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Development and validation of a SNPs panel used for beef traceability throughout the food chain

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Abstract

Meat labelling is important to avoid unfair competition practices of producers, processors and sellers aiming gain an economic advantage from misrepresenting cheaper meat products as premium quality ones. Genetic traceability is an ideal tool for the detection and prevention of fraud in the sale of beef. In this work, a method for validating protected status based on a combination of single nucleotide polymorphisms (SNPs) and a high-throughput real-time PCR protocol was developed. A total of 1,911 beef samples were used to develop and validate an OpenArray® panel consisting of 26 SNPs selected from an ISAG-ICAR panel. The applicability of the method was determined by comparing 143 blind pairs of samples. The combined probability of identity (PI) with the 26 SNPs selected was 2.09-11 with 22 SNPs showing an individual PI lower than 0.4. In all tests, the blind pair of samples were correctly as-signed. The panel designed and validated in this study is of great utility to confirm genetic content of the specific genetic group tested and detect fraud in a routine way that requires a minimum amount of time and is cost effective.

Keywords: SNPs; fraud; beef; traceability; real-time PCR; OpenArray®.

Practical Application: Ensure traceability of beef from slaughterhouse to fork.

1 Introduction

Meat specification is important to avoid unfair competition practices of producers, processors and sellers aiming gain an economic advantage from misrepresenting cheaper beef products as premium quality ones (Espiñeira & Santaclara, 2016). One of the main characteristics that can be used to distinguish meat quality, and its market value is the breed and place of origin of the product. In Spain, Rubia Gallega (RG) is one of the principal local cattle breeds., In 1996, the European Union (EU) allowed the creation of the Protected Geographical Indication (PGI) "Ternera Gallega", which is comprised of pure RG and it crosses (European Commission, 1996). In 2017, under Regulation 1151/2012 (European Parliament, 2012) the PGI "Vaca e boi de Galicia" was created. Under this framework, beef of high quality with the PGI designation may garner higher prices than unprotected beef. In this sense, traceability is essential to avoid fraud. But in some occasions, conventional tracing techniques are susceptible to error through the loss of correspondence between a registration number and the related animal (Aung & Chang, 2014; Capoferri et al., 2006). It is therefore necessary to develop new methods of traceability that are not susceptible to any type of manipulation.(Badia-Melis et al., 2015). In this context, genetic analyses represent a useful tool allowing regulators to determine if two different pieces of beef correspond to the same animal and detect fraudulent labelling (Zhao et al., 2018).

Genetic traceability is based in the comparison of specific regions of the DNA between samples. There are different DNA markers than can be used for genetic tracing. From those, multi-allelic microsatellites or short tandem repeats (STRs) has been widely used for individual identification and parenterage (Zhao et al., 2018). In a recent study, STRs haven used for parentage and traceability purposes of Pirenaica Cattle in comparison to other breeds with positive results (Gamarra et al., 2020). However, in recent years, diallelic single nucleotide polymorphism (SNPs) appear to be effective alternatives to SRTs. Although less informative, since 2–2.25 SNPs are required to have the same exclusion power as a microsatellite with five alelles, SNPs have some advantages over microsatellites (Weller et al., 2006). Due to their simple form, a single nucleotide change at one position of the genetic code, SNPs have lower mutation rates, allowing for more robust genotyping and data interpretation. They are suitable for the standardized representation of genotyping results as digital signatures of DNA and are suitable for various genotyping techniques (Negrini et al., 2009). One such techniques is real-time PCR. The use of probes labelled with different fluorophores in this technique has provided researcher with the ability to perform genotyping reactions with high specificity.

Thus SNPs analysis is a promising tool to be routinely implemented in beef traceability. In a recent article, Zhao et al. (2019) developed a PCR-capillary electrophoresis method based on the analysis of 12 SNPs for genetic traceability in China large-size beef company and distinguish individuals with a matching probability of 1.70×10^{-5}. Similarly, Capoferri et al. (2006) developed a method for genetic control of conventional beef labeling based in the analysis of 12 selected SNPs by real-time PCR. In this

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study, the probability that two random individuals presented the same genotype was 7.67 10^{-5}. This last study demonstrated that combination of SNPs and real-time PCR can be used routinely for genetic monitoring in the food chain. However, the main limitation of these studiesis the number of assays that can be performed at once and time and regent requirements. In order for this method to be routinely introduced into PGI monitoring programs, it is necessary to keep the costs per sample analysed as low as possible. In this sense, the recent development of high-throughput real-time PCR platforms as OpenArray® technology have allowed researchers to overcome these limitations. With this specific platform is possible to perform 3,072 reactions in the same plate with minimum sample and reagent consumption as the volume of reaction is 33 nL. (Lamas et al., 2016). This technology also allow increases the number of SNPs that can be analyzed per sample while maintaining a lower cost per sample than conventional real-time PCR. Finally this technology also allows for the simultaneous analysis of approximately 100 samples, which makes it a good option to be used routinely for genetic traceability control.

This provides researchers with a great opportunity of use real-time PCR to perform as a method to routinely trace the genetics of bovine meat within the production chain by processing a wide range of samples within one real-time PCR run. Therefore, the aim of this work was to use OpenArray® technology to develop and validate a panel of bovine SNPs that may be used for genetic control practices to verify the individual traceability of "Ternera Gallega" beef.

2 Materials and Methods

2.1 Sample collection and SNPs selection

A total of 1,911 samples of "Ternera Gallega" beef were collected from different points of the food processing chain, which included slaughterhouses and sale points. Specifically, 143 pairs of samples (each pair was composed by a muscle and an auricular pavilion sample from the same animal)) of PGI "Ternera Gallega" beef were collected in slaughterhouses by PGI veterinarians and submitted to the laboratory for a blind analysis. These samples were used to determine the applicability of the panel developed for individual identification. Additionally, 94 pairs of those samples were sent to an external laboratory that carried out correspondence assays using the STRs technique. The rest of the samples included in the study were those collected routinely at slaughterhouses and points of sale by PGI in the frame of genetic traceability control programme.

The selection of 26 SNPs for use in the study (Table 1) was based on analysis of a panel of 100 SNPs recommended by the International Society for Animal Genetics (ISAG) International Committee for Animal Recording (ICAR) for parenterage and individual identification. The panel has information regarding the minor allele frequency (MAF) of SNPs for different lineages of cattle. However, for the specific case of Rubia Gallega there was no information either within the ISAG panel or the literature. Therefore, the selection of the SNPs was based on the MAF of a type of cattle permitted to crossbreed with Rubia Gallega (Limousine, Blonde Aquitaine or Holstein). SNPs with a MAF higher than 0.3 of these breeds were preferably selected.

2.2 DNA isolation and quantification

Genomic DNA was extracted from 20 mg of muscle or auricular pavilion by using PureLink® Genomic DNA Mini Kit (Invitrogen™, ThermoFisher Scientific, CA, USA) according to the manufacturer´s protocol. DNA was quantified by using Qubit™ dsDNA BR Assay Kit (InvitrogenTM, ThermoFisher) in combination with the Qubit™ fluorometer (InvitrogenTM, ThermoFisher) according to the manufacturer´s protocol. DNA samples were normalized to 25-75 ng/μL.

2.3 Nanoliter volume, high-throughput, real-time PCR

Genotypic profiling was carried out using a TaqMan® OpenArray® system (Applied Biosystems™, Thermo Fisher Scientific). The primers and TaqMan® probes (Table 1) labelled one with VIC (Allele 1) and one with FAM (Allele 2) were preloaded on plates by the company. A design consisting of 26 SNPs and 96 samples per array was selected. In each array we included a negative control sample and a positive sample that consisting of a sample analyzed in a previous array. The purpose of including the positive sample was to confirm the repeatability of the method. Real-time PCR reactions were performed according to the TaqMan® OpenArray® protocol. Briefly, in a 384-well plate, 2.5 μL of each DNA sample (25-75 ng/μL) was mixed with 2.5 μL of TaqMan® OpenArray® Genotyping PCR Master Mix (Applied BiosystemsTM, Thermo Fisher Scientific). The PCR reaction mixtures were loaded automatically into the OpenArray® plates using an OpenArray® AccuFill™ System (Applied BiosystemsTM, Thermo Fisher Scientific). The following real-time PCR protocol was used: a Pre PCR hold of 10 min at 93 °C, 50 cycles at 95 °C for 45 s, 94 °C for 13 s, and 53.5 °C for 2 min 14 s.

2.4 Data analysis

The OpenArray® experiment files were uploaded to the online software Cloud (Thermo Fisher Scientific). Alleles are automatically assigned by the software according the fluorescence values obtained for each Taqman™ Probe. The allelic discrimination plot for each of the SNPs was visualized and samples in which a genotype was not automatically assigned by the software were manually revised. Subsequently, data was downloaded and correspondence between samples was determined using Microsoft Excel 2010 worksheet (Microsoft Office, WA, USA). Samples with the same alleles from each SNP were considered to be derived from the same animal. Samples with one or more alleles differences were considered to be derived from different animals.

The probability of identity (P_I) is defined as the probability that two randomly selected unrelated individuals would possess identical SNP genotypes. It was calculated for each SNP form the genotype frequencies by the following Formula 1:

$$P_I = (X_{aa})^2 + (X_{ab})^2 + (X_{bb})^2 \quad (1),$$

Table 1. SNPs included in this study and the primers and probes designed for OpenArray® plates.

Chromosome	Accession Nº NCBI	Locus identifier	Assay ID	Primer and probe sequences	Context Sequence	Alelles
18	AY914316.1	BTA18 3144	AHQJU6T	**For** GAGGAGAGCTCTTCTCAGATTGC	ATGAAAGGAAT[A/C]GGAAATC	A,C
				Rev TGCTCTCCTTGTCTGGGAAGA		
				VIC ATGAAAGGAAT**A**GGAAATC		
				FAM AAAGGAAT**C**GGAAATC		
1	DQ404149.1	BTA01 BES6	AHI155I	**For** GGGCTCATAAGATAAAGCTTTTGCT	TCAGCATTTCTT[T/C]GAGATTA	T,C
				Rev GGGCATAGAACTCCTTGAAAATGG		
				VIC TCAGCATTTCTTT**G**AGATTA		
				FAM AGCATTTCTT**C**GAGATTA		
16	DQ846693.1	BTA16 349064_338	AHS1RI9	**For** AGGGTTTTCAGGCTTTATGTCTGT	TTTGCCTATG[A/G]TTGCCTG	A,G
				Rev AGACCAGCTTCTTGGCTTCTG		
				VIC TTTGCCTATG**A**TTGCCTG		
				FAM TGCCTATG**G**TTGCCTG		
28	EF042091.1	BTA28 186736_5402	AHUAPPH	**For** TGATGAAGCAATAAGGCACTGATGA	CCCAGCCCTT[T/C]ACCACA	T,C
				Rev GGTAAACTCATCACCTCCAAACTTTTG		
				VIC CCCAGCCCTTT**T**ACCACA		
				FAM CCAGCCCTT**C**ACCACA		
3	DQ489377.1	BTA03 240852_12041	AHVJNVP	**For** CTCTGTACAGCAGGAGAAATAGCAA	CTGCATCA[A/G]ACAAACC	A,G
				Rev TGTGCCGACCTCACGTG		
				VIC CTGCATCA**A**ACAAACC		
				FAM TGCATCA**G**ACAAACC		
23	AY937242.1	BTA23 5279	AHWSL1X	**For** AAAACAGAGCAAAAATCCTTGCCTT	CCCATTCCCTA[T/C]CCCATG	T,C
				Rev ACACAAAAAATACATTTAGTTATTTATTATGTATATTGTCTGTCT		
				VIC CCCATTCCCTA**T**CCCATG		
				FAM CCATTCCCTA**C**CCCATG		
11	AY851162.1	BTA11 IL1B	AH0JGKL	**For** CTCTCTCCCTAAAGAAAGCCATACC	TGAATAACCCC[A/G]AGGACTG	A,G
				Rev GCTACATTCTTCCCTTCCCTTCTG		
				VIC TGAATAACCCC**A**AGGACTG		
				FAM AATAACCCC**G**AGGACTG		
1	DQ381153.1	BTA01 135_21121	AH1SEQT	**For** CTCCCAAGGGCATCTCTCTAC	CCAGGCATTA[A/C]TATTAC	A,C
				Rev TGTCCTTTGTCTGTCAGAATGGAT		
				VIC CCAGGCATTA**A**TATTAC		
				FAM CCAGGCATTA**C**TATTAC		
5	DQ500958	BTA05 BES11	AH21CW1	**For** GCACTCCCTTCCCAGAACAG	CTCTGCTAATG[T/C]TTTCAG	T,C
				Rev ACCCAGACAAAGGTCAATGCTT		
				VIC CTCTGCTAATGTTTTCAG		
				FAM TCTGCTAATGCTTTCAG		
8	DQ916058.1	BTA08 302326_838	AH4AA29	**For** ACCAGGGCATTACCTATCACAGA	TGTCCGTTTCC[T/C]TTAAAAT	T,C
				Rev GGTTAGACTTGGCGCTCTTAATAGA		
				VIC TGTCCGTTTCC**T**TTAAAAT		

Table 1. Continued...

Chromosome	Accession Nº NCBI	Locus identifier	Assay ID	Primer and probe sequences	Context Sequence	Alelles
				FAM TCCGTTTCC**C**TTAAAAT		
18	EF028073.1	BTA18 186240_12002	AH5I89H	**For** CAAGATGATAAGGATTGTATTTAAGGTTGTCT	TCGCATTAATTAGT[A/G]TCTACC	A,G
				Rev GTCCTGCCTGTCAATAATCAAGTTG		
				VIC TCGCATTAATTAGT**A**TCTACC		
				FAM CGCATTAATTAGT**G**TCTACC		
6	DQ647190.1	BTA06 106320_3036	AH6R7FP	**For** GTCCAGGGACACCAGTCTATG	CTCCATGTC[A/G]TTGGAAC	A,G
				Rev CCCAAGTACGAAAGGGTTAGTGAT		
				VIC CTCCATGTC**A**TTGGAAC		
				FAM TCCATGTC**G**TTGGAAC		
29	EF034080.1	BTA29 50303_4748	AH705LX	**For** CACCACGGTCACATGATCCT	CAATATGGC[A/G]GTGCTTC	A,G
				Rev AGCTATTAATACGTATTCCATTCTATAGTGT		
				VIC CAATATGGC**A**GTGCTTC		
				FAM AATATGGC**G**GTGCTTC		
22	EF093509.1	BTA22 190836_5512	AHABIXY	**For** CTGTGTGATTGGCCAAAAGGTATT	CAAACAATACTAAAATC[A/T] ATTATC	A,T
				Rev TGGTGTCCTTAGCAAATAAATGAATGAATAAAT		
				VIC AACAATACTAAAATC**A**ATTATC		
				FAM CAAACAATACTAAAATC**T**ATTATC		
12	DQ786763.1	BTA12 215881_4945	AHBKG36	**For** AGAAGCTTCTGGCCCAGAGA	TGTTTTAGCAAAC[T/A]ACATTTG	T,A
				Rev GTCTTTTCCTGAGTCCCCACATTAT		
				VIC TGTTTTAGCAAAC**T**ACATTTG		
				FAM TTAGCAAAC**A**ACATTTG		
25	EF034083.1	BTA25 60020_10226	AHCTFAE	**For** CGGAGGTTTGGAGTCTTGTTTTCT	AGTCAATCTTCA[A/C]GCAAAAT	A,C
				Rev GCCAGCACCTCCCATTCTAT		
				VIC AGTCAATCTTCA**A**GCAAAAT		
				FAM CAATCTTCA**C**GCAAAAT		
29	DQ404153.1	BTA29 11629	AHD2DGM	**For** TCCTGCAGTTCTCTCCTGTCT	TCAGGGCTC[A/G]GTGAGC	A,G
				Rev GGACCATGTGGCCAAGCA		
				VIC TCAGGGCTC**A**GTGAGC		
				FAM AGGGCTC**G**GTGAGC		
6	DQ789028.1	BTA06 35310_9826	AHFBBMU	**For** TCAAAGCAACACATCTGGTCATCA	CAACCTGCT[T/C]GGCTCT	T,C
				Rev AATTAACTCTCTCAAATCTGAGTGAAGTG		
				VIC CAACCTGCT**T**GGCTCT		
				FAM AACCTGCT**C**GGCTCT		
15	DQ866817.1	BTA15 BES7	AHGJ9S2	**For** GTCCAGCATCTTTAACCCTGACTAA	CAATCTACT[A/G]AAATTCC	A,G
				Rev GTGGTGATCTCAAAGAGGCTTTCA		
				VIC CAATCTACT**A**AAATTCC		
				FAM AATCTACT**G**AAATTCC		
26	AY943841	BTA26 12063	AHHS7ZA	**For** ACTCTAGAGGGCCTAAACTCCAAAT	CTTCTGTCCC[A/G]AGTCAAG	A,G

Table 1. Continued...

Chromosome	Accession Nº NCBI	Locus identifier	Assay ID	Primer and probe sequences	Context Sequence	Alelles
				Rev GGCCTGCTTCTCACAAAGACA		
				VIC CTTCTGTCCC**A**AGTCAAG		
				FAM TCTGTCCC**G**AGTCAAG		
4	DQ647186.1	BTA04 116028_6113	AHKA4BQ	**For** TGGCTAGGGATCTGACTGCTT	CCTCACAGCT[A/G]TGTCTTT	A,G
				Rev GAAGACAGGGAGAAGGGAAAGTTTA		
				VIC CCTCACAGCT**A**TGTCTTT		
				FAM TCACAGCT**G**TGTCTTT		
19	AY916666.1	BTA19 5067	AHLJ2HY	**For** ATCTGAGAGGTGGGACAAGGA	TGCGGC[T/C]AAGCCAC	T,C
				Rev CCTTTCCCAGAGACTCAGACTTG		
				VIC TGCGGC**T**AAGCCAC		
				FAM CGGC**C**AAGCCAC		
26	EF150946.1	BTA26 90532_852	AHZAIED	**For** CACTTCTCTCTCTGCTTTCCCA	CCACCGC[A/G]TGGTTAT	A,G
				Rev TTCCAGAAGGGCACCAAGAAAA		
				FAM CCACCGC**A**TGGTTAT		
				VIC CACCGC**G**TGGTTAT		
5	AY844963.1	BTA05 16075	AHX1J75	**For** TCTTTGTATGTTTTAAATCTTGTTTTTCCTTCTGT	CCCTTTTGTACA[T/C]TGGTTAC	T,C
				Rev TCAGATATCCTGAAGGTGAGATCCAA		
				VIC CCCTTTTGTACA**T**TGGTTAC		
				FAM CCTTTTGTACA**C**TGGTTAC		
20	DQ888313.1	BTA20 BES1	AH893R5	**For** CTGGCAGAGTTCGGGTTTAGT	CACCATGCCA[T/C]GTAAAT	T,C
				Rev GCTTCTGGAGCTCACCCA		
				VIC CACCATGCCA**T**GTAAAT		
				FAM CCATGCCA**C**GTAAAT		
10	DQ786761.1	BTA10 135062_12754	AHRSTC1	**For** CATATACCATAGTATTTTTTCCCTTAGCTTACTGA	TGCTTTTTATAAG[A/G]TTTAAC	A,G
				Rev CTAGACCCTTTTCAAGTTTCTTTTGTTTCTT		
				VIC TGCTTTTTATAAG**A**TTTAAC		
				FAM TGCTTTTTATAAG**G**TTTAAC		

Where X_{aa}, X_{ab}, X_{bb} were the genotype frequencies. The P_I of the set of SNPs selected in this study was represented by the product of P_I of each individual marker (Heaton et al., 2005). P_I calculations only included samples that corresponded to different individuals. Duplicate samples resulting from the same individual were not used when performing these types of calculations.

3 Results and Discussion

All primers and probes designed for this study were effective and redesign was not necessary. In Table 2, the number of failed reactions from each assay has been provided. From the 49,686 total reactions carried out, only 2,223 (4.47%) failed. A previous study that used OpenArray® for cattle rustling produced a failure rate of 21.14% for beef samples (Fernández et al., 2014). The authors suggested that improving the design of the Taqman® probes would decrease the number of failures. For example, they observed that SNP assay rs17872223 (assay AHWSL1X in our study) had a success rate of 70%, while in this study, 98.17% of reactions were successful. Therefore, the results of this study SNP assays designed for this study performed better than the previous one. The failed reaction rates for this study varied between 1-6% for all assays with the exception of assay AHABIXY, which produced a 15% failure rate (Table 2).

The OpenArray® technology is characterized by its low consumption of sample and reagents. Thus, in a 384-well plate, 2.5μL of DNA sample and 2.5μL of master mix are mixed and loaded in the array, were the 26 SNPs assays are preloaded, using an automatic robot and special tips (Van Doorn et al., 2007). In the previous method developed using conventional real-time PCR, 6.25 μL of master mix were used for the analysis of a single SNP (Capoferri et al., 2006). In the method proposed in this work, it is possible to analyze 26 SNPs using only 2.5 μL of master mix. This lower cost of consumables makes it possible to reduce the price per sample and, therefore, this method can be better implemented in the meat sector. But this method also has some limitations. Due to the minimal volume loaded in each well (33 nL), arrays are very sensitive to evaporation until they are completely sealed. Wells situated on the borders of the array are especially susceptible to evaporation. The AHABIXY assay was located on the border of the array, which could explain its why its reaction failure was higher than the other assays tested.

The allele and genotype frequency of each assay was calculated based on results obtained. A total of 22 assays produced P_I values lower than 0.4 (Table 2), meaning that they had great discriminatory potential. The assay AHBKG36 produced the highest P_I value (0.600), with a MAF of 0.04. It is worth mentioning that this assay showed produced a good discriminatory potential value of 0.392 in genetic identification procedures carried out

Table 2. Allele and genotype frequencies observed in this study.

Assay	UND/NOAMP*	Allele frequencies		Genotype frequencies observed			P_I
		a	b	a,a	a,b	b,b	
AH0JGKL	86 (4.5%)	0.28	0.72	0.08	0.40	0.52	0.438
AH1SEQT	78 (4.08%)	0.50	0.50	0.25	0.51	0.24	0.381
AH21CW1	100 (5.23%)	0.47	0.53	0.23	0.47	0.29	0.364
AH4AA29	108 (5.65%)	0.50	0.50	0.26	0.48	0.26	0.365
AH5I89H	95 (4.97%)	0.51	0.49	0.25	0.50	0.24	0.378
AH6R7FP	90 (4.7%)	0.40	0.60	0.17	0.47	0.37	0.381
AH705LX	90 (4.7%)	0.70	0.30	0.50	0.40	0.10	0.419
AH893R5	87 (4.55%)	0.60	0.40	0.36	0.50	0.15	0.394
AHABIXY	288 (15.00%)	0.55	0.45	0.31	0.47	0.22	0.367
AHBKG36	112 (5.86%)	0.41	0.59	0.04	0.74	0.22	0.600
AHCTFAE	87 (4.55%)	0.39	0.61	0.15	0.48	0.37	0.389
AHD2DGM	58 (3.03%)	0.54	0.46	0.30	0.48	0.22	0.370
AHFBBMU	78 (4.08%)	0.61	0.39	0.37	0.48	0.15	0.387
AHGJ9S2	45 (2.35%)	0.49	0.51	0.24	0.49	0.27	0.370
AHHS7ZA	26 (1.36%)	0.50	0.50	0.24	0.51	0.25	0.379
AHI155I	105 (5.49%)	0.49	0.51	0.24	0.51	0.26	0.379
AHKA4BQ	92 (4.81%)	0.51	0.49	0.26	0.50	0.24	0.376
AHLJ2HY	84 (4.39%)	0.35	0.65	0.13	0.44	0.43	0.396
AHQJU6T	87 (4.55%)	0.47	0.53	0.24	0.47	0.29	0.364
AHRSTC1	36 (1.88%)	0.47	0.53	0.25	0.45	0.31	0.355
AHS1RI9	21 (1.09%)	0.66	0.34	0.43	0.47	0.10	0.414
AHUAPPH	92 (4.81%)	0.46	0.54	0.20	0.52	0.28	0.389
AHVJNVP	115 (6.01%)	0.55	0.45	0.29	0.52	0.19	0.388
AHWSL1X	35 (1.83%)	0.59	0.41	0.36	0.47	0.18	0.377
AHX1J75	101 (5.28%)	0.47	0.53	0.22	0.49	0.28	0.374
AHZAIED	27 (1.41%)	0.55	0.45	0.33	0.45	0.22	0.359
Combined							2.09E-11

using Angus herbs (Fernández et al., 2013). This highlights the importance of selecting SNPs appropriate for the breed tested in order to produce assays with highest power of exclusion. The assay AH0JGKL and AH705LX had a P_I values of 0.438 and 0.42 and MAF values of 0.08 and 0.10, respectively. These assays produced the lowest level of discriminatory power. AHRSTC1 produced a low P_I value (0.355), but had a higher exclusion capacity. Combining results using all 26 SNPs resulted in a P_I value of 2.09 x 10^{-11}, demonstrating the great discriminatory potential of the panel of SNPs selected for this study. Even, assays with lower P_I values failed, the global P_I would remain 3.39 x 10^{-9}. Karniol et al. (2009) obtained similar results with a panel of 25 SNPs (10^8 to 10^{10}) in different breeds typically dedicated to meat production.The results of the present study confirm that the 26 SNPs panel developed have a similar capacity for individual identification as a panel of 11–12 STRs (~10^{-11}) (Allen et al., 2010; Dalvit et al., 2008; Heaton et al., 2005). That exclusion power is sufficient to resolve simple cases of genetic identification (Fernández et al., 2013).

To determine the applicability of this methodology to trace meat, a total of 143 pairs of samples (143 muscle samples and 143 auricular pavilions) were analyzed to determine if each pair of samples corresponded to the same animal. The 143 samples were assigned correctly with 132 pairs of samples corresponding to the same animal and 11 pairs of samples were determined to be from different animals. Therefore, the specificity and sensibility of the method was determined to be 100%. From the 143 pairs of samples tested, 90 were also analyzed using the STR methodology and the results obtained from samples analyzed by both techniques were the same. This provided evidence that the 26 SNPs panel designed for individual animal identification was accurate. In each array a previously analyzed sample was included to determine the repeatability of the methodology. The 100% of repeated samples showed the same genotypic code. The SNPs included in this study were selected from the 100 SNPs panel recommended by ISAG-ICAR for parentage and individual identification according their allele distribution in the main cattle breeds (i.e Brangus, Limousine, Brahman, Charolaise, Holstein, Blonde d'Aquitaine). Therefore, the panel developed in this work could be successfully used for individual identification in the main cattle breed used in beef production. In this sense, Rogberg-Muñoz et al. (2016) were able to certify Angus, Hereford and Japanese Black meat by using 95 SNPs of the panel proposed by ISAG-ICAR. However, this panel failed with the native Chinese yellow cattle, probably because it is genetically more distant from the other breeds.

Genetic traceability is a powerful tool to complement conventional methods for tracing food products through the production chain. The use of this genetic tool is especially important for products with high-added value, for which fraud is common (Aprile et al., 2012; Felderhoff et al., 2020). By using this tool, producers can verify that their products are correctly labeled throughout each step of the production of a marketable product. This is especially important regarding retail selling points where it is difficult to trace products using conventional methods. In such cases, meat can be labeled in a fraudulent way to increase profit through selling standard products at premium quality rates. Therefore, through the use of genetic tools, producers can elucidate if a simple steak belongs to the expected animal or has been fraudulently labelled. The development of inexpensive methods that require minimal laboratory work will make it possible to generalize the use of genetic traceability by the meat industry. In this sense, Zhao et al. (2019) combined PCR with capillary electrophoresis to develop a panel of 12 SNPs for individual identification. Although the results were promising with a P_I 1.70×10^{-5}, this method use two techniques in genotyping analysis, increasing working time and reagent expense. In the other hand, the method developed by Capoferri et al. (2006) has the limitation of reagent consumption , which means that the number of SNPs evaluated must be kept to a minimum and lower P_I values. In addition, these two methods are not high throughput, which limits the number of samples that a laboratory can process at a time Thefore, the present study represents a step forward in the use of real-time PCR and SNPs for enhance our ability to genetically trace samples by increasing the number of assays that can be performed at the same time and by reducing the volume of reagents required. Thus, the OpenArray® design selected for this study facilitated the analysis of 96 samples in less than 4 h.

4 Conclusion

A panel of 26 SNPs selected from ISAG-ICAR core panel was successfully designed to genetically evaluate "Ternera Gallega" beef through the meat production process. The selection of a high-throughput, real-time PCR platform to perform the Taqman® assays allowed the simultaneous analysis of a wide range of SNPs from a wide range of samples simultaneously. The development of this panel will allow researchers to carry out the genetic evaluation a routinely way that requires a minimal quantity of reagents and time, making it very useful for tracing PGI. Due to the simplicity of the evaluation of SNPs in comparison with STRs, the results can be easily interpreted as a simple genetic code, which reduces the post-analysis time requirement. The economic return from the implementation of genetic traceability programmes by PGIs is much higher than the total cost of the program. In one hand, this control avoid that retail sell beef of lower quality as a PGI beef, avoiding unfair competition and boosting sales. In other hand, genetic control also reduces reputational problems by preventing consumers from consuming low-quality meat labelled as PGI, which could result in the consumer getting the wrong impression of the product. Finally, advertising at points of sale that PGI carries out this type of genetic control enhances brand reputation and consumer confidence. Future studies should be focused in the development and validation of panel designed to identify breed label fraud.

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