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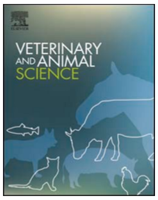

Genetic diversity of meat quality related genes in Argentinean pigs

VR Rodriguez [a], JI Maffioly [a], LA Zdanovicz [a], RM Fabre [a], ME Barrandeguy [b,c,d], MV García [b,c,d], M Lagadari [a,d,*]

[a] *Facultad de Ciencias de la Alimentación – Universidad Nacional de Entre Ríos. Argentina*
[b] *Departamento de Genética. Facultad de Ciencias Exactas, Químicas y Naturales. Universidad Nacional de Misiones, Argentina*
[c] *Instituto de Biología Subtropical – Nodo Posadas (UNaM – CONICET), Argentina*
[d] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*

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ABSTRACT

Genetic influence on pork quality exists between breeds and within a breed. The variation is caused by a large set of genes, and pork quality traits have a multifactorial background. Research into the genetics of meat quality found causative mutations associated with marked effects on pig meat value. This study aimed to investigate the segregation of meat quality-related SNPs and compare their diversity and genetics in commercial and Creole pigs from different farms in the North-West of Argentina. A screen for SNPs in *RYR1*, *PRKAG3*, *CAST*, and *SOX6* candidate genes and the differentiation of their genotypes by PCR–RFLP was conducted. All genes were characterized by a high level of polymorphism and heterozygosity, and populations showed no differences in the genetic structure for the analyzed SNPs. These results highlighted the role of pig genotypes as a source of basic variability potentially affecting processed meat products and fresh meat.

Abbreviations

SNP	single nucleotide polymorphisms
FAO	Food and Agriculture Organization
ISAG	International Society for Animal Genetics
WHC	water holding capacity
IMF	intramuscular fat
HAL	Halotane gene
PSS	Porcine stress syndrome
PSE	pale, soft and exudative meat
RN	Rendement Napole gene
PRKAG3	γ subunit of adenosine monophosphate-activated protein kinase
CAST	Calpastatin gene
QLT	quantitative trait loci
PKA	adenosine cyclic 3′, 5′-monophosphate- dependent protein kinase
CTAB	cetyl-trimethyl ammonium bromide
TE	Tris-EDTA
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
AR	allelic richness
MAF	minor allele frequency
HO	observed heterozygosity
HE	expected heterozygosity
HWE	Hardy-Weinberg equilibrium
PCA	Principal Component Analysis
NJ	Neighbour-joining tree
AMOVA	analysis of molecular variance
FST	Wright fixation index
FIS	inbreeding coefficient
AT	Annealing temperature
AS	amplicon size
RE	restriction enzyme
LD	Linkage Disequilibrium
N	sample size
Na	allelic number per locus

Background

Given the increasing global demand for meat, fast-growing species with a high food conversion rate, such as pigs, can contribute greatly to the development of the livestock subsector. According to The Food and Agriculture Organization (Food and Agriculture Organization of the

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[*] Corresponding author.
E-mail address: mariana.lagadari@uner.edu.ar (M. Lagadari).

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United Nations (FAO) 6 Sep 2020), pork is a major source of protein for humans and accounts for a large percentage of world meat production. In this way, commercial pig production has increased significantly in recent decades. And more pigs, of the same small number of breeds, are raised on fewer and fewer farms, with an increase in the yield of products of animal origin. Large-scale production systems have reached a high level of uniformity as they are based on the same genetic material and therefore provide the same type of feed and infrastructure to animals. However, in developing countries, a large percentage of current pig herds continue to be kept under traditional small-scale production systems. These traditional production methods are a sample of the viability of alternative production systems, usually mixed agricultural systems linked to local markets. Nevertheless, in both types of production, there has not been significant focus on meat quality until the last few years. Meat quality depends on consumers' subjective perceptions, who are demanding not only a carcasś high lean content but also optimal tenderness, marble, aroma and acidity along with an attractive color and water holding capacity (WHC). Furthermore, the quality concept is related to sensory, nutritional, hygienic, technological and genetic components, as well as factors of cellular metabolism that influence meat attributes. Therefore in view of the market́s increasing requirements, the pork industry must focus on controlling every quality parameter along the whole production chain.

As already mentioned, genetic improvement strategies have focused on the production of animals with a rapid transformation of the feed consumed into lean meat and accelerated animal growth, which often affect negatively the organoleptic characteristics of the meat (Wood et al., 2008). Meat quality is a complex polygenic trait and this genetic influence exists between breeds and within a breed (Andersson, 2001; Gispert et al., 2000). Research into the genetics of meat quality found causative mutations associated with marked effects on pig meat value. Specifically, mutations in two major genes referred to Halothane (HAL) and Rendement Napole (RN). The one commonly known as the Halothane gen is the *RYR1* gene encoding the calcium release channel in the skeletal muscle sarcoplasmic reticulum called ryanodine receptor (Gene ID: 396,718) (Fujii et al., 1991). Porcine stress syndrome (PSS) or malignant hyperthermia is an autosomal recessive disease originated by a mutation that causes a substitution $C > T$ at 1843 nucleotide position in the *RYR1* gene. Homozygous recessive animals (TT) turn in a pale, soft and exudative meat (PSE), which results in high losses in the industry. *RYR1* also affects the quality of heterozygote (CT) swine carcasses (Sather, Jones, Tong & Murray, 1991). Furthermore, RN or *PRKAG3* gene is also known to have a negative effect on meat quality; it is associated with the pork acidity ((Naveau, 1986); Milan et al., 1996, 2000). Non-synonymous single nucleotide polymorphisms (SNPs) in this gene such as 199I>*V* and 200R>*Q* are associated with important pork quality traits (WHC and pH) (Ciobanu et al., 2001; Granlund, Jensen--Waern & Essén-Gustavsson, 2011; Josell et al., 2003; Lindahl et al., 2004). Calpastatin *(CAST*, Gene ID: 397,135) is also important in terms of the quality traits of pork. *CAST* is a specific inhibitor of μ- and m-calpain proteases, which are responsible for early postmortem muscle proteolysis (Goll, Thompson, Li, Wei & Cong, 2003; Huff-Lonergan & Lonergan, 2005). Several *CAST* polymorphisms have been described including *CAST* 638Ser>Arg-and *CAST* 76,872 *G*>*A* which have been associated with pork tenderness (Ciobanu et al., 2004; Gandolfi et al., 2011). *SOX6* codes for a transcription factor and the versatility of this gene plays an important role in the specification of slow fiber during skeletal muscle differentiation by inhibiting the transcription of several sarcomeric genes (Hagiwara, 2011; Quiat et al., 2011); in addition it is associated with muscle growth and quality characteristics. Polymorphisms' at porcine *SOX6* sequence (Gene ID: 397,173) have been related to meat quality traits in commercial breed population (Pietrain and Duroc × Pietrain F2 population) (Zhang et al., 2015). In particular, two substitution (rs81358375:*G*>*A* and rs321666676:G>C.) were described at SSC2 intronic sequence here named *SOX6*A and *SOX6*B, respectively.

Considering the link between genetic background and quality attributes as an important step towards management of pork quality, the aim of this study was to analyze the segregation of meat quality related SNPs and compare their diversity and genetic structure across Creole and commercial crossbred populations.

Methods

Animals and sample collection

A total of 242 unrelated animals including commercial and Creole pigs from commercial and family farms at the North-West of Entre Rios state of Argentina (Northeast: 54° 54 50.64 S and 57°49 54.02 W Southeast: 32° 28 23.74 S and 58° 15 12.55 W Southwest: 32° 28 05.36 S and 59° 07 39.97 W and Northwest: 30° 52 42.18 S and 59° 03 43.23 W) were included in the present study. 153 were commercial hybrid breeding stock animals from 12 different producers (the main of the tested animals are hybrids derived from crossing hybrids females Landrace x Yorkshire and a percentage of Chinese breeds with terminal hybrids males composed by different proportions of Duroc, Pietrain, Hampshire, Yorkshire and Landrace). These farms are middle to large scale farms (15–250 dams and 2–4 sires per farm) and, three of these farms only use artificial insemination. A total of 89 were Creole breeding stock animals from 10 different small scale-farms (5–50 dams, 1–2 sires per farm). These local Creole pigs have not been the subject of any conservation or breeding program. The term Creole ("Criollo" in Spanish) is used to refer to descendants from the Iberian Peninsula (Elliott, 2007). The Creole pigs population in North-West Argentina, which is supposed to originate in the animals introduced by the Spaniards during the colonization having received since then numerous contributions from other exotic breeds **(**Revidatti et al., 2014). Hair bulbs samples were collected from the back of pigs, pulling strongly with the thumb, index and middle fingers. The hair bulbs of approximately 50 hairs were removed from each pig. Samples were labeled, transported and stored in plastic bags at room temperature until processed in the laboratory.

DNA extraction

Genomic DNA was extracted using the cetyl-trimethyl ammonium bromide (CTAB) method (Murray & Thompson, 1980, Sambrook and Russell, 2001). Briefly, about 15 bulbs were incubated in TE buffer, 10% SDS and proteinase K (1 mg / ml) for 15 min at 37 °C. Then, 5 M NaCl and CTAB (0.7 M NaCl, 10% CTAB, Genbiotech) were added and incubated at 65 °C for 10 min. Subsequently, chloroform: isoamyl alcohol was added in a 24: 1 ratio, and after centrifugation, DNA was precipitated from the aqueous phase with cold isopropanol. Then, washes were carried out with 70% ethanol, pellet allowed to dry at room temperature and resuspended in 15 μl of TE buffer. DNA concentration and purity (A260/A280 ratio) for each sample was assessed using a spectrophotometer. The measured DNA samples were stored at −80 °C until further analysis.

PCR–RFLP analysis

In the present study seven SNPs of porcine meat quality-related genes were analyzed. All animals were genotyped for a C1843T point mutation in the *RYR1* gene (M91451.1:g.1843C>T), two functional mutation at the *PRKAG3* gene where the SNP at codon 199 cause an *I*>*V* amino acid substitution and the SNP at codon 200 a *R*>*Q* substitution (NM_214,077.1:c.596G>*T, I*>*V* and NM_214,077.1:c.599G>C, *R*>*Q*); two SNP in CAST gene, CAST 638 Ser>Arg (EU137105.1:g.114650A>C) and CAST 76,872 *G*>*A* (EU137105.1:g.76872G>*A*), and two SNP at the transcription factor *SOX6, SOX6*A (rs81358375:*G*>*A*) at 42,812,066 nucleotide position and *SOX6*B (rs321666676:G>C) at 43,023,574. Genotyping of SNPs was done by PCR–RFLP procedure. PCR mix comprised: 1 nM dNTPs, forward and reverse primers (10 pmol), nuclease free water, 1X green buffer and 0.6 U GoTaq DNA polymerase and 1 µl de DNA template (30 ng/µl) in a final volume of 25 µl. Detailed information about SNPs identification is given in Table 1. PCR amplification was performed in a conventional ESCO AERIS PCR thermocycler with the following cycling program: initial denaturation at 94 °C for 5 min; 38 cycles of 94ºC for 30 s, specific annealing temperature for each pair of primers, for 30 s. and 72ºC for 30 s, and a final extension at 72ºC for 7 min. The amplified and digested DNA fragments of SNPs were separated on 3% agarose gel with 0.1 µg / ml ethidium bromide visualized with UV transilluminator and photographed. The genotype of the individuals was determined for each polymorphism by analyzing the size of the fragments in RFLP.

Genetic diversity and population genetic structure analyses

Allelic and genotype frequencies were calculated and a χ^2 test was used to verify the independence of allele frequencies. After that, over $n>5$ animals per producer, allelic richness (A_R), minor allele frequency (MAF), observed heterozygosity (H_O), expected heterozygosity (H_E) and Hardy-Weinberg equilibrium test (HWE) were estimated using GenAlEx software (Peakall & Smouse, 2012). Genetic variability among different animal populations was analyzed by a Principal Component Analysis (PCA) using Genalex software (Peakall & Smouse, 2012). The genetic structure was determining implementing Bayesian simulation procedure by STRUCTURE software (Pritchard, Stephens & Donnelly, 2000). Also, a Neighbour-joining (NJ) tree ((Saitou and Nei, 1987) was performed based on the observed genotypes of the animals from the different farms, assuming unrelated animals and no common ancestry, using the MEGA X platform (Kumar, Stecher, Li, Knyaz & Tamura, 2018). An analysis of molecular variance (AMOVA) was performed attending to different sources of variation: Model I) Between populations and within populations: Model II) Between populations, between subpopulations within populations and within subpopulations, whereas populations refers Creole and Hybrid lines animals and subpopulations to each farm. Both models included a level within individuals. Also, Wright fixation index (F_{ST}) and inbreeding coefficient (F_{IS}) were estimated. AMOVA analysis, F_{ST} and F_{IS} indexes together to its statistical significance *p*-values were estimated by Arlequin software (Excoffier & Lischer, 2010). The statistical significance for the difference between populations of H_E, H_O, A_R and F_{IS} was evaluated by a pairwise *t*-test using the FSTAT software (Goudet, 1995). Linkage Disequilibrium (LD) was calculated with Arlequin and based on Lewontin and Kojima (1960), Slatkin (1993), and Excoffier and Slatkin (1995); using the EM algorithm with 2 initial condition and 10,000 permutations. The differences were considered significant when $P < 0.05$.

Results

Polymorphism profiles

The 242 pigs were genotyped for the mentioned SNPs by PCR–RFLP procedure. PCR products of the expected size were obtained for each marker. All SNPs were segregating in both populations. Fig. 1 shows electrophoresis gel images of PCR–RFLP profile for *RYR1, PRKAG3, CAST* and, *SOX6*. Particularly, due to the absence of recombination between I199V and R200Q neighboring codons at *PRKAG3* locus (Milan et al., 1996), these two mutations yield three haplotypes for the RN gene: RN^- (199 V/200Q), rn+ (199 V/200R) and rn* (199I/200R) (Josell et al., 2003; Lindahl et al., 2004).

Allelic and genotypic frequencies

The allelic and genotypic frequencies of the studied markers for hybrids and Creole animals are summarized in Table 2. In all cases, the p-value was greater than 0.05 by the χ^2 test, no incidence of the populations analyzed on the allele frequencies was observed. In both populations, the *RYR1* SNP homozygote genotype TT was absent. Remarkably enough, it turns out that the commercial population showed a high percentage of CT individuals (29.87%). Even so, the lower frequency of negative allele 1843T (T) indicates the possibility of PSE meat in both populations (22.41% and 14.945% for Creole and commercial pigs, respectively). For the *PRKAG3* gene, three allelic variants were identified and the pigs studied showed the following six diplotypes: RN^-/RN^-, RN^-/rn+, RN^-/rn*, rn+/rn+, rn+/rn* or rn*/rn*. The deleterious allele RN^- was observed at *PRKAG3* with 36.77% in local populations and with 24.35% in commercial pigs. Again, such an incidence was not expected in the last one. Concerning the two *CAST* polymorphisms, *CAST* 638 Ser>Arg-and *CAST* 76,872 *G>A*, heterozygote's genotypes were the most numerous in both populations. And for *SOX6* gene, allele A of *SOX6*A SNP, described as favorable for fresh meat, was the most frequent in both populations and allele C of the *SOX6*B SNP, associated with meat color and pH, was observed mainly in heterozygosis (Zhang et al., 2015; Rodriguez et al., 2020).

Genetic diversity parameters and population genetic structure

Within-population genetic diversity. In order to determine the genetic diversity of commercial and Creole populations in the seven analyzed SNP at 4 loci considered, standard indices of genetic diversity including average number of alleles per locus (Na), observed and expected heterozygosity (H_O and H_E) minor Allele Frequency (MAF) and Hardy-Weinberg equilibrium (HWE) were estimated (Table 3). All SNPs were polymorphic; the number of allele per locus was 2, with the particular exception of RN, which showed 3 alleles (RN^-, rn+ and rn*). The MAF

Table 1
Details of SNPs.

Gene SNP	Primer sequence (5′–3′)	AT (°C)	AS (bp)	RE	PCR–RFLP pattern (bp)	References
RYR1 $_{1843C>T}$	F:GTGCTGGATGTCCTGTGTTCCCT R: CTGGTGACATAGTTGATGAGGTTTG	52.0	134	*Hha*I	134//90+44	Brenig & Brem, 1992
$RN_{200R>Q}$	F:GGAACGATTCACCCTCAACT R: AGCTCTGCTTCTTGCTGTCC	52.0	114	*Mbi*I	114//82+32	Martínez-Quintana et al.; 2006
$RN_{199I>V}$	F:GGAACGATTCACCCTCAACT R: AGCTCTGCTTCTTGCTGTCC	52	114	*Hsp*91	114//81+33	Martínez-Quintana et al.; 2006
CAST $_{638Ser>Arg}$	F:CCTTTGTTGTGTTCTCTGAGG R:AAACCTATTTTCAGGGATATGGG	52.5	183	*Pvu*II	183//142+41	Ciobanu et al.; 2004
CAST $_{76872G>A}$	F:TTCCCATAGCCCACAAGAAG R:AATGAGCAGCCAACATCAGA	50.0	376	*Hinf*I	376//247+129	Gandolfi et al.:2011
*SOX6*A $_{42812066G>A}$	F:CCAGTCCATCCTTTCCTTGA R:GTTTCCAAAAGGGAATGCAG	58.0	402	*BSMB*I	402//305+91	Zhang et al.; 2015
*SOX6*B $_{43023574G>C}$	F:CAATGCCATCGTTGAGTCTG R: GTTGTACTGCACATCTTCTCCCTGTTGGATCGTCT	50.6	258	*BSMB*I	258//217+41	Zhang et al.; 2015

Annealing temperature (AT), amplicon size (AS), restriction enzyme (RE), and PCR-RFLP pattern of each primer used.

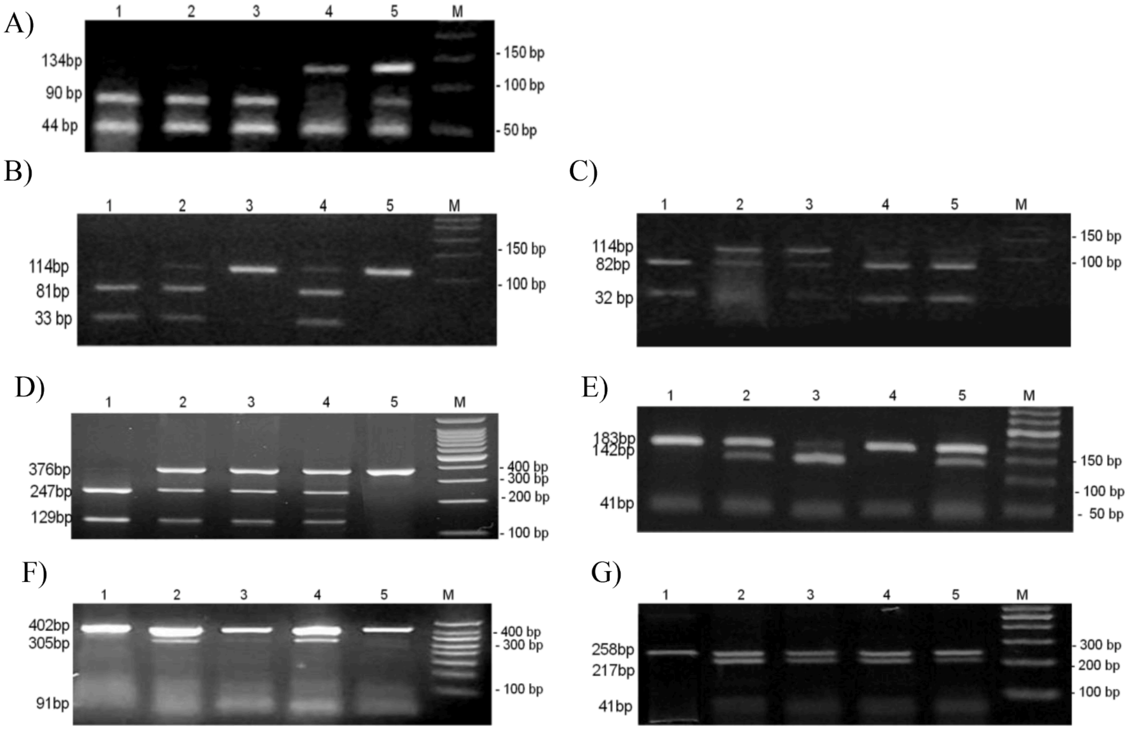

Fig. 1. SNPs PCR-RFLP profile on 3% agarose gel.

A) PCR-RFLP profile of *RYR1*$_{1843C>T}$ SNP by using HhaI. Lane M: 50 bp DNA Ladder (Genbiotech, Cat# B041–50). Lanes 1–3: CC genotypes. Lanes 4, 5: Ct genotype.

B) PCR-RFLP profile of *PRK3G* RN$_{199I>V}$ SNP by using Hsp91. Lane M: 50 bp DNA ladder (Genbiotech, Cat #L00607). Lane 1: VV genotype. Lanes 2, 4: VI genotype and, lanes 3, 5: II genotype.

C) PCR-RFLP profile of *PRK3G* RN$_{200R>Q}$ SNP by using MbiI. Lane M: 100 bp DNA ladder (Genbiotech, Cat #L00607). Lanes 1, 4, 5: RR genotype. Lanes 2,3: RQ genotype,

D) PCR-RFLP profile of *CAST*$_{76872G>A}$ SNP by using HinfI. Lane M: 100 bp DNA ladder (Promega). Lane 1: GG genotype. Lanes 2–4: GA genotype. Lane 4: AA genotype.

E) PCR-RFLP profile of *CAST* $_{638Ser>Arg}$-SNP by using PvuII. Lane M: 50 bp DNA Step Ladder (Genbiotech, Cat# B041–50). Lane 3: SS genotype. Lanes 2, 5: SA genotype. Lanes 1, 4: AA genotype.

F) PCR-RFLP profile of *SOX6*A SNP by using BSMBI. Lane M: 50 bp DNA ladder (Genbiotech, Cat #L00607). Lanes 2, 4: AG genotype. Lanes 1, 3, 5: AA genotype.

G) PCR-RFLP profile of S *SOX6*B SNP by using BSMBI. Lane M: 50 bp DNA ladder (Genbiotech, Cat #L00607). Lane 1: CC genotype. Lanes 2–5: GC genotype.

Table 2
Allelic and genotypic frequencies at different SNPs sites in pigs from North-West of Argentina.

		Hybrid animals(N = 153)		Creole animals(N = 87)	
GeneSNP	Genotype	Genotypefrequency (%)	Allelicfrequency (%)	Genotypefrequency (%)	Allelicfrequency (%)
RYR1	CC	70.13	C = 85.06	55.17	C = 77.59
$_{1843C>T}$	Ct	29.87	t = 14.94	44.83	t = 22.41
PRKAG3	RN^{-}/rn*	36.36	RN^{-}=24.35	50.57	RN^{-}=36.77
$_{199I>V/200R>Q}$	rn^{+}/rn*	23.38	rn*=42.21	14.94	rn*=37.35
	rn^{+}/rn^{+}	16.23	rn^{+}= 33.44	9.19	rn^{+}=25.86
	RN^{-}/rn^{+}	11.04		18.39	
	rn*/rn*	12.34		4.59	
	RN^{-}/RN^{-}	0.65		2.29	
CAST	GG	33.12	G = 62.67	40.23	G = 66.09
$_{76872G>A}$	GA	59.09	A = 37.33	51.72	A = 33.91
	AA	7.79		8.05	
CAST	CC	5.85	C = 39.29	1.15	C = 27.59
$_{638Ser>Arg}$	CA	66.88	A = 60.71	52.87	A = 72.41
	AA	27.27		45.98	
*SOX6*A	AA	48.05	A = 74.03	55.17	A = 77.59
$_{42812066G>A}$	AG	51.95	G = 25.97	44.83	G = 22.41
*SOX6*B	GG	24.03	G = 53.25	13.78	G = 49.41
$_{43023574G>C}$	GC	58.44	C = 46.75	71.26	C = 50.59
	CC	17.53		14.94	

was higher than 0.2 for all the SNPs analyzed in Creole animals however was lesser than 0.2 only in *RYR1* locus in hybrids animals. The average He was 0.455 for commercial animals and ranged from a maximum value of 0.498 for the *SOX6*B locus to a minimum value of 0.255 for the *RYR1* locus, whereas at the Creole animals the average He was 0.452, ranged from a maximum value of 0.659 for the RN locus to a minimum of 0.342 for *RYR1* locus. The total heterozygosity values (Ho) exceeded the average heterozygosity level of 0.5 indicating high genetic diversity

Table 3
Genetic diversity.

Gene SNP	Commercial pigs ($N = 153$) Na	MAF	H_O	H_E	A_R	HWE	Creole pigs ($N = 89$) Na	MAF	H_O	H_E	A_R	HWE
RYR1$_{1843C>T}$	2.000	0.150	0.301	0.255	2	0.029	2.000	0.219	0.438	0.342	2	0.008
PRKAG3$_{199IV/200R>Q}$	3.000	0.242	0.706	0.650	3	0.000	3.000	0.264	0.843	0.659	2	0.000
CAST$_{76872G>A}$	2.000	0.373	0.588	0.468	2	0.001	2.000	0.343	0.528	0.451	2	0.104
CAST$_{638Ser>Arg}$	2.000	0.392	0.667	0.477	2	0.000	2.000	0.281	0.539	0.404	2	0.002
*SOX6*A$_{42812066G>A}$	2.000	0.258	0.516	0.383	2	0.000	2.000	0.230	0.461	0.355	2	0.005
*SOX6*B$_{43023574G>C}$	2.000	0.467	0.582	0.498	2	0.037	2.000	0.494	0.719	0.500	2	0.000
Averange	2.167	0.313	0.560	0.455	2.167		2.167	0.305	0.588	0.452	2.167	

N=Sample size, Na= allelic number per locus, MAF= minor allele frequency, H_O= observed heterozygosity, H_E= expected heterozygosity, A_R= allelic richness, and HWE= Hardy-Weinberg equilibrium.

in both populations. Almost all loci deviated from HWE ($P < 0.05$) in both populations, ranged between 0 and 0.037 in commercial and 0–0.104 in Creole animals, whereas CAST$_{G76872A}$ locus at Creoles resulted in equilibrium with $P>0.05$. Significant deviations in HWE in the studied loci correspond to an observed heterozygosity higher than expected heterozygosity. Therefore, both populations showed heterozygous excess and an F_{IS} value of −0.244 for commercial and −0.310 Creole populations (Table 4). Within-populations genetic diversity was reflected at the allelic richness (AR) observed 2.167 for both commercial and Creole animals. The same richness was observed when the subpopulation of each farm pigs were analyzed (data not showed).

Between population genetic differentiation. The values of F_{ST} and F_{IS} indexes for each locus and for the set of loci for all populations are shown in Table 5. Genetic differentiation between and within breeds were evaluated (Model I). According to the values obtained by Weir & Cockerham estimators (Weir and Cockerham, 1984), the populations were not separated from a random mating model since the average F_{IS} for all loci was −0.252. Negative F_{IS} values observed in all loci are indicators of an excess of heterozygous although in no case were significant indicating no evidence of *inbreeding trends* between and within breeds. The global F_{ST} value was 0.007 ($P = 0.002$) suggesting a low genetic differentiation between subpopulations

Model II shows differentiation between, within populations and between subpopulation. F_{ST} highest values were observed for *RYR1* and RN locus (0.156 and 0.109 respectively) (Table 5), and the global F_{ST} for this hierarchical model was 0.078 showing again the low genetic differentiation.

Table 5
Genetic differentiation: F_{IS} and F_{ST} values.

Locus	Model I F_{ST} (*P*)	F_{IS} (*P*)	Model II F_{ST} (*P*)	F_{IS} (*P*)
RYR1$_{1843C>T}$	0.012(0.036)	-0.218 (1)	0.155 (0.000)	0
PRKAG3$_{199I>V/200R>Q}$	0.014 (0.006)	-0.153 (0.999)	0.109 (0.000)	0
CAST$_{76872G>A}$	0	-0.223 (1)	0.023 (0.002)	0
CAST$_{638Ser>Arg}$	0.022 (0.002)	-0.374 (1)	0.049 (0.000)	0
*SOX6*A$_{42812066G>A}$	0	-0.327 (1)	0.029 (0.000)	0
*SOX6*B$_{43023574G>C}$	0	-0.264 (1)	0.108 (0.000)	0
Global	0.007 (0.002)	-0.252 (1)	0.078 (0.000)	0

F_{ST}= fixation index, F_{IS} = inbreeding coefficient, P = p value. F_{IS} and F_{ST} were calculated by locus considering an AMOVA with the following hierarchical levels: Model I: between populations and within populations. Model II: Between populations, between subpopulations within populations and within populations. Both models include a level within individuals to estimate the inbreeding coefficient.

Population genetic structure analysis

Principal component analysis (PCA) was employed to explore the clustering of individuals of different populations. The first three principal components explained 22.60%, 19.93% and 17.22% of the total variation and the accumulated contributions of these three principal components explained 59.76% of genetic variation. The heterogeneous distribution of genotypes in the PCA analysis indicates no differences in the genetic structure of the two populations, given the information from these seven analyzed markers. Both populations are completely overlapped, which may suggest their genetic closeness for the analyzed SNPs. PCA was used in cluster analysis based on the genotype of each individual (Fig. 2). The contributions of the first two principal components (pc) were 22.6% and 19.93% of the total variation, respectively, and their accumulated contributions, 42.53%. PCA showed no separate groups of genotypes. In well accordance with results obtained in the PCA, similar results were obtained for a Bayesian analysis using the multilocus genotype data was implemented in STRUCTURE software (data not shown) and for a NJ tree (Fig. 2). Pairwise linkage disequilibrium between SNPs was used to measure the linkage disequilibrium (LD). This study provides an overview of LD patterns between SNPs related to meat quality in different subpopulations of Creole (11) (Table 6); commercial pigs were not included because they are not pure lines. Only, 6 statistically significant associations out of 15 comparisons between pairs of SNPs were observed. These allele pairs may be undergoing co-selection due to animal breeding schemes in the domestication process (Table 6).

Table 4
Genetic variability between populations.

Parameter	Commercial pigs	Creole pigs	*P*
$\mathbf{H_O}$	0.559	0.584	1.000
$\mathbf{H_E}$	0.449	0.446	0.674
$\mathbf{A_R}$	2.167	2.167	0.160
$\mathbf{F_{IS}}$	-0.244	-0.310	0.663

H_O= Observed Heterozygosity, H_E= Expected Heterozygosity, A_R= Allelic Richness and F_{IS}= inbreeding coefficient, P = p value.

Discussion

Genetic characterization of pig breeds is essential to preserve their genetic variability, to advance conservation policies and to contribute to

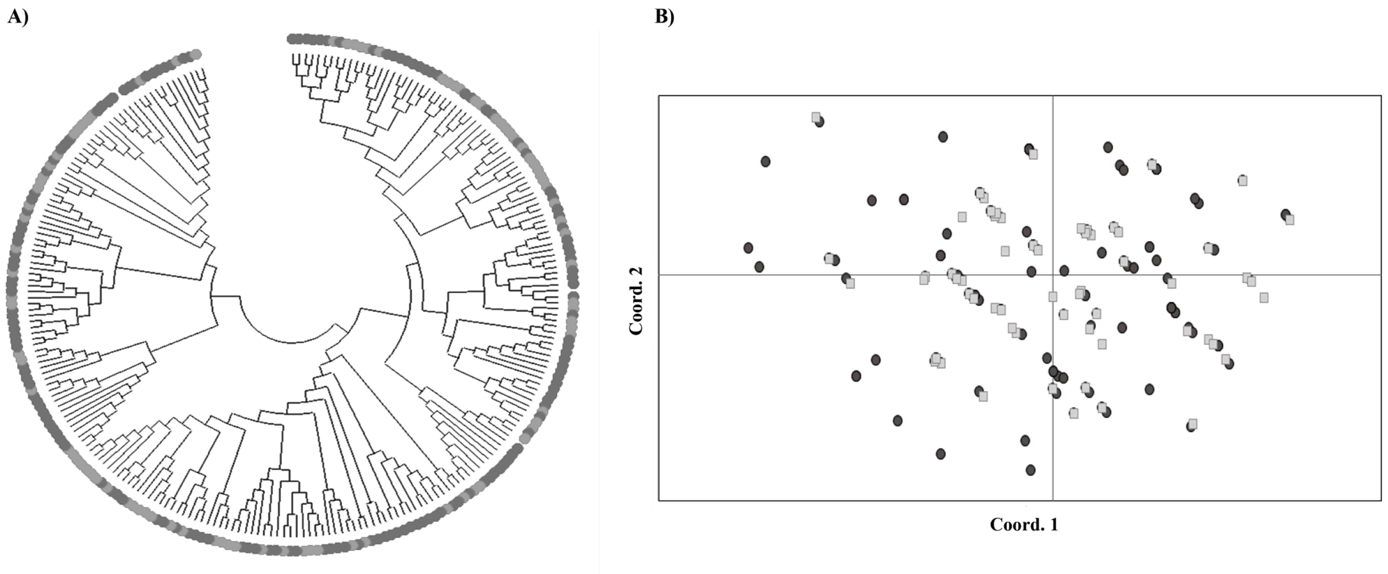

Fig. 2. Population structure analyses for all pig individuals.
(A) Neighbor-joining tree for all individual pigs. (B) First and second principal components from a principal component analysis of all populations. The contributions of the first two principal components (Coord.) were 44.19% and 22.6%; (●) represents commercial breeds and (☐) Creole pigs.

Table 6
Pairwise LD of SNP loci.

SNP Pair	Creole pigs LD (*P*)	X^2 (*P*)
RYR1$_{1843C>T}$ x *PRKAG3*$_{199I>V/200R>Q}$	0.010	9.962 $^{(**)}$
RYR1$_{1843C>T}$ x *CAST*$_{76872G>A}$	0.014	6.447 $^{(*)}$
PRKAG3$_{199I>V/200R>Q}$ x *CAST*$_{76872G>A}$	0.074	5.339
RYR1$_{1843C>T}$ x *CAST*$_{638Ser>Arg}$	0.009	5.339 $^{(**)}$
PRKAG3$_{199I>V/200R>Q}$ x *CAST*$_{638Ser>Arg}$	0.191	3.525
CAST$_{76872G>a}$x *CAST*$_{638Ser>Arg}$	0.003	8.896 $^{(**)}$
RYR1$_{1843C>T}$ x *SOX6*A$_{42812066G>A}$	0.153	2.279
PRKAG3$_{199I>V/200R>Q}$x *SOX6*A$_{42812066G>A}$	0.010	9.979 $^{(**)}$
CAST$_{76872G>A}$ x *SOX6*A$_{42812066G>A}$	0.468	0.579
CAST$_{638Ser>Arg}$-x *SOX6*A$_{42812066G>A}$	0.365	0.875
RYR1$_{1843C>T}$ x *SOX6*B$_{43023574G>C}$	0.005	0.010 $^{(**)}$
PRKAG3$_{199I>V/200R>Q}$x *SOX6*B$_{43023574G>C}$	0.130	4.380
CAST$_{76872G>A}$ x *SOX6*B$_{43023574G>C}$	0.285	1.244
CAST$_{638Ser>Arg}$-x *SOX6*B$_{43023574G>C}$	0.400	0.786
*SOX6*A$_{42812066G>A}$ x *SOX6*B$_{43023574G>C}$	0.504	0.503

LD= linkage disequilibrium. LD significance level (X^2 and p value) between each pair of SNPs under study. Significance level (*) $P < 0.05$; (**) $P < 0.01$ and, (***) $P < 0.001$.

their promotion and sustainability. Genetic diversity studies generally focus on candidate genes related with reproduction, lipid, carbohydrate and protein metabolism, growth and development, cellular homeostasis, locomotor behavior and response to nutrient and a list of microsatellite markers recommended by the International Society for Animal Genetics / Food and Agriculture Organization of the United Nations working group (FAO, 2011). However, the present study aimed to analyze the segregation of meat quality-related SNPs and compare their diversity and genetic structure across Creole and commercial crossbred populations. Seven polymorphisms were considered at four different genes, including the so-called "Halothane" or RYR1 gene (g.1843C>T), "Rendement Napole" or *PRKAG3* gene (199I>*V* and 200R>*Q* substitutions in *PRKAG3*), Calpastatin (638C>A and 76872G>*A*) and *SOX6* gene (*SOX6*A and *SOX6*B) (Table 2). Based on PCR-RFLP fragment patterns results, alleles and genotypes frequencies were established. For *RYR1* (1843C>T) SNP, genotypic frequencies observed were 55.17% and 70.13% homozygous normal CC, 44.83% and 28.87% of individuals heterozygous (CT) for Creole and commercial animals respectively, whereas no homozygous TT were detected in any of the analyzed populations. The frequency of allele T found for Creole (22.41%) and commercial pigs (14.945%) was noticeably high, even if the possibility to generate PSE meat is considered. In four different provinces of Argentina (Córdoba, Santa Fe, Chaco and Tucumán), Marini et al. reported similar frequency for T allele (19.6%) in a hybrid animal's population derived from crossing hybrid females Landrace x Yorkshire with terminal hybrid males composed by different proportions of Duroc, Pietrain, Hampshire and Yorkshire (Marini et al., 2012) where 4.2% resulted homozygous susceptible (TT). Also, a report describing the meat quality of commercial hybrid pigs in Argentina showed some little evidence of PSE on synthetic boar line (Lloveras et al., 2008). Similar results for *RYR1* SNP frequency were reported for commercial pig populations in Brazil, which is considered the biggest Latin America producer country (Bastos, Federizzi, Deschamps, Cardellino & Dellagostin, 2001, Band et al.; 2005; Silveira et al., 2011). Among European local pig as in most commercial European pigs (Fujii et al., 1991) the c.1843T mutant allele is scarce since many initiatives have been carried out to eliminate this allele (Muñoz et al., 2018). The three functionally alleles have been identified at the *PRKAG3* locus: 199V–200R (wildtype, rn+), 199V–200Q (RN^{-}) and 199I–200R (rn*). The *RN*$^{-}$ allele was present in 36.77% of the Creole pigs with the RN^{-}/rn* genotype as the most frequent (50.57%). For hybrids, 24.35% had RN^{-} allele and also the RN^{-}/rn* genotype was the most representative (36.36%). RN^{-}/RN^{-} genotype was the least frequent in both populations (2.29 Creole and 0.62% hybrids). High frequency of the mutated dominant *RN*$^{-}$ *allele* (RN^{-}/RN^{-}, RN^{-}/rn+) *in both populations indicated the possibility of acid meat and reflex that the SNP has not been eliminated from breeding populations yet.* The *PRKAG3* R200Q SNP appears in Hampshire breed or derived synthetic lines and the mutant allele was absent in several European local porcine breeds (Muñoz et al., 2018). *On the other hand, the I allele (rn*) is widely reported to have a positive effect on pork quality* (Ciobanu et al., 2001; Lindahl et al., 2004; Otto et al., 2007), is highly represented in *both populations* (37.35% and 42.21%). High frequencies of the rn* allele, were also reported in the bibliography for Iberian pigs (Muñoz et al., 2019). Mexican pig populations showed similar RN gene frequency in Creole as well as certain commercial pigs such as Yorkshire and Hampshire suggesting that no changes have arisen by artificial or natural selection (Carr, Morgan, Berg, Carter & Ray, 2006; González Sarabia et al., 2011). Thus, *selection against the two major genes* RYR1 (*T*) and *PRKAG3 (RN*$^{-}$*) alleles by genomic selection can potentially reduce the* *frequencies of the defective genes with high accuracy to enhance pork quality. Also, for the* CAST *gene*, Ser638Arg SNP showed three genotypes in both populations, being the heterozygote AC the most frequent (52.87 Creole and 66.88% Commercial pigs) and the CC under-represented (1.15 and 5.85%). Allele A of *CAST* p.Ser638Arg was the most frequent, as reported in French (Santé-Lhoutellier et al., 2012), Spanish (Gou et al., 2012) and Italian commercial pigs (Davoli et al., 2017). In contrast, studies conducted in Latin America, Mexican Creole pigs showed a lower frequency of the favorable allele A for CAST$_{638G>A}$ than in cuinos and Yorkshire pigs (González Sarabia et al., 2011). Allele frequencies in Creole pigs were 66.09 and 33.91% for *CAST* g.76872 G and A allele, respectively, and 62.67 and 37.33% for commercial breed populations. In both cases the homozygous genotype AA was the least abundant (8.05 and 7.79%). Based on the published literature, 638Arg/638Arg genotypes were associated with lower firmness desirable for fresh meat (Ciobanu et al., 2004) and *CAST* g.76872 *G>A* genotype had suggestive effect on drip loss, with a lower drip loss in pigs carrying AA genotype compared with the GG genotype (Gandolfi et al., 2011). In this context, the populations studied here would require molecular marker-assisted selection (MAS) strategies to maximize meat quality. It was also observed that pig *SOX6*, is embedded in or close to many reported QTLs (Ai et al., 2012; Harmegnies et al., 2006; Lee et al., 2003; Stearns et al., 2005a); T. M. Stearns et al., 2005(b); Thomsen, Lee, Rothschild, Malek & Dekkers, 2004; Ruckert and Bennewitz, 2010). Even the available studies about porcine *SOX6* are very limited; two SNPs located at intronic sequence have been reported to be related to growth, carcass, and meat quality traits (Zhang et al., 2015). Allele frequencies for *SOX6*A, A and G, were 77.59 and 22.41% in Creole population, and 74.93 and 25.97% in commercial population, respectively. Genotype frequencies for *SOX6*A, AA and AG, were 55.17, 44.83% in Creole population, and 74.03 and 25.97% in commercial population, respectively. The GG genotype was missing in both populations, which might be explained by masked selection by farmers for this polymorphism. For *SOX6*B allele frequencies G and C were 49.91 and 50.59 in the Creole population. For commercial pig populations they were 53.25 and 46.75%, respectively. Genotype frequencies for *SOX6*B, GG, GC, and CC, were 13.78, 71.26, and 14.94% in Creole population and 24.03, 58.44 and 17.53% for commercial populations, respectively. SOX6A was associated to pH, CRA and color in Pietrain and DuPi (Duroc x Pietrain) populations and the Pi pigs carrying genotype AA of *SOX6*A have high pH and thick backfat (Zhang et al., 2015). We have previously reported that *SOX6*A may influence pH and CRA, whereas allele C of *SOX6*B may be linked just to pH in *Halothane free* animals. So, the selection of A allele for *SOX6*A and C for *SOX6*B could improve the production of good quality fresh meat (Rodriguez et al., 2020). These are the first results on *SOX6* allele segregation reported in Argentine pig populations. However, available studies on *SOX6* in pigs are still limited and, more work is needed to elucidate the role of *SOX6* in pork quality and production. Pig genetic diversity within populations is variable and consequently had quite variable heterozygosities on different chromosomal regions that may reflect the relatively long time of breeding and selection for the pig (Zhang & Plastow, 2011). When all analyzed loci were considered, the average expected heterozygosity (H$_E$) values were 0.452 and 0.455 for Creole and commercial animals and the average of observed heterozygosity (H$_O$) were 0.588 and 0.56, respectively, indicating hybridization. Genetic variability at the different loci in each population may suggest a low level of artificial selection for the meat-quality related SNPs analyzed here. In several reported studies for local Latin American breeds, the genetic diversity analysis is mostly based on microsatellite markers recommended by FAO/ISAG. Although the heterozygosity obtained here mediated by RFLPs analysis is not equivalent, the values resembled those found for other local populations such as North East Argentina Creole pigs (MA Revidatti, 2009, Ph.D. thesis, University of Córdoba, Spain), Mexican Hairless pigs (Canul et al., 2005), Cuban Creole pigs (Martínez et al., 2005) Uruguayan pig breed Pampa Rocha (Montenegro et al., 2015) and Brazilian breeds such as Monteiro, Moura, and Piau (Sollero et al., 2009). In a recent study performed by Muñoz et al. (2019), European autochthonous breeds values for H$_O$ and H$_E$ were 0.297 and 0.303, respectively, values considerably lower than those reported previously for European cosmopolitan and Chinese pig breeds (Laval et al., 2000; Luetkemeier, Sodhi, Schook & Malhi, 2010), but similar to those reported for some European local breeds (Herrero-Medrano et al., 2014). Also, the average expected heterozygosity was above 0.63 for Portugal native breeds and Landrace, ranging between 0.56 and 0.59 for other native breeds, Large With and Pietrain pigs, and below 0.5 in Duroc populations (Vicente et al., 2008). Chinese population had much higher diversity, ranging from 0.700 to 0.876 from 18 Chinese pig breeds (Yang et al., 2003). In contrast to many reports where the H$_E$ was much higher than the H$_O$, in the present study the He was lower in both populations when meat quality related SNPs were analyzed. Considering European populations, Muñoz et al. (2018) described the diversity of several polymorphisms on meat production candidate genes in European local pig breeds. In those population they reported *RYR1*$_{1843C>T}$ with a H$_O$: 0.048 H$_E$: 0.053; *PRKAG3* 199I>*V* H$_O$: 0.399 H$_E$: 0.388 and CAST$_{76872G>A}$ H$_O$: 0.295 H$_E$: 0.389. In addition, Mexican hairless pigs showed similar average heterozygosities for the *RYR1*$_{1843C>T}$ and *CAST*$_{G76872A}$ loci, but lower for the *PRKAG3*$_{199I>V/200R>Q}$ and Mexican commercial Yorkshire breeds showed a He value of 0.49 for *RYR1*$_{1843C>T}$, and *PRKAG3*$_{199I>V/200R>Q}$ and 0.23 for *CAST*$_{76872\ G>A}$ (González Sarabia et al., 2011).

The average within-breed MAF ranged from 0.15 for *RYR1* to 0.46 for *SOX6*B in commercial pigs and 0.2 for *RYR1* to 0.49 for *SOX6*B in Creole pigs, suggesting that the 7 SNPs analyzed are polymorphic. In accordance with the well-known negative effect of the T allele, lowest MAF values corresponded to *RYR1* in both populations. Selection *against* porcine stress syndrome is currently performed in commercial hybrid pig (*RYR1 free* lines) and farmers as well. Considering the SNPs with two (*RYR1, CAST* and *SOX6*) and three loci (RN), within-breed genetic diversity was reflected at the allelic richness (AR) observed as 2.167 for both commercial and Creole animals.

In both populations, SNPs genotypic frequencies do not agree with Hardy-Weinberg expectations for $P<0.01$, except for local pigs-*CAST*$_{76872G>A}$ loci ($P = 0.104$), which showed reduced heterozygosity. The deviations for HWE together with the F$_{ST}$ values for *RYR1* and *PRKAG3* loci differ from zero significantly (0.155 and 0.109) may suggest some genetic differentiation that may derivate from artificial selection effect over the two major genes (Table 5, global F$_{ST}$ = 0.078, Model II). The average F$_{ST}$ of all loci was 0.007, which means that most of the genetic variation was kept within populations and only a little of the genetic variation exists between populations (Model I). Negative F$_{IS}$ coefficients ranging from −0.370 to −0.150, were estimated for commercial and local population, suggesting an excess of heterozygous in well accordance with the Ho obtained. Therefore, here both populations had excess heterozygous and a negative inbreeding coefficient which may indicate *absence* of *inbreeding for the loci analyzed.*

Domestic animal diversity is the basic material for genetics and breeding studies and it is an important form of insurance which enables responses to as-yet-unknown future challenges. To analyze what has happened to the pig's population history (e.g., breeding history, selection, genetic drift, mutation), linkage disequilibrium (LD) - the nonrandom association of alleles at different loci - for each marker was estimated. In contrast to hybrid pigs, Creole pigs are commonly obtained by crossing closer lines driving to a small effective population size. This fact together with the possible hybridization and the negative F$_{IS}$ value observed, may explain the LD obtained (Table 6).

According to the SNPs analyzed, a neighbor-joining tree was constructed in which it was not possible to differentiate the local and commercial populations under study. Consistent with NJ tree results, the PCA also showed no differences in the genetic structure of the two populations.

Based on the results for the Creole population in the current study, the possibility of admixture with commercial animals has to be considered. As Burgos-Paz and col. data suggest that Creole pigs have undergone a dramatic introgression with international-breed pigs. Modern village pigs in the Americas are the result of much independent colonization and introgression events, maybe including a direct Chinese introgression (Burgos-Paz et al., 2013). This could explain the excess of heterozygosis in the Creole population studied here and may lead to reconsideration of the term "Creole" to locally adapted pigs.

An evaluation of divergence between these two populations based on just 7 SNPs, especially considering that the commercial population includes a wide range of crosses may result not be informative enough but the high degree of variability reported here is valuable information for the crossbreeding program's point of view.

Conclusion

Pig populations from the North-West of Argentina analyzed in this work show a high genetic variability at the level of the meat quality markers *RYR1*$_{1843C>T}$, *PRKAG3*$_{199I>V/200R>Q}$, *CAST*$_{76872G>}$, *CAST*$_{638C>A}$, *SOX6*A and *SOX6*B, and slightly pronounced genetic differentiation. Even many initiatives have been carried out to eliminate the *RYR1* and *PRKAG3* deleterious alleles, in the present study high incidence of T and RN^{-} alleles have been found in both populations analyzed. The meat quality markers studied here may be used in genetic selection programs. Consequently, these results highlighted the role of swine genotypes as a source of variability that can affect both processed meat products and fresh meat.

Declarations

None

Author contributions

Conceptualization: M.L., V.R.R, M.E.B and M.V.G.; Data curation: V.R.R. and M.L.; Formal analysis: V.R.R., M.L., R.F., M.E.B and M.V.G.; Funding acquisition: M.L; Investigation: V.R.R., J.I.M., L.A.Z., R.F. and M.L.; Methodology: V.R.R, J.I.M., L.A.Z. and M.L.; Project administration: M.L.; Resources: M.L. and V.R.R; Supervision: M.L.; Writing, review & editing: M.L., V.R.R., M.E.B and M.V.G. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author or reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

No conflict of interest declared.

Declaration of competing interests

The authors declare that they have no competing interests.

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