.g.*, RG, CH, GA, IN95 or MA, the phenotype was clearly explained by the *myostatin* genotype, but for a large number of individuals of the AV and PA breeds the observed phenotype was different from that expected from the genotype.

4. DISCUSSION

4.1. New mutations

The high level of polymorphism already described for the *myostatin* gene in humans [12] and cattle [15] was confirmed when a large number of individuals

Table II. Haplotypes found in the myostatin gene when screening a total of 28 European bovine breeds.

	nt267(A-G)	F94L	S105C	nt324(C-T)	nt374–51(T-C)	nt374–50(G-A)	nt374–16(del 1)	nt387(G-A)	nt414(C-T)	nt419(del7-ins10)	D182N	Q204X	E226X	nt747 + 7(G-A)	nt747 + 11(A-G)	nt748–78(del 11)	nt821(del11)	E291X	C313Y
1		+			+														
2																	+		
3					+														
4					+	+	+		+							+			
5					+	+	+		+			+				+			
6					+														+
7													+						
8				+	+														
9																			
10					+	+	+		+	+						+			
11		+			+													+	
12			+		+														
13					+	+	+	+	+							+			
14					+						+								
15	+				+	+	+		+							+			
16	+				+														
17				+	+	+	+		+							+			
18					+										+				
19					+									+					
20														+			+		

randomly sampled from different European bovine breeds were screened. The average number of SNP in human genes has been found to be 1 out of every 185 bases [37] and was larger here (1 out of every 100 bases) which suggests that the high level of polymorphism shown in this gene is the result of the selection of new occurring mutations thus increasing the variability at this locus.

All known mutations were identified and in addition seven new polymorphisms were described. Two of these were missense mutations in the first two exons, which are spliced into the mature peptide. Since *myostatin* is a member of the TGFβ family where nine cysteines are highly conserved across members

Table III. Genotype and phenotype association in 13 different European bovine beef breeds. Genotype is represented both by haplotype frequencies (only the most relevant are shown) and by presence or absence of any disruptive mutation. Phenotype is measured through the Phenotypic Score (PS) ranging from 0 to 2 and classified in four categories.

Haplotype frequencies (%)								Phenotype	PS ≤ 0.5			0.5 < PS ≤ 1			1 < PS ≤ 1.5			1.5 < PS ≤ 2		
1	2	3	4	5	6	7	10	Genotype	+/+	+/mh	mh/mh	+/+	+/mh	mh/mh	+/+	+/mh	mh/mh	+/+	+/mh	mh/mh
1	90	2	6					Asturiana Valles			1		5	10		2	12		2	12
	10	40	46					Asturiana Montaña	20	5										
2	69	16	12					Rubia Gallega				2	4	3	2	13	16			
44	25	22					5	Pirenaica							18	13	2	2	3	2
		100						Bazadaise	15			9			19			5		
	49	25	20					BBBelge mixte	1	26			16			9				
5	2	89		3				Blonde d'Aquitaine										30	3	
8		23		62				Charolaise	10	1						1	1		3	14
		25	16		59			Gasconne	10	10			1			1	3	1	2	17
100								Limousine										9		
8	7	31		50	1		1	Inra 95				8	12			8	9			6
		27				52	16	Maine-Anjou	6	11							8			18
2	87					7	1	Parthenaise		3	22		1	15			12			

of the gene family and among species, it would be interesting to study the influence of the addition of a tenth cysteine residue in individuals carrying the S105C mutation. The additional cysteine residue may influence intra- and inter-peptide binding and have an effect on function and hence on the phenotype of the bearing individual (work currently in progress).

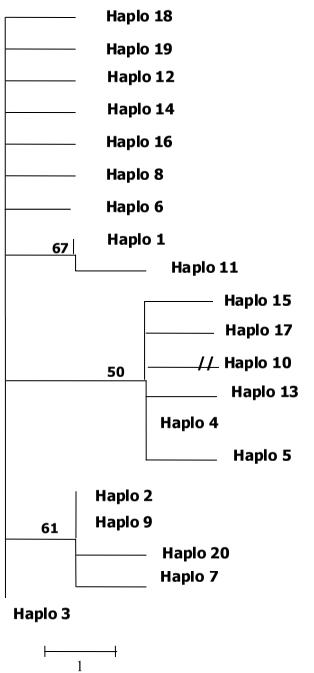
4.2. OLA

The OLA technique was set up according to the procedure of Grossman [16] and Karim [22], with some modifications which allowed flexibility to screen all different mutations as well as other polymorphisms which may be found in the future. Nine mutations (seven already described, [4, 15] and two mutations detected using the SSCP technique), were selected for screening by the OLA technique. From these mutations six were disruptive, and three produced an amino acid change affecting Exon 1 and 2. The reason for including these mutations was to confirm that when the protein adopts a particular structure, it will have a different activity which will result in a different phenotype.

4.3. Haplotypes

Grobet *et al.* (1998) [15] described the wild type as the haplotype lacking any mutation (haplotype 9 in this study) as found in the Holstein breed. However, here we found that the Holstein breed shows a significant high frequency of haplotype 4 (data not shown), in concordance with the results of Smith *et al.* (2000a) [34]. Moreover, haplotype 3 exclusively bearing the nt374–51(T-C) mutation was found in 34% of the total meiosis analysed (Fig. 2) and should then be considered as the wild type allele, being much more frequent than haplotype 2 (26% and the most frequent among disruptive haplotypes), haplotype 4 (10.5%) and haplotype 9 (1.5%). Also, it should be noted that the unique mutation present in haplotype 3 was found in nearly all breeds.

The disruptive haplotypes other than haplotype 2 were present in a much lower frequency across several breeds and some were exclusive of one or a few related breeds as is the case of haplotype 7 only found in the Maine-Anjou or haplotype 5 in the Charolaise. Some of the disruptive haplotypes were never associated with other mutations, for example nt821(del11) (haplotype 2) and E226X (haplotype 7) and also among the breeds, some haplotypes never combined (1 and 4 for example) or conversely were frequently found together (2 and 9) also indicating the history of the breeds. Some breeds showed a high polymorphism at the *myostatin* gene as for example PI probably due to its condition as a frontier breed intensively crossed with BA individuals, or the British LH breed [33] where a surprisingly high level of different haplotype combinations can be found in such a small breed. In other breeds however,



HAPLOTYPES	Asturiana Valles	Asturiana Mont.	Pirenaica	Rubia gallega	Aubrac	Bazadaise	Blonde Aquitaine	Bretonne PieNoire	Charolaise	Gasconne	Limousine	Inra95	Maine-Anjou	Normande	Parthenaise	Salers	Aberdeen-Angus	Ayrshire	Beef Shorthorn	Devon	Galloway	Hereford	Hereford trad.	Longhorn	South Devon	BBB mixte	BBB culard	Marchigiana
18																									3			
19																								1				
12															2													
14													2															
16							1									1												
8					1				2			1	1			2												
6										63		1																
1	1		35	2	24		3		8		18	7			2					3	1							
11																												4
15					1																							
17																1												
10			4									1	15		1													
13													1			2		1			2							
4	5	23		10	9			3	3	16			1			18	3	5		2	5	7	2	3	7	21		
5							2		58			43			1													
2	79	5	20	55	10		1		1			6		2	96									2	14	51	10	
9	1	2	3		1											1							3	1	3	6		
20																				1				1				
7													49		8													
3	2	20	18	13	8	98	59	7	22	27		27	25	32		25	7	4	10	4	2	3	5	2	13	26		

Figure 2. Frequencies of the 20 haplotypes in the 28 European bovine breeds considered and the relationships between the different haplotypes deduced from the complete coding sequence using Kimura distance and the construction of a Neighbour Joining tree. Bootstrap values higher then 50 are shown. Scale bar refers to the number of nucleotide substitutions per site.

there was a very low variability (*e.g.*, CH or GA), reflecting a high selection on this gene.

Rare haplotypes are more likely to be recently derived than those that are frequent [39], and if we observe the haplotype pairs 15 and 16, 8 and 17, 19 and 20 we will see that they share rare but also more common mutations; this can be the result of recombination events between existing haplotypes or show that some sites have undergone repeated mutational events (Fig. 2).

When observing the non-disruptive haplotypes, haplotype 1 appeared in the Aubrac, Limousine and Pirenaica breeds; the latter two breeds were surprisingly those in which most individual phenotypes are not explained by a disruptive mutation in the *myostatin* gene. This indicated a higher phenotypic influence than expected for a conservative mutation (work currently in progress).

4.4. Introducing the phenotype

The pattern of haplotype sharing is an indicator of the history of the different bovine populations, or breeds, so the distribution of shared haplotypes is very useful to investigating population relationships. In the last century, different explanations on the origin of the double-muscled phenotype in different continental beef breeds were proposed. One hypothesis is the extensive dissemination of individuals of the Shorthorn breed used in the late 19th century to improve most western European bovine breeds which would explain the presence of the trait [10,25], and the other being the Friesian breed [9,20,31] or more generally milk purpose black pied bovine populations from the Baltic plain (Hanset, pers. comm.), being responsible for spreading the mutation all over western Europe [25]. In order to test the hypothesis of the introduction of the double muscling alleles from a single breed, we studied the relationships between the different haplotypes deduced from the complete coding sequence, using the Kimura distance (which allows to consider transitions as well as transversions) and the construction of a Neighbour Joining tree (Fig. 2). The examination of these relationships between the haplotypes showed haplotype 3 as the wild type from which all mutations have arisen in four defined groups: the first cluster was missing intronic 374–51 (haplotypes 2, 9, 7 and 20); the second cluster groups haplotypes 4, 5, 10, 13, 15 and 17 by sharing a set of intronic mutations found together (nt374–51, nt374–50, nt374–16 and nt414), a third group included haplotypes 1 and 11, and finally the fourth was integrated by 19, 12, 18, 14, 6, 8 and 16. Among these four groups, one set of "old" haplotypes appeared at equivalent times, that is those integrating the last group and also 1, 2 and 9, while haplotypes 7, 20, 4 and 11 arose later and more recently those haplotypes belonging to the second cluster, with 10 being the most recent (Fig. 2).

The consideration of these relationships between haplotypes should make the evolution of the *myostatin* gene easier to understand. The existence of many different haplotypes that are not rooted in a common mutated ancestor seems to definitely refute a Shorthorn-Durham origin for the muscular hypertrophy phenotype. Although there was an important introduction of individuals belonging to this breed across Europe at the end of the 19th century and at the beginning of the 20th, especially in grassland (oceanic) territories, the analysis of the *myostatin* gene of this breed at the present indicates the lack of any mutation. However, the theory of a founder mutation (nt821(del11)) spreading from an epicentre localised in the Friesian or Black Pied breed, much before its large specialisation into milk production seems much more congruent. There is much evidence of a hypertrophic phenotype in this population [20,38] before the organisation of the breed and selection for high milk specialisation and before the migration of this population into several milk breeds, (*e.g.*, Normande or Parthenaise) before 1950. It is important to note that during these years, most breeds in Europe were dual purposes, and they have only recently been selected specifically for beef or milk production. This can explain the introduction of haplotype 2 from the Friesian breed to dairy (Normande), and beef breeds (Aubrac, Blanc Bleu Belge, Parthenaise, Asturiana de Valles, and Rubia Gallega), after a large diffusion of this breed before the nineteen-fifties and later. In an attempt to improve the beef characters of some breeds, the selection of individuals that were heterozygotes for the disruptive mutation may have occurred through all northern and western Europe including Spain, France, the Netherlands, Belgium, Germany and Austria. For instance, the Moyennne and Haute Belgique breed, issued from this milk purpose population, became the Blanc Bleu breed through fixation of mutation nt821(del11) and later selection for ***mh*** expression [17,18]. This phenomenon has been performed through different waves made evident when observing the large linkage disequilibrium found in Asturiana de Valles showing a more recent introduction [11]. Other breeds have been left apart from these migratory movements: this is the case for the Maine-Anjou, Charolaise and Gasconne located geographically in the French continental grasslands, in which particular mutations arose later with no spreading to the surrounding populations, or even in some cases (*e.g.*, Limousine) have never appeared. According to this hypothesis, the results found for the British breeds were congruent since only individuals belonging to the South Devon showed a disruptive mutation corresponding to haplotype 2 which could have been introduced from exchanges with the Friesian or Black pied breed which have been highly documented in this country [20].

4.5. Phenotype-genotype comparison

When genotypes and phenotypic scores (Tab. III) are correlated, it seems most likely that other loci might play a role in the development of the double

muscled phenotype. The high level of incomplete penetrance or variations in modifier genes present in some breeds like PA (37 individuals of 53) or AV (11 individuals of 44) showed that the expression of the muscular hypertrophy phenotype does not arise from a single disruptive mutation in a major gene. The high variability of phenotypic scores (ranging from 0 to 1.74 in the Charolais culard strain for example) seems to confirm ([11,15]) the theory of a loci heterogeneity.

Given the high influence of the *myostatin* gene in the good muscular conformation of most European bovine beef breeds, and although there have been many efforts to identify other influencing genes and QTLs with smaller effects [5–7,35], it seems necessary to systematically screen most individuals in order to allow the management of this particular locus, and to possibly evaluate the effect of this and other genes in the phenotype.

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