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A. Soumillion, J.H.F. Erkens, Johannes A. Lenstra, G. Rettenberger ...+1 more authors

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Soumillion, Ann; Erkens, Jo H. F.; Lenstra, Johannes A.; Rettenberger, Günther; Te Pas, Marinus F. W.

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## Genetic variation in the porcine myogenin gene locus

Ann Soumillion,<sup>1</sup> Jo H.F. Erkens,<sup>1</sup> Johannes A. Lenstra,<sup>2</sup> Günther Rettenberger,<sup>3</sup> Marinus F.W. te Pas<sup>1</sup>

<sup>1</sup>DLO-Institute for Animal Science and Health (ID-DLO), P.O. Box 65, 8200 AB Lelystad, The Netherlands

<sup>2</sup>Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands

<sup>3</sup>Swiss Federal Institute of Technology, Department of Animal Science, ETH-Zentrum TAN, 8092 Zurich, Switzerland

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**Abstract.** The myogenin (*MYOG*) gene fulfills a key function in muscle differentiation by controlling the onset of myoblast fusion and the establishment of myofibers. In meat-producing animals like pigs and cattle, myofiber numbers have been related to growth capacity. We have characterized the porcine *MYOG* gene to detect genetic variation at this locus and to relate it to growth characteristics. *MYOG* gene fragments were isolated by PCR on genomic DNA and by screening a genomic library with a mixture of the four human MyoD cDNA fragments. Both the exons and promoter region were very similar to the human and mouse genes. Southern blot analysis of 105 unrelated pigs revealed three polymorphic *MspI* sites, located in the promoter region, the second intron, and at the 3' side of the gene. PCR-RFLP tests detecting four *MYOG* alleles were developed. PCR analysis of a panel of pig-rodent somatic cell hybrids confirmed the genetic localization of *MYOG* on pig Chromosome (Chr) 9. The PCR-RFLP tests and microsatellite markers on Chr 9 offer the possibility to genotype large numbers of pigs for studies of genetic linkage to meat deposition and growth characteristics.

### Introduction

The MyoD gene family consists of four structurally related genes, *MYOD1*, *MYOG*, *MYF5*, and *MYF6*. These genes encode basic helix-loop-helix (bHLH) proteins and are involved in muscle cell determination and differentiation in vitro (Olson 1990; Weintraub et al. 1991) as well as in vivo (Lyons and Buckingham 1992). *MYOG* is the only MyoD gene expressed in all skeletal muscle cell lines (Wright et al. 1989; Edmondson and Olson 1989). Knock-out experiments in mice have shown that this protein fulfills an essential function in muscle differentiation by regulating the onset of myoblast fusion and the formation of functional muscle fibers (Hasty et al. 1993; Nabeshima et al. 1993).

Breeding in meat-producing animals focuses on growth and lean meat deposition. In pigs, the number of muscle fibers at birth appears to determine the maximal lean meat growth capacity (Händel and Stickland 1984, 1988). Meat production capacity seems to be determined also in cattle by the number of embryonically developed myocytes, since double-musled cattle show a higher number of prenatally developed myofibers than other cattle (Swatland and Kiefer 1974; Hanset et al. 1982). The central role of *MYOG* in the process of muscle differentiation suggests that genetic variation in this locus may be associated with differences in myoblast and myofiber numbers. To initiate the investigation into

the influence of different *MYOG* alleles on the muscle development and growth characteristics of pigs, we have isolated genomic *MYOG* fragments and characterized four haplotypes based on *MspI* sites. A fast PCR-RFLP test now offers the possibility to perform association studies with growth characteristics (Te Pas and Visscher 1994).

### Materials and methods

**Isolation of genomic DNA.** Genomic DNA was isolated from EDTA-treated blood samples and stored at  $-80^{\circ}\text{C}$ , following standard procedures (Sambrook et al. 1989).

**PCR amplification of genomic *MYOG* fragments.** *MYOG*-specific PCR amplifications were performed on 50 ng genomic DNA in 50- $\mu\text{l}$  reactions containing 1.25 U *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.) or 0.2 U Super Tth polymerase (SphaeroQ, Leiden, The Netherlands) in the appropriate buffer, 20 pmole of both primers, and 0.2 mM of each dNTP (Boehringer Mannheim, Germany). Primer sequences and reaction conditions are specified in Table 1.

**Cloning and sequencing of *MYOG* fragments.** PCR1 and PCR2 amplification products (Table 1) were cloned into pUC18 or pBS<sup>-</sup> plasmid and sequenced by standard molecular biology technology (Sambrook et al. 1989).

**Screening of a porcine genomic library.** A total of  $0.5 \times 10^6$  plaque-forming units (pfu) of a porcine genomic library in the phage vector EMBL3/SP6/T7 (Clontech Laboratories Inc., Palo Alto, Calif.) were screened by hybridization to a mixture of the four radioactive labeled human MyoD cDNA fragments (ATCC, Rockville, Md.; Braun et al. 1989a, 1989b, 1990). Positive clones were isolated by three successive rounds of plaque purification. Phage DNA was isolated according to the manufacturer's recommendations by standard molecular biology technology (Sambrook et al. 1989).

*MYOG*-containing phage clones were identified by PCR amplification on 1  $\mu\text{l}$  of 1:1000 dilution of the DNA stocks, by use of PCR1 or PCR3 (Table 1). Fragments of two phage clones were subcloned for sequence analysis by standard molecular biology technology (Sambrook et al. 1989).

**Southern blotting and RFLP analysis of porcine genomic DNA.** Ten microgram of porcine genomic DNA was digested with 100 U *MspI* (Boehringer) for 16 h at  $37^{\circ}\text{C}$ , electrophoresed in a 0.8% (wt/vol) agarose gel, and transferred to positively charged nylon membranes (Boehringer) by Southern blotting (Southern 1975). Membranes were hybridized to radioactive labeled *MYOG* genomic fragments following the manufacturer's instructions.

**Mapping and PCR-RFLP detection of polymorphic *MspI* sites.** *SacI* subclones of *MYOG* phage clones were digested with *MspI* to determine the presence and localization of the *MspI* sites. Sequences flanking the *MspI* sites were determined. PCR tests were developed for the amplifica-

Correspondence to: M.F.W. te Pas

The nucleotide sequence data reported in this paper have been submitted to the EMBL data base and have been assigned the accession numbers X89007 (porcine myogenin gene), X89209 (3' polymorphic site), and X89210 (CA-repeat).

**Table 1.** Primer sequences, annealing temperatures, and fragment characteristics for PCR amplification reactions of different regions of the pig *MYOG* gene.

Amplified region	Primer sequences <sup>a</sup>	Annealing temperature (°C)	Predicted size of amplified product (bp)
PCR 1 Coding regions and introns	FW: 5' - GGAATTCATGGAGCTGTATGARACATC REV: 5' - GGAATTC AATCTCAGTTGGGCATGGT	61	2099
PCR2 Exon 1	FW: 5' - GGAATTCATGGAGCTGTATGARACATC REV: 5' - TCRGCTCCTCCTGGTTGA	61	442
PCR 3 Exon 1-Exon 2	FW: 5' - CAACCAGGAGGAGCGGATCTCCG <sup>b</sup> REV: 5' - AGGCGCTGTGGGAGTTGCATTCACT <sup>b</sup>	59–61	871
PCR4 3'-SIDE	FW: 5' - TCAGGAAGAACTGAAGGCTG REV: 5' - GTTTCCTGGGGTGTTC	60	353
PCR 5 5'-SIDE	FW: 5' - TCTTGACCTTGTATTGTGG REV: 5' - CTTCTCACACCACCTTAC	60	364
PCR 6 5'-SIDE	FW: 5' - GAGTCTCATCTGACTGACAC REV: 5' - TCRGCTCCTCCTGGTTGA	60	990

<sup>a</sup> FW: forward primer; REV: reverse primer.

<sup>b</sup> Montarras et al. (1991).

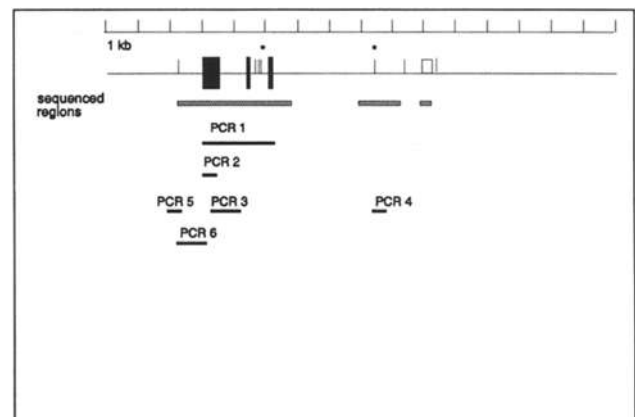
tion and detection of these sites: PCR4 detecting a polymorphic *MspI* site at the 3' end of the gene, PCR5 detecting a nonpolymorphic *MspI* site in the promoter region, and PCR6 amplifying a promoter fragment diagnostic for the 2.3-kb allele. Ten microliter of the amplification products were digested with 10 U *MspI* enzyme at 37°C and analyzed on an agarose gel.

**Chromosomal localization by somatic cell hybrid analysis.** A pig-rodent hybrid cell panel consisting of 21 hybrids has been established and characterized by QFQ-banding, FISH, and PCR, as previously described (Rettenberger et al. 1994a, 1994b, 1994c, 1995b). This cell panel was tested for the presence of the *MYOG* gene by PCR3, and the distribution pattern of these signals was compared with the distribution pattern of the porcine reference loci for each hybrid. The percentage of hybrids that are identical for the presence of a reference locus and the presence of the porcine *MYOG* PCR product is the concordance value for a given chromosome. Concordance and correlation phi ( $\phi$ ) were statistically evaluated as described by Chevalet and Corpet (1986). In a panel of 20 hybrids, a marker is syntenic with a chromosome/reference locus, with a probability of 2.5% of making an incorrect decision, if  $\phi > 0.74$ . If  $\phi < 0.59$ , synteny can be excluded, and if  $0.59 \leq \phi \leq 0.74$ , no decision can be made.

## Results

**Amplification, cloning, and sequence analysis of the porcine *MYOG* gene.** Specific PCR reactions were developed to determine the porcine *MYOG* sequence (PCR 1 and 2). Primers were based on an alignment of human (Salminen et al. 1991) and mouse *MYOG* sequences (Edmondson et al. 1992). Fragments of 2081 and 442 bp respectively, covering the complete coding sequences and two introns, were obtained by PCR amplification on pig genomic DNA. These fragments were cloned in pUC18 and sequenced (Fig. 1). Like the human and mouse genes, the pig *MYOG* gene has three exons. The first exon encodes the bHLH domains. Exon 2 encodes 27 amino acids of the transactivation domain (Schwarz et al. 1992) and exon 3 the conserved C-terminal segment, common to the four MyoD proteins (Fujisawa-Sehara et al. 1990). The amino acid sequence of porcine *MYOG* is for 97% and 96% identical to the sequence of human and mouse *MYOG* respectively, while the bHLH region has the same amino acid sequences as in human, mice, and pigs. The porcine introns (785 and 639 bp) are larger than the corresponding introns of mice (513 and 526 bp) and humans (131 and 125 bp).

The *MYOG* promoter and 3' noncoding sequences were isolated and sequenced with *MYOG*-positive phage clones. This revealed 571 bp of the promoter sequences and 856 bp of the 3' untranslated region, containing a putative polyadenylation signal (AATAAA) at positions 3397 to 3402 (numbers refer to nucleotide numbers in EMBL database entry X89007). Analysis for the presence of transcription factor binding consensus sequences revealed three E-box sequences (CANNTG) located at positions 508–513

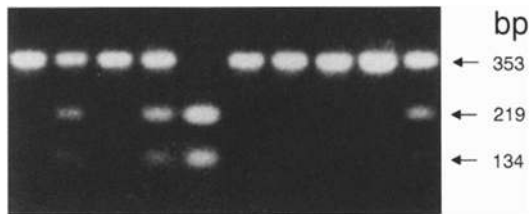


**Fig. 1.** Structure of the pig *MYOG* gene. Coding sequences are indicated in black. Open box indicates the position of the CA-repeat. Vertical lines indicate *MspI* restriction sites. Polymorphic *MspI* restriction sites are indicated by asterisks. The positions of the PCR reactions are indicated.

(E3), 210–215 (E2), and 22–27 (E1). A myocyte-specific enhancer factor 2 (MEF-2) site (456–464), a nuclear factor 1 (NF-1) site (469–481), and a TATA-box sequence (494–498) were identified within 70 bp of the putative transcription start sites. Comparison of the pig sequence with the human and mouse sequences revealed more than 96% sequence identity within the proximal 160 bp upstream from the transcription start sites.

**Characterization of a CA-repeat at the porcine *MYOG* gene locus.** Hybridization of the *MYOG* phage inserts with a (CA)<sub>25</sub>-repeat probe revealed the presence of a CA-repeat sequence at about 5 kb downstream of the TGA termination codon (Fig. 1). Sequence analysis of this region demonstrated that the CA-repeat was interrupted by CCC stretches.

**Characterization of *MspI* haplotypes at the porcine *MYOG* gene locus.** The presence of a variable 4.2/4.9-kb *MspI* fragment at the *MYOG* gene locus has been previously described by Ernst and associates (1993). Southern blot analysis with a probe specific to the 3' side of the *MYOG* gene comprising 195 bp of intron 2 and exon 3 and 4 kb of 3' untranslated sequences (Fig. 1), and restriction mapping of *MYOG* subclones, enabled us to localize the described polymorphic *MspI* site at the 3' side of the gene and to sequence its flanking regions. A PCR-RFLP test (PCR4) for detection of this polymorphic site was developed and used for genotyping a panel consisting of 105 unrelated pigs from seven breeds (Fig. 2, Table 2, columns 9–11). The Meishan population under



**Fig. 2.** Detection of a polymorphic *MspI* site at the 3' side of the *MYOG* gene in eight Great Yorkshire (GY) boars. A fragment of 353 bp is amplified (PCR4) and digested with *MspI*, resulting in the 353-bp fragment, or in 134-bp and 219-bp fragments.

investigation does not contain the 4.2-kb allele, but this allele is predominant in Duroc and Wild Pigs. Both alleles were equally present in Pietrain, Dutch Landrace (DL), and Hampshire, while the Great Yorkshire (GY) population was characterized by a high frequency of the 4.9-kb allele.

Furthermore, *MspI* digestion of PCR1 products of the 105 unrelated pigs revealed sequence variation at position 2394 in the second intron (Fig. 1, Fig. 3) for the Meishan, DL, and Duroc breeds (Table 2, columns 6–8). Southern blotting with the PCR1 fragment as a probe detected in all breeds a 2.3-kb *MspI* fragment (Table 2, column 1–3). Only in the Meishan population was a second 1.8-kb fragment also visualized that is coupled to the absence of the 2394 *MspI* site (see Fig. 3A and Fig. 3B). This indicates a deletion between the *MspI* sites or a third variable *MspI* site in the promoter region. Since the *MspI* site located at the 5' site of the gene (Fig. 1) can be detected by means of PCR5/*MspI* digestion in all animals tested, the polymorphic site must be located between this site and the coding region. Amplification of this region appears to be associated with the 2.3-kb allele (Fig. 3). Autosomal Mendelian segregation of the 1.8-kb and 2.3-kb pig *MYOG* fragments and of the *MspI* polymorphism in the second intron was checked in 58 Meishan pigs from 4 two-generation families and in a DL heterozygous  $\times$  homozygous cross (nine animals, data not shown). Analysis of the genotypes of the 105 unrelated pigs and of the progeny of the DL cross suggests the presence of at least four different *MYOG* haplotypes in pigs (Fig. 4). The allele frequencies depend on the breed, with allele 4 specific for the Meishan breed, allele 3 only in a few DL and Duroc animals, and alleles 1 and 2 predominant in the Western breeds.

**Chromosomal assignment of the porcine *MYOG* gene.** We analyzed a pig–rodent somatic cell hybrid panel for the presence or absence of the porcine *MYOG* gene by *MYOG*-specific PCR3 amplification on genomic DNA. A pig *MYOG* fragment with a predicted size of 873 bp was amplified, together with hamster or mouse fragments of 650 and 600 bp respectively. The porcine PCR3 fragment was further identified by *EcoRI* and *PvuII* digestion, generating fragments of respectively 313 + 560 bp and 219 + 654 bp, consistent with the expected length.

The distribution pattern of the porcine *MYOG*-specific PCR products (Table 3) was compared with the distribution patterns of reference loci corresponding to pig chromosomes (Rettenberger et al. 1994a, 1994b, 1994c, 1995b). The highest concordance percentage (90%) and phi ( $\phi$ ) value (0.81) were found for Chr 9.

## Discussion

The isolation and sequence analysis of the porcine *MYOG* gene described here have led to the characterization of genetic variation at this gene locus at three different sites and the identification of at least four different *MYOG* haplotypes that can be distinguished by fast PCR-RFLP tests.

We observed high sequence identity in both the coding and

regulatory regions, indicating high conservation between pigs, human, and mice. The proximal 160 bp of the promoter region contains the E1-box, which is present in the *MYOG* gene of human, mice, and pigs and which has been demonstrated to be the only *MYOG* binding E-box sequence in the mouse promoter (Edmondson et al. 1992). Funk and Wright (1992) identified MEF-2 and NF-1 as stabilizing factors for the in vitro binding of *MYOG* to E-box sequences. The presence of binding sites for both factors in the highly conserved minimal proximal promoter sequences of the *MYOG* genes of different species indicates that these factors are involved in the regulation of the *MYOG* expression. Outside this minimal promoter region, some species-specific differences can be found, such as the E2-box, which is conserved only between human and porcine genes. We found no variable sites within the coding and proximal promoter regions, probably because of their functional importance. Comparison of the sequence we describe here with another recently reported sequence of the porcine *MYOG* gene (Briley et al. 1995) also shows differences only outside these regions (see the EMBL data base entry X89007).

Association studies require the genetic screening of many animals, showing the need for fast methods like PCR-based tests, rather than time-consuming methods such as Southern-RFLP or sequencing (Te Pas and Visscher 1994). Fast and easily detectable PCR-RFLP tests detecting polymorphic sites near to, but outside the coding and regulatory regions of a gene can be associated with functional sequence divergence of a gene. The number of recombinations between the detected polymorphism and a functional polymorphism depends on their distance. Within a few thousand bp, the recombination percentage will be almost zero. So a PCR-RFLP detection of gene-specific haplotypes allows a fast detection of functional polymorphisms in which the same gene is involved (Te Pas and Visscher 1994). We describe the localization of four haplotypes within the porcine *MYOG* gene locus that can be detected by PCR-RFLPs with *MspI*. One *MspI* site is located 3' to the gene, one is situated within the second intron, and a Meishan-specific RFLP is found within the promoter region. The 1.8-kb allele detected by Southern blotting in Meishan appears to be coupled to a failure of the PCR6 reaction, suggesting that the 2.3/1.8-kb polymorphism is explained by a deletion within the 2.3-kb fragment.

Analysis of a panel of 105 unrelated pigs from seven different breeds shows that the 3' polymorphism is found in all breeds tested except the Meishan population, in which only the large allele was found. Ernst and colleagues (1993) found the large allele as the predominant *MYOG* allele in different Chinese pig breeds (Fengjing, Meishan, Minzu); this might be explained by the very small numbers (<10) of founder animals of these populations in the U.S. and in Europe (E.W. Brascamp, personal communication). The polymorphism in the second intron can be detected in Meishan, Duroc, and Dutch Landrace. In Meishan animals this polymorphism is coupled with another polymorphism in the promoter region. Because of the limited distribution of the polymorphic sites in promoter and second intron, the 3' polymorphism is best suited for the study of association between PCR-RFLP alleles and the variation of general meat production traits within different pig breeds, while the other two sites can be used for studying the inter-breed variation of meat characteristics. Large numbers of commercial European pigs will be genotyped by use of the PCR-RFLP tests for the *MspI* sites a and b (Fig. 4), and the resulting genotypes will be tested for the association with growth characteristics.

By using a cell hybrid panel, we assign *MYOG* to pig Chr 9, confirming a former mapping obtained by linkage analysis with an *MspI* RFLP (Archibald et al. 1995). In the human, *MYOG* maps to Chr 1q31-q41. Our results are in agreement with the conserved synteny between segments of human Chr 1 and porcine Chr 9, as has been demonstrated by linkage mapping of CR2 (Johansson et al. 1994) and Zoo-FISH analysis (Rettenberger et al. 1995a). From

**Table 2.** Frequency of *MYOG* genotypes in seven pig breeds, based on three polymorphic *MspI* sites, in the promoter, second intron, and 3' site of the gene.

Breed	No. <sup>a</sup>	Promoter <sup>b</sup> (%)			Intron 2 <sup>c</sup> (%)			3' end <sup>b,d</sup> (%)		
		1.8/1.8	1.8/2.3	2.3/2.3	2/2	2/3	3/3	219/219	353/219	353/353
Meishan	11	9	36	55	9	36	55	0	0	100
Pietrain	5	0	0	100	0	0	100	40	20	40
Duroc	10	0	0	100	0	10	90	50	50	0
Wild pig	10	0	0	100	0	0	100	60	40	0
GY <sup>e</sup>	40	0	0	100	0	0	100	2.5	20	77.5
DL <sup>e</sup>	20	0	0	100	0	10	90	30	40	30
Hampshire	9	0	0	100	0	0	100	22	56	22

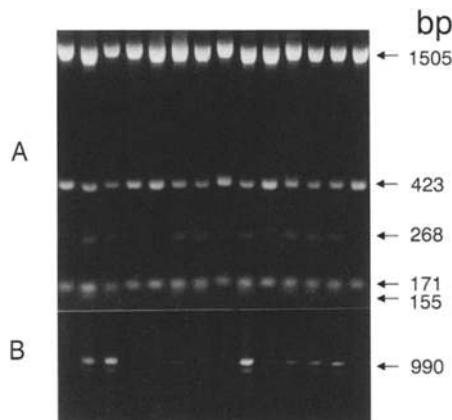
<sup>a</sup> Number of unrelated pigs tested.

<sup>b</sup> A 2.3-kb/1.8 kb *MspI* polymorphism is detected in Meishan pigs by Southern blot hybridization to the *MYOG* PCR 1 probe.

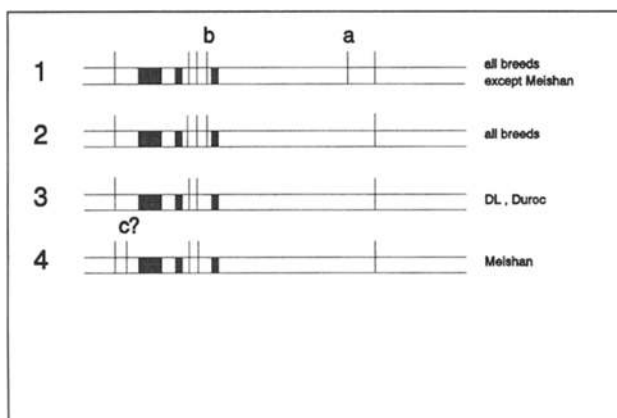
<sup>c</sup> 2 denotes the absence, 3 the presence of the *MspI* site at position 2394.

<sup>d</sup> PCR 4/*MspI* RFLP analysis detecting either a fragment of 353 bp for the absence of the *MspI* site, or fragments of 219 and 134 bp for the presence of the *MspI* site.

<sup>e</sup> GY: Great Yorkshire; DL: Dutch Landrace.



**Fig. 3.** Detection of two *MspI* polymorphisms at the pig *MYOG* gene locus in Meishan pigs; association between a polymorphic site in the promoter and a polymorphic site in the second intron. (A) PCR1/*MspI* analysis, detecting an *MspI* polymorphism in the second intron of the *MYOG* gene at position 2394; the amplified fragment of 2099 bp is digested by *MspI*, resulting in four fragments of 1505, 171, 155 and 268 bp, or in three fragments of 1505, 171, and 423 bp. (B). PCR6 analysis, showing the amplification of a fragment of 990 bp that is coupled to the 268-bp fragment in 3A.



**Fig. 4.** Schematic representation of the four *MYOG* haplotypes that were distinguished in the pig breeds and crosses tested, based on the observed combinations of three polymorphic *MspI* fragments. The polymorphic sites are indicated by a, b, c. Shaded areas indicate coding sequences. Vertical lines indicate *MspI* sites.

the Zoo-FISH data, localization of *MYOG* on porcine Chr segment 9q21-qter can indirectly be predicted. Chromosomal assignment of *MYOG* offers the possibility to use a couple of microsatellites as an alternative or complementary set of polymorphic markers. A CA-

**Table 3.** Concordance percentages and values of the correlation coefficient phi ( $\phi$ ) for *MYOG* and pig chromosomes/reference loci in a pig-rodent somatic cell hybrid panel.

Chromosome/reference loci	con	$\phi$
1/IFNA	67	0.41
2/S0091	67	0.41
3/APOB	81	0.63
4/S0001	52	0.04
5/S0092	67	0.36
6/RYR1	62	0.35
7/TNFB	76	0.51
8/ALB	71	0.37
9/S0095	90	0.81
10/S0038	81	0.60
11/S0009	52	0.04
12/GH	81	0.59
13/S0076	43	-0.16
14/DAO	48	0.01
15/S0088	38	-0.23
16/S0006	52	-0.01
17/ENDO	62	0.28
18/S0062	52	0.14
X/S0022	46	-0.08
Y/SRY	62	0.44

repeat sequence of variable length (19 and 22 repeats) has been identified by Edmondson and coworkers (1992) and Yee and Rigby (1993) between positions -603 and -560 in the mouse *MYOG* promoter. We also identified a CA-repeat sequence within the porcine *MYOG* gene locus. However, since this CA-repeat is interrupted by CCC stretches, it is unlikely that this microsatellite will be polymorphic.

In summary, we have isolated the porcine *MYOG* gene, assigned it to Chr 9, and found four haplotypes based on *MYOG* polymorphisms. In a panel of 105 unrelated pigs and in several Meishan families, at least four *MYOG* alleles were identified, some of which were breed specific. PCR tests for rapid identification of these alleles were developed. This allows association studies with the growth and meat deposition performances of pigs.

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