Developpement of a genetic traceability test in pig based on single nucleotide polymorphism detection

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Abstract

In order to assure traceability along the meat transformation chain, a powerful system is required. The administrative traceability shows limits that the use of genetic markers could overcome. The individual genome contains sequence differences, basis of the genetic polymorphism of which the genetic markers are the witnesses. Among them, two classes seem to dominate on the traceability field: the microsatellites and the single nucleotide polymorphisms (SNP). The aim of this work was to develop a genetic traceability test in pig based on SNPs mainly located in 5' and 3' untranslated regions. We selected a set of 21 SNP markers including new SNPs identified in this study and SNPs previously described. A genotyping assay was performed on 96 individuals from the major crossbred pig population in Belgium. Results showed that all individuals tested presented a different genotype. We concluded that such a genetic treaceability approach might help the administrative system.

Pig / SNP / traceability

1. Introduction

During the last years in Belgium, the food industry was affected by several scandals and crisis such as the hormones, the polychlorinated biphenyl (PCB) (unjustly called "dioxin crisis") and the bovine spongiform encephalopathy (ESB), resulting in a mistrust of the consumer for the Belgian meat and a lost of export dealings [4]. In order to restore the brand image of Belgian meat products, it is important to assure a traceability along the meat transformation chain. In Belgium, several administrative traceability system exist, the principal is the SANITEL system including an automatic treatment of data related to animal identification and registration [4]. The main disadvantage of this system is that the traceability stopped at the slaughterhouse. It is therefore almost impossible to link a piece of meat with an animal. Moreover, the administrative traceability is not unfailing, the lost of documents and the risk of cheating are always possible. So, the administrative traceability presents limits that the use of genetic markers could overcome.

Indeed, the genome of individual animals differs from each other (with the exception of monozygotic twins). In other words, it is possible to distinguish all individuals of the same species since they possess differences in their DNA sequence. Today, the genetic markers used for the individual identification and parentage control are almost exclusively microsatellite

markers, which occur once every 30-46 kb in pigs [16]. Single nucleotide polymorphism (SNP) markers are more abundant with an occurrence of about one SNP per kb in human [14] and about one SNP per 500 bp in mice [12] and cattle [8]. SNPs present following advantages over microsatelllite markers: 1) relatively stable from generation to generation [10], 2) more easily in laboratory handling and interpretation [10], 3) usable for standardized representation of genotyping results as a digital DNA signatures [6], and 4) compatible with automation [11]. One disadvantage is that SNP is generally a biallelic marker as a result a lower information content compared with a highly polymorphic microsatellite. But, this disadvantage can be compensated by a higher number (at least 20) of markers [15].

The first step of this study was to find new SNPs present in the 5' and 3' untranslated region (UTR) of pig genes. Next, we selected the more informative SNPs to integrate a set of SNP markers. After all, we estimated the potential utility of these markers in animal identification. The final aim of this work was to develop a powerful genetic traceability test for use in the major crossbred pig population found in Belgium in order to improve the administrative system used now.

2. Materials and methods

2.1. Animals and DNA samples

The panel of pigs consisted of 96 crossbred (Large White, Piétrain, Landrace) originated from 5 different channels of pig production in Belgium. DNA was extracted from pieces of cheek collected at the slaughterhouse, using the Wizard SV Genomic DNA Purification System (Promega) following the recommendation of the manufacturer.

2.2. PCR Primer design

One hundred and forty primer pairs were used for amplification and SSCP analysis (not shown). Primers used for sequencing and polymorphism identification are listed in Table I.

All primers were designed with Oligo 6.6 software (Medprobe). For SSCP analysis, PCR products length was comprised between 100 and 300 base pairs.

2.3. PCR amplification

The PCR reactions contained 5 μl of purified DNA, 1 U of Taq DNA polymerase (Amersham Biosciences), 5 μl of 20mM dNTP mix (Eurogentec), 5 μl of 10X PCR buffer and 0,5 μl of each primer (40 μM) in a total volume of 50 μl. Reactions were performed in a Flexigene thermal cycler (Techne) with the following conditions: initial denaturation at 94°C for 5 min., followed by 35 cycles at 94°C for 30 sec., annealing temperature (see Tab. I) for 30 sec. and 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were analyzed by 2% agarose gel electrophoresis in 1X TAE buffer.

2.4. Single strand conformation polymorphism (SSCP) analysis

For SSCP, 5 μl of the PCR product was mixed 1:1 with denaturating buffer (95% formamide, 0,025% xylene cyanol and 0,025% bromophenol blue), heat-denaturated at 95°C for 5 min., and then chilled on ice. Electrophoresis was carried out at 12°C constant temperature on a GeneGel SSCP gel (Amersham Biosciences) in a GenePhor Electrophoresis Unit (Amersham Biosciences). The following conditions were used: 6mA 90V for 25 min. and 14 mA 500V for 50 min. Bands were visualized with the DNA Silver Staining Kit (Amersham Biosciences).

2.5. DNA sequencing

Amplification products were purified on a KingFisher Magnetic Particle Processor (Thermo LifeSciences) using the Wizard MagneSil PCR Clean-Up System (Promega). Sequencing reactions were performed in 20 µl containing 5 µl of purified PCR product, 8 µl of DYEnamic ET Terminator sequencing premix (Amersham Biosciences) and 1 µl of primer (4 mM). Upper and/or lower PCR primers were used as sequencing primer. The cycling conditions were: 25 cycles at 95°C for 20 sec., 50°C for 15 sec., and 60°C for 1 min. Sequencing reactions were purified with AutoSeq96 (Amersham Biosciences) and automatic reading was performed using a MegaBACE 500 sequencer (Amersham Biosciences). The sequence data were analyzed with the Sequence Analyzer 3.0 software (Amersham Biosciences) and the sequence comparisons were performed with ClustalW (http://www.ebi.ac.uk/clustalw/).

2.6. Single nucleotide primer extension (SNuPe)

Amplification products were purified as described in DNA sequencing. For the primer extension reaction the following mix was used: 5 µl of purified PCR product, 1 µl of primer (2 pmol) and 4 µl of SNuPe premix (Amersham Biosciences) in a total volume of 10 µl. Following conditions were applied: 25 cycles at 96°c for 10 sec., 50°C for 5 sec. and 60°C for 10 sec. Reaction products were purified as decribed in DNA sequencing. Five µl of the reaction products were finally combined 1:1 with a multi-injection marker for separation and detection on the MegaBACE 500. Data were analyzed with SNP Profiler 1.0 software (Amersham Biosciences). Extension primers are presented in the Table II.

3. Results

3.1. PCR amplification and SSCP analysis

In order to find new SNPs, a first screening by the SSCP method was realized on 20 5'UTR and 42 3'UTR. Thirty-nine primer pairs of 140 did not give the expected result by PCR (no amplification or non specific amplifications). Then, 101 primer pairs were tested on 20 DNA samples out

from 96 for amplification and subsequent SSCP analysis of the PCR product. A polymorphism in the migration profile was observed with 38 primer pairs. Presence of SNP(s) was confirmed or invalidated in these PCR products by DNA sequencing.

3.2. Polymorphism identification

In order to determine the type and the position of the SNP(s), PCR products presenting a SSCP polymorphism were submitted to sequencing on both strands. Presence of SNP was confirmed with 18 primer pairs. In total, 39 new SNPs were discovered (Tab. III), and 1 SNP in the H-FABP gene (GenBank X98558) was already described. So, over 23382 base pairs amplified, 40 SNPs were identified, representing an average of 1 SNP per 585 bp. A 509 bp fragment of the 5'UTR of the steroidogenic acute regulatory (StAR) protein gene (GenBank AF038553) contained 11 SNPs, and a new SNP was identified in the 5‘UTR of the heart fatty acid-binding protein (H-FABP) gene (GenBank X98558). Thirty SNPs of 40 were transitions and 8 were transversions, and 2 polymorphisms showed more than 2 alleles.

3.3. Determination of the polymorphism rate

For this step, new PCR primers were designed. Forty-eight DNA samples out from 96 were submitted to PCR and sequencing. For each SNP, allele frequency was estimated and the polymorphism rate (heterozygosity) was calculated as follow: $H = \sum_{i=1}^{n} ip_i^2$. Results are presented in Table III. We considered that a SNP is highly informative when the H value is comprised between 0,3 and 0,5. Only 21 SNPs out of 40 presented this criterion. But, SNPs in the same sequence were sometimes highly associated, and in this case we selected only one of them. Therefore, to obtain a minimum of 20 highly informative SNPs usable in a genotyping test, we completed our panel with SNPs already described in 5'UTR and 3'UTR and others various SNPs in porcine genes. Polymorphism rate for these SNPs was also determined (Tab. III). For 4 SNPs, no polymorphism was observed in the DNA sequence of the samples tested. After this first discrimination based on the H value, 22 SNPs remained available.

3.4. Probability of identity in crossbred pig population and genotyping assay

The SNP genotyping method was based on primer extension reaction and multiple injection system. The design of the SNP primers was done according to the recommended guidelines by Amersham Biosciences with

the consequence that for some SNPs no SNP primer could be selected (i.e. in X98558 sequence). Finally, 21 SNPs were included for the genotyping test (Tab. III). The utility of the set of these 21 SNPs to identify all animals in a population was evaluated. The average for the H value for these 21 SNPs was 0,44. The probability that an individual A selected randomly in a population is identical to an individual B selected randomly in the same population was calculated as follow [13]:

$$P(A=B)=\prod_{i=1}^{r}\left(\sum_{i=1}^{n_i} q_{ij}^{4}+4\sum_{j=1}^{n_i-1}\sum_{k=j+1}^{n_i} q_{ij}^{2}q_{ik}^{2}\right)$$

This probability is approximately 7 x 10^{-9} for the 21 SNPs selected.

To test our SNP set in a real case, we performed a genotyping assay on 96 individuals representing the major crossbred pig population in Belgium and originated from 5 different pig production channels. Results showed that not any pigs tested possessed the same genotype (data not shown).

4. Discussion

Following the recent crisis in the animal production area, the public has emphasised the need for the improved identification of animals and animal products that can guaranty the traceability from the producer to the consumer. A beef carcass has a unique identity, linked to the identity of the live animal. However after processing from the slaughterhouse to the retail point, the carcass may be disassembled into a lot of separate pieces. To maintain identity through this processing and distribution chain using conventional labelling system is difficult [3]. Only DNA could help the administrative traceability as DNA sequence in each nucleic cell of an individual is identical and this sequence is specific to the individual (excepted for monozygotic twins).

To develop a traceability test in pig based on single nucleotide polymorphism, two choices presented to us: working with SNPs already present in GenBank or searching new SNPs in the major crossbred pig population in Begium. We chose the second solution. We studied 5' and 3'UTR of pig genes by PCR amplification and SSCP analysis. Seventy-two percent of the primer pairs designed gave the expected PCR product. For the others, either PCR conditions used were not optimal, or primers annealed in regions where DNA forms secondary structures, or primers were not specific for the region to amplify. Migration polymorphisms were observed

for only 38% of the primer pairs tested. Presence of SNPs was confirmed from PCR products obtained with only 18% of the primer pairs.

In total, we identified 40 SNPs, representing an average of 1 SNP per 585 bp. This value is low compared with a similar study [9]. By directly sequencing PCR amplification products from genes on the porcine chromosome 2, these authors found 1 SNP per 108 bp. This strong difference could be explained by the fact that we have missed SNPs when performing a first sreening by SSCP analysis. Interestingly, the first third of the 5'UTR of the StAR protein gene contained 11 SNPs representing an average of 1 SNP per 46 bp. Such regions with a high concentration of SNPs were already described in others porcine genes [1, 7]. These high polymorphic regions were used to develop a genotyping test by sequencing [2]. Composition of the 39 new SNPs identified in this study was: 74% C/T or A/G, 21% A/T or G/C or A/C or G/T, and 5% others. This observation is comparable with previous studies [5, 9].

The heterozygosity value for each new SNP and for SNPs previously described was calculated. The H value varied from 0 to 0,63. The H values > 0,5 are explained by the fact that two polymorphisms showed more than 2 alleles. Furthermore, some SNPs previously described were not observed in the pig population used in this study. This observation underlined the fact that SNP could be population specific. Twenty-one informative SNP markers (H>0,3) were selected for the genotyping test.

The probability that 2 individuals selected in the same population are identical was approximately 7 x 10^{-9}. Since the Belgian pig population is 7 10^{6}, the test was considered as sufficiently discriminant. A real case scenario was realized by genotyping 96 individuals originated from 5 different pig production channels in Belgium. All individuals possessed a unique "DNA fingerprinting". Therefore, it seems that our genotyping test might be usable for a genetic traceability system. The main SNP detection techniques used for a high throughpout are DNA microarrays, mass spectrometry or OLA (oligonucleotide ligation assay). In the next future, we will change or improve our genotyping method to minimize the laboratory handling and to reduce the cost of the test.

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Table I : PCR primers used in this study.

GenBank accession no.	Primer	Sequence	Fragment size (bp)	Anealing temp..
AF329087	F5B	5' TCAGTACCCAGCCAGTT 3'	365	58
	F6B	5' GGGTTTTGAGTCTTACACTTG 3'		
AF342812	F17	5' GCGCAGGGATGGTTTCAC 3'	422	61
	F20	5' GGTGGCATCATGAGAACCTGA 3'		
AF451836	F47B	5' TGGCGTGTCTGAGTGCATCA	407	58
	F50B	5' CCCTCTAGCCCTATCAAGTTCAA 3'		
AF139178	F53B	5' CAGCGAACCTCCACAGCATCT 3'	401	63
	F54B	5' CGGGATCCCAGACGAGA 3'		
AF038553	STAR1	5' CCCAACCCACTGAGAGTGAAG 3'	509	63
	STAR2	5' GGTCAAATCTGAGCCAAATCT 3'		
U12627	F89	5' TGCCGAGTGACACAGATTTGT 3'	273	63
	F90	5' AGTCACCAGTCAGGGCAATG 3'		
E01557	F103B	5' TAGGGCTAAAATGAATGT 3'	340	50
	F104B	5' TAACCCAAGTTTTGTGCTT 3'		
E08096	F109B	5' GAATTTCAATTTCTTTCCCAATG 3'	165	58
	F110B	5' AAGCAGAGTGGAGAGATAGAGCC 3'		
AF034974	F119B	5' CCCACCTCGCATCACTCTAGCTG 3'	391	63
	F120B	5' GCGTCACAATTAAAGGAGCC 3'		
AF034974	F147	5' GGCGTAGGAGTCCTCAG 3'	277	53
	F148	5' CCTGCGAAATCGGAAAT 3'		
Y15710	F151B	5' AACACACAGTACACACAGCACGTAT 3'	380	55
	F152B	5' GCCAATTTAAAAGTCCCTAAG 3'		
AF426435	F165B	5' GCCGTGCTCCTCTGTAATTTG 3'	386	58
	F166B	5' GATCTTTGCCTCAGCCTGGAT 3'		
X98558	F243	5' CTCTCCTTAATCCTGCGACCT 3'	493	64
	F246	5' GCTTTAAATGGCCCTAGCGTC 3'		
AF020322	F249	5' ACGCTTGAATGGTAGTGGAAC 3'	401	61
	F250	5' TTTACACCCGAAGCAC 3'		
AF327369	F277	5' AGACTCCAGCCCAAACTG 3'	214	58
	F278	5' CGGGTTGGAATGAATTTCT 3'		
AJ000928	CMYCF	5' ATCACTACTGCGGTACAACAG 3'	195	63
	CMYCR	5' GGATTTGGTGGTGGCAAGC 3'		
AJ493461	MYH4F	5' AGTGAAGAGTAATTCATCTAAA 3'	124	55
	MYH4R	5' GATTGCAAAATTCTCTGTAGA 3'		
AF473820	PKCTF	5' GATATCCTGAGACTTGCCTCT 3'	364	61
	PKCTR	5' GCGTCCACACATATTTCATT 3'		
Y16181	HFABPF	5' CTGCCCTAATCTGACCCTC 3'	241	58
	HFABPR	5' CAACAAGAACCGGAACTGAAC 3'		
AF227686	GNRHRF/2	5' CCGAAATGGTAAACAGGGTGT 3'	599	55
	GNRHRR/2	5' TCATATGGGCAGGGAGA 3'		
U70883	FUT1F	5' CATTGCCCACCTGTTCTTC 3'	595	61
	FUT1R	5' CTACCTACCGTTGGCAGTTTG 3'		
M29939	SLADQA152	5' CGACCATGTTGCCTCCTA 3'	238	55
	SLADQA377	5' CGCGGTGTTGTTGGAAC 3'		
X68247	RYR1336	5' TCTTGCCTCCGACTTCTCA 3'	493	58
	RYR1810	5' ACCGGAGTGGAGTCTCTGA 3'		
Y16180	F311	5' TTTCACCAGACCCGATCATTC 3'	592	63
	F312	5' GGACTCTGCCCTGTATTCCTA 3'		

AJ251197	M19 M20	5' GTGAGGGGGACATTGGAAAC 3' 5' GGGCAGAGCGGGTGA 3'	723	58

Table II : extension primers used for the SNuPe reaction.

GenBank accession no.	SNP		Primer	Sequence	Sense
	Type	Position			
AF329087	Y	618	619L21	5’ TTGAGTTAGGACCACGAT 3’	Reverse
	S	642	621U21	5' CGTGGTCCTAACTCAATTGGA 3'	Direct
AF451836	Y	139	118U21	5’ CCCTTTAGGTCTCAATTTCCT 3’	Direct
	H	159	160L21	5’ GTCAGGTCATCCGCAATCCTC 3’	Reverse
	K	317	296U21	5’ AGCTCAGCTCAAGTCCTAAAA 3’	Direct
AF038553	R	308	287U21	5’ TAGAAAGAAAAGCAGAAAATC 3’	Direct
U12627	K	88	89L18	5’ GCTCATAGGAACACAGAC 3’	Reverse
	R	208	209L18	5’ CGCCTGCCTCCCCCCAAC 3’	Reverse
	V	251	233U18	5’ GACCATCTCCATCCTTAT 3’	Direct
E08096	R	1350	273L18	5’ AACACAAGGATCTGGATA 3’	Reverse
AF034974	R	848	232U21	5’ CCCTTCGCGGCGTTACTCAGC 3’	Direct
	Y	864	270L21	5’ GACCAAGCCCAGTCATGAAAC 3’	Reverse
	R	994	400L21	5’ CCAATTCTTTCCTGTGGCGTA 3’	Reverse
AF426435	Y	501	502L21	5’ CCTACCCATCAAGCCAGTGGA 3’	Reverse
AF020322	Y	615	284U21	5’ ATTATTTTCAGAGGAAAAAGT 3’	Direct
AF473820	R	224	225L22	5' TCTTTTCCAGGTTGAAAAGGAA 3'	Reverse
U70883	Y	2148	2129U19	5' GCAAGCACTCACCAACCCC 3'	Direct
M29939	Y	245	224U21	5' TGATGGCGACGAGGAATTCTA 3'	Direct
X68247	Y	1666	COP5	5' ATGAGATCTTGGTTGGAGC 3'	Reverse
Y16180	R	1929	1930L21	5' ACCGTGACTGAGCAGGCTTAA 3'	Reverse
AJ251197	Y	384	363U21	5' GTAACACCTTGGGCAAGTCAC 3'	Direct

Table III : type and position of SNPs

GenBank accession no.	Chr.	SNP		Allele frequency			H
		Position	Variation	Allele 1	Allele 2	Allele 3	
AF329087		618	Y	0,67	0,33		0,44*
(5'UTR)		642	S	0,4	0,6		0,48*
AF342812		116	M	0,1	0,9		0,17
(3'UTR)		375	Y	0,15	0,85		0,26
AF451836		103	R	0,84	0,16		0,27
(5'UTR)		139	Y	0,8	0,62		0,47*
		159	H	0,19	0,33	0,48	0,63*
		317	K	0,25	0,75		0,38*
AF139178	7	393	Y	0,95	0,05		0,10
(5'UTR)							
AF038553	15	308	R	0,73	0,27		0,39*
(5'UTR)		338	R	0,73	0,27		0,39
		388	W	0,85	0,15		0,25
		397	Y	0,73	0,27		0,39
		431	Y	0,875	0,125		0,22
		495	Y	0,85	0,15		0,25
		553	Y	0,875	0,125		0,22
		579	S	0,85	0,15		0,25
		614	Y	0,85	0,15		0,26
		615	R	0,87	0,13		0,23
		621	Y	0,88	0,12		0,21
U12627	7	88	K	0,62	0,38		0,47*
(3'UTR)		199	S	0,5	0,5		0,5
		208	R	0,26	0,74		0,39*
		251	V	0,57	0,29	0,14	0,58*
E01557		2989	R	0,98	0,02		0,04
(3'UTR)							
E08096		1350	R	0,73	0,27		0,39*
(3'UTR)							
AF034974	1	848	R	0,62	0,38		0,47*
(3'UTR)		864	Y	0,79	0,21		0,33*
		994	R	0,74	0,26		0,39*
		2284	R	0,27	0,73		0,4
Y15710	2	1420	Y	0,85	0,15		0,25
(3'UTR)		1486	R	0,86	0,14		0,23
AF426435		359	S	0,17	0,83		0,28
(5'UTR)		390	Y	0,16	0,84		0,27
		501	Y	0,18	0,82		0,30*
X98558	6	1306	R	0,66	0,34		0,45[1]
(5'UTR)		1324	Y	ND	ND		ND[1]
		1524	Y	0,65	0,35		0,45
AF020322		615	Y	0,67	0,33		0,44*
(3'UTR)		739	Y	0,84	0,16		0,26
AF327369	4	1947	Y	0,98	0,02		0,04
(3'UTR)							
AJ000928		1189	R	0,97	0,03		0,06[1]
(5'UTR)							

AJ493461 (3'UTR)	12	26	W	1	0	0[1]
AF473820 (3'UTR)	10	171	Y	0	1	0[1]
		222	K	0,67	0,33	0,44[1]
		224	R	0,67	0,33	0,44[1]*
		339	Y	0,04	0,96	0,08[1]
Y16181 (3'UTR)	6	310	R	1	0	0[1]
AF227686 (3'UTR)	8	661	Y	ND	ND	ND[1]
		755	K	ND	ND	ND[1]
		1632	W	1	0	0[1]
		1721	S	0,07	0,93	0,13[1]
U70883 (3'UTR)	6	915	R	ND	ND	ND[1]
		1465	R	ND	ND	ND[1]
		2148	Y	0,64	0,36	0,46[1]*
M29939		245	Y	0,75	0,25	0,38[1]*
		254	R	0,94	0,06	0,11[1]
		278	M	0,93	0,07	0,13[1]
		279	R	0,5	0,5	0,5[1]
		293	Y	0,64	0,36	0,46[1]
		294	R	0,38	0,62	0,47[1]
		296	W	0,98	0,02	0,03[1]
		301	S	0,98	0,02	0,03[1]
		308	W	0,96	0,04	0,07[1]
		314	R	0,96	0,04	0,07[1]
		332	Y	0,62	0,38	0,48[1]
		339	R	0,9	0,1	0,1[1]
		342	K	0,78	0,22	0,34[1]
		352	M	0,89	0,11	0,19[1]
		353	Y	0,92	0,08	0,15[1]
		363	Y	0,88	0,12	0,22[1]
		364	S	0,81	0,19	0,32[1]
		368	Y	0,87	0,13	0,22[1]
X68247	6	1666	Y	0,45	0,55	0,44[1]*
Y16180	6	647	Y	ND	ND	ND[1]
		737	Y	ND	ND	ND[1]
		861	M	ND	ND	ND[1]
		1489	Y	0,07	0,93	0,12[1]
		1776	K	0,6	0,4	0,48[1]
		1811	S	0,58	0,42	0,49[1]
		1929	R	0,61	0,39	0,47[1]*
		1970	Y	0,61	0,39	0,47[1]
		2767	Y	ND	ND	ND[1]
AJ251197	4	202	R	0,47	0,53	0,5[1]
		254	R	0,44	0,56	0,49[1]
		384	Y	0,48	0,52	0,5[1]*
		438	R	0,47	0,53	0,5[1]

Legend : M = A or C, R = A or G, W = A or T, S = C or G, Y = C or T, K = G or T, V = A or C or T, V = A or C or G

* : SNPs selected

1 : SNP present in GenBank

ND: not determined