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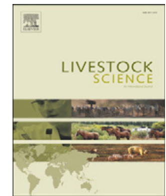

A Primer-Extension Assay for simultaneous use in cattle Genotype Assisted Selection, parentage and traceability analysis

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ABSTRACT

The use of genotype information as an aid to selection can be a cheap and effective way to improve the genetic progress in beef cattle breeds, specially in the case of high cost phenotypic recording which is true for many economic traits in beef cattle. SNPs located at candidate genes underlying economic traits allow prediction of the genetic merit of individuals and, combined with parentage and traceability analysis, guarantee consumer protection. Here we present a cost-effective technology, the Capillary Primer-Extension Assay, to genotype validated mutations which identify differences between individuals in candidate genes associated directly or potentially with meat tenderness, marbling and muscle growth, milk yield, protein and fat content, sex or coat colour. We genotyped 70 SNPs in 8 beef, 3 dairy and one semi-feral (never selected for any production trait) breeds and present a panel of 53 SNPs with the aim of enabling a reasonable tool for parentage analysis, animal identification and production of markers usable in GAS in small local breeds for which other tools are unaffordable.

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1. Introduction

The recent sequencing of the bovine genome has generated a large number of single nucleotide polymorphisms (SNP), but only a few tens of mutations at different genes have been associated so far to different traits such as milk, meat, growth performance or coat colour in cattle (see Ibeagha-Awemu et al., 2008 for revision). Genomic selection (Meuwissen et al., 2001) based on genome-wide non-associated SNPs is being used currently in dairy cattle for its application in selection as a way to overcome traditional progeny testing schemes and the difficulties of using such a small list of causative mutations in marker-assisted selection (MAS) for cattle. However, this approach is hardly usable in beef cattle, mostly due to the reduced family sizes and scarcity of phenotypes for which measuring is difficult and expensive (e.g. meat quality traits). Although the use of MAS has been shown to increase the rate of genetic gain (Meuwissen and Goddard, 1996; Hayes and Goddard, 2003) and seems a more powerful selection strategy for inbreeding management (Pedersen et al., 2009), it is currently poorly used in common improving selection schemes. In beef breeds it represents an important alternative to pedigree selection as bulls are selected uniquely on the basis of their molecular information.

Another problem preventing the widespread use of molecular markers in MAS is the fact that polymorphisms identified in particular breeds are not present in all cases in European breeds and the association between this eventual polymorphism and meat quality traits is not always true in the production systems used in different countries (e.g. Quaas et al., 2006; Schenkel et al., 2006). Most of the published associations in local breeds need to be validated before it can be used for improvement.

The purpose of the present study is to propose a tool based on Capillary Primer Extension to simultaneously analyse a set of polymorphisms located in different genes, either associated with economic traits like meat quality, growth or production or with coat colour, allowing its use for

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Table 1
Sixty-nine polymorphisms tested in 60 different genes, SNP location, allele frequencies, and exclusion probabilities for each breed.

Locus symbol and SNP position [1]	GenBank [2]	Allele1/ Allele2	Frequency of allele 1											
			Pas [3]	Jer [3]	Hol [3]	Cha [3]	Lim [3]	Sim [3]	A A [3]	A V [3]	Pir [3]	Lid [3]	Cas [3]	ANI [3]
CAPN1 g.6545 C<T*	AF248054	C/T	0.565	0.578	0.660	0.546	0.431	0.222	0.552	0.667	0.532	0.750	0.403	0.567
CAPN1 g.4558 G<A*	AF248054	G/A	0.677	0.719	0.827	0.756	0.613	0.333	0.750	0.617	0.452	0.550	0.790	0.750
CAPN1 g.5709 G<C*	AF252504	C/G	0.290	0.465	0.480	0.102	0.145	0.056	0.138	0.052	0.000	0.000	0.100	0.217
CAST g.2959 G<A*	AF159246	A/G	0.565	0.782	0.692	0.689	0.677	0.778	0.883	0.733	0.645	0.975	0.839	0.850
CHRNE g.1145del20*	NC_007317	C/del	1.000	0.982	1.000	0.938	0.936	0.694	0.983	0.983	0.952	1.000	0.914	0.933
c-KIT g.115 G<T*	AJ243060	G/T	0.048	0.032	0.192	0.386	0.433	0.029	0.310	0.460	0.532	0.474	0.413	0.296
CRH g.22 C<G*	AF340152	C/G	–	0.694	0.542	0.421	0.371	0.467	0.700	0.268	0.462	–	0.467	0.333
CSN3 g.12947 G<A*	AY380229	G/A	1.000	0.968	1.000	1.000	1.000	1.000	1.000	1.000	0.958	1.000	1.000	1.000
CSN3 g.13100 C<A*	AY380229	C/A	0.483	0.887	0.212	0.683	0.581	0.361	0.093	0.467	0.333	0.500	0.385	0.614
CSN3 g.13120 A<G*	AY380229	A/G	0.936	1.000	0.904	1.000	1.000	1.000	0.911	1.000	1.000	1.000	1.000	1.000
DGAT1 g.6829 A<G*	AY065621	A/G	0.000	0.607	0.442	0.095	0.097	0.094	0.379	0.328	0.333	0.083	0.250	0.267
FADS1*	ss63322537	T/C	0.855	0.259	0.481	0.667	0.419	0.333	0.518	0.617	0.694	0.679	0.597	0.650
GDF8*	ss77831865	G/del	1.000	1.000	1.000	1.000	1.000	1.000	0.967	0.317	0.887	1.000	0.984	1.000
GDF8*	ss77831863	C/A	1.000	1.000	1.000	0.988	0.016	1.000	1.000	1.000	0.694	1.000	1.000	1.000
GDF8*	ss77831864	C/T	1.000	0.969	1.000	0.826	0.984	1.000	1.000	1.000	0.936	1.000	1.000	1.000
GH1*	ss77831800	T/G	0.855	0.984	0.900	0.978	0.919	0.972	1.000	1.000	1.000	1.000	1.000	0.966
GHR 4962 g.T<A*	AM161140	T/A	–	0.844	0.865	0.950	0.984	0.917	0.833	0.897	0.952	–	1.000	0.967
LEP g.198 C<T*	AF120500	C/T	–	0.625	0.600	0.700	0.581	0.778	0.500	0.567	0.554	–	0.200	0.533
LGB g.5864 C<T*	Z48305	C/T	0.387	0.532	0.539	0.556	0.550	0.556	0.765	0.617	0.452	1.000	0.516	0.583
LOX g.7548 C<T*	NW_001495344	C/T	0.629	0.241	0.712	0.554	0.565	0.528	0.396	0.648	0.677	0.211	0.550	0.655
MC1R g.422 T<C*	S71017	T/C	0.037	0.339	0.977	0.163	0.177	0.000	0.889	0.517	0.242	0.975	0.048	0.891
MC1R p.G104V*	S71017	G/ins	0.645	0.317	0.180	0.882	1.000	1.000	0.517	0.214	1.000	0.175	0.167	0.350
POMC g.437del1*	J00021	C/T	0.931	1.000	1.000	0.826	0.636	0.583	–	0.929	0.650	–	0.929	0.844
PPARGC1A g.19 C<T*	AY547554	C/T	0.855	0.433	0.567	0.841	0.942	0.958	0.929	0.600	0.050	0.500	0.375	0.593
RORC g.3290 T<G*	DQ667048	T/G	0.710	0.812	0.731	0.900	0.717	0.778	0.367	0.733	0.613	0.900	0.500	0.817
SCD g.10329 T<C*	AY241932	T/C	0.177	0.125	0.385	0.442	0.419	0.306	0.155	0.333	0.436	0.625	0.742	0.667
SILV g.92 G<A*	EF065525	G/A	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
TG g.1696 C<T*	M35823	T/C	–	0.250	0.175	0.273	0.571	0.786	0.423	0.333	–	–	0.364	0.263
TYR g. 981in1*	AY162287	G/T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TYRP1 g. 1300 C<T*	AF400250	G/A	1.000	0.984	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
UCP2 g.812 G<A*	XM_614452	C/T	–	0.578	0.019	0.143	0.290	0.083	0.214	0.250	0.267	–	0.224	0.308
ABCA1**	ss28451692	C/A	–	0.871	0.885	0.552	0.783	0.444	0.621	0.850	0.823	–	0.567	0.500
ACAT2**	ss65658764	T/C	–	0.185	0.500	0.250	0.419	0.667	0.550	0.586	0.435	–	0.733	0.586
ALDH2**	ss77831990	T/C	0.581	0.783	0.731	0.792	0.613	0.333	0.567	0.741	0.419	0.421	0.717	0.500
CAST**	ss77832278	C/G	0.462	0.031	0.346	0.333	0.355	0.583	0.345	0.267	0.323	0.658	0.113	0.500
CAV3**	ss62797050	C/T	0.581	0.375	0.365	0.359	0.355	0.250	0.276	0.350	0.258	0.605	0.323	0.300

CFL1**	ss77831721	T/C	0.387	0.531	0.135	0.620	0.468	0.306	0.117	0.367	0.258	0.342	0.306	0.133
CRI1**	ss77832128	T/G	0.117	0.034	0.091	0.052	0.184	0.167	0.000	0.036	0.135	0.050	0.150	0.260
CTSF**	ss77831844	G/A	0.967	0.815	1.000	0.813	0.891	0.559	0.583	0.683	0.886	0.868	0.946	0.974
CYP1A1**	ss77832034	A/G	0.274	0.063	0.019	0.067	0.177	0.056	0.017	0.133	0.274	0.000	0.323	0.033
DNAJA1**	ss65351307	T/C	–	0.323	0.540	0.655	0.783	0.833	0.683	0.683	0.862	–	0.903	0.500
FABP4**	ss77831857	G/C	0.258	0.387	0.462	0.557	0.483	0.625	0.414	0.433	0.483	0.658	0.321	0.333
FIT2**	ss61961642	C/A	0.339	0.242	0.154	0.233	0.306	0.250	0.086	0.433	0.435	0.550	0.339	0.196
HSPB1**	ss63015930	T/C	–	0.094	0.038	0.217	0.016	0.607	0.133	0.317	0.183	–	0.129	0.185
IGF2R**	ss77831877	A/G	0.194	0.031	0.058	0.200	0.145	0.333	0.483	0.167	0.290	0.075	0.065	0.150
ME3**	ss77831909	A/G	0.548	0.242	0.673	0.467	0.371	0.278	0.333	0.350	0.065	0.184	0.433	0.533
PCSK1**	ss77831755	T/C	0.633	0.688	0.135	0.422	0.484	0.389	0.683	0.617	0.500	0.425	0.694	0.345
PLTP**	ss77832104	A/G	0.387	0.609	0.288	0.407	0.710	0.417	0.800	0.586	0.482	0.175	0.290	0.638
PPM2C**	ss77831758	T/C	0.371	0.032	0.481	0.163	0.306	0.361	0.117	0.117	0.150	0.100	0.032	0.350
PRKAG2**	ss77832378	G/A	0.367	0.233	0.385	0.091	0.210	0.143	0.133	0.150	0.327	0.237	0.081	0.117
SREBP1C**	ss62543518	T/C	–	0.375	0.500	0.233	0.161	0.708	0.426	0.233	0.258	–	0.400	0.345
SUSP1**	ss77831761	A/G	0.919	0.656	0.904	0.783	0.968	0.694	0.750	0.650	0.855	1.000	0.758	0.800
VIM**	ss77831736	T/C	0.532	0.266	0.385	0.644	0.887	0.583	0.600	0.717	0.887	1.000	0.387	0.667
ACACA	ss64381883	G/A	–	1.000	0.827	1.000	1.000	1.000	1.000	0.983	0.984	–	1.000	0.967
CPT1	ss65363345	G/C	–	1.000	1.000	0.983	0.983	1.000	1.000	1.000	0.977	–	1.000	0.914
CRYAB	ss62086225	T/C	–	0.371	0.120	0.283	0.210	0.250	0.241	0.233	0.121	–	0.177	0.233
GLUT4	ss62538460	G/A	–	1.000	0.827	1.000	1.000	1.000	1.000	0.983	0.984	–	1.000	0.967
INSIG2	ss62463931	A/T	–	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	1.000	1.000
LEP	ss77832159	T/A	0.125	0.000	0.038	0.081	0.065	0.083	0.050	0.069	0.097	0.025	0.150	0.224
LPL	ss65478732	T/C	–	0.000	0.096	0.100	0.000	0.056	0.063	0.000	0.016	–	0.083	0.086
MGAT1	ss65425229	T/C	–	1.000	1.000	1.000	1.000	1.000	0.983	0.983	1.000	–	0.968	1.000
MMP1	ss77831916	G/A	0.790	1.000	0.846	0.957	0.952	1.000	0.750	0.800	0.887	0.850	0.984	0.933
PLOD3	ss77831757	A/G	0.016	0.078	0.077	0.278	0.048	0.222	0.117	0.133	0.113	0.000	0.000	0.133
PPARA	ss65362714	C/T	0.710	0.859	0.846	0.886	0.914	0.861	0.933	0.800	0.750	0.925	0.903	0.862
PPARG	ss62850198	G/A	0.839	0.875	0.885	0.837	0.823	0.861	0.724	0.900	0.839	1.000	0.897	0.944
RORA	ss65549854	A/G	0.065	0.375	0.000	0.156	0.017	0.028	0.000	0.100	0.033	0.150	0.000	0.052
SCAP	ss62839002	G/A	–	0.828	0.846	0.983	1.000	0.971	0.933	0.850	0.968	–	0.887	0.850
SOCS2B	ss77832234	T/C	0.242	0.000	0.096	0.159	0.065	0.111	0.268	0.217	0.097	0.025	0.267	0.121
STX17 g.897 T<G	NW_001503623	T/G	0.968	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Excl P1 (2) [4]			0.93625176	0.95417063	0.96075856	0.96727130	0.97047482	0.96746170	0.96376927	0.97883043	0.97546442	0.86317936	0.96034088	0.97340052
Excl 2P (2) [5]			0.99983648	0.99997514	0.99997482	0.99999088	0.99998758	0.99998449	0.99998267	0.99999461	0.99999329	0.99860737	0.99998039	0.99999165

[1] Locus symbol along with SNP position according to the sequence indicated in the next column. * SNPs associated with different traits (31); ** SNPs with minor allele frequency (MAF) exceeding a 0.3 threshold in at least one breed added to the Previous group to improve the power for parentage analysis and animal traceability (22).

[2] GenBank Accession Numbers for *Bos taurus* sequences including the interrogated SNPs or dbSNPs accession number.

[3] Complete breed names: Pasiega (Pas), Jersey (Jey), Holstein (Hol), Charolais (Cha), Limousin (Lim), Simmental (Sim), Aberdeen Angus (A A), Asturiana de los Valles (A V), Pirenaica (Pir), Lidia (Lid), Casina (Cas), Avileña-Negra Ibérica (ANI).

[4] Combined exclusion probability of the 53 polymorphisms group tested when only one parent was genotyped at the breed level.

[5] Combined exclusion probability of the 53 polymorphisms group tested when the parent pair genotypes were available at the breed level.

traceability, parentage test together with validation of polymorphisms and production of markers usable in MAS or, more specifically, in Genotype Assisted Selection (GAS) in small local cattle breeds.

2. Materials and methods

2.1. Sample collection and DNA extraction

We used 356 individuals from 12 populations belonging to three dairy breeds (two local – 31 Pasiega and 32 Jersey – and one highly selected — 26 Holstein); six beef breeds, some of them disseminated throughout the world (46 Charolais, 31 Limousin, 18 Simmenthal and 30 Aberdeen Angus) or more locally used (30 Asturiana de los Valles and 31 Pirenaica); one semi-feral corresponding to the Lidia breed (n = 20) which has never been selected for any production trait; and finally two local maternal breeds (31 Casina and 30 Avileña-Negra Ibérica). All breeds were blood sampled in Magic Buffer® (BIOGEN Diagnóstica, Spain) and DNA was extracted using a standard phenol chloroform method (Sambrook et al., 1989).

2.2. Choice of the genes

A total of 70 different polymorphisms belonging to 61 genes have been chosen to use in a Primer-Extension Assay multiplex. Among them, 24 genes have been associated with different traits (Table 1). These are: Dairy Genes*: casein* (*CSN3*) (Kaminski, 1996), *β-Lactoglobulin* (*LGB*) (Aschaffenburg and Drewry, 1957), *diacylglycerol O-acyltransferase* (*DGAT1*) (Grisart et al., 2001), *fatty acid desaturase 1* (*FADS1*) (unpublished data), *growth hormone* (*GH*) (Schlee et al., 1994) and *peroxisome proliferator-activated receptor gamma coactivator 1 alpha* (*PPARGC1A*) (Weikard et al., 2005). Tenderness genes: *calpain* (*CAPN1*) (Page et al., 2002; White et al., 2005), *calpastatin* (*CAST*) (Barendse, 2002) and *lysyl oxidase* (*LOX*) (Barendse, 2002); Muscle growth genes: *cholinergic receptor nicotinic epsilon* (*CHRNE*) (Thompson et al., 2003), *corticotropin releasing hormone* (*CRH*) (Buchanan et al., 2005), *growth hormone receptor* (*GHR*) (Blott et al., 2003), *uncoupling protein 2* (*UCP2*) (Sherman et al., 2008), *proopiomelanocortin* (*POMC*) (Thue and Buchanan, 2002) and *myostatin* (*GDF8*) (Grobet et al., 1997); Marbling genes: *DGAT1* (Grisart et al., 2001), *leptin* (*LEP*) (Buchanan et al., 2002), *thyroglobulin* (*TG*) (Barendse, 1999) and *retinoic acid receptor-related orphan receptor C* (*RORC*) (Barendse et al., 2007); Fatty acids profile genes: *stearoyl-CoA desaturase* (*SCD*) (Taniguchi et al., 2004) and *FADS1* (unpublished data); Coat colour genes: *melanocortin 1 receptor* (*MC1R*) (Klungland et al., 1995), *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (*c-KIT*) (Klungland et al., 2000), *tyrosinase* (*TYR*) (Schmutz et al., 2004), *tyrosinase-related protein 1* (*TYRP1*) (Berryere et al., 2003) and *silver* (*SILV*) (Oulmouden et al., 2005).

Nineteen SNPs were randomly chosen among the polymorphisms gathered by Williams et al. (2009) (Table 1). These are: *ALDH2* ss77831990, *CAST* ss77832278, *CFL1* ss77831721, *CRI1* ss77832128, *CTSF* ss77831844, *CYP1A1* ss77832034, *FABP4* ss77831857, *IGF2R* ss77831877, *LEP* ss77832159, *ME3* ss77831909, *MMP1* ss77831916, *PCSK1* ss77831755, *PLOD3* ss77831757, *PLTP* ss77832104, *PPM2C* ss77831758, *PRKAG2* ss77832378, *SOCS2B* ss77832234, *SUSP1* ss77831761, *VIM* ss77831736.

Finally, a set of 19 polymorphisms located in candidate genes was added in order to test their association with different traits and to increase the traceability and paternity analysis power (Table 1): *ABCA1* ss28451692, *ACACA* ss64381883, *ACAT2* ss65658764, *CAV3* ss62797050, *CPT1* ss65363345, *CRYAB* ss62086225, *DNAJA1* ss65351307, *FIT2* ss61961642, *GLUT4* ss62538460, *HSPB1* ss63015930, *INSIG2* ss62463931, *LPL* ss65478732, *MGAT1* ss65425229, *PPARA* ss65362714, *PPARG* ss62850198, *RORA* ss65549854, *SCAP* ss62839002, *SREBP1C* ss62543518, *STX17* g.897T<G NW_001503623. Additionally, we included an amelogenin (*AMEL*) gene polymorphism for gender assignment inferred by GenBank *AMELX* and *AMELY* sequence alignment which generated a new GenBank accession number: ss244244317.

2.3. SNP multiplex and Primer Extension (PE) amplifications

The 70 target sequences were amplified in four multiplex reactions, an 18-plex (Multiplex 1), a 21-plex (Multiplex 2), a 15-plex (Multiplex 3) and a 16-plex (Multiplex 4) (Table S1). The SNP multimix for each amplification reaction consisted of oligonucleotide primers at varying concentrations ranging between 0.5 μM and 1.5 μM (Table S1), 1.25 μl of QIAGEN® Multiplex PCR (Izasa, Spain) and 10 ng of DNA with a final volume of 3.5 μl. The thermal cycling and the multiplex purification procedures are described elsewhere (Sevane et al., 2010).

Using the technique protocol previously applied in this laboratory (Sevane et al., 2010), four PE reactions were developed and purified. Table S1 shows the different concentration of PE primers and their final lengths, including a variable number of nucleotides and/or a neutral oligonucleotide region TAAACTAGGTGCCACGTCGTGAAAGTCTGACAA totally or partially added at the 5′ end to generate longer products, following a test with BLAST to avoid any match with other *Bos taurus* sequences present in the multiplex.

Two μl of multiplex cleaned extension product was added to 10 μL Hi-DiTM Formamide (Applied Biosystems) and 0.17 μl of GeneScanTM-120 LIZTM internal size standard (Applied Biosystems), before injection in an ABI 3130 sequencer using POP-7® (Applied Biosystems). Data were analyzed using GeneMapper v4.0 (Applied Biosystems). Duplication of all SNPs has been performed in 5% of the samples for repeatability purposes; also accuracy has been checked through genotype coincidence in animals genotyped either with Taqman technique or Sanger sequencing; and finally Mendelian inheritance has been checked in four trios for reliability.

2.4. Data analyses

Basic genetic parameters including allele frequencies, expected heterozygosity (H_e), observed heterozygosity (H_o), polymorphic information content (PIC), and deviation from Hardy-Weinberg equilibrium (HWE) were estimated using CERVUS 3.0.3 (http://www.fieldgenetics.com/pagesabout-Cervus_Overview.jsp; Marshall et al., 1998). This program was also used to calculate the probability of identity (PI) and sib identity (PSI), and polymorphism exclusion probabilities

for the situation where genotypes were available for only one parent (Excl P1) or both parents were genotyped (Excl P2).

The allocation test was performed by the Bayesian method of Baudouin & Lebrun (Baudouin and Lebrun 2001) using the software Geneclass2 (http://www.montpellier.inra.fr/URLB/geneclass/geneclass.html) with an assignment threshold value of 0.05 (Piry et al., 2004). The results of this test were evaluated by three indexes: (i) sensitivity, or Rate of Correct Assignment (number of samples correctly allocated to category "j"/number of animals sampled from category "j"); (ii) average assignment probability (average of the probability of any correct assignment calculated per category); and (iii) specificity (number of correct assignment to category "j"/ total (correct + incorrect) assignment to category "j".

3. Results

Table 1 includes the 24 genes tested in this study and their polymorphisms considered by others to be associated to meat traits such as tenderness (*CAPN3*, *CAST*, *LOX*), marbling (*DGAT1*, *RORC*, *LEP*, *TG*), fatty acid composition and muscle growth (*CHRNE*, *GHR*, *CRH*, *GDF8*, *UCP2*, *POMC*), milk yield, protein and fat content, (*CSN3*, *DGAT1*, *BLACT*, *FADS1*, *PPARGC1A*, *GH*) or coat colour traits (*MC1R*, *c-KIT*, *SILV*, *TYR*, *TYRP1*). Frequencies are also included indicating the general prevalence of the alleles in a range of breeds.

The rest of the markers chosen are shown also in Table 1. In addition to these 69 SNPs, one SNP (*AMEL*) inferred by GenBank *AMELX* and *AMELY* sequence alignment, was used for gender assignment distinguishing chromosomes X and Y. The 70 target sequences were amplified in four multiplex reactions, an 18-plex (Multiplex 1), a 21-plex (Multiplex 2), a 15-plex (Multiplex 3) and a 16-plex (Multiplex 4) (Table S1). The complete set of 70 markers was resolved by capillary electrophoresis in an automatic sequencer enabling a clear reading of the different peaks (Fig. 1) and has been used for the following results.

3.1. Parentage and traceability analysis

The utility of the collection of SNPs presented in this study was evaluated to estimate the power for parentage analysis and animal traceability of four different sets of polymorphisms (Table 2): i) a set that comprises all SNPs tested except for the sex gene *AMEL* (69 SNPs); ii) a sub-set exclusively containing the polymorphisms associated to different traits (31 SNPs); iii) a group including only the SNPs with minor allele frequency (MAF) exceeding a 0.3 threshold in at least one breed (46 SNPs); iv) and, finally, a panel which combines the polymorphisms from the last two groups (53 SNPs). Table 1 shows the SNPs included in each group.

One measure of the utility of a genetic system is the probability of identity (PI), defined as the probability that two individuals selected at random would possess identical genotypes (Holt et al., 2000). Based on allele frequencies for the 12 breeds, the estimated PI in these SNP panels ranged between 9.6^{-19} and 4.35^{-9} (Table 2). The probability of exclusion (probability that a random candidate parent will be excluded from paternity) when only one parent was genotyped (ExclP1), varied from 88% for the 31 SNPs panel to 98.6% for the whole SNPs tested. When both putative parents were genotyped (ExclP2), all panels had enough statistical power to detect almost all cases of incorrect parenthood showing a probability of exclusion above 99.99%, except for the 31 SNPs panel with 99.89%.

Table 2
Parentage analysis and animal identification powers.

	69 SNPs [1]	31 SNPs [2]	46 SNPs [3]	53 SNPs [4]
H_e [5]	0.2975	0.3089	0.3892	0.3451
PIC [6]	0.2387	0.2447	0.3068	0.2730
ExclP1 [7]	0.98549942	0.87551349	0.98116966	0.98159433
ExclP2 [8]	0.99999964	0.99888508	0.99999661	0.99999761
PI [9]	9.63^{-19}	4.35^{-9}	2.17^{-16}	9.88^{-17}
PSI [10]	5.91^{-10}	0.00004898	8.57^{-9}	5.81^{-9}

[1] Total polymorphisms tested.
[2] Polymorphisms associated with different traits.
[3] Polymorphisms with minor allele frequency (MAF) exceeding a 0.3 threshold in at least one breed.
[4] Combined panel of polymorphisms from 2 and 3.
[5] Mean expected heterozygosity.
[6] Mean polymorphic information content.
[7] Combined exclusion probability when only one parent was genotyped.
[8] Combined exclusion probability when the parent pair genotypes were available.
[9] Combined probability identity.
[10] Combined probability sib identity.

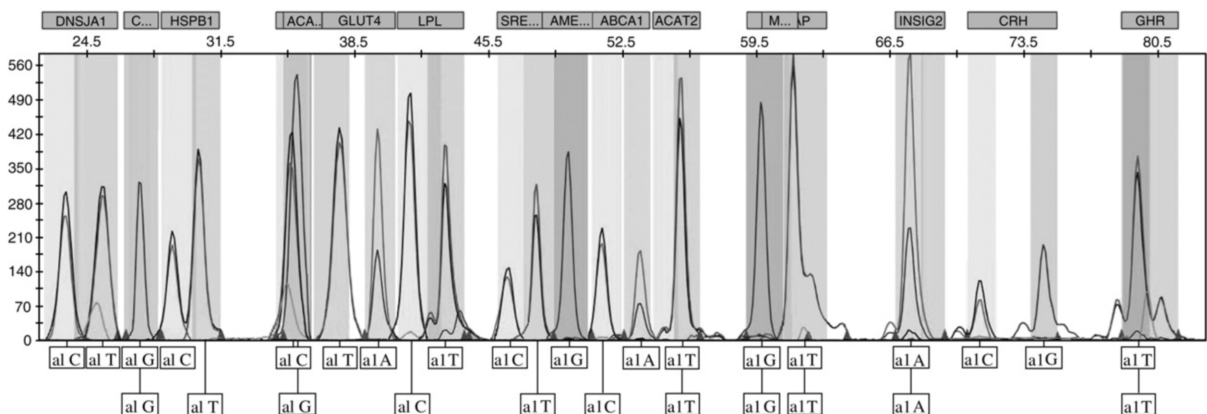

Fig. 1. GeneMapper v4.0 analysed electropherogram of Multiplex 4 Capillary Primer-Extension Assay.

Table 3
Descriptive statistics calculated at the marker level for the 69 polymorphisms tested.

Locus symbol and SNP position [1]	GenBank [2]	N° of animals	H_O	H_E	PIC [3]	Excl P1 [4]	Excl P2 [5]	HW [6]
CAPN1g.6545 C<T*	Af248054	355	0.449	0.499	0.374	0.124	0.281	NS
CAPN1 g.4558 G<A*	AF248054	365	0.405	0.445	0.346	0.099	0.263	NS
CAPN1 g.5709 G<C*	AF252504	346	0.166	0.269	0.233	0.036	0.193	***
CAST g.2959 G<A*	AF159246	365	0.398	0.365	0.298	0.067	0.235	NS
CHRNE g.1145del20*	NC_007317	346	0.075	0.081	0.077	0.003	0.073	ND
c-KIT g.115 G<T*	AJ243060	328	0.352	0.416	0.329	0.086	0.253	NS
CRH g.22 C<G*	AF340152	252	0.339	0.485	0.367	0.117	0.276	***
CSN3 g.12947 G<A*	AY380229	289	0.008	0.008	0.008	0.000	0.008	ND
CSN3 g.13100 C<A*	AY380229	287	0.375	0.489	0.369	0.119	0.278	**
CSN3 g.13120 A<G*	AY380229	357	0.033	0.037	0.036	0.001	0.035	ND
DGAT1 g.6829 A<G*	AY065621	335	0.272	0.343	0.284	0.059	0.226	**
FADS1*	ss63322537	342	0.417	0.495	0.372	0.122	0.279	NS
GDF8*	ss77831865	360	0.084	0.146	0.135	0.011	0.121	ND
GDF8*	ss77831863	349	0.036	0.175	0.159	0.015	0.141	ND
GDF8*	ss77831864	360	0.053	0.070	0.067	0.002	0.064	ND
GH1*	ss77831800	346	0.072	0.074	0.071	0.003	0.067	ND
GHR 4962 g.T<A*	AM161140	290	0.178	0.180	0.164	0.016	0.144	ND
LEP g.198 C<T*	AF120500	294	0.426	0.501	0.375	0.125	0.281	NS
LGB g.5864 C<T*	Z48305	329	0.441	0.479	0.364	0.114	0.274	NS
LOX g.7548 C<T*	NW_001495344	336	0.461	0.497	0.373	0.123	0.280	NS
MC1R g.422 T<C*	S71017	325	0.286	0.462	0.355	0.106	0.269	***
MC1R p.G104V*	S71017	278	0.316	0.479	0.364	0.115	0.274	***
POMC g.437del1*	J00021	137	0.179	0.217	0.193	0.023	0.165	ND
PPARGC1A g.19 C<T*	AY547554	251	0.196	0.394	0.316	0.078	0.246	***
RORC g.3290 T<G*	DQ667048	363	0.427	0.427	0.336	0.091	0.257	NS
SCD g.10329 T<C*	AY241932	354	0.469	0.482	0.366	0.116	0.276	NS
SILV g.92 G<A*	EF065525	363	0.000	0.183	0.166	0.017	0.146	ND
TG g.1696 C<T*	M35823	96	0.193	0.417	0.330	0.087	0.254	***
TYR g. 981in1*	AY162287	361	0.000	0.000	0.000	0.000	0.000	ND
TYRP1 g. 1300 C<T*	AF400250	365	0.011	0.011	0.011	0.000	0.011	ND
UCP2 g.812 G<A*	XM_614452	305	0.272	0.449	0.348	0.101	0.265	***
ABCA1**	ss28451692	292	0.326	0.403	0.321	0.081	0.249	*
ACAT2**	ss65658764	269	0.451	0.498	0.374	0.124	0.280	NS
ALDH2**	ss77831990	344	0.430	0.460	0.354	0.106	0.268	NS
CAST**	ss77832278	330	0.393	0.435	0.340	0.094	0.260	NS
CAV3**	ss62797050	362	0.438	0.456	0.352	0.104	0.267	NS
CFL1**	ss77831721	365	0.402	0.436	0.341	0.095	0.260	NS
CRI1**	ss77832128	287	0.188	0.188	0.170	0.018	0.149	ND
CTSF**	ss77831844	262	0.214	0.345	0.285	0.059	0.227	***
CYP1A1**	ss77832034	365	0.202	0.207	0.186	0.021	0.160	NS
DNAJA1**	ss65351307	292	0.392	0.432	0.338	0.093	0.259	NS
FABP4**	ss77831857	350	0.438	0.499	0.374	0.124	0.281	NS
FIT2**	ss61961642	357	0.377	0.410	0.325	0.084	0.251	NS
HSPB1**	ss63015930	290	0.278	0.278	0.239	0.039	0.197	NS
IGF2R**	ss77831877	364	0.295	0.367	0.300	0.067	0.236	**
ME3**	ss77831909	362	0.449	0.487	0.368	0.118	0.277	NS
PCSK1**	ss77831755	360	0.435	0.500	0.375	0.125	0.281	NS
PLTP**	ss77832104	355	0.445	0.499	0.374	0.124	0.281	NS
PPM2C**	ss77831758	356	0.310	0.339	0.281	0.057	0.224	NS
PRKAG2**	ss77832378	339	0.308	0.290	0.248	0.042	0.203	NS
SREBP1C**	ss62543518	283	0.425	0.423	0.333	0.089	0.256	NS
SUSP1**	ss77831761	365	0.260	0.284	0.244	0.040	0.200	NS
VIM**	ss77831736	365	0.428	0.476	0.362	0.113	0.273	NS
ACACA	ss64381883	297	0.074	0.071	0.068	0.003	0.065	ND
CPT1	ss65363345	280	0.020	0.025	0.025	0.000	0.025	ND
CRYAB	ss62086225	293	0.377	0.333	0.277	0.055	0.222	NS
GLUT4	ss62538460	298	0.063	0.066	0.063	0.002	0.060	ND
INSIG2	ss62463931	298	0.000	0.000	0.000	0.000	0.000	ND
LEP	ss77832159	347	0.134	0.142	0.132	0.010	0.119	ND
LPL	ss65478732	276	0.075	0.083	0.080	0.003	0.075	ND
MGAT1	ss65425229	298	0.024	0.024	0.024	0.000	0.023	ND
MMP1	ss77831916	365	0.216	0.197	0.177	0.019	0.154	NS
PLOD3	ss77831757	365	0.161	0.186	0.168	0.017	0.147	ND
PPARA	ss65362714	358	0.257	0.251	0.220	0.032	0.184	NS
PPARG	ss62850198	353	0.260	0.240	0.211	0.029	0.178	NS
RORA	ss65549854	359	0.166	0.179	0.163	0.016	0.143	ND
SCAP	ss62839002	297	0.215	0.192	0.174	0.018	0.151	ND
SOCS2B	ss77832234	354	0.231	0.243	0.213	0.030	0.180	NS
STX17 g.897 T<G	NW_001503623	357	0.005	0.005	0.005	0.000	0.005	ND

The same parameters gathered in Table 2 along with Hardy-Weinberg equilibrium are shown in Table 3 referred to each marker. The test statistic for some SNPs deviates significantly ($p<0.05$) from the expectation under the neutral mutation hypothesis, even when Bonferroni correction is applied, and the deviation from Hardy-Weinberg equilibrium could not be calculated for 23 polymorphisms due to the poor allele frequency variability (the Hardy-Weinberg tests were only carried out on polymorphisms for which the expected frequency of rare allele homozygotes exceeded 0.05). Allele frequencies of genetic markers vary significantly between populations, thus affecting the power of genetic identification as shown in Table 1, where the combined exclusion probability of the 53 polymorphisms group are presented at the breed level. It is expected that over time the panel of 53 SNPs will be less informative due to the fact that some of the linked markers will be selected in different populations and thus frequencies will change to MAF values <0.3, lowering exclusion probabilities. To test this hypothesis we have changed in two breeds (Charolais and Pirenaica) the MAF of six SNPs, resulting in a worse scenario which drops exclusion probabilities to 0.94694699 (Charolais) and 0.95362469 (Pirenaica) when only one parent is known (Excl P1), and 0.99995467 (Charolais) and 0.99995774 (Pirenaica) for two known parents (Excl 2P) (Table S2).

3.2. Assignment test

Results of GeneClass 2 allocation tests are reported in Table 4. The overall rate of correct assignment was ~95%, the average assignment probability 96% and the average specificity 96% when using the 69 SNPs panel. For the 53 SNPs subset, the rates are slightly lower (89%, 93% and 90%, respectively) but suitable for the probabilistic assignment of individuals to their origin breed (Negrini et al., 2008).

4. Discussion

The present study sought to develop a low-medium throughput genotyping assay which comprises polymorphisms associated in some bovine breeds to different traits, along with SNPs in candidate genes potentially linked to economic traits. This tool will allow the easy screening of previously associated polymorphisms but also genetic traceability and breed authentication, which represents a valid marketing strategy and operative tool to support and protect high quality products from local breeds, and parentage analysis simultaneously to generalize its use in small bovine populations.

Capillary Primer-Extension Assay (Fig. 1) enables genotyping in reduced numbers of samples for a small number of SNPs, has proven reliability – see e.g. in humans where a 52plex is being used for forensic purposes in different labs all over the world (Sanchez et al., 2006) – is efficient (low amounts of DNA and reagents are needed for good readability), lower cost than other currently available alternatives, and flexible – changes in the multiplex are readily feasible at any time if information on new SNPs arises. The need for an assay allowing the rapid, flexible and cost-effective genotyping of a reduced number of mutations (<100) is an important issue, particularly in small census populations. Indeed, most of the local beef breeds present reduced family size and problems when rationalising a cost-effective recovering economic trait strategy. Also high density genotyping of economically important genes is difficult and costly, preventing its application for genomic selection (Meuwissen et al., 2001). Although new tools are now underway (e.g. Illumina 3 k chip), the technique and SNPs included in this study are a more affordable possibility for small local breeds, as few of these can use medium throughput genotyping with their limitations in census and their short economical resources.

The multiplexes described here, included SNPs associated with milk (as related to maternal ability for calf weaning), carcass and meat traits, coat colour and sex determination. They have been applied to six highly selected beef breeds, two local maternal beef breeds, three dairy breeds and one semiferal breed in order to produce genotyping results usable in adult or embryo DNA of individuals belonging to small populations, allowing the introduction of new mutations in the test, if necessary, at small additional cost, thereby implementing the genetic signature at any time. Moreover, GAS allows for accurate selection of favourable traits and is specially interesting for traits for which selection has been historically difficult, such as those complicate to measure or which are measured postmortem (e.g. technological meat properties or carcass data) (White et al., 2005). Here the set of 53 SNPs allows validation of 31 SNPs in genes and detection of other associations; also high power paternity testing and traceability can be performed with this set of markers in all breeds, resulting in a tool which can promote the interest of local breed products.

An interesting result of the genes analysed here is the fact that F94L mutation (ss77831863) (Grobet et al., 1997) at the *GDF8* gene (a substitution which occurs at exon 1, a region known to be the inhibitory domain of the myostatin propeptide) is highly present in the Limousin and Pirenaica breeds and has been shown to increase meat weight and to reduce fat depots in Australian and USA Limousin crosses (Sellick et al., 2006, 2007; Esmailizadeh et al., 2008).

Another datum of interest, based on the associations found in other populations, is that of the dairy Pasiega breed showing fixation of q allele for *DGAT1* and also for *PPARGC1A*,

Notes to Table 3:

[1]Locus symbol along with SNP position according to the sequence in the next column. * SNPs associated with different traits (31); ** SNPs with minor allele frequency (MAF) exceeding a 0.3 threshold in at least one breed added to the Previous group to improve the power for parentage analysis and animal traceability (22).

[2]GenBank Accession Numbers for *Bos taurus* sequences including the interrogated SNPs or dbSNPs accession number.

[3]Polymorphic information content.

[4]Polymorphism exclusion probability when only one parent was genotyped.

[5]Polymorphism exclusion probability when the parent pair genotypes were available.

[6]Significance of deviation from Hardy-Weinberg equilibrium: NS = not significant, * = significant at the 5% level, ** = significant at the 1% level, *** = significant at the 0.1% level, ND = not determined.

Table 4
Assignment efficiency (Sens.), average probability (%) (Av. Prob.), specificity (Spec.), and number of animal not correctly assigned to each breed (Not Ass.) calculated with GeneClass 2 (assignment method Baudouin and Lebrun (2001), threshold of scores 0.05).

Breed	Nº Ind.	69 SNPs				53 SNPs			
		Sens.	Av. Prob.	Spec.	Not Ass.	Sens.	Av. Prob.	Spec.	Not Ass.
Pasiega	31	0.97	99.07	0.97	1	0.97	97.75	0.91	1
Jersey	32	1.00	98.63	1.00	0	1.00	93.28	0.97	0
Holstein	26	1.00	92.76	0.93	0	0.88	93.14	0.77	3
Charolais	46	0.96	96.39	0.96	2	0.89	96.20	0.98	5
Limousin	31	0.97	98.42	0.97	1	0.94	96.76	0.97	2
Simmental	18	0.94	98.11	0.94	1	1.00	94.20	0.82	0
Aberdeen Angus	30	0.97	93.79	0.97	1	0.80	87.88	0.96	6
Asturiana de los Valles	30	0.93	97.41	0.93	2	0.90	94.38	0.87	3
Pirenaica	31	0.90	86.38	0.93	3	0.77	86.39	0.80	7
Lidia	20	1.00	99.97	1.00	0	1.00	98.38	1.00	0
Casina	31	0.90	96.19	0.90	3	0.81	92.81	0.89	6
Avileña-Negra Ibérica	30	0.87	91.05	0.96	4	0.73	85.90	0.81	8
Overall	356	0.95	95.68	0.96	18	0.89	93.09	0.90	41

which means an overall low milk fat content (Tupac-Yupanqui et al., 2004; Weikard et al., 2005), in contrast to the case of the Jersey and Holstein dairy breeds, which show quite balanced frequencies for both alleles. Following with the milk characteristics, the presence of allele B and E at the *CSN3* locus reflects good ability for cheese production (Barroso et al., 1999) in Pasiega and Jersey. Other genes are implicated in meat fat content, as is the *SCD* locus which affects the MUFA content (Taniguchi et al., 2004) and consequently carcass flavour; Aberdeen Angus, Jersey and Pasiega should produce flavored beef carcasses, as shown by the higher frequency of the C allele. *DGAT1* and *TG* have been shown to increase meat marbling (Barendse, 1999; Thaller et al., 2003) and the mutated allele shows a high contribution in the differentiation of the populations, as can be seen with the possible high meat marbling of Holstein or the low one of Simmenthal. Some genes have been linked to tenderness, as are *CAPN1* (Page et al., 2002; White et al., 2005) and recently *DNAJA1* (Bernard et al., 2007; Marty et al., 2010), and results show a medium prevalence of the wild type allele suggesting medium toughness of the meat in most breeds.

Coat colour alleles can allow discrimination between populations: concerning the *MC1R* locus, the e/e genotype appeared fixed in most individuals of Simmental, Limousin, and Charolais breeds, whereas the E^D/E^D genotype was fixed in Aberdeen Angus, Holstein, and Lidia breeds. The *SILV* locus shows a mutation which explains the particular coat of the Charolais breed (Oulmouden et al., 2005). Finding the mutated dominant allele fixed in this breed is useful for traceability purposes. Presence of one SILV allele (which confers a certain dilution of the original coat colour) is an example of how coat colour loci can be a tool for tracing introgression from other breeds in an individual. Unique alleles at particular loci are a good tool to determine the breed an individual belongs to, but nevertheless, other SNPs can be used for traceability in a "farm to fork" basis, as the choice of a panel allowing genetic traceability represents a valid marketing strategy and operative tool to support and protect high quality products from local breeds, usually linked to traditional farming and production methods vital to their survival, and often to the economic sustainability of rural areas (Schwagele 2005; Smith et al., 2005).

The populations which are more informative are Limousin, Holstein, Charolais and Pirenaica, mostly due to the mutated alleles of *GDF8* and *MC1R* (Table 1 and data not shown). The semi-feral breed corresponds to a population of the Lidia breed which has never been selected for dairy or for meat traits as the interesting traits in this population are related to behaviour. That makes this breed a reference for most of the loci genotyped here.

With regard to the utility of SNPs for traceability and paternity purposes, we propose the 53 polymorphisms subset containing the SNPs associated with different traits along with 22 SNPs with minor allele frequency (MAF) exceeding a 0.3 threshold in at least one breed in order to increase statistical power. This assay allows the combined analysis for GAS application plus pedigree information, gathering the genetic information necessary for predicting the genetic merit of individuals, as well as guaranteeing the final product traceability. The 53 SNP set shows a high PI (9.88^{-17}) and 99.99% of exclusion probability (Excl P2), a statistical power in different tested scenarios equivalent to, or better than, the currently used microsatellite panels based on the data of many studies (Heaton et al., 2002; Werner et al., 2004; Fisher et al., 2009; Karniol et al., 2009). Morever, Fisher et al. (2009) states that 40 SNP would be at least as effective for parentage matching as the current 14-microsatellite panel used in New Zealand for dairy cattle.

Although the use of this tool for GAS needs further studies in each population to specify the estimate of each SNP effect, the information which will be given by this Capillary Primer-Extension Assay is very valuable to decide a breed orientation. With time, those SNPs used for GAS will change their frequencies generally tending to the fixation of one allele and resulting in less informative markers for paternity testing. Moreover, this situation will be very variable among breeds due to the specific interests each can have (e.g. DGAT1 where one allele or the other can be selected depending on the country specific interests). However, as 22 of the SNPs present in the panel are not located in associated genes, values of Excl P1 and Excl 2P stay in the range to be used for paternity testing (Table S2). Also, it should be noted that the multiplexes showed here are based on a high flexibility tool as any SNP can be dropped or added at any time with small expenses.

In conclusion, the assay developed here should allow parentage, traceability analysis and Genotype Assisted Selection at an affordable cost and low effort to be performed in any cattle population and would provide a methodology to complement the phenotypic information registered under the traditional schemes. Also, different associations between mutations at other loci and economic traits are underway, and this Capillary Primer-Extension Assay admits the introduction of new polymorphisms at small additional cost, thereby implementing the genetic signature at any time.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at doi:10.1016/j.livsci.2010.10.011.

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