GMR

Study of genetic variability in pigs after the traditional breeding program

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ABSTRACT. Molecular markers are tools used to improve genetic gains. The objective of this study was to analyze the security of alleles of molecular marker genes for characteristics of economic interest in a pure population of pigs. After the extraction of DNA from the hair of 272 Large White matrices, the allele and genotype frequency of single nucleotide polymorphism was performed using the ARMS-PCR Multiplex technique in the DGAT1, LEPR, H-FABP, MC4R, and SREBF1 genes using RFLP-PCR for the GH gene. After capillary electrophoresis in an automated DNA sequencing of the DGAT1, LEPR, H-FABP, and SREBF1 genes, no polymorphisms were found. Only the MC4R marker presented 100% heterozygosity. For the GH gene, 209 of the initial population samples were genotyped. The PCR product (605 bp) was digested with the restriction enzyme *Dde*I, with fragments being of 335, 148, and 122 bp for the D_1 allele and 457 and 148 bp for the D_2 allele. The genotypic frequency obtained of D_1D_2 was

88% and of D_2D_2 was 22%. The D_1 allele presented a frequency of 11% and the D_2 allele of 89%. The high intensity of selection for commercial breeds justifies the absence or the low number of polymorphisms for the genes studied.

Key words: DNA; SNPs; Polymorphism; Molecular markers; Gene-assisted selection

INTRODUCTION

The use of candidate genes related to economic interest traits such as markers has been widely applied in genetic breeding programs (Rotschild, 2000; Chen et al., 2007; Coutinho et al., 2010). However, the increase in these contributions is restricted when one is dealing with characteristics of low heritability or those that are difficult to measure, for example, carcass yield, disease resistance, longevity, gender/sex-related, or in the case of phenotypes that are difficult to measure (Dekkers, 2004; Goddard and Hayes, 2009; Coutinho et al., 2010).

According to Hayashi et al. (2004) and Faleiro (2007), single nucleotide polymorphisms (SNPs) come about by mutation in a single-base pair in the DNA sequence between members of the same species that can cause phenotypic differences. The advantages of SNPs as genetic markers are the availability of genotype protocols, which are fast, reliable, and reproducible, with high-performance or high-density protocols and substantially lower costs (Lenstra et al., 2012). These marker genes are potential candidates due to their important physiological effects related to characteristics of economic importance (Franco et al., 2005). This information can be incorporated into traditional breeding methods (Li et al., 2006; Bižienė et al., 2011).

Among the molecular markers associated with characteristics of economic interest are the genes, among which we can mention: DGAT1 (diacylglycerol acyltransferase 1), a gene that encodes the enzyme of the same name acting in the metabolism of intestinal fat absorption, lipoprotein synthesis, adipose tissue formation, and lactation (Nonneman and Rohrer, 2002; Cui et al., 2011); LEP (leptin) and LEPR (leptin receptor) are important regulators of appetite, metabolism, reproduction (Wylie, 2011; Georgescu et al., 2014), and the deposition of intramuscular fat (Tyra and Ropka-Molik, 2011); H-FABP (heart-type fatty acid binding protein) is related to the regulation of fatty acid uptake and intracellular transport (Gerbens et al., 1999; Tyra et al., 2011; Chao et al., 2012); MC4R (melanocortin 4 receptor gene) is an important genetic marker for characteristics related to backfat thickness, feed intake, and growth rate (Jokubka et al., 2006; Óvilo et al., 2006); SREBF1 (sterol regulatory element binding transcription factor) involved in the differentiation of adipocytes, as well as cholesterol and fatty acids (Stachowiak et al., 2013); and GH (growth hormone) that influences economically important characteristics such as carcass weight and fat thickness (Franco et al., 2003; Bižienė et al., 2011).

All the genes mentioned above are involved in the process of lipid metabolism and growth. The lipids are involved in the production of steroid hormones and are also important signaling molecules involved in the regulatory mechanisms of maturation and, therefore, in the acquisition of oocyte competence (Prates et al., 2014). Therefore, they may be related to reproductive characteristics.

In this way, the objective of this study was to analyze in a pure population of Large White pigs, which is under selection effect for the amount of meat in the carcass, backfat

thickness, age and weight at slaughter, number of piglets weaned per sow/year, and the allelic and genotypic frequencies of the genes DEGAT1, LEPR, H-FABP, MC4R, SREBF1, and GH.

MATERIAL AND METHODS

The hair samples were collected from animals coming from a breeding/improvement program in the final phase of genetic stabilization. Mutations (SNPs) were used in genes associated with lipid metabolism available in GenBank, but with no or only a very few polymorphism studies.

DNA extraction

Two hundred and seventy-two (272) females were used in this research for the genes H-FABP, DGAT1, LEPR, MC4R, and SREBF1, and of these, 209 for the GH gene. To extract the DNA from each sow, 10 hairs with bulbs were used, and the procedure was carried out according to the protocol adapted from Miller et al. (1988).

DNA amplification

DNA amplification was carried out using ARMS (amplification-refractory mutation system) by PCR (polymerase chain reaction) multiplex of 5 genes with M13 tail tagged (Table 1).

PCRs were carried out using 20 ng DNA, 1X buffer [200 µM dNTP, 2.5 mM $MgCl_2$, 2 U Taq DNA polymerase, 4 pmol of each primer (Table 1), 20 pmol of each probe M13-FAM and M13-NED] and the volume adjusted to 20 µL with ultrapure water. All reagents, except the primers and probes, were from Invitrogen®.

Three primers were designed for each of the genes using the sequences deposited in GenBank. They were designed using the Primer3 (http://frodo.wi.mit.edu/edu/primer3/) program, and their quality was verified using Oligo Analyzer 3.1 (http://www.idtdna.com/calc/analyzer).

Table 1. Primers designed to amplify the target regions of the genes DGAT1, LEPR, H-FABP, MC4R, SREBF1, and an M13 probe.

Gene	Primer	Amplicon (bp)	GenBank No.
DGAT1	F*: 5'-M13-1**AGC CAG CGC CCC CGG TC**C**-3'		XM_005655311.2
	F*: 5'-M13-2**- AGC CAG CGC CCC CGG TC**T**-3'	143	1680(C/T)
	R*: 5'-CTG TGC CTG CCT GCC ATC-3'		
LEPR	F: 5'-GTG ATA ACT GCA TTT GAC TTG GC-3'		
	R: 5'-M13-1 AGT TTG ATA AGT AGG TAC CAC TT**A T**-3'	209	GQ268934
	R: 5'-M13-2 AGT TTG ATA AGT AGG TAC CAC TT**G A**-3'		228(A/G); 229(T/A)
H-FABP	F: 5'-M13-1- CTA GCC CAG CCT CAC CAT GG**T**-3'		
	F: 5'-M13-2 CTA GCC CAG CCT CAC CAT GG**C**-3'	183	JN646857
	R: 5'-TGA GTC CCC ATT CAC TTC GAT G-3'		48(C/T)
MC4R	F: 5'-TAC CCT GAC CAT CTT GAT TG-3'		
	R: 5'-M13-2 GAG TGC ATA AAT CAG GGG AT**C**-3'	196	NM_214173.1
	R: 5'-M13-1 GAG TGC ATA AAT CAG GGG AT**T**-3'		1425(T/C)
SREBP1	R: 5'-M13-2 CAG CAC ACG CGC CTC CAC G**A**-3'		
	R: 5'-M13-1 CAG CAC ACG CGC CTC CAT G**G**-3'	263	AB686492.1
	F: 5'-GCC GTG GTG AGA AGC GGA CGG CTC AC-3'		13334(G/A)

F: forward; R: reverse; **M13-1 5'-FAM-GTC AAG ATG CTA CCG TTC, **M13-2 5'-NED-5'-ACT CAT CGG AAT CGT ATG.

The amplification of the DNA was carried out in a thermocycler (Thermal Applied BioSystems® Cycler 2720), under the following conditions: 95°C for 10 min, 5 cycles of 95°C for 15 s, 55°C for 45 s, 70°C for 1 min and 56 s, 31 cycles at 95°C for 15 s, 53°C for 45 s, 70°C for 1 min, a half and extension at 70°C for 30 s, and a final extension at 25°C for 10 min.

Genotyping using capillary electrophoresis

For capillary electrophoresis genotyping, 1 µL of the PCR product plus 9 µL Formamide Hi-Di™ (Applied Biosystems) and 0.3 µL of the LIZ® 600 (Applied Biosystems) marker were used. The reactions were carried out in a thermocycler (Thermal Applied BioSystems® Cycler 2720) at 95°C for 7 min for denaturation. Then, the samples were subjected to capillary electrophoresis on the ABI 3130 sequencer (Applied Biosystems), and the fragments were analyzed using the GeneMapper 3.0 software.

From the results of ARMS-PCR multiplex for DGAT1, LEPR, H-FABP, MC4R, and SREBF1 genes the polymorphisms were identified by the presence of a different color peak for each homozygote in each gene and two different color peaks in the electropherogram in heterozygous individuals.

PCR-RFLP

PCR-RFLP was carried out for the GH gene using 1X buffer [2 mM $MgCl_2$, 0.4 mM dNTP, 10 pmol of each primer (forward and reverse), 1 U Taq polymerase, 1 µL genomic DNA], and ultrapure water to a final volume of 20 µL. The sequence, amplicon, and GenBank registration number of the primers used are in Table 2.

Table 2. Primers designed to amplify target regions of the GH gene.

Gene	*Primer	Amplicon (bp)	GenBank No.
GH	F: 5'-TTATCCATTAGCACATGCCTGCCAG-3'	605	M17704
	R: 5'-CTGGGGAGCTTACAAACTCCTT-3'		

*Primer designed by Larsen and Nielsen (1993), from the sequence of Vize and Wells (1987). F: forward; R: reverse.

The reactions were carried out in a thermocycler (T100™ Thermal Cycler) under the following conditions: 95°C for 3 min, 35 cycles at 95°C for 45 s, 60°C for 40 s, 76°C for 1 min, and a final extension at 76°C for 4 min. After the amplification 5 µL of the PCR product was applied to an agarose gel at 2%.

The remainder of the amplification was digested with 1 U of the *Dde*I (Promega) enzyme and incubated for 16 h at 37°C, and then the product was applied to an agarose gel at 2.5%. For the GH gene after digestion of the samples, two alleles were obtained: D_1 with fragments of 335, 148, and 122 bp and D_2 with 457 and 148 bp.

RESULTS

For the DGAT1 gene, the frequency of the C allele and the CC genotype was 100% in the population. LEPR and H-FABP presented a 100% frequency of GAGA and TT genotypes and 100% of GA and T alleles, respectively. In the MC4R gene, 100% of the genotype

frequency (TC) and 50% of the frequency of each allele (T and C) were observed. Concerning the SREBF1 gene, 100% of genotypic and allelic frequencies were also found (AA and A, respectively). GH presented 22% of the D_1D genotype and 78% of the D_2D_2, with an 11% frequency of D_1 allele and 89% of D_2 (according to Table 3).

For the amplification reaction of the GH gene, a 605-bp amplicon was obtained (Figure 1). After digestion with the *Dde*I enzyme, 457-, 335-, and 148-bp fragments were observed (Figure 2). It was not possible to visualize fragment 122.

Table 3. Genotype and allelic frequencies of DGAT1, LEPR, H-FABP, MC4R, SREBF1, and GH genes in Large White breeders.

Genes	Genotype	Genotype frequency (%)	Alele	Allele frequency (%)
DGAT1	TT	0	T	0
	TC	0	C	100
	CC	100		
LEPR	GAGA	100	GA	100
	GAAT	0	AT	0
	ATAT	0		
H-FABP	TT	0		
	TC	0	T	0
	CC	100	C	100
MC4R	TT	0		
	TC	100	T	50
	CC	0	C	50
SREBF1	GG	0	G	0
	GA	0	A	100
	AA	100		
GH	D_1D_1	0	0	0
	D_1D_2	22	D_1	11
	D_2D_2	78	D_2	89

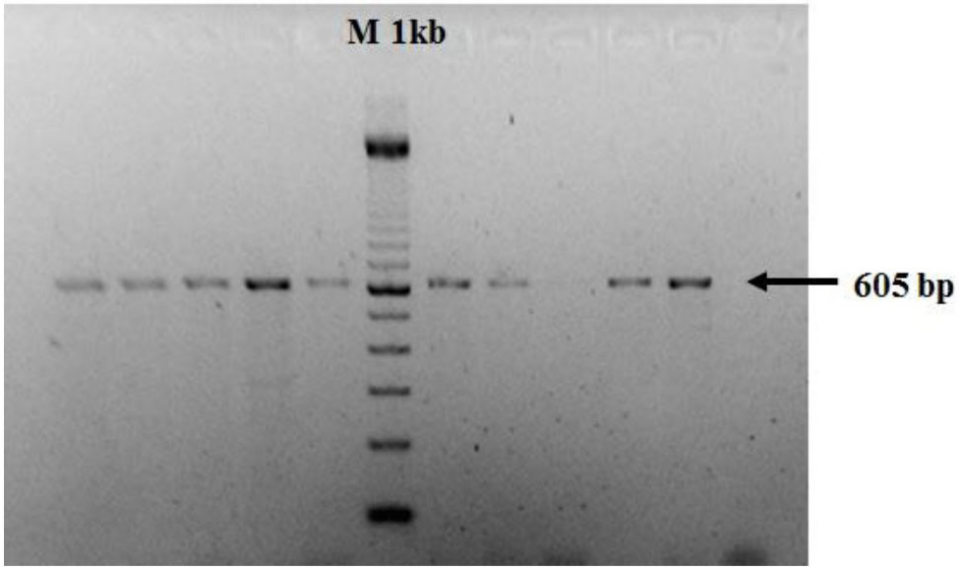

Figure 1. Amplification of the GH gene on 2% agarose gel showing an amplicon of 605 bp and the marker (M) of 1 kb.

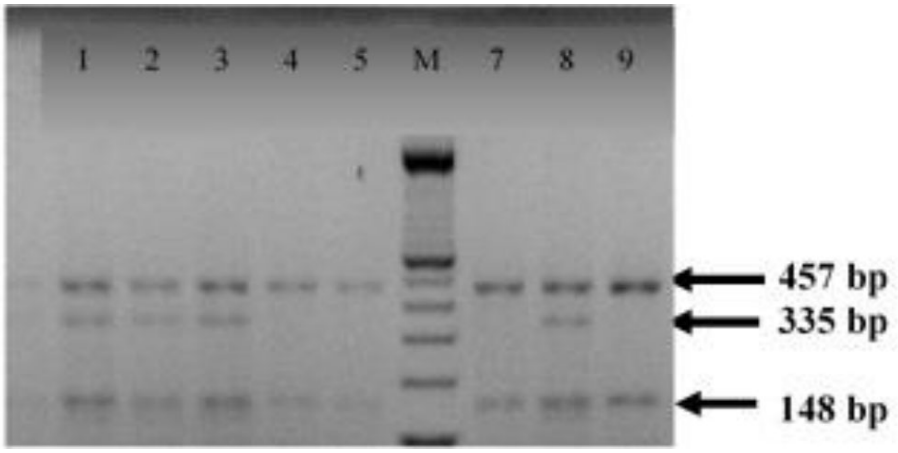

Figure 2. GH gene digested by *Dde*l enzyme on 2.5% agarose gel. *Lane M* = 1-kb marker; *lanes 1*, *2*, *3*, and *8* = fragments 457, 335, and 148 (D_1 D_2) and *lanes 4*, *5*, *7*, and *9* = showing fragments 457 and 148 (D_2 D_2).

DISCUSSION

The results found for the DGAT1, LEPR, H-FABP, and SREBF1 genes showed that they reached the fixation of their alleles, indicating the absence of genetic variation. This may be justified by the high selection pressure experienced by the animals over several years. Besides, selection for other traits or characteristics may have indirectly generated a decrease in the genetic variability of the population (Ayres et al., 2006).

Gondim (2015) when working with the DGAT1 gene in distinct populations (Large White x Landrace, Piau, Large White x Landrace, Jiaxing x Meishan) concluded that the C allele is practically fixed in these populations meaning that they are in a pronounced selection process, since they were animals from nucleus and multiplier farms. Zang et al. (2016), when working with the same gene but with a deletion in the 3'-UTR region, determined the existence of an allelic dominance. In the present study, the C allele presented itself as homozygous for all individuals analyzed (Table 3).

In regards to the LEPR gene, the findings of this study corroborate with Gondim (2015) who did not find any variation in the genotypes in the studied populations. According to the same author, the allelic frequencies in a finite population stem or derive from fixation or exclusion by decreasing the heterozygosity of the population. Balatsky et al. (2016), when working with a different SNP but in the same gene, found three genotypes (CC, CT, and TT), with the C allele having a 66% frequency.

For the H-FABP gene, a 100% homozygous for the TT genotype was found, contradicting or disagreeing with the results found by Gondim (2015), where the highest frequency was reported for the CC genotype. Gondim (2015) also reported that SNPg.240T>C when homozygous for the T allele decreases the first period of coverage, and the delivery/parturition interval, showing that the result of the population studied in this experiment reached the apex for selection. Chen et al. (2014) analyzed the H-FABP polymorphisms using PCR-RFLP using 3 restriction enzymes in six swine breeds and as the results of this study found a high predominance of one allele in at least 50% of the analyzed loci. Li et al. (2006),

when studying which would be the best markers for native Chinese breeds that would help in selection and conservation, showed significant differences in the frequencies of genotypes and alleles between breeds. When using the PCR-RFLP, a frequency of 100% of the AA genotype was found for the Meishan breed.

The only marker found in this study in heterozygosis was MC4R, which indicates that there are at least two distinct pure lineages for the formation of this population, as each one contributed with identical alleles. Gondim (2015) observed a greater frequency of the T allele regarding the C allele for the three genetic groups evaluated. The TT genotype of Piau and LWLD had a frequency of 52 and 45%, respectively; however, the LLJM gene group had a higher genotypic frequency of the heterozygous CT (49%). Li et al. (2006), using another polymorphic region of the 6 breeds studied, found 100% of homozygous BB in the Meishan breed. A study performed by Óvilo et al. (2006) in commercial hybrid pigs (Landrace x Taihu x Large-White) found two polymorphisms of the MC4R gene and the AA, AG, and GG genotypes with the following frequencies 8, 36, and 56%, respectively, and observed that none of the alleles would have a clear advantage in selection processes.

Chen et al. (2008) carried out experiments to clarify the role of SREBF1 in the deposition of intramuscular fat (IMF) in crossbred Erhualian x Duroc animals, called Sutai. They found three AA genotypes with a frequency of 50%, AB (36%), and BB (14%). The authors also correlated that both the level of mRNA in SREBF1 and IMF in the muscles were greater in the AB and BB genotypes than in AA animals. With these results, they suggest that the SNP of the SREBF1 gene could be used as a genetic marker to improve the IMF in swine. Renaville et al. (2010, 2013), when studying swine populations using the same SNP as studied here, found the allele A with a frequency of 76 and 74.45%, whereas in the population studied here 100% of the allele A was observed (Table 3).

Franco et al. (2005) found the genotype frequencies of 0.662 for the D_1D_1 genotype and 0.338 for the D_1D_2 genotype and the allelic frequencies of 0.831 for D_1 and 0.169 for D_2 for the GH gene in a Landrace population, differing from the latter study with the Large White population, in which the D_1D_1 allele was not found. The referred authors considered that the animals with the D_1D_2 genotype had a lower fat thickness and a greater average daily gain of weight. The possible explanation for the result could be that this polymorphism leads to an amino acid alteration in the protein sequence, thereby interfering with the efficiency or amount of GH secretion.

In the study carried out by Franco et al. (2001) with 3 swine populations (Pietran, Large White, and Landrace), two polymorphisms (GHD with the enzyme *Dde*I and GHC with enzyme *HhaI*) were found. Large White GHD genotypes showed a greater number of individual heterozygous as well as an increase in heterozygotes for the GHC polymorphism in the Landrace and Pietran breeds. The phenotypic frequencies may be influenced by the selection for reproductive and performance characteristics as against the halothane gene according to the authors. Although no high genetic variability was obtained, the markers can be used in genetic studies of populations under lower selection pressure (Franco et al., 2001).

In general, world literature has shown that even when using different populations and/or different genetic markers with diverse polymorphism identification techniques, it is often the determination of high allelic predominance, as was observed in most of the genes studied here, that holds true (Franco et al., 2001; Li et al., 2006; Bosse et al., 2012; Renaville et al., 2010, 2013).

This leads to the confirmation that the traditional improvements and selection

processes of superior genotypes throughout the domestication process led to the fixing of genes of interest and consequently other related genes, reducing genetic variability and demanding more efficient programs in the selection of the best genotypes and in the preservation of the genome bank, so that in the future it will be possible to introduce new genotypes and recover genetic diversity. Every day the need for the use of molecular markers is becoming more evident, whether it is to help in assisted breeding programs or genetic analyses and the determination of population inbreeding (Herrero-Medrano et al., 2014; Schiavo et al., 2016; Peripolli et al., 2017).

CONCLUSION

In general, low polymorphism was found in the GH gene, and no polymorphisms were found in the other genes, which indicate the good efficiency in generating homozygous animals in traditional breeding programs.

Conflicts of interest

The authors declare no conflict of interest.

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