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Simultaneous genotyping to detect myostatin gene polymorphism in beef cattle breeds

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Summary

The myostatin gene codes for a growth factor involved in muscle development, and polymorphism in this gene can have important economic consequences. Nine mutations affecting the amino-acidic sequence have already been described, six of which are disruptive, inactivating the protein and causing bovine muscular hypertrophy. As the number of known mutations grows, it is necessary to develop a simple, routinely usable technique able to screen individuals in all populations. The oligonucleotide ligation assay (OLA) is proposed here for the rapid genotyping of the nine mutations known affecting the coding sequence in the main breeds of beef cattle. This technique showed its ability to reveal the genotype of individuals being a good tool to determine the frequency of each mutation in a population. The procedure is very flexible as new mutations can be added and removed at any time. Depending on the genotype of each individual, the technique allows breeders to make quick decisions on matings and general selection tendencies.

Zusammenfassung

Simultane Genotypisierung von Polymorphismen im Myostatin-Gen in Fleischrinderrassen

Das Myostatin-Gen kodiert für einen Wachstumsfaktor, der in die Muskelentwicklung eingebunden ist und Polymorphismen in diesem Gen können daher wichtige ökonomische Konsequenzen haben. Bisher wurden neun Mutationen, die Auswirkungen auf die Aminosäuresequenz haben, beschrieben. Sechs davon inaktivieren das Protein und verursachen bovine muskuläre Hypertrophie. Da die Anzahl der bekannten Mutationen in diesem Gen steigt, ist es notwendig, eine einfache, in der Routine einsetzbare Methode zu entwickeln, um Individuen in allen Populationen untersuchen zu können.
Zur schnellen Genotypisierung der neun bekannten Mutationen, welche die kodierende Sequenz in den Hauptfleischrinderrassen betreffen, wird hier der Oligo-Ligationsassay (OLA) vorgeschlagen. Durch diese Technik ist es möglich, den Genotyp jedes Individuums und die Frequenz jeder einzelnen Mutation in der Population festzustellen. Die Prozedur ist sehr flexibel, da zu jedem Zeitpunkt neue Mutationen hinzugefügt bzw. weggelassen werden können. Diese Methode erlaubt dem Züchter, in Abhängigkeit vom Genotyp jedes Individiums schnelle Entscheidungen über die Anpaarung und die allgemeine Selektionsrichtung zu treffen.

Introduction

Since its first appearance (Culley 1807), bovine muscular hypertrophy has extended widely among European beef cattle. The phenotype mainly involves increased muscle development (giving rise to the name *double muscling*), leading to greater growth rates and carcass value (Ménissier 1982a). In many populations where the trait appears, extreme expression may or may not be desirable, depending on local economic circumstances (which include the distocia prevalence) and as a consequence the management of muscular hypertrophy phenotypes can vary. The myostatin gene has been identified as the locus

underlying the character, and several inactivating mutations of this gene are responsible for the phenotype. These studies showed there to be a surprising genetic variability, in disagreement with the classical explanation of a single mutation introduced into European bovine populations by the Shorthorn breed (Ménissier 1982b). Six different disrupting mutations were known until recently (Fig. 1): C313Y, nt821 (del11), nt419 (del7-ins10), Q204X, E226X and E291X (Cappucio et al. 1998; Grobet et al. 1998), in addition to a conservative missense mutation, F94L. After studying 28 European cattle breeds, two new missense mutations, known as S105C and D182N have also been described (Miranda et al. 2000).

The phenotypic influence of some mutations, although studied in several populations (Casas et al. 1999; Keele and Fahrenkrug 2001) remains unclear, making the generalized mutation diagnostic necessary to deepen on their phenotypic effects. The oligonucleotide ligation assay (OLA; Grossman et al. 1998) is a simple and rapid genotyping method based on the ligation of two consecutive probes which specifically hybridize to a target sequence (in this work the three amplified exons of the myostatin gene). The technique might be regarded as an optimal solution for the screening of important myostatin polymorphisms in different beef populations, and should provide breeders with the necessary information to make decisions on the best management solutions for their circumstances.

Materials and methods

DNA was extracted from blood samples using a standard protocol (Jean-Pierre 1987; Sambrook et al. 1989). The three exons of the myostatin gene were simultaneously amplified by Polymerase chain reaction (after a 5 min denaturation step at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, followed by a final extension of 15 min and a 45 min extra step at 99°C to inactivate residual polymerase activity) by adding 40 ng DNA, 3 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 μM of each primer and 0.5 U Taq DNA Polymerase (Promega Corporation, Madison, WI, USA) to the final reaction. The primers used are listed in Grobet et al. (1998) and Karim et al. (2000).

All nine mutations are detected using a single OLA assay. Three probes were designed for each: a common 3′ probe phosphorylated at both ends, and two allele-specific 5′ probes labelled with distinct fluorophores (Table 1). The ligands migrate differently depending on their conformation, allowing the simultaneous screening of mutations in an automatic capillary sequencer. After hybridizing to 5 μl of the previously amplified fragments, the different probes (30–100 fmol) were simultaneously allowed to ligate (5 U of T4 DNA

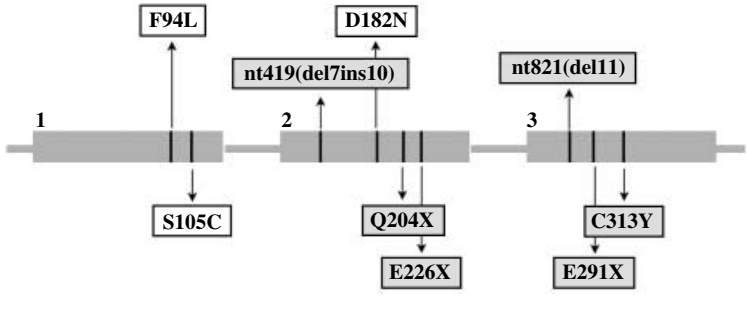

Fig. 1. The three exons of the myostatin gene and the location of the studied mutations are shown. Disruptive mutations are shown in grey boxes. Missense mutations which do not inactivate the protein are shown in white

Table 1. **Probes used in a single OLA assay**

Probes	Sequence	Size (bp)	Electrophoretic size
S105C-Hex	ATG CCA GCA GTG ACG GCT C	19	35
S105C-Fam	ATG CCA GCA GTG ACG GCT G	19	34.1
S105C-2p	p CTT GGA AGA CGA TGA CTA CCA CG p	23	
F94L-Hex	TCC TGG AAC TGA TTG ATC AGT TC	23	37.3
F94L-Fam	TCC TGG AAC TGA TTG ATC AGT TA	23	36.7
F94L-2p	p GAT GTC CAG AGA GAT GCC AGC p	21	
nt821-Hex	GGC TTG ATT GTG ATG AAC ACT CC	23	40
nt821-Fam	GAG AGA TTT TGG GCT TGA TTG TG	23	39.4
nt821-2p	p ACA GAA TCT CGA TGC TGT CGT TA p	23	
Q204X-Hex	AAC CCA GGC ACT GGT ATT TGG C	22	43
Q204X-Fam	AAC CCA GGC ACT GGT ATT TGG T	22	42.5
Q204X-2p	p AGA GCA TTG ATG TGA AGA CAG TGT TG p	26	
nt419-Hex	AAC CCA AAT GTT GTT TCT TTA AAT T	25	45
nt419-fam	CCA AAT GTT GTT TCT AAG CAT ACA A	25	44.2
nt419-2p	p TAG CTC TAA GAT ACA ATA CAA TAA ACT p	27	
E226X-Hex	AAC AAC CTG AAT CCA ACT TAG GCA TTG	27	46
E226X-Fam	AAC AAC CTG AAT CCA ACT TAG GCA TTT	27	45.4
E226X-2p	p AAA TCA AAG CTT TAG ATG AGA ATG G p	25	
C313Y-Hex	GGC CAA TTA CTG CTC TGG AGA ATG	24	48
C313Y-Fam	GGC CAA TTA CTG CTC TGG AGA ATA	24	47.3
C313Y-2p	p TGA ATT TGT ATT TTT GCA AAA GTA TCC TCA p	30	
E291X-Hex	TGC TGT CGT TAC CCT CTA ACT GTG GAT TTT T	31	52.7
E291X-Fam	TGC TGT CGT TAC CCT CTA ACT GTG GAT TTT G	31	51.9
E291X-2p	p AAG CTT TTG GAT GGG ATT GGA TTA TT p	26	
D182N-Hex	TCC TGA GAC TCA TCA AAC CCA TGA AAG	27	49.7
D182N-Fam	TCC TGA GAC TCAT CAA ACC CAT GAA AA	27	48.5
D182N-2p	p ACG GTA CAA GGT ATA CTG GAA TCC GAT CT p	29	

Three probes are necessary for each mutation: a common 3′ probe (2p), and two 5′ probes, one specific for the wild allele (labelled Hex) and the other for the mutated allele (labelled Fam). The size of each probe and the electrophoretic migration size of all allele-specific ligands are shown.

Ligase-Promega Corporation, Madison, WI, USA) in a final 10 µl volume, following a 2 min denaturation step (35 cycles of denaturation [94°C, 3 s] and ligation [60°C, 3 min], and a final 10 min 99°C inactivating step).

Twenty microlitre formamide and 0.5 µl size standard (Gene Scan®-350 size standard 350, PE Applied Biosystems, Foster City, CA, USA) were mixed with 1 µl of the ligation reaction products. After 5 min denaturation they were then loaded into an automatic capillary sequencer (ABI PRISM™ 3700 or 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA, USA) and interpreted using Genescan software (PE Applied Biosystems, Foster City, CA, USA).

Results and discussion

Other genotyping methods have been proposed for one (Antoniou et al. 1999) or more myostatin mutations (Fahrenkrug et al. 1999). The OLA technique has already proved its worth in the diagnosis of myostatin mutations (Karim et al. 2000), but some modifications are here proposed as avoiding the use of expensive spacers and the adaptation to capillary electrophoresis. Furthermore this new approach with more polymorphisms (including two newly described) provides more information when analysing beef cattle populations.

Several mutations can be diagnosed thanks to the variable size of the ligands, which differ at least in one nucleotide. The discrimination between the two possible alleles of a mutation is based on the fluorophore labelling: green for the wild allele and blue for the

Table 2. **Control samples genotypes**

	Control samples genotypes																
Mutations	AV 1	AV 2	PA 1	PA 2	PA 3	CH 1	IN 1	IN 2	MA 1	MA 2	MA 3	PI 1	PI 2	PI 3	PI 4	MR 1	GA 1	GA 2
S105C			+/−															
F94L								+/ −				+/−		+/−	+/+	+/+		
D182N											+/−							
nt419									+/−				+/−	+/−				
E226X				+/−					+/−	+/+								
Q204X					+/−	+/+	+/−	+/−										
nt821	+/ −	+/+	+/−	+/−	+/−							+/−	+/−					
C313Y							+/−										+/−	+/+
E291X																+/+		

Eighteen samples belonging to the breeds: Asturiana de Valles (AV), Charolaise (CH), Parthenaise (PA), INRA 95 (IN), Maine Anjou (MA), Pirenaica (PI), Marchigiana (MR) and Gasconne (GA) have been used as control genotypes. The presence of the different mutations is indicated as (+/+) in mutated homozygotes, (+/−) for heterozygotes and empty for wild type homozygotes.

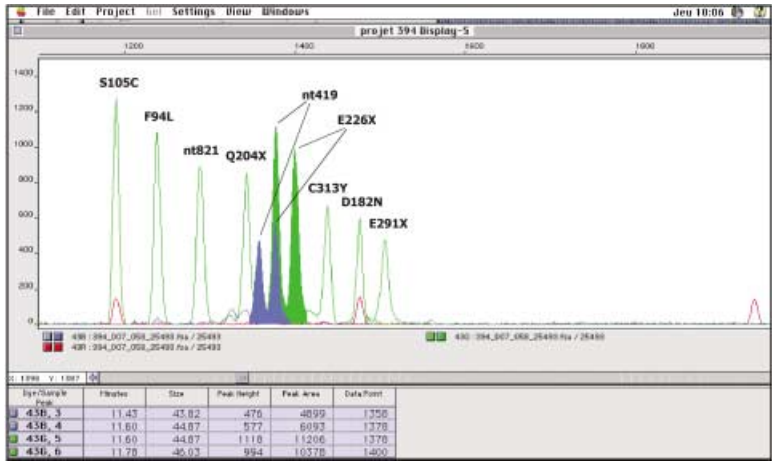

Fig. 2. OLA image of an individual heterozygous for two mutations (nt419 and E226X) obtained in a ABI PRISM 3700 automatic sequencer. The green peak refers to wild type and blue peaks refer to mutated alleles. Red peaks belong to the TAMRA size standard

mutated allele. The assay is internally controlled by the necessary detection of at least one of the alleles for each mutation.

To validate its accuracy a total of 18 specimen representatives of eight breeds (Asturiana de los Valles, Parthenaise, Charolaise, INRA95, Maine Anjou, Pirenaica, Marchigiana and Gasconne) were previously genotyped by sequencing and used to provide positive and negative genotype controls for all nine mutations (Table 2). Results of both techniques agreed perfectly, demonstrating the capacity of the OLA assay to effectively genotype the nine selected mutations. Fig. 2 shows a typical capillary electrophoresis image of an OLA analysis.

The analysis described is an effective genotyping tool which can be used with the different beef cattle breeds showing the most important genetic variability at the myostatin gene level. When the correlation between some mutations of this gene and expression of the character is required, this assay is an excellent tool: it screens populations quickly (each electrophoresis takes only 20 min), is flexible (allowing the introduction of new mutations at any time), and it provides information useful when taking decisions on matings. Following its refinement, the technique is now offered by G.I.E. LABOGENA (Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France), allowing breeders to obtain more information on the genetic composition of their populations at the myostatin gene level. Selection for or against certain genotypes and their expression can then be undertaken, and specific mating programmes designed depending on each breeder's needs. The systematic use of this assay in different breeds will help in the management of the myostatin gene and will facilitate the on-going knowledge of other loci implicated in growth traits.

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