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## Development of a SNP parentage assignment panel in some North-Eastern Spanish meat sheep breeds

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### Abstract

**Aim of study:** To validate two existing single nucleotide polymorphism (SNP) panels for parentage assignment in sheep, and develop a cost effective genotyping system to use in some North-Eastern Spanish meat sheep populations for accurate pedigree assignment.

**Area of study:** Spain

**Material and methods:** Nine sheep breeds were sampled: Rasa Aragonesa (n=38), Navarra (n=39), Ansotana (n=41), Xisqueta (n=41), Churra Tensina (n=38), Maellana (39), Roya Bilbilitana (n=24), Ojinegra (n=36) and Cartera (n=39), and these animals were genotyped with the Illumina OvineSNP50 BeadChip array. Genotypes were extracted from the sets of 249 SNPs and 163 SNPs for parentage assignment designed in France and North America, respectively. Validation of a selected cost-effective genotyping panel of 158 SNPs from the French panel were performed by Kompetitive allele specific PCR (KASP). Additionally, some functional SNPs (n=15) were also genotyped.

**Main Results:** The set of 249 SNPs for parentage assignment showed better diversity, probability of identity, and exclusion probabilities than the set of 163 SNPs. The average minor allele frequency for the set of 249, 163 and 158 SNPs were  $0.41 + 0.01$ ,  $0.39 + 0.01$  and  $0.42 + 0.01$ , respectively. The parentage assignment rate was highly dependent to the percentage of putative sires genotyped.

**Research highlights:** The described method is a cost-effective genotyping system combining the genotyping of SNPs for the parentage assignment with some functional SNPs, which was successfully used in some Spanish meat sheep breeds.

**Additional key words:** autochthonous breeds; pedigree; genotyping; animal breeding

**Abbreviations used:** AI (artificial insemination); EBV (estimated breeding value); ISAG (International Society for Animal Genetics); KASP (kompetitive allele specific PCR); LOD (logarithm of the odds); MAF (minor allele frequency); PE (exclusion probability); PI (probability of identity); SNP (single nucleotide polymorphism)

**Authors' contributions:** Conceived the research and designed the experiments: JHC, MS, SF, FT, BL; performed the experiments: PS, LPI; collected and codified the data: JHC, MS, JLA, MAJ, PS, LPI; analyzed the data: JHC, MS, MAJ; discussion and writing the manuscript: JHC, MS, FT, JF, JLA, SF, BL. The manuscript was proofread by all the co-authors.

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## Introduction

Breeding programs have the purpose to get sustainable genetic gains in one or several traits while controlling the loss of genetic variation. Traditional pedigree

based-BLUP (Best linear unbiased prediction) selection (Henderson, 1984) is used to calculate estimated breeding values (EBVs) obtained from performance records and pedigree information. However, the success of genetic evaluations systems is directly affected by the accuracy

of pedigrees. Complete pedigree information is a prerequisite to get accurate EBVs, correctly rank parents and offspring and maximize the genetic gain (Israel & Weller, 2000; Raoul *et al.*, 2016). In this sense, the proportion of known sires is very low in Spanish meat sheep populations because the management (extensive or semi-extensive farming) relies very little on artificial insemination (AI) or natural mating with a single ram per group of ewes. Moreover, a number of these populations are considered as endangered breeds with reduced effective population size, and reared in small-sized flocks. Therefore, the implementation of a mating scheme based in pedigree information can control the inbreeding that is greatly affected by population structure (Gutiérrez *et al.*, 2008).

In this situation, the number of ewes belonging to a breeding program nucleus remains limited, because only some of them are inseminated by or mated to a single identified ram. Furthermore, another source of incorrect pedigree record information is usually due to ewes failing to keep their litter together, or lamb desertion, that may lead to limit the selection response (Barnett *et al.*, 1999; Visscher *et al.*, 2002).

Therefore, genomic information like DNA markers can contribute to reconstruct the phylogenetic relationships of populations. Microsatellite markers have been used extensively for parentage control in sheep (Arruga *et al.*, 2001; Glowatzki-Mullis *et al.*, 2007; Saberivand *et al.*, 2011; Visser *et al.*, 2011; Souza *et al.*, 2012; da Silva *et al.*, 2014) and are recommended by the International Society for Animal Genetics (ISAG) as they are highly abundant and informative, relatively inexpensive to use, and generate satisfactory results in tests for paternity exclusion. However, as DNA markers in genomic selection studies (Meuwissen *et al.*, 2013), single nucleotide polymorphisms (SNPs) are now largely developed on SNPs chip arrays allowing high throughput genotyping (Heaton *et al.*, 2002; Werner *et al.*, 2004; Hayes, 2011). Recently, various SNP panels have been developed for sheep of different international breeds specifically for parentage assignment (Bell *et al.*, 2013; Clarke *et al.*, 2014; Heaton *et al.*, 2014; Tortereau *et al.*, 2017). The SNPs panel developed from French breeds was the first panel based on European sheep breeds (Tortereau *et al.*, 2017). These authors pointed out that four Spanish breeds (Churra, Ojalada, Castellana and Rasa aragonesa) belonging to the Sheep HapMap breeds of the International Sheep Genomics Consortium (Kijas *et al.*, 2012a) had similar minor allele frequency (MAF) values for the selected SNPs to that described in the French breeds, suggesting that this panel should perform well in these Spanish breeds.

In addition, in Spain, a national breeding program for resistance to classical scrapie was implemented. In the breeding programs, animals are genotyped, and those carrying favorable *Prnp* alleles for resistance are used as breeding animals (Hunter *et al.*, 1997; Acin *et al.*, 2004).

In the same way, a selection program for prolificacy in Rasa Aragonesa breed implements the genotyping of reproducers for alleles associated to prolificacy (Calvo *et al.*, 2020), as well as that related to reproductive seasonality (Calvo *et al.*, 2018). Apart from these SNPs, some other SNPs are of interest to genotype for validation of their effects in these Spanish breeds, such as atypical scrapie susceptibility (Moum *et al.*, 2005), lentivirus susceptibility infection (Heaton *et al.*, 2012; Sider *et al.*, 2013), or other alleles found in other breeds and related to prolificacy (Bodin *et al.*, 2007; Drouilhet *et al.*, 2013).

The objective of this study was to validate two existing SNP panels for parentage assignment in sheep, including the French panel, and develop a cost-effective genotyping system of a reduced set of SNPs in an open platform to use in some North-Eastern Spanish meat sheep populations for accurate pedigree assignment. In a second stage, we tested and validated the performance of the cost-effective genotyping system together with some functional SNPs in replacements lambs from different farms and breeds.

## Material and methods

### Samples and genotyping

#### *OvineSNP50 BeadChip array genotyping*

Three hundred and thirty-five ewes from nine Spanish sheep breeds were sampled: Rasa Aragonesa (n=38), Navarra (n=39), Ansotana (n=41), Xisqueta (n=41), Churra Tensina (n=38), Maellana (n=39), Roya Bilbilitana (n=24), Ojinegra (n=36) and Cartera (n=39). Sheep breeds considered in the current work are specialized in meat production. The Churra Tensina, Ansotana, Maellana, Roya bilbilitana, Xisqueta and Cartera are considered endangered sheep breeds, having the first four a very low census ranging between 8,000 and 13,000 heads (<https://www.mapa.gob.es/es/ganaderia/temas/zootecnia/razas-ganaderas/razas/catalogo/>). Animals were selected as unrelated as possible. In this sense, the maximum number of animals selected from each flock was 4, and they were unrelated based on their pedigree records. Furthermore, to check Mendelian inheritance 11 duos (2, 1, 2, 2, 2 and 2 from Rasa aragonesa, Navarra, Ansotana, Xisqueta, Churra Tensina, and Cartera, respectively) and 3 trios (2 and 1 from Navarra and Rasa Aragonesa sheep breeds, respectively) were also selected.

Genomic DNA was extracted from blood samples of the 335 ewes using the FlavorPrep Genomic DNA mini kit (Flavorgen, Ibián, Zaragoza, Spain). DNA samples were genotyped with the Illumina (San Diego, California, USA) OvineSNP50 BeadChip array designed by the International Sheep Genome Consortium (Kijas *et al.*,

2012b). SNP genotyping services were provided by the "Xenetica Fontao" company ([www.xeneticafontao.com](http://www.xeneticafontao.com)).

visualized with SNP Viewer 2 software version 4.0 (LGC, 2013). Genotype data for each animal were exported for the statistical analysis.

### Validation by kompetitive allele specific PCR (KASP)

A total of 2,018 replacement ewe lambs from 12 farms and 3 breeds, and their putative fathers were sampled (428 sires). Only the candidate parents and their offspring were genotyped to perform paternity assignment. Furthermore, the 11 duos, the 3 trios, and 5 randomly selected samples from each breed (45 samples in total) were also genotyped to check the consistency between both genotyping approaches: OvineSNP50 BeadChip array and KASP (design and assays were performed by the LGC company, Biotools, Madrid, Spain). Validation of a selected cost-effective genotyping panel of SNPs from the French panel was performed by KASP. Additionally, some functional SNPs (n=15) were also genotyped located in BMP15 (n=3; Bodin *et al.*, 2007; Martinez-Royo *et al.*, 2008; Demars *et al.*, 2013), B4GALNT2 (n=1; Drouilhet *et al.*, 2013), MTNR1A (n=1; Calvo *et al.*, 2018), PRNP (n=7; Hunter *et al.*, 1997; Acín *et al.*, 2004; Moum *et al.*, 2005), TMEM154 (n=2; Heaton *et al.*, 2012; Sider *et al.*, 2013) and HSP90AA1 (n=1; Salces-Ortiz *et al.*, 2015). The functional SNP coordinates and affected traits are indicated in the Table 1. Results were

### Analysis of genotypic data

#### Selection of the SNP panel for parentage assignment

Firstly, we applied the quality control (QC) criteria on the raw genotypes obtained from the OvineSNP50 BeadChip array using PLINK 1.9 (Chang *et al.*, 2015) as follows: i) Individuals with low call rate (< 0.97) were excluded from additional analysis; ii) SNPs with unknown location of the marker in the ovine chromosomes were excluded; iii) SNPs were also excluded if they showed a low call rate (< 0.97), a MAF < 0.05, or significant deviations from Hardy-Weinberg equilibrium (HWE) ( $p$ -value < 0.001) within breed. The subsequent analysis focused on two sets of SNPs; a first set of 249 SNPs published from the French panel for parentage assignment (Tortereau *et al.*, 2017) and the 163 SNPs panel described in Heaton *et al.* (2014) used in the North American and globally diverse breeds. Paternity assignment effectiveness does not only depend on the number of SNPs used but also on the level of informativeness

**Table 1.** Functional SNPs jointly genotyped with the subset of 158 SNPs from the French panel for the validation by Kompetitive allele specific PCR (KASP). Information about the location, dbSNP name in Ensembl variation database, gene, and associated phenotype. References associated to these genes and phenotypes are indicated by using a superscript.

CHR	SNP position in Oar3.1 and allele variation	dbSNP name	Gene	Phenotype
11	11:g.36938224T>A	rs588626728	B4GALNT2	Prolificacy/FecLL <sup>[1]</sup>
13	13:g.46225659G>A	rs601660229	PRNP	Classical scrapie susceptibility <sup>[2,3]</sup>
13	13:g.46225660C>T	rs591379086	PRNP	Classical scrapie susceptibility <sup>[2,3]</sup>
13	13:g.46225674C>T	rs598580733	PRNP	Atypical scrapie susceptibility <sup>[4]</sup>
13	13:g.46225714G>A	rs605048948	PRNP	Classical scrapie susceptibility <sup>[2,3]</sup>
13	13:g.46225764A>C	NA	PRNP	Classical scrapie susceptibility <sup>[2,3]</sup>
13	13:g.46225765G>A	rs160575103	PRNP	Classical scrapie susceptibility <sup>[2,3]</sup>
13	13:g.46225766G>T	rs400844237	PRNP	Classical scrapie susceptibility <sup>[2,3]</sup>
17	17:g.4857244 G>A	rs408593969	TMEM154	Lentivirus susceptibility <sup>[5,6]</sup>
17	17:g.4857350 T>A	rs427737740	TMEM154	Lentivirus susceptibility <sup>[5,6]</sup>
19	19:g.65645462C>G	rs397514116	HSP90AA1	Sperm DNA fragmentation <sup>[7]</sup>
26	26:g.15118464 G>A	rs403212791	MTNR1A	Reproductive seasonality <sup>[8]</sup>
X	X:g.50971644-50971660indel	rs421419167	BMP15	Prolificacy/FecXR <sup>[9]</sup>
X	X: g. 50971170C>T	NA	BMP15	Prolificacy/FecXGR <sup>[10]</sup>
X	50971158C>T	NA	BMP15	Prolificacy/FecXL <sup>[11]</sup>

CHR: chromosome. NA: not available. <sup>[1]</sup>Drouilhet *et al.*, 2013; <sup>[2]</sup>Hunter *et al.*, 1997; <sup>[3]</sup>Acín *et al.*, 2004; <sup>[4]</sup>Moum *et al.*, 2005; <sup>[5]</sup>Heaton *et al.*, 2012; <sup>[6]</sup>Sider *et al.*, 2013; <sup>[7]</sup>Salces-Ortiz *et al.*, 2015; <sup>[8]</sup>Calvo *et al.*, 2018; <sup>[9]</sup>Martinez-Royo *et al.*, 2008; <sup>[10]</sup>Demars *et al.*, 2013; <sup>[11]</sup> Bodin *et al.*, 2007

that these markers provide. To study the informativeness of the SNPs included in this work, three informative indexes were calculated for both sets of SNPs and for each population included in this study: the MAF, the exclusion probability (PE), and the probability of identity (PI) (Schütz & Brenig, 2015; Tortereau *et al.*, 2017). PE is the probability to exclude one (PE1) or two (PE2) randomly sampled parent(s) from the parentage of an individual which is truly unrelated to them. PE1 assumes that genotypes are known for the offspring and a putative parent, but genotypes are not available for a known parent (one parent missing). PE2 assumes genotypes are known for the offspring, one confirmed parent, and one putative parent (both parents genotyped). PI is the probability that two randomly selected individuals in a population have identical genotypes for all the SNPs genotyped.

A reduced panel of 158 SNPs from the French panel was chosen to use in an open platform for a cost-effective genotyping for parentage assignment. Only SNPs with a MAF >0.3 and a call rate >0.97 in the 9 breeds were selected.

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#### Parentage assignment validation

We carried out the paternity assignment in each of the ten farms by using the CERVUS software (Kalinowski *et*

*al.*, 2007). CERVUS uses a simulation procedure to determine the distribution of the critical values of logarithm of the odds (LOD) or Delta score for 80% and 95% confidence levels for the candidate father–offspring pairs. LOD score was used for paternity assignment. The simulation parameters were as follows: 10,000 simulated offspring, the number of candidate parents and the sampled sires was provided by the breeders' association (varying between 50% and 100%), at least 90% loci having allele calls, with an estimated 5% genotyping error rate. We allowed one SNP genotype mismatch between offspring and its assigned sire because of technical genotyping failures.

## Results and discussion

We selected two sets of SNPs for parentage assignment described in sheep. All SNPs from these panels fulfill the QC criteria. Tortereau *et al.* (2017) reported that the panel of 249 SNPs used for parentage assignment in the French breeds had similar medium MAF values in four Spanish breeds (Churra, Ojalada, Castellana and Rasa Aragonesa) belonging to the Sheep HapMap breeds (Kijas *et al.*, 2012a), suggesting that this set of SNPs should perform well in these breeds. Furthermore, the North American panel of 163 SNPs for parentage testing (Heaton *et al.*, 2014) also found that the Rasa Aragonesa sheep breed had the highest MAF value (0.40) of all breeds.

In the nine North-Eastern Spanish meat sheep populations included in this study, no mendelian inheritance errors were detected in verified family trios or duos for

**Table 2.** Major statistics for two parentage panels (French, North American and globally diverse breeds), and a subset of 158 SNPs from the French panel on the 9 Spanish populations: MAF, PI (Probability of identity), PE1 and PE2 (exclusion probabilities considering the exclusion of one or the two parents respectively).

Breeds	French panel (249 SNPs)				North American and globally diverse breeds (163 SNPs)				French panel subset (158 SNPs)			
	MAF	PI	1-PE1	1-PE2	MAF	PI	1-PE1	1-PE2	MAF	PI	1-PE1	1-PE2
Ansotana	0.42	2.36E-102	4.28E-14	1.53E-35	0.40	7.88E-65	4.96E-09	6.70E-23	0.43	1.09E-64	2.38E-09	5.90E-23
Churra Tensina	0.39	5.02E-102	3.29E-14	1.17E-35	0.37	2.11E-63	1.95E-08	2.75E-22	0.41	3.38E-65	4.62E-09	1.03E-22
Xisqueta	0.43	1.07E-100	4.62E-14	1.63E-35	0.40	1.14E-64	6.13E-09	7.83E-23	0.44	2.57E-66	2.20E-09	5.58E-23
Navarra	0.43	1.23E-98	1.34E-13	4.18E-35	0.40	8.88E-65	5.68E-09	7.02E-23	0.44	2.01E-65	1.98E-09	5.08E-23
Rasa Aragonesa	0.42	2.75E-100	1.38E-13	4.61E-35	0.40	9.57E-66	4.79E-09	6.08E-23	0.43	1.22E-64	2.44E-09	6.06E-23
Roya Bilbilitana	0.40	1.44E-100	9.67E-14	3.27E-35	0.38	4.60E-62	1.35E-08	1.67E-22	0.42	4.94E-64	3.84E-09	8.85E-23
Maellana	0.40	2.75E-100	1.38E-13	4.61E-35	0.38	7.39E-63	1.65E-08	2.26E-22	0.42	1.58E-64	3.24E-09	7.66E-23
Ojinegra	0.41	1.44E-100	9.67E-14	3.27E-35	0.39	7.82E-64	1.01E-08	1.33E-22	0.42	3.50E-65	2.79E-09	6.76E-23
Cartera	0.41	2.61E-102	9.58E-14	3.26E-35	0.39	2.98E-64	1.01E-08	1.38E-22	0.43	2.38E-66	2.67E-09	6.56E-23

both panels. SNPs were in Hardy-Weinberg equilibrium. There were not any uninformative SNPs in each breed group (MAF=0) in any breed group. The average MAF for the sets of 249 and 163 SNPs were  $0.41 + 0.01$ , and  $0.39 + 0.01$ , respectively. The set of 249 SNPs had better diversity, PI, PE1 and PE2 values than the set of 163 SNPs (Table 2). PI, PE1 and PE2 values are highly dependent on the number of SNPs (Jamieson & Taylor, 1997), but Tortereau *et al.* (2017) demonstrated that these values were better on these populations with 150 SNPs randomly selected from the French panel than those of the North American panel.

For the reasons described above, we decided to select a reduced panel of 158 SNPs from the French panel for a cost-effective genotyping. Only, SNPs with a MAF value greater than 0.3 and with a call rate  $> 0.97$  in all the nine populations were retained. The SNPs were distributed over the 26 autosomes. The major statistics for this panel of 158 SNPs (MAF, PI, PE1 and PE2) are shown in Table 2. The names, MAFs, and other features of the SNPs of each panel are shown in Tables S1-S3 [suppl]. The average MAF for the set of 158 SNPs was  $0.42 + 0.01$ , having better values than the other two sets of SNPs. Slightly better values were found for the reduced panel (158 SNPs) compared to the American one (163 SNPs) for the PI, PE1 and PE2 values, although Ansotana and Rasa Aragonesa showed lower PI values with the set of 163 SNPs. At the population level, the lowest and greatest average MAF values were obtained respectively in Churra tensina, and in Xisqueta and Navarra breeds whatever the panel. In general, all breeds showed good PI, PE1, and PE2 values. For the set of 158 SNPs, the probability (PI) that two randomly selected individuals have identical genotypes within breed was very low, reaching its lowest and highest values in the Cartera (2.38E-66) and the Roya Bilbiliana populations (4.94E-64), respectively. However, in the Rasa Aragonesa breed the lowest PI value was found with the set of 163 SNPs (9.57E-66) compared to the set of 158 SNPs (1.22E-64).

The set of 158 SNPs was also used to perform parentage assignment validation using KASP technology. Furthermore, the 15 functional SNPs were also genotyped in conjunction with those used for parentage assignment for a total of 173 SNPs. KASP technology was chosen because is a very cost-effective genotyping platform. In this sense, the total cost per sample for a set of 192 SNPs assay (DNA extraction and genotyping a maximum of 192 SNPs) was €9 when dealing with more than 1,500 individuals (all-inclusive service from the LGC, Genomics Hoddesdon, UK). The price goes down around €2 when genotyping more than 3,000 samples. Five SNPs from the reduced panel failed or had a call rate  $< 0.95$  in KASP genotyping. However, MAF, PI, PE1 and PE2 had similar values (Table S4 [suppl]). Functional SNPs were genotyped successfully. For example, we could genoty-

pe efficiently for the numerous alleles of the *PRNP* gene (Table 1) at codons 136 (p.A136V,T), 141 (p.L141F), 154 (p.R154H) and 171 (p.Q171R,H,K), identifying 3, 2, 2, and 4 alleles for each codon, respectively. This validation was performed in 12 commercial farms from three different breeds. Farmers declared a proportion of putative sires sampled from the farm because not all the putative males were available, mainly because some sires were dead. Table 3 shows the assignment rate in different farms from the three breeds. As expected when the list of putative sires was completely (or almost) genotyped in a farm, a very high assignment rate was obtained. In two farms, a 100% assignment rate was achieved. In general, the assignment rate is highly dependent to the percentage of putative sires genotyped. We only found one out of 2,018 replacement ewes (farm G) with two possible parents, a father-offspring pair. This problem has been previously pointed by Tortereau *et al.* (2017) recommending to genotype at least 180 SNPs given the number of false-positive results when the dam is not genotyped and the true sire is not among the candidate sires or are highly-related. Because this is an open genotyping platform we could complete the panel with more SNPs to increase the parentage assignment power; or add new validated functional SNPs.

The total cost per sample for this set of 173 SNPs for parentage assignment and genotyping some functional genes (the same price is for the genotyping a maximum of 192 SNPs) is similar to those used with microsatellites. In this way, this panel is routinely used in Rasa Aragonesa and Ojinegra sheep breeds for parentage assignment and genotyping of functional SNPs by KASP. Marker- or gene-assisted selection (MAS/GAS) is been applied in these breeds for pre-selection of replacement animals for increasing frequency of favorable alleles of a major gene, for example *PrnP* alleles for scrapie resistance or *BMP15* alleles for litter size. However, a balance over time between selection for polygenes and the major gene for a given trait is needed to avoid inbreeding, and maintain the genetic variability within the breed.

In conclusion, the described method is successfully used in some meat Spanish sheep breeds, combining the genotyping of SNPs for the parentage assignment with some functional SNPs that can be used for pre-selection of replacement animals. The described method is a cost effective genotyping system, which is routinely used in Rasa Aragonesa and Ojinegra meat sheep breeds in their selection schemes by KASP genotyping technology. In addition, the SNPs for the parentage assignment could be genotyped using other genotyping platforms such as, for example, a custom low density array (these SNPs are included in the Illumina OvineSNP50 BeadChip array) or by Sequenom technology as described by Tortereau *et al.* (2017).

**Table 3.** Assignment rate of replacement ewes in different farms from the Rasa Aragonesa, Navarra and Cartera breeds. The number of replacement ewes, sires and declared proportion of sires sampled and genotyped are also indicated.

Breed	Farm	Replacement ewes	Sires	Assignment rate (%)	Proportion of candidate parents sampled (%) <sup>[1]</sup>
<b>Rasa Aragonesa</b>					
	A	35	5	100	100
	B	140	26	74	80
	C	160	73	100	95
	D	343	70	74	80
	E	295	56	80	80
	F	125	56	88	90
	G	625	73	86	90
	H	60	25	92	90
<b>Navarra</b>					
	I	116	12	67	75
	J	50	10	96	95
<b>Cartera</b>					
	K	39	5	87	80
	L	30	17	85	80

<sup>[1]</sup>According to the information provided by farmers.

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