Universidade Federal de Uberlândia

Faculdade de Medicina Veterinária

Uso de seleção assistida por marcadores moleculares em melhoramento genético de suínos.

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Médica Veterinária

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Tese apresentada ao Programa de Pós-Graduação da Faculdade de Medicina Veterinária, como parte das exigências para a obtenção do título de Doutor em Ciências Veterinárias

Orientador: Prof. Dr. Robson Carlos Antunes

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Tese de doutorado de Isaura Maria Ferreira, submetida ao programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal de Uberlândia como parte dos requisitos necessários para a obtenção do título de doutora em Ciências Veterinárias.

Área de concentração: Produção animal
Subárea: Manejo e eficiência de produção dos animais, seus derivados e subprodutos.

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"Tudo o que é seu encontrará uma maneira de chegar até você"

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Resumo: Com o avanço do conhecimento sobre o genoma animal, a produção de suínos se beneficia das novas ferramentas moleculares na busca de melhores resultados zootécnicos, como eficiência alimentar, número de leitões desmamados/porca ano, maior produção de carne na carcaça, melhor rendimento dos cortes cárneos. Assim a utilização dos marcadores moleculares seleciona os melhores genótipos para as características desejadas, permitindo um menor tempo entre as gerações, menor custo para os produtores, maior rendimento de produtos para indústria e melhor qualidade para os consumidores. O trabalho foi dividido em duas etapas onde no primeiro estudo procurou-se avaliar em uma população de 272 matrizes Large White, os polimorfismos dos genes DGAT1, LEPR, HFABP, MC4R, SREBF1 pela técnica do ARMS-PCR e para o polimorfismo do gene GH empregou-se a técnica do PCR-RFLP. O DNA genômico foi extraído do bulbo do pelo. Os *primers* foram desenhados para cada um dos genes utilizando as sequências depositadas no GenBanK. Após o sequenciamento do DNA não foi encontrado polimorfismos, com exceção do marcador MC4R que apresentou 100% de heterozigose. Da população inicial, 209 amostras foram genotipados para o gene pGH, onde um *amplicon* de 605 pares de base foi digerido pela enzima de restrição DdeI, resultando em dois alelos. O alelo D_1 com 335,148 e 122 pb e o alelo D_2 com 457 e 148 pb. A frequência genotípica de D_1D_2 foi de 88% e para D_2D_2 de 22%. O alelo D_1 apresentou frequência de 11% e D_2 89%. A alta intensidade de seleção para raças comerciais, justifica a ausência ou o baixo número de polimorfismos para os genes estudados. Para a segunda parte da pesquisa objetivou-se associar o polimorfismo do pGH/DdeI com características de carcaça e qualidade de carne. Utilizou-se uma população de 476 animais provenientes do cruzamento do macho AGPIC 415 e fêmeas LW X LD livres da mutação do gene halotano, machos castrados e fêmeas com idade de 150 a 180 dias. Foram coletadas as informações como peso da carcaça quente, comprimento de carcaça, pH 45min e pH ± 16h *pós mortem* dos músculos *Longíssimus cervicis*, *Longíssimus dorsi* e *Semimebranosus,* espessura de toucinho, profundidade do músculo *LD* e cor pelo sistema CIELAB. A capacidade de retenção de água foi realizada pelo método do papel filtro no músculo *Semimebranosus* e calculado a porcentagem de carne magra. Encontrou-se um SNP na posição g365G> A, com a troca do núcleotideo guanina por uma adenina, e em consequência, o aminoácido arginina por uma glutamina. Apresentou o alelo D_1 com os fragmentos de 335 pb, 148 pb e 122 pb e o alelo D2 com 457 genotípicas pb e 148 pb com as frequencias de 0,43 e 0,57 respectivamente. As frequências encontradas foram as seguintes: D_1D_1 (22,09%), D_1D_2 (49,82%), D_2D_2 (28,09%). Após a realização do teste de qui-quadro a população se apresentou em equilibrio de Hardy-Weinberg. O polimorfismo do gene pGH/DdeI apresentou relação com as características de carne magra e pH a 45, propriedades estas importantes para a comercialização da carne fresca bem como produtos processados.

PALAVRAS CHAVES: SNPs, Hormônio do crescimento, Características econômicas, Carne magra.

ABSTRACT: With the advancement of knowledge about the animal genome, the production of pigs benefits from the new molecular tools in search of the best zootechnical, results such as feed efficiency, number of piglets weaned / sow year, higher meat production in the carcass, better yield of meat cuts. Thus, the use of molecular markers selects the best genotypes for the desired characteristics, allowing shorter generation time, lower cost to producers, higher yields of products for industry and better quality for consumers. The work was divided into two stages where the first study sought to evaluate in a population of 272 Large White matrices the polymorphisms of the DGAT1, LEPR, HFABP, MC4R, SREBF1 genes by the ARMS-PCR technique and for the pGH gene polymorphism the PCR-RFLP technique was used. Genomic DNA was extracted from the hair bulb. The *primer*s were designed for each of the genes using the sequences deposited in GenBanK. After DNA sequencing no polymorphisms were found, with the exception of the MC4R marker which showed 100% heterozygosity. Considering the initial population, 209 samples were genotyped for the pGH gene, where a 605 base pair amplicon was digested by restriction enzyme DdeI, resulting in two alleles. The D1 allele with 335,148 and 122 pb and the D2 allele with 457 and 148 pb. The genotypic frequency of D_1D_2 was 88% and for D_2D_2 22%. The D_1 allele had a frequency of 11% and for D_2 of 89%. The high intensity of selection for commercial breeds justifies the absence or the low number of polymorphisms for the genes studied. For the second part of the research, we aimed to associate pGH / DdeI polymorphism with carcass characteristics and meat quality. A population of 476 animals from the crossing of the AGPIC 415 male and LW X LD females free from the halothane gene mutation, castrated males and females aged 150 to 180 days. The information were collected as warm carcass weight, carcass length, pH 45min and pH ± 16h postmortem of the muscles *Longíssimos cervicis*, *Longíssimos dorsi* and *Semimebranosus,* backfat thickness, LD muscle depth and color by the CIELAB system. The water holding capacity was determined by the filter paper method in the *Semimebranosus* muscle and the percentage of lean meat was calculated. A SNP at position g365G> A was found, with the exchange of the guanine nucleotide by an adenine and consequently the amino acid arginine by a glutamine. It presented the D_1 allele with fragments of 335 bp, 148 bp and 122 bp and the D_2 allele with 457 bp and 148 bp and with frequencies of 0.43 and 0.57 respectively. The genotypic frequencies found were as follows: D_1D_1 (22.09%), D_1D_2 (49.82%), D_2D_2 (28.09%). After the chi-square test the population presented in Hardy-Weinberg equilibrium. The polymorphism of the pGH / DdeI gene was related to the characteristics of lean meat and pH to 45, properties that are important for the commercialization of fresh meat as well as processed products.
KEYWORDS: Assisted selection, SNPs, DdeI, Economic characteristics, Lean meat

Sumário

CAPÍTULO 1

1. Introdução

Nos últimos anos a cadeia produtiva suinícola se esforça na busca pela melhoria na produção e qualidade da carne. Para isto, alia-se ao melhoramento genético das raças já existentes, investindo na sanidade dos rebanhos com novos medicamentos, diagnósticos mais precisos e aperfeiçoamento do manejo nutricional. Preocupa-se também com a ambiência animal, a qual influencia no comportamento e no desempenho zootécnico em todas as fases de produção, além de alternativas para o uso dos resíduos sólidos, como fonte de energia (biogás), tornando autossustentáveis as propriedades, e com isto, reduzindo o custo de produção.

Os investimentos na tecnologia de abate, o cuidado na rede de frio, a logística de distribuição da carne e seus derivados são responsabilidades de todo sistema produtivo, o qual tem por objetivo a elaboração de alimento com qualidade em todos os seus aspectos: nutricional, tecnológico, sensorial, ético e outros. Tais parâmetros podem influir nas características finais da carne suína, conforme observados por Rosenvald e Andersen (2003) e Pena et al., (2016), haja vista sua complexidade, e os vários fatores e interações existentes ao longo da sua produção até o consumo final.

Com os esforços para consecução em quantidade e qualidade da carne, desenvolveu-se o estudo da genômica suína, ou seja, o conhecimento dos genes e suas interações com o ambiente (ROTHSCHILD, 2000; SILVA et al., 2003), sendo possível identificar genes associados às características desejáveis de produção como prolificidade, resistência a determinadas patologias que afetam a criação, conversão alimentar e menor teor de gordura na carne. Os resultados desejados são obtidos com o emprego de técnicas com a identificação de cada indivíduo e de seu valor genético.

Associados a esse conhecimento, os genótipos superiores são selecionados, transmitindo para sua descendência os atributos de interesses econômicos, os quais possibilitam realizar a seleção mais precoce das características desejadas, de um rebanho em menor tempo e com um melhor retorno financeiro (SINGH et al., 2014).

Os genes utilizados como marcadores moleculares são ferramentas usadas para melhorar os ganhos genéticos e possibilitam estudar características, anteriormente, impossíveis de serem trabalhadas (COUTINHO; ROSÁRIO; JORGE, 2010). São potenciais candidatos, devido aos seus efeitos fisiológicos importantes, genes relacionados ao crescimento, reprodução, lactação, resistência às doenças e qualidade da carne, permitindo que criadores e produtores comerciais de suínos tomem decisões quanto à produção. A suinocultura tem investido nesses conhecimentos e principalmente colocando-os em prática juntamente com os processos tradicionais de seleção.

2 REFERENCIAL TEÓRICO

2.1 Marcadores genéticos

Marcadores genéticos ou descritores genéticos são quaisquer características morfológicas, bioquímicas ou moleculares que diferenciam indivíduos e que sejam facilmente detectáveis. Segundo Regitano e Veneron (2009); Coutinho; Rosário; Jorge, (2010) e Borém e Caixeta (2016), os marcadores genéticos são características de herança mendeliana simples, que possibilitam identificar o genótipo a partir do fenótipo do indivíduo. Em animais de produção as características relevantes são determinadas pela combinação de diferentes genes (herança poligênica) de grande e ou pequeno efeito individual, sob grande interferência de fatores ambientais. O mapeamento dessas características pode ser feito com base nos *Quantitative Trait Loci* (QTLs), que podem ser definidos como uma região do genoma que abriga um ou mais genes que afetam as características quantitativas, ou seja, as que apresentam distribuição contínua (altura, peso e comprimento) dos valores fenotípicos (TENEVA; PETROVI, 2010; FALEIRO et al., 2011; HAYWARD et al., 2015). A seleção assistida por marcadores (SAM), segundo Silva et al., (2003), permite selecionar reprodutores com características favoráveis, por meio do genótipo. Isso possibilita que as características de baixa herdabilidade ou aquelas que só podem ser mensuradas após o abate sejam utilizadas com frequência, permitindo um melhoramento animal mais eficiente (DEKKERS; ROTHSCHILD; MALEK, 2001; SINGH et al., 2014; BORÉM; CAIXETA, 2016).

As três principais classes de marcadores genéticos são baseadas em: 1) características morfológicas e produtivas (visualmente avaliadas); 2) marcadores bioquímicos (baseado em produtos dos genes) e 3) marcador molecular (determinado pela análise de ácido desoxirribonucleico-DNA).

2.1.1 Marcadores Morfológicos

Os marcadores morfológicos foram os primeiros a serem usados e são dependentes da observação das características fenotípicas, ou seja, escolhendo os animais com melhor desempenho e melhor adaptação ao meio ambiente, iniciando-se assim os programas de melhoramento (COUTINHO; ROSÁRIO; JORGE, 2010; AL-SAMARAI; AL-KAZAZ, 2015). Bueno-Silva (2012) relatam que a utilização destes marcadores muitas vezes não é precisa, pois, a relação entre o genótipo e o fenótipo de um indivíduo pode ser influenciada por fatores ambientais, embora algumas características fenotípicas estejam sobre o estrito controle genético, indicando uma ligação entre o fenótipo de interesse e um gene específico.

2.1.2 Marcadores Bioquímicos

O surgimento dos marcadores bioquímicos, na década de 1970, representou um avanço para o melhoramento genético, possibilitando o conhecimento sobre as distâncias entre os genótipos e sua variabilidade. Os marcadores bioquímicos são proteínas que podem ser separadas por eletroforese devido sua propriedade de migração de suas cargas elétricas. As enzimas isoenzimas são enzimas que têm as mesmas funções metabólicas (isoformas) e diferem entre si em algumas propriedades, devido às variações alélicas dos genes codificadores, segundo Hoffmann (2006) e Faleiro et al. (2011). Quando estas isoenzimas são produtos de alelos de um mesmo loco passam a ser chamadas de aloenzimas (PIMENTEL, 1988; BUENO-SILVA, 2012).

2.1.3 Marcadores moleculares

Os marcadores moleculares são sobretudo úteis, especialmente, para a seleção das caraterísticas difíceis de serem selecionadas pelos métodos convencionais. O marcador molecular ou marcador genético é um gene ou sequência de DNA, que define um fenótipo (Ferreira e Grattapaglia , 1998), ou seja, são características herdadas geneticamente e que diferem dois ou mais indivíduos. Estes são numerosos e espalhados por todo o genoma, evidenciam caráter codominante e muitas vezes são multialélicos (COUTINHO; ROSARIO; JORGE, 2010; POLIDO et al., 2012; YANG et al., 2013). O potencial genético de um animal pode ser determinado com precisão, antes mesmo da expressão do fenótipo com a utilização de marcadores moleculares. Mathur, Liu e Muir ([2018]) descrevem que o DNA pode ser extraído de um número pequeno de células e muitos genes podem ser analisados simultaneamente, e assim possível gerar os dados para o programa de melhoramento (KESAWAT; DAS, 2009).

O aprimoramento conforme Faleiro et al. (2011), e o surgimento de técnicas de biologia e genética molecular para a obtenção dos marcadores moleculares, juntamente ao conhecimento da bioinformática, resultam em análises mais detalhadas e precisas, possibilitando obter maior quantidade de polimorfismos, demandando menor tempo, utilizando-se de infraestrutura mais simples e menor quantidade de reagentes. Portanto, a escolha de um ou mais marcadores requer o conhecimento de suas aplicações e propriedades (SEMAGN; BJØRNSTAD; NDJIONDJOP, 2006; KESAWAT; DAS,2009). Com base nas características de cada uma das inúmeras técnicas de genotipagem, a questão é selecionar aquela que melhor se ajusta às necessidades do programa de melhoramento, em termos do número de marcadores, da cobertura do genoma, do tempo para obtenção e processamento dos dados e dos custos por amostra ou por *data point* (variáveis do processo) (BORÉM; CAIXETA, 2016). Alguns marcadores moleculares são apresentados no Quadro 1.

Quadro 1 - Principais tipos de marcadores moleculares

Marcador	Vantagens	Desvantagens
Bioquímico- Isoenzimas	Baixo custo Determinação genotípica dos *locus* pode ser feita em qualquer parte da planta Vários *locus* de isoenzimáticos podem ser analisados simultaneamente Não demandam ampla amostragem do genoma	Reduzida cobertura nos genomas investigados Baixo nível de polimorfismo identificado por *locus* Influência do ambiente no estádio da planta
PCR - Reação em Cadeia pela Polimerase (*Polymerase Chain Reaction)*	Permite amplificação de pequena quantidade de DNA Rápida, segura	Facilidade de contaminação Não automatizado
RAPD-Polimorfismo do DNA Amplificado ao Acaso *(Random Amplified Polymorphic DNA)*	Baixo custo Produz um grande número de bandas que podem ser caracterizadas individualmente Rapidez na obtenção dos dados Possibilidade de automatização do processo.	Baixa reprodutibilidade Baixo conteúdo de informação genética em cada loco Genótipos homozigotos não se diferenciam diretamente dos heterozigotos
AFLP-Polimorfismos de Comprimento de Fragmento Amplificado (*Amplified Fragment Length Polymorphism)* SFLA-Amplificação do Comprimento do Fragmento Seletivo (*Selective Fragment Length Amplification)* SRLA- Amplificação seletiva do fragmento de restrição *(Selective Restriction Fragment Amplification)*	Alta reprodutibilidade. Capacidade de analisar simultaneamente várias regiões do genoma Cobertura ampla do genoma Detecção de altos índices de polimorfismos, sem a necessidade do conhecimento prévio de dados de sequência	Dificuldade em identificar variantes alélicas

Continua

Quadro 1 - Principais tipos de marcadores moleculares

Continuação

Microssatélite SSR- Sequências Simples Repetidas *(Simple Sequence Repeat)* STR - Repetições Curtas em Tandem *(Short tandem Repeat)*	Altamente informativo Alta taxa de mutação de alelos, alta heterozigosidade Útil no mapeamento comparativo Requer pequena quantidade de DNA Identificação de clones Identificar espécies, independente do estado de vida do animal.	Técnica complexa Alto custo Difícil de automatizar Requer a construção de biblioteca genômica Custo e trabalho empreendidos no desenvolvimento dos *primers*

Fonte: Adaptado de Schlötterer (2004); Faleiro et al., (2011); Borém e Caixeta, (2016)

2.1.4 Tipos de marcadores moleculares

2.1.4.1 - ARMS – PCR

Sistema de amplificação refratária a mutação (*Amplification Refractory Mutation System*) também conhecido como Reação em Cadeia da Polimerase Alelo- Específico (ASPCR), ou amplificação por PCR de alelos específicos, é um método confiável para detectar qualquer mutação envolvendo alterações de base única ou pequenas deleções (NEWTON et al.,1989). A técnica baseia-se na utilização de *primers* com uma sequência específica para PCR, que permite a amplificação do teste de DNA, quando o alelo alvo estiver contido na amostra.

Após uma reação de ARMS, a presença ou ausência de um produto de PCR é diagnóstica para a presença ou ausência do alelo alvo. Para análise de duas ou mais mutações tem-se o Multiplex ARMS-PCR (REGITANO; VENERONI, 2009).

A utilização ARMS-PCR quando se usa mais de um *primer* é interessante, pois pode-se pesquisar vários genes candidatos em uma mesma reação ou vários polimorfismos no mesmo gene. Estudo assim foi realizado por Ferreira et al. (2017) com cinco genes de interesse econômico em uma população de matrizes suínas, o que possibilitou genotipar de uma vez só todos os animais, para os genes de interesse. Chai et al. (2010) utilizaram quatro pares de *primers* para pesquisa de

polimorfismos no gene SERCA-1, relacionado com o controle de entrada e saída de cálcio na membrana do retículo sarcoplasmático, sugerindo que a mutação poderia estar relacionada com a qualidade de carne.

2.1.4.2 - RFLP

Polimorfismo no comprimento do fragmento de restrição ou *Restriction Fragment Length Polymorphism* (RFLP) é um recurso molecular eficaz na identificação de variações genéticas. De acordo com Regitano e Veneroni (2009) os fragmentos de restrição de tamanhos diferentes obtidos após digestão por uma endonuclease de restrição permite a denominação de alelos e podem ser facilmente comparados entre as populações. Estes polimorfismos são caracteristicamente codominantes e de natureza bialélica, em genes de cópias únicas podem estar em regiões codificantes. É uma técnica que consiste na amplificação de uma região de DNA conhecida, por meio da PCR, e que utiliza um par de *primer*s complementares a sítios específicos. Para obtenção das sequências dos nucleotídeos realiza-se a pesquisa das mesmas em bancos de dados disponibilizados em sites específicos disponíveis na internet. A base molecular do RFLP é que as substituições, inserções, deleções, duplicações e inversões de base nucleotídicas podem remover ou criar novos sítios de restrição (YANG et al., 2013).

Os sítios de restrição são reconhecidos pelas endonucleases, que são obtidas da extração de bactérias e de outros organismos. Estas os reconhecem e clivam a fita dupla de DNA em 4 a 8 pares de base, podendo ocorrer eventos de mutação nestes locais. Também podem ser obtidas pelo sequenciamento de fragmentos de DNA de interesse, que pode ser um gene específico, pequenos genomas de micro-organismos ou sequências expressas. Os fragmentos são separados por tamanho após a eletroforese em gel de agarose ou poliacrilamida (FERREIRA; GRATTAPAGLIA, 1998; BEUZEN; STEAR; CHANG et.al., 2000; CHEN et al., 2008; RAMALHO et al., 2012; BORÉM; CAIXETA, 2016).

PCR-RFLP tem sido utilizado em vários trabalhos de investigação, como exemplo a dos pesquisadores Guan et al. (2018), que usaram um par de *primer* universal para distinguir em uma mistura de produto cárneo, diferentes espécies animais, conseguindo reconhecer as oito espécies misturadas intencionalmente.

Segundo os autores o método apresentou-se sensível, específico e confiável na diferenciação das espécies. Para a identificação de genes candidatos para características de produção, Hunyadi-Bagi et al. (2016); Laliotis; Marantidis; Avdi, (2017) e Ferreira et al. (2017) também utilizaram esta técnica. Assim como Faria et al. (2006); Gol et al. (2016); Balatsky et al. (2016) e Michelle-Fong et al. (2018) com a associação dos genes de diferentes raças com a qualidade da carne e carcaça.

2.1.4.3 – SNPs

Polimorfismos de nucleotídeo único (SNPs) ou *Single Nucleotide Polymorphism* envolvem a substituição de um nucleotídeo por outro ou a adição ou exclusão de um ou alguns nucleotídeos (RODRIGUEZ-MURILO; SALÉM, 2013; BORÉM; CAIXETA, 2016). Em uma população essa alteração no genoma para ser considerada um SNP, tem que ocorrer com uma frequência alélica mínima de 1%. De acordo com Vignal et al. (2002); Regitano e Veneroni (2009) os SNPs são marcadores interessantes, por se encontrarem em qualquer região do genoma ou em qualquer sequência de interesse. São preferidos em relação a outros marcadores pela facilidade de genotipagem e pela baixa taxa de mutação (Faleiro et al., 2011). Uma das vantagens desse marcador é que em situações que exige a análise de grande número de indivíduos e/ou de polimorfismos se mostra eficiente, permitindo sua utilização em grande escala. Em modelos de predição, o uso de marcadores para uma maior quantidade de diferentes polimorfismos em animais de produção deu origem aos *chips* de SNP (COUTINHO; ROSÁRIO; JORGE, 2010; FALEIRO et al., 2011). Como a avaliação é simultânea, e de um grande número de marcadores, a seleção é realizada não mais com informações pontuais, mas sim baseado no genoma (REGITANO.; VENERON, 2009; RAMOS et al. 2009).

Na maioria dos casos a substituição de um único nucleotídeo leva à troca de um aminoácido da proteína e, por consequência, à modificação fenotípica, podendo ter a perda da funcionalidade da proteína (YANG et. al., 2013; BORÉM; CAIXETA, 2016).

2. 2 Definição de genes candidatos

Nos últimos anos grande número de genes foram identificados e mapeados usando a tecnologia molecular em suinocultura. Para a maioria dos fenótipos de herança quantitativa, a seleção dos genes candidatos para estudo continua muito complexa. Faleiro et al. (2011) chama a atenção para os genes que podem ter efeito significativo sobre a expressão das características, mas que não fazem parte de importantes processos fisiológicos conhecidos. Essa seleção demanda conhecimentos de genética, bioquímica, fisiologia, muitas vezes não disponíveis mesmo para fenótipos bem caracterizados ou rotas metabólicas conhecidas. O mais adequado é selecioná-los com base em evidências que os correlacionem com o fenótipo-alvo (BORÉM; CAIXETA, 2016). Segundo Coutinho; Rosário; Jorge (2010) são utilizadas três estratégias para identificar genes de interesse: mapeamento de QTL, genes candidatos e sequenciamento de DNA e ácido ribonucleico mensageiro (mRNA), incluindo expressão gênica (GARCIA; PORTO-NETO, 2006)

Alguns genes utilizados como marcadores de interesse na produção e na qualidade de carne de suínos são apresentados no Quadro 2.

Quadro 2 - Genes candidatos para características de interesse econômico.

Gene	Cromossomo	Efeito	Fonte
Hal Halotano	6	Associado com carne pálida, flácida e exsudativa - PSE	Fuji et al. (1991)
ESR Hormônio receptor do estrógeno	1	Aumento do tamanho da Leitegada	Rothschild et al. (1996) Suwanasopee; Koonawootrittriron (2011)

Continua

Quadro 2 - Genes candidatos para características de interesse econômico.

Continuação

DGAT Diacylglicerol acyltransferase	4	Absorção de gordura e lactação	Nonneman e Rohrer (2002)
MC4R Receptor da Melanocortina 4	1	Regulação da temperatura corporal, resposta imune e ingestão alimentar; Espessura de Toucinho	Ovilo et al., (2006)
H-FAB Proteína de ligação de ácidos graxos – coração	6	Deposição de gordura intramuscular; Transporte de ácidos graxos intracelular	Gerbens et al. (1999 Chen et al., (2014)
SREBF1 Factor 1 de transcrição do elemento regulador do esterol	12	Diferenciação dos adipócitos	Chen et al., (2008) Stachowiak et al., (2013)
LEP Gene Leptina	6	Estimula a produção de neurotransmissores, neuropeptídios, hormônios hipotalâmicos	Zhang et al. (2014) Barb (2001)
GH Hormônio do crescimento	12	Eficiência alimentar; Taxa de crescimento; Síntese de proteína	Lan et al., (2018)

Fonte: A autora.

Os avanços da tecnologia molecular juntamente com a bioinformática permitiram a identificação de muitas regiões cromossômicas. As quais afetam diversas características como resistência a doenças, aumento do número de leitões, diminuição da deposição de gordura na carcaça (SILVA et al., 2 0 0 3 ; KIMIEĊ et al., 2010; OKAMURA et al., 2012). Em concordância com Cheng et al. (2000); Gol et al., (2016); Bertolini et al., (2018), estudos sobre os polimorfismos de nucleotídeos têm grande potencial para melhorar as características de ganho

diário de peso, conversão alimentar e desempenho, assim como massa muscular, dentre outras características).

Neste sentido, a primeira pesquisa teve como objetivo associar características de interesse econômico com a fixação de alelos de genes candidatos em uma população de matrizes puras e na segunda pesquisa o objetivo foi de identificar o polimorfismo do gene do crescimento com a endonuclease DdeI de animais provenientes de cruzamento comercial e relacioná-lo com qualidade de carne e característica de carcaça.

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CAPÍTULO 2

Study of genetic variability in pigs after the traditional breeding program

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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 (Rothschild, 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 *primer*s and probes, were from Invitrogen®.

Three *primer*s were designed for each of the genes using the sequences deposited in GenBank. They were designed using the *Primer*3 (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. *Primer*s designed to amplify the target regions of the genes DGAT1, LEPR, H-FABP, MC4R, SREBF1, and an M13 probe.

Gene	*Primer*	Amplicon (bp)	GenBank number
	F*: 5'-M13-1**AGC CAG CGC CCC CGG TC**C**-3'		XM_005655311.2
DGAT1	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'		
	F: 5'-GTG ATA ACT GCA TTT GAC TTG GC-3'		
LEPR	R:5'-M13-1AGTTTGATAAGTAGGTACCACTT**AT**-3'	209	GQ268934
	R:5'-M13-2AGTTTGATAAGTAGGTACCACTT**GA**-3'		228(A/G); 229(T/A)
	F: 5'-M13-1- CTA GCC CAG CCT CAC CAT GG**T**-3'		
H-FABP	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)
	F: 5'-TAC CCT GAC CAT CTT GAT TG-3'		
MC4R	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)
	R: 5'-M13-2 CAG CAC ACG CGC CTC CAC G**A**-3'		
SREBP1	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 *primer*s used are in Table 2.

Table 2. *Primer*s designed to amplify target regions of the GH gene.

Gene	**Primer*	Amplicon (bp)	GenBank number
GH	F: 5'-TTATCCATTAGCACATGCCTGCCAG-3' R: 5'-CTGGGGAGCTTACAAACTCCTT-3'	605	M17704

**Primer* designed by Larsen and Nielson (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 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 10

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, HFABP MC4R, SREBF1 and GH genes in Large White breeders.

GENES	GENOTYPE	GENOTYPE (%)	FREQUENCY	ALELE	ALLELE FREQUENCY (%)
	TT	0		T	0
DGAT1	TC	0		C	100
	CC	100			
	GAGA	100		GA	100
LEPR	GAAT	0		AT	0
	ATAT	0			
	TT	0			
HFABP	TC	0		T	0
	CC	100		C	100

	TT	0		
MC4R	TC	100	T	50
	CC	0	C	50
	GG	0	G	0
SREBF1	GA	0	A	100
	AA	100		
	D_1D_1	0	0	0
GH	D_1D_2	22	D_1	11
	D_2D_2	78	D_2	89

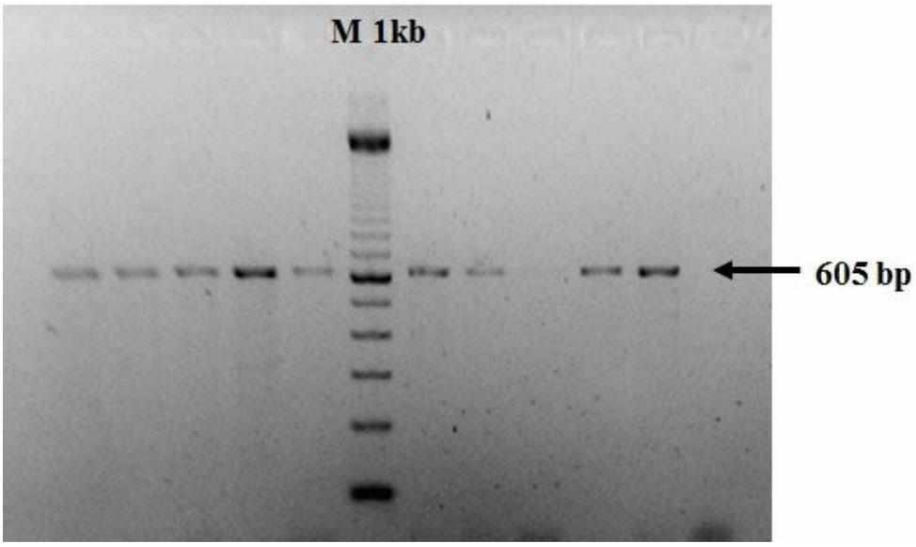

Figure 1. Amplification of the GH gene on 2% agarose gel showing an amplicon of 605 bp and the marker (M) of 1 kb.

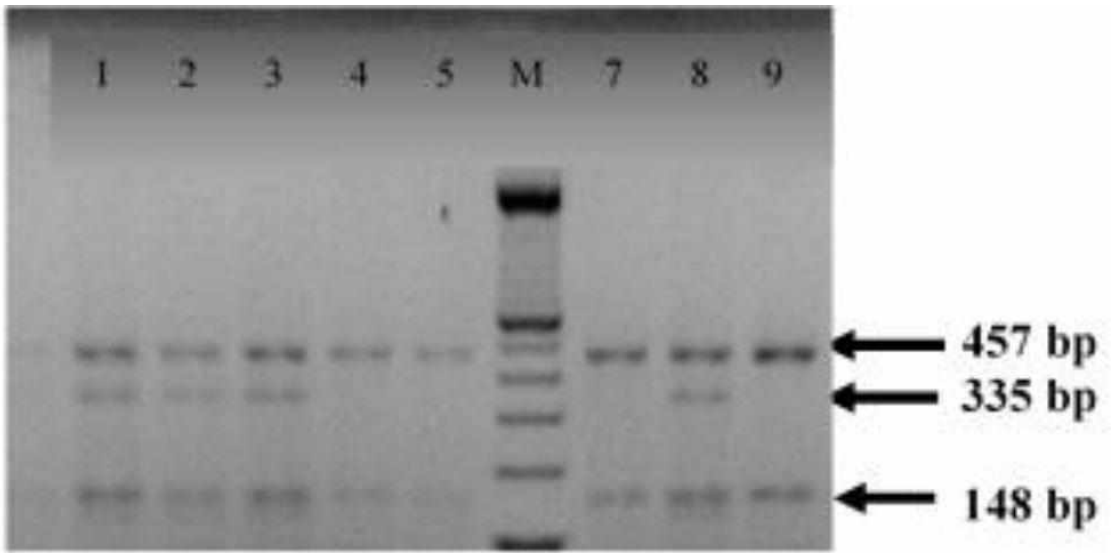

Figure 2. GH gene digested by *Dde*l enzyme on 2.5% agarose gel. *Lane M* = 1-kb marker; *lanes 1*, *2*, *3*, and *8* = fragments 457, 335, and 148 ($D_1 D_2$) and *lanes 4*, *5*, *7*, and *9* = showing fragments 457 and 148 ($D_2 D_2$).

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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CAPÍTULO 3

Artigo que será submetido a revista Genetics Molecular Reserch.

A DdeI polymorphism in the growth hormone gene is associated with higher lean meat yield and pH 45 min in pigs

A DdeI polymorphism in the growth hormone gene is associated with higher lean meat yield and pH 45 min in pigs

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ABSTRACT: Marker-assisted selection has been widely used in breeding programs. The use of single nucleotide polymorphisms as genetic markers enables identification of genotypes that best contribute to quantitative characteristics. Therefore, we evaluated the association between the DdeI growth hormone gene polymorphism and meat and carcass traits. A total of 476 halothane-free animals were genotyped. Animals were originated from the crossing of a AGIPIC®415 male and Large White (LW) x Landrace (LD) females. Animals were castrated males and females, and were slaughtered between 150 and 180 days old. Hot carcass weight, carcass lenght, pH 45 min min, and pH ± 16 h *postmortem* of *Longissimus cervicis*, *Longissimus dorsi*, and *Semimembranosus* muscles, backfat thickness, *Longissimus dorsi* muscle depth, and color by the CIELAB system were collected and the percentage of lean meat was calculated. Water holding capacity was determined by the filter paper method in the *Semimembranosus* muscle. A 605-base pair (bp) amplicon was obtained by polymerase chain reaction and a DdeI polymorphism was genotyped and two alleles identified, D_1 with 335, 148, and 122 bp and D_2 allele with 457 and 148 bp. Allele frequencies were 0.43 for D_1 and 0.57 for D_2. Genotypic frequencies were as follows: D_1D_1 (22,09%), D_1D_2 (49,82%), and D_2D_2 (28,09%). The Chi-Square test showed that the population was in Hardy-Weinberg equilibrium. This DdeI SNP was associated with lean meat percentage and pH 45 min min value in the *Longissimus cervicis* muscle, properties that are important for the commercialization of fresh meat as well as processed products.

KEYWORDS: Single nucleotide polymorphism, Growth Hormone, Carcass traits, Meat quality, Swine

INTRODUCTION

The analysis of pork quality is constant and considers cultural, sensorial, nutritional, and hygienic properties, economic issues, improvement in *ante* and *postmortem* handling, technological processes, and genetic variables (Rosenvald; Andersen, 2003; Joo et al., 2013; Pena et al., 2016). However, to meet the need for increased production and improve carcass and meat quality, producers and the swine industry have begun using molecular techniques. With the information obtained by DNA analyses, it is possible to select genes that significantly affect production, enabling the use of marker-assisted selection (Samarai; Kazaz,

2015; Pena et al. 2016). Many genes known to affect carcass and meat quality have been mapped in swine, among which porcine growth hormone (pGH) is highlighted.

pGH has more than 300 functions (Waters et al., 2006) and is involved in the growth of skeletal muscles, bone, and adipose tissue (Parr et al., 2016; Lan et al., 2018) and stimulates the production of insulin-like growth factor 1 in the liver (Cheng et al., 2016). Studies to improve meat quality and performance characteristics with variants of the pGH gene were described by Schellander et al. (1994); Knorr *et al.* (1997); Cheng *et al.* (2000); Song et al. (2003); Franco *et al.* (2008); Bižienė *et al.* (2011); and Lyubov *et a*l. (2016).

The allelic frequencies of pGH polymorphism between breeds may not be the same, because these populations undergo different selection pressure, for distinct characteristics, such as carcass quality and meat. 'For example, Wenjun et al. (2003) found that European breeds had high B allele frequencies, whereas Chinese native breeds showed high frequencies of the A allele for the ApaI when studying pGH gene polymorphism. Franco et al. (2005) observed that the D_1D_2 genotype was associated with a higher percentage of lean meat and lower backfat thickness in a Landrace swine population. Carcass length was related to the pGH/DdeI polymorphism in Large White swine (Putnová et al., 2001). However, Rybarczyk et al. (2007) observed no relationship between the identified pGH genotypes with the MspI, HaeII, CfoI, and ApaI enzymes with carcass and meat quality. Additionally, Casas-Carrillo et al. (1997) did not detect an association between pGH/DdeI and pGH/HaeII gene polymorphisms with carcass and meat quality determined based on pH.

The aim of this study was to evaluate the association between the DdeI polymorphism in the pGH gene with meat quality and carcass traits in a crossbred population of pigs.

MATERIAL AND METHODS

Biological samples

A total of 476 left half carcasses from 150 to 180 days-old female and castrated male pigs from an industrial crossing (AGIPIC®415 males × LW and LD females) were used. All animals were halothane-free and were raised under the same conditions and from two commercial farms. Animals were shipped and transported in accordance with animal welfare standards and slaughtered in a local slaughterhouse according to the Brazilain legislation.

Carcass traits and meat quality

Temperature and pH were measured at two time points (45 min and 16 ± 3 h *postmortem*) in the *Semimembranosus* (SM), *Longissimus dorsi* (LD), and *Longissimus cervicis* (LC) muscles using a portable pH meter (model 205, brand Testo, West Chester, PA, USA) with digital identification and automatic temperature compensation. Carcass length (CL) was measured in the still hot half carcasses, considering as the initial point of measurement the ventral skull edge of the Atlas and end point the cranial edge of the pubic symphysis, according to the Brazilian carcass evaluation method (ABCS, 1973), using a precision measuring tape with 0.05 cm increments. Data of color was obtained on the SM and LC muscles using the Delta Vista® portable spectrophotometer (GE Healthcare, Little Chalfont, UK) at 16 ± 3 h *postmortem*, where L* corresponds to the flesh luminosity, a* to the red content, and b* to the yellow content, according to the CIELAB system. Weight of the hot carcass was obtained before being placed in the cold chamber and the cold weight was determined after 16 ±3 Backfat thickness (BT) was measured between the last lumbar vertebra and first sacral vertebra in the median sagittal plane using a caliper. The *Longissimus dorsi* depth (LDD) and backfat depth (BD) were determined at the last lumbar vertebra, perpendicularly to the dorsal-lumbar line, at 6 cm from the vertebral column with a caliper. All measurements were performed on cold carcass. To calculate the percentage of lean meat (LM), the equation described by Antunes was used (2018), as follow: Lean meat yield (%) = 67,31240 – 0,47691 × backfat thickness

The water holding capacity (WHC) was measured using a modified compression method described by Grau and Hamm (1953) in the *Semimembranosus* muscle. This procedure was performed in duplicate. Next, the liquid and meat areas were photographed and processed with the **ImageJ** 1.8® image analysis tool (NIH, Bethesda, MD, USA). The WHC was expressed as the ratio of the compressed area of the meat divided by the exudate area in the filter paper.

Molecular analyses

Samples of the SM muscle were collected and stored in sterile plastic tubes at -80°C until processing. To isolate the genomic DNA, the PureLink® Genomic Kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's recommendations. The NanoDrop® ND-1000 spectrophotometer (Waltham, MA, USA) was used for DNA

quantification. For genotyping, a pair of primers was used (forward 5′ TTATCCATTAGCACATGCCTGCCAG 3′ and the reverse 5′ CTGGGGAGTTACAAACTCCTT 3′ (Genbank Accession number: M17704)), as designed by Larsen and Nielsen (1993) to amplify a region of 605 bp. PCR was performed in a final volume of 20 μL. Each reaction contained the following: 1X buffer, 2 mM of $MgCl_2$, 0.4 mM dNTPs, 7.5 x 10^{-6}μM each primer (forward and reverse), 1 U of Taq polymerase, genomic DNA (50–200 ng), and ultrapure water to the final volume. The thermocycler conditions were as follows: initial denaturation at 95°C for 3 min, 35 cycles at 95°C for 45 s, 61°C for 40 s, 76°C for 1 min, and final extension at 76°C for 4 min. After amplification, 5 μL of the PCR product was added to 2 μL of loading buffer and applied to a 2.0% agarose gel for electrophoresis. The amplicon of 605-bp of length was confirmed under UV light and photodocumented. The remaining 15 μL was digested with 3 U of the restriction enzyme DdeI overnight at 37°C. After digestion, 3 μL of loading buffer was added to each sample and applied to a 2.5% agarose gel and stained with ethidium bromide, which was visualized under UV light and photodocumented. For the GH gene after digestion of the samples, two alleles were obtained: D1 with fragments of 335, 148, and 122 bp and D2 with 457 and 148 bp.

Statistical analysis

Allele frequencies were calculated by simple allele counting (Falconer; Mackay, 2012).Chi-square test was used to determine if the frequencies were in Hardy-Weinberg equilibrium. To test the association between genotypes and carcass and meat quality traits, the partial quantile covariate was used; analysis of equality of variances by the Levene test and a normality test of the residues in the analysis of variance mathematical model were performed. Considering that data do not show normality, the Kruskal Wallis test was performed. Farm was initially considered in the statistical model, but as the result was not significant, it was removed as an effect in the analysis. The data were analyzed using SPSS software (SPSS, Inc., Chicago, IL, USA).

RESULTS

DdeI restriction enzyme digestion of the 605-bp PCR amplicon revealed two alleles: D_1 (335, 148, and 122 bp) and D_2 (457 and 148 bp) (Figure 1).

Figure 1.

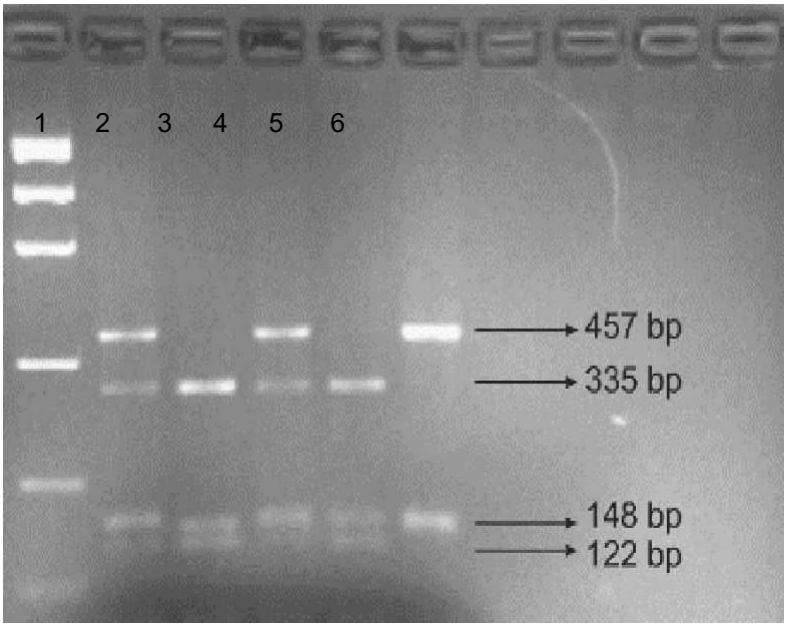

Figure 1. DdeI polymorphism in the growth hormone gene detected by PCR-RFLP. PCR products were runin a 2.5% agarose gel. Lane 1: 1 Kb DNA ladder (Invitrogen); lanes 2 and 4: D_1D_2, genotype lanes 3 and 5: genotype: D_1D_1 genotype and lane 6 D_2D_2 genotype. bp – base pair.

Table 1 shows genotypic and allelic frequencies of Gh DdeI polymorphism. Chi-square test ($\chi^2 = 4,97$; $p < 0,05$) indicated that the observed and expected frequencies of the pGH/DdeI genotypes were in Hardy-Weinberg equilibrium.

Table 1: Genotypic and allelic frequencies of the DdeI pGH gene polymorphism

Genotype pGH /DdeI		Observed Frequency (%)	Expected Frequency (%)	Allele D_1 (%)	Allele D_2 (%)
D_1D_1	93	19.53	22.09		
D_1D_2	262	54.83	49.82	47	53
D_2D_2	122	25.00	28.09		

Data of meat quality and carcass traits according to the genotypic effect of the pGH/DdeI gene, are shown in Table 2.

Table 2. Meat quality and carcass traits according to the GH genotypes

Variables	D_1D_1		D_1D_2		D_2D_2	
	Mean	SD	Mean	SD	Mean	SD
HCW	79.72[a]	11.2	83.27[a]	10.66	82.12[a]	10.87
% LM	61.6 [a]	2.78	62.80[a]	2.24	63.13[b]	2.34
CL (cm)	93.70[a]	4.38	94.63[ab]	3.89	94.16[a]	4.73
BT (mm)	8.95[a]	4.8	10.14[a]	5.15	9.52[a]	5.25
BD (mm)	18.65a	6.48	19.41[a]	6.14	18.10[a]	7.47
pH_{45} LC	6.51[a]	0.25	6.45[ab]	0.26	6.43[b]	0.26
pH_{45}LD	6.63[a]	0.26	6.60[a]	0.31	6.6 [a]	0.33
pH_{45} SM	6.59 [a]	0.49	6.62 [a]	0.308	6.63 [a]	0.31
ºC 45'-LD	31.43[a]	4.17	31.33[a]	4.04	31.85[a]	4,57
CCW	77.72[a]	10.9	81.1[a]	10.43	80.07[a]	10.63
$pH_{\pm16}$-LC	6.42[a]	0.54	6.40[a]	0.66	6.51[a]	0.48
$pH_{\pm16}$ LD	6.20[a]	0.50	6.14[a]	0.56	6.74[a]	5.99
$pH_{\pm16}$ SM	5.93[a]	0.7	5.96[a]	0.62	6.01[a]	0.48
LDD	65.82[a]	14.18	66.66[a]	10.70	66.82[a]	11.25
WHC- SM	0.40[a]	0.08	0.41[a]	0.09	0.40[a]	0.09
L* SM	38.29[a]	11.15	38.16[a]	8.0	38.74[a]	5.39
Color a* SM	-2.22[a]	1.76	-2.0[a]	2.16	-2.09[a]	1.99
Color b* - SM	5.74[a]	2.43	5.30[a]	1.79	5.33[a]	2.31

SD- Standard deviation, LC- *Longissimus cervicis*, HCW- hot carcass weight, LM- lean meat, CL- carcass length, LD- *Longissimus dorsi*, CCW- cold carcass weight, SM- *Semimembranosus* muscle, LDD- *Longissimus dorsi* depth, BT- backfat thickness, BD- backfat depth, WHC- water holding capacity, L*- luminosity.
Different letters in the same line indicate a significant difference ($p< 0.05$) by the Kruskal Wallis test.

DISCUSSION

After analyzing the χ^2 test, the studied population was found to be in Hardy-Weinberg equilibrium, thus obtaining the allelic frequencies of D_1 (0.47) and D_2 (0.53), demonstrating that in this population, this polymorphism can be used as a selection tool. Franco et al. (2001), identified the allelic and genotypic frequencies for the DdeI polymorphism in the GH gene showing allelic frequencies for Landrace (D_1: 0.69 and D_2: 0.31), Large White (D_1: 0.25 and D_2: 0.75) and Pietrain (D_1: 0.72 and D_2: 0.28). It is interesting to note that the frequencies of the Pietrain and Landrace breeds are close, suggesting that they may be selected for the same characteristics. Demonstrating that the genotypic frequency of these populations was influenced by the selection of quantitative characteristics.

Kaushik et al. (2014), studying the DdeI polymorphism, found the presence of both, D_1 and D_2 alleles. The D_1 allele was most frequent in Hampshire (0.55), while the D_2 was found in the crossing between Hampshire × Ghungroo (0.6).

When the GH genotypes were evaluated in this work, differences were found for lean meat characteristics, with the D2D2 genotype presenting the highest percentage. According to the research conducted by Franco et al. (2008) who concluded the existence of a direct effect of the D2 allele (GH / DdeI) on pork quality. The trend toward lean meat consumption has increased the selection of animals for this characteristic.

Regarding pH_{45} we showed that the D_2D_2 animals had a lower value than the others, Kauffman (Kauffman, 1991; Ramos et al., 2009) Although pH 45 min is not a good indicator of the final quality of the meat, according to Kauffman (1991) and Tomovic et al (2014), pH_{45} < 6 may be an indicator of pale, soft, and exudative meat and values above 6.3 are desirable for obtaining reddish, firm, non-exudative meat in the case of loin and leg muscles, which predominantly contain white fibers. However, the LC muscle consists predominantly of red fibers and is used as an indicator of dark, firm, and dry (DFD) meat. Variations of pH_{45} have multifactorial causes such as the hottest season of the year, fights between animals that did not fast, and type of muscular fiber (Dalla Costa et al., 2016; Rey-Salgueiro et al., 2018). Due to these factors, pH decline in the transformation of the muscle in meat directly influences attributes such as color, WHC, softness, and flavor, according to Rübensam (2000); Ramos; Gomide (2007); Scheffler et al. (2013); thus, meat should not be classified using a single parameter. Meat, which can also be classified by the WHC as pale, soft, and exudative or as DFD, was analyzed in this study using the filter paper method in the SM muscle, which revealed no significant difference among genotypes considered normal for the evaluated muscle (Houfmann, 1982). Using the same method, Tomovic et al. (2014) introduced for the Landrace and Large White breeds and WHC is influenced by the muscular type. WHC is an important attribute in the industry as it is important for the yield of processed and stained products of fresh meat

The color of pork is influence by multiple factors such as feed, pre-slaughter handling, genetics, age, anatomical function of the muscle, amount of oxidative, glycolytic, mixed fibers, and sex (Kim et al., 2013; Arkfeld et al. 2017; Faucitano, 2018). A study of the color of some muscles showed that the SM muscle of castrated males and females produced under the same conditions did not show differences in L*, a*, and b*, indicating no effect of sex (Overholt et al., 2016). In this study, lots of animals for slaughter included castrated males and females, reared under the same conditions, possibly the same results as those described in the previous work would be observed above would likely be in this study, lots of animals for

slaughter included castrated males and females, reared under the same conditions, possibly the same results as those described in the previous work would be observed.

Some studies observed an association between variants in the pGH gene with meat quality attributes (Putnová, et al., 2001; Faria et al., 2006; Bižienė et al., 2011; Lyubov, et al., 2016). Rybarczyk et al. (2007), studying four SNPs, and Kmieć et al. (2010), evaluating the HaeII polymorphism, observed that this association was weak or non-existent for the characteristics associated with meat quality. Bižienė et al. (2018), investigated SNPs in 143 animals from 5 different breeds and found that GH polymorphism (M17704.1: g.316G>A) is significant for daily weigh gain, highest value for CC genotype pigs. They also suggested that this list is due as different races.

Balatisky et al. (2015) suggested that using pGH as a molecular marker in assisted selection is effective only in some populations. The differences are related both to the peculiarities of their specialization in genetic improvement as to productivity and origin, due to the number of QTL. Confirming this work, because both the paternal and the maternal lineage are selected for different characteristics.

CONCLUSION

The polymorphism of the pGH / DdeI gene was related to the characteristics of lean meat and pH to 45, properties that are important for the commercialization of fresh meat as well as processed products.

Conflicts of interest

The authors declare no conflict of interest.

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Anexo A

Polimorfismo do Hormônio do crescimento suíno (pGH) utilizando a enzima de restrição DdeI- 2231pares de base - Genbank M17704

CCCGGGGGACATGACCCCAGAGGAGGAGCGGGAACAGGATGAGTGGGAGGAGGTTCTAAA***TTATCCAT***
TAGCACATGCCTGCCAGTGGGCCATGCATAAATGTATAGAGAAAATAGGTGGGGGCAGAGGGAGAGAG
AAGAGGCCAGGG**TATAAAA**AGGGCCCAAAAGGGACCAATTCC**A**GAATCCCAGGACCCAGCTCCCCAGA
CCACTCAGGGACCTGTGGACAGCTCACCGGCTGTG*ATG*GCTGCAGGCAAGTGCCCCTAAAATCCCAGT
GGCTTGGTGTGTTCTGAAGGGTGACGTGGGGGCCATGCAGATGGATGGGGCACCAACCTTGGGCTTTG
GGGTTTCCGAATGTGAGCATGGATATCTACTCCTAGATATGAGGCCAAGTTTTAAATGTCCCTGGGGG
AGGGGGAGGAGAAGGGACAGGGCTGGTGGAGCCAGGCCTCTTGTCTCTGGGATCCCTCTCTCACGGGC
CCTCCTGGTCTCTAGGCCCTCGGACCTCCGCGCTCCTGGCTTTCGCCCTGCTCTGCCTGCCCTGGACT
C**G**GGAGGTGGGCGCCTTCCCAGCCATGCCCTTGTCCAGCCTATTTGCCAACGCCGTGCTCCGGGCCCA
GCACCTGCACCAACTGGCTGCTGACACCTAC***AAGGAGTTTGTAAGCTCCCCAG***GGAGGGTGCTGGAGG
GGGGTGGTGGAGAGGGGTGAATTCGTCCCTCTCTGCCTAGTGGGAGGAAAATGAGGGGTTCTGGAGTA
TTGAGGCCAACCGAAGATGCTATCAGGTGAGTGTAAACTGAAGGGGATTCCCAAGAAAAGCAGCAAGG
AGAACCGCGCCCCAGTGTAGACCTGGATGGCTGTCCCCTCTCCCAGGAGCGCGCCTACATCCCGGAGG
GACAGAGGTACTCCATCCAGAACGCCCAGGCTGCCTTCTGCTTCTCGGAGACCATCCCGGCCCCCACG
GGCAAGGACGAGGCCCAGCAGAGATCGGTGAGTGGCCACCTGCCACCTGCCAGCCGGGGAGCAGGGGC
CTCCCTCTTCCTAAGAAGGCTGCCCCATCTCTCATCATCAGGGCCTTGGGCGGCCTTCTCCCCGAGCT
GGTGGGGGTGATGGTGGCAGAGGGCGGGGTGGTGAGGGGGACGCCCACCGGCGGAGGCAGCGCCCCCC
ATCCACGCATCTGCCCGCAGGACGTGGAGCTGCTGCGCTTCTCGCTGCTGCTCATCCAGTCGTGGCTC
GGGCCCGTGCAGTTCCTCAGCAGGGTCTTCACCAACAGCCTGGTGTTTGGCACCTCAGACCGCGTCTA
CGAGAAGCTGAAGGACCTGGAGGAGGGCATCCAGGCCCTGATGCGGGTGGGGAGGCGCGCTCGGGTCC
CGCACACTGGGGCCCATGCCGGCTCTCTCCCGGCTGAGCGGAGCGGTGGGGGGACGCACGTGGGCTGG
GGGAGAGGGTCCCGATGCTCTCTCTGTAGCAGTTCACTCTCGACCCGGAGAAATCTTTTCCTCATTTC
CCCCTGCGGAGTCTTCCCTCTTTGTCCTTCTCCAAGCATGGAGGGGAGGGTGGAAGACGGAGGGGACA
GGAGAGCGCCGCTGCCAAGGACTCGGCCTCTGTCTCTCTCTCCCTTTTGCAGGAGCTGGAGGATGGCA
GCCCCCGGGCAGGACAGATCCTCAAGCAAACCTACGACAAATTTGACACAAACTTGCGCAGTGATGAC
GCGCTGCTTAAGAACTACGGGCTGCTCTCCTGCTTCAAGAAGGACCTGCACAAGGCTGAGACATACCT
GCGGGTCATGAAGTGTCGCCGCTTCGTGGAGAGCAGCTGTGCCTTCTAGTTGCTGGGCATCTCTGTTG
CCCCTCCCCAGTACCTCCCCTGACCCTGGAAAGTGCCACCCCAATGCCTGCTGTCCTTTCCTAATAAA
ACCAGGTTGCATCGTATTGTCTGAGTAGGTGTCACTCTGCGATGGAGGGAGGTGGGGCAGTAGGGCAA
GGGGTGGGGGTGGGAAGACAACCTGCAGGCATCCTTGGGGGTCTCCTGGGGACCTAGACACTGAATGA
TGGTTGACCCGGCTTCTTCCTGGGCTTGAAAGAGCAGGCACATTACCTTCTCTCTGTTACACACCCAC
TGCACCCACTGCTCAGGTCTGCAGTCCAGCTTGCTGGGCACTCATAGGTCAGGACCACCCCCCATCCT
GCTACACCCCCCGCCTCCCATAAAGTACCCAAGAATGGAAAGAGATGAAAGCAAG

Forward: ***TTATCCATTAGCACATGCCTGCCAG*** Reverse: ***CTGGGGAGCTTACAAACTCCTT*** –
GACCCCTCGAATGTTTGAGGAA

O SNP se dá pela troca de uma Guanina por uma Adenina e em consequência a substituição do aminoácido Arginina (R) por uma Glutamina (Q)

ANEXO B - Normas da Revista GMR para submissão do artigo

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