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Association of microsatellite markers with fiber diameter trait in Peruvian alpacas (*Vicugna pacos*)

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ABSTRACT

A total of 140 Peruvian alpacas of two breeds (Huacaya and Suri) were analyzed for 69 microsatellite markers, in order to make a first approximation to the association analysis with the alpaca fiber diameter, which is the main trait related to alpaca fiber quality. A total of 599 alleles were observed across the two breeds, with a global average of 8.68 alleles per locus. Mean gene diversity in the total population was 0.701, meanwhile both breeds exhibited similar values of 0.686 (Suri) and 0.695 (Huacaya). On the other hand, the values from the inbreeding coefficient (F_{IS}) were 0.154 (Suri) and 0.162 (Huacaya) in both breeds, being the genetic differentiation low between these populations ($F_{ST}=0.022$), and with a gene flow (*Nm*) value of 9.3. The hierarchical AMOVA corroborated that the differentiation between both breeds only explained the 2.5% of genetic variability.

The analysis of association between the microsatellite markers panel and fiber diameter trait was done following an innovative methodology, which was focused in two steps. In the first one, animals were sampled according to a selective genotyping strategy, resulting in six microsatellite markers (LCA68, GLM6, LGU50, VOLP59, LCA85 and LCA90) associated with the genotypes carrying the hypothetical major gene. In the second step, the analysis revealed four significant associations of microsatellite loci (LCA68, VOLP59, LCA9O and GLM6). The subsequent DUNCAN test was used to determine the statistical differences among the fiber diameter EBV means, that corresponded to the different alleles. Eleven out from fourteen alleles of the following loci LCA68 (199, 189, 201, 197, 203 and 205), VOLP59 (112 and110) and LCA90 (243, 229 and 227), showed positive effect (decreasing fiber diameter), and only three alleles for the following loci LCA68 (195) and LCA90 (231 and 249), gave a negative effect (increasing fiber diameter).

1. Introduction

The livestock production in Peruvian high-Andean area is based on South American domestic camelids (alpacas and llamas). Two alpaca breeds (Huacaya and Suri) are the main animal production in this area due to the high altitude (over 3500 m.a.s.l). The Huacaya represents more than 85% of the alpaca population in Peru (Quispe et al., 2009) and its fiber is the most demanded product by the textile industry. One of the main goals in alpaca breeding is the selection based on the fiber diameter (thinner fiber) (Gutiérrez et al., in press), although Allain and Renieri (2010), indicated that there are many factors controlling the quantity and quality alpaca fiber. Some studies have been carried out for the identification of genes associated with the fleece color (Cransberg and Munyard, 2011).

The microsatellite markers are an useful tool for genetic identification, molecular relationships, parentage testing,

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development of genomic maps used for localizing loci associated to productive traits and others (Aranguren and Jordana, 2001). QTL studies using commercial populations may result in direct economic benefit, those results could be used immediately on the target population (Boichard et al., 2006). The knowledge of genetic markers and the linkage maps on sheep and cattle have allowed the identification of quantitative trait loci (QTL) (Maddox et al., 2001; Ihara et al., 2004), which explained a significant fraction of genetic variance of the phenotypes of interest (Weller, 2001). QTL related to the staple strength, staple length, fiber diameter and variation coefficient from the fiber diameter were detected on sheep (Rogers et al., 1994; Allain et al., 2006; Bidinost et al., 2008; Itenge-Mweza et al., 2007; Parsons et al., 1994; Ponz et al., 2001). Around 100 and 50 microsatellite markers of alpaca are published, although many more markers are needed to saturate the genome (Munyard et al., 2009). According to this, almost 4000 microsatellite markers were needed to create a high density of genome coverage in the bovine (Ihara et al., 2004) and the mouse (Watanabe et al., 1999).

Due to the rapid progress of molecular genetics research, new genomic tools are now available. The use of the increasingly cheaper and high-density SNP (single-nucleotide polymorphisms) will allow the fine mapping studies and dissection of many production traits including fiber and fleece characteristics (Allain and Renieri, 2010). However, there are few research groups working with this new genomic tool (SNP) (Guridi et al., 2011), and the linkage map has not still been reported in alpacas. Further studies using molecular tools in alpaca populations would allow using a high-throughput SNP or gene chip. The microsatellite markers are still considered useful for most of the genetic studies and currently a SNP markers panel is available for some livestock species (Munyard et al., 2009).

The study performed by Pérez-Cabal et al. (2010) in one alpaca population (*Vicugna pacos*) from both breeds (Huacaya and Suri) reported for the first time the segregation of major genes that may be affecting the fiber traits: mean fiber diameter (MFD), standard deviation of fiber diameter (SDFD), variation coefficient of fiber diameter (CVFD), and comfort factor (CF). This study concluded that there were two major genes involved: one major gene affecting the fiber diameter (FD and CF), and the second major gene affecting the diameter variability (SDFD and CV).

The objective of the present work was to investigate associations between a panel of 69 microsatellite markers with a major gene affecting the fiber diameter trait and the EBV (expected breeding value) of the fiber diameter. This study was performed using a population of elite alpacas from Peru, which was integrated by both breeds Huacaya and Suri.

2. Materials and methods

2.1. Samples

The animals sampled for this study were selected following a selective genotyping strategy, in which those animals that exhibited either the highest or the lowest probability of being homozygote for a hypothetical beneficial allele which would reduce the fiber diameter were considered, according to the results obtained by Pérez-Cabal et al. (2010). Taking into account this selective genotyping strategy a total of 140 animals were sampled from the Huacaya ($N=74$) and Suri ($N=66$) breeds corresponding to 46 and 49 homozygous animals carrying the favorable and unfavorable allele, respectively.

The alpaca samples were provided by Pacomarca experimental ranch, which belongs to Inca Tops S.A. one of the most important fiber processers in Peru. This experimental ranch is carrying out a very advanced program for the genetic improvement of the alpaca. Pacomarca is located in Puno at an altitude of 4060 m above sea level, with a temperature below -15 °C in winter, being the maximum temperature 15 °C during summer (Gutiérrez et al., 2009).

2.2. Collected phenotypic and genetic data

The phenotypic and genetic data used in this work belongs to the previous studies carried out by Pérez-Cabal et al. (2010) and Cervantes et al. (2010). Pérez-Cabal et al. (2010), shows the segregation of a major gene affecting fiber diameter (fiber diameter FD and comfort factor CF). This study also used the fiber diameter measures and the EBV data of FD, which were obtained by Cervantes et al. (2010).

2.3. Genomic DNA preparation

The blood samples were collected by vein puncture on filter paper. From a total of 140 animals. A volume of 100 μl genomic DNA (20 ng/μl approximately) was obtained, using the i-genomic CTB® Kit, following the manufacturer protocol.

2.4. Microsatellite typing

A total of 69 microsatellite markers, previously reported in South American camelids (Lang et al., 1996; Obreque et al., 1998, 1999; Penedo et al., 1998a,b, 1999; Bustamante et al., 2003; McPartlan et al., 1998; Sarno et al., 2000) and *Camelus dromedarious* (Mariasegaram et al., 2002), were used in this study (Table 1).

The microsatellite markers were amplified in 22 multiplex reactions. The PCR conditions consisted of an initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, annealing at 55.2–57.8 °C for 90 s, 72 °C for 60 s; and a final extension at 72 °C for 30 min. PCR reactions were performed in a Mastercycler Ep gradient (Eppendorf®) thermocycler. The total reaction volume of 25 μl contained approximately 20 ng of DNA. The final concentrations were: 1 × reaction buffer, $MgCl_2$ 2.0 mM, dNTPs 4 mM, Taq DNA polymerase 5U (BIOTOOLS B&M LABS.S.A) and 5 mM of each primer (forward primers labeled with fluorochromes at its 5′end, 6-FAM, NED, PET and VIC).

The PCR reactions were run in an automated DNA sequencer (Applied Biosystems, ABI 3130 Avant), the internal DNA standard used was the GENESCAN 500 LIZ. The DNA fragments were scored with the aid of the Gene Mapper software (Version 4.01, Applied Biosystems).

Table 1
Summary data for PCR conditions and fragment size ranges of 69 microsatellite markers, amplified in two breeds (Huacaya and Suri) from Peruvian alpacas.

Locus	Size range (bp)	Fluorescent dye	Annealing temperature (°C)	Multiplex PCR
LCA68	188–206	VIC	55.2	1
YWLL59	91–132	NED		
LGU75	178–197	NED		
YWLL46	84–109	VIC	55.2	2
VOLP68	133–146	VIC		
YWLL43	138–150	PET		
YWLL36	142–175	6-FAM		
YWLL29	210–224	6-FAM	55.2	3
YWLL44	80–119	6-FAM		
YWLL38	171–178	VIC		
LCA19	76–113	6-FAM	55.2	4
LCA54	139–151	6-FAM		
LGU93	183–211	6-FAM		
LCA77	233–264	PET		
VOLP72	163–176	VIC	55.2	5
LCA66	216–258	VIC		
LCA22	101–115	NED	55.2	6
LCA63	192–219	NED		
LCA82	103–120	PET		
LCA85	192–209	PET		
LCA33	118–122	6-FAM	55.2	7
LCA86	162–168	6-FAM		
YWLL40	174–187	6-FAM		
LCA90	224–251	6-FAM		
LCA37	124–156	NED	55.2	8
LCA36	201–206	NED		
LCA8	223–249	NED	55.2	9
LCA56	129–165	PET		
LGU52	179–205	PET		
VIAS A1	137–144	VIC	55.2	10
VIAS A2	194–203	VIC		
VOLP59	103–134	NED		
VOLP67	145–163	NED		
LAB13	178–187	NED	55.2	11
LAB7	140–166	PET		
VOLP33	176–184	PET		
LCA83	190–209	PET		
VOLP10	228–234	PET	55.2	12
VOLP03	128–166	6-FAM		
VOLP32	185–247	6-FAM		
VOLP12	151–163	PET	55.2	13
CVRL06	213–235	PET		
VOLP01	248–266	PET		
LCA23	127–157	6-FAM	55.2	14
LCA65	162–190	6-FAM		
CVRL01	168–236	VIC	55.8	15
CVRL05	124–144	VIC	55.2	
VOLP04	219–253	6-FAM		
CVRL08	199–213	NED		
LGU76	232–265	PET	55.2	16
LAB3	106–162	6-FAM		
GLM5	142–168	VIC	55.2	17
GLM7	185–207	VIC		
GLM2	130–135	NED	55.2	18
GLM6	149–158	NED		
GLM4	181–208	NED		
LGU56	157–181	6-FAM	55.2	19
LGU68	202–230	6-FAM		
VOLP77	143–167	VIC		
LGU50	170–192	VIC	57.8	20
LGU49	220–244	VIC	55.2	
LCA71	132–148	NED		
LAB1	154–190	NED	55.2	21
LGU51	192–208	NED		
YWLL08	126–184	PET		
LGU79	197–223	PET		
LCA24	100–119	VIC	55.2	22
LCA94	184–203	VIC		
LCA5	176–205	6-FAM		

2.5. Statistical analysis

The genetic variability in the total population and in each breed was measured using the alleles number per locus, the observed (H_O) and the expected (H_E) heterozygosity (Nei, 1978), the Hardy–Weinberg (HW) equilibrium based on 1000 permutations, the polymorphism information content (PIC) value and the allele frequency values. The data were analyzed using the CERVUS 3.0 software (Marshall et al., 1998; Kalinowski et al., 2007). The statistical package GENETIX 4.05 (Belkhir et al., 2004) was used to estimate the fixation indices, (F_{IS}, F_{ST} and F_{IT}) per locus and to determine the population genetic structure related to the differentiation within and between the alpaca breeds. The fixation indices were calculated according to Weir and Cockerham (1984), with the Jackknife procedure applied over the loci with a confidence interval of 95% computed with 1000 iterations. The genetic structure of the alpaca populations were analyzed with Wright's *F*-statistics (Wright, 1965) and the gene flow (number of migrants in each generation, *Nm*), was also found. To evaluate the partitioning of genetic variability between and within the two breeds, a hierarchical analysis of molecular variance (AMOVA) was conducted using a GenAlEx v6.5 software package (Peakall and Smouse, 2012).

Due to the large number of microsatellite markers analyzed (69), the significance level of association analyses is smaller than the limit (e g. for a confidence level of 95%, the type I error would decrease to 0.00072), and there is a high probability that a microsatellite loci can appear as significant and shows a spurious association (Curran-Everett, 2000). On the other hand, associations may also occur by linkage disequilibrium between different microsatellite loci (although it cannot be ensured, because the location of these microsatellites on the alpaca chromosomes are not reported yet). Taking into account, these facts we decided to include a preliminary step for preselected microsatellite loci and to have a more clear association.

Following Reynolds et al. (1983) methodology, a first step was the construction of a genetic distance matrix, in which the two alpaca breeds were considered as a single population. Based on this genetic distance matrix, a phylogenetic tree was reconstructed using the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) algorithm and the NEIGHBOR program from the PHYLIP 3.65 package (Felsentein, 2004). On the second step, some microsatellite markers previously selected from the cluster analysis were used. The GLM procedure from SAS 9.0 (SAS Institute Inc., 2003) was applied to analyze the influence of these microsatellite markers on the FD EBV by means of a general linear model with two effects (the microsatellite marker and the breed).

A posteriori DUNCAN's multiple range test was applied to detect significant differences in the FD EBV for significant microsatellites. Although, this test attempts to correct the error rate in a similar manner to Bonferroni correction to avoid the declaration of false positive parameters when a large number of contrasts are analyzed. The Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995), was carried out to correct the corresponding *P* values (with a false discovery rate of 5%). This methodology is an efficient way of controlling the false discovery rate in multiple testing, considering that it is more powerful than the classical Bonferroni correction (Thissen et al., 2002).

The EBV were obtained using a BLUP animal model with the Pacomarca dataset (updated at December 31, 2011), according to the model described in the previous study performed by Cervantes et al. (2010). The model fitted for genetic analyses included the following fixed effects: month–year of recording as contemporary group, colour (only in Huacaya breed, white and others), sex (male or female) and age at shearing in days as a linear and quadratic covariant. The breeding values were rescaled to a mean value of 100 and standard deviation of 20 to jointly analyze both breeds. According to the previous study performed by Cervantes et al. (2010), the former additive genetic variances for the fiber diameter were 5.4819 (Huacaya breed) and 3.0180 (Suri breed).

3. Results

3.1. Genetic variability and population structure

Sixty nine microsatellite markers were successfully amplified in two Peruvian alpaca breeds detecting a total of 599 alleles (Table 2). The number of alleles per locus varied from 2 (LCA33) to 25 (YWLL08) with a global average of 8.68 alleles per locus. Most of the markers were highly polymorphic, with only one locus (LCA33) exhibiting two alleles and five microsatellite markers (GLM2, LCA36, LCA86, VIAS A1 and VOLP10), showing three alleles. The observed heterozygosity (H_O) ranged from 0.014 (GLM2) to 0.862 (LCA8). The expected heterozygosity (H_E) per locus varied from 0.056 (GLM2) to 0.929 (LAB3), being the average values observed and expected heterozygosity of 0.582 and 0.701, respectively. Deviations from the Hardy–Weinberg equilibrium (HWE) were found in 12 loci (LAB13, LCA37, LCA68, LCA77, LCA85, LCA90, LGU76, VOLP01, VOLP10, VOLP67, VOLP72 and YWLL43) in the total population, and across both breeds Huacaya and Suri in 3 (LCA90, LGU76 and VOLP01) and 4 (LAB13, VOLP01, VOLP10 and YWLL43) loci, respectively. The Polymorphic Information Content (PIC) values ranged from 0.055 (GLM2) to 0.919 (LAB3) and the average value was 0.663.

Some parameters of genetic variability in both Huacaya and Suri breeds are showed in Table 3. The allelic variability of the two alpaca breeds were relatively high and similar. The Huacaya breed showed the highest allelic diversity (8.06), whereas the Suri breed exhibited a lower value (7.74). The observed heterozygosity values (H_O) ranged from 0.581 (Suri) to 0.583 (Huacaya), and the expected heterozygosity values were 0.686 (Suri) and 0.695 (Huacaya); being the mean observed heterozygosity (0.582) lower than the expected heterozygosity (0.701) in the total population. The values of inbreeding coefficient (F_{IS}) for both breeds were 0.154 (Suri) and 0.162 (Huacaya). The overall heterozygote deficit (F_{IS}) of 16.1% was observed within the breeds; whereas, the total population exhibited a heterozygote deficit (F_{IT}) of 18%, see Table 2.

According to the global F_{ST} value, the 2.2% of the genetic variation was considered for the breed differences and the 97.8% was for the variation among individuals (Table 2).

Table 2
Variability parameters for 69 microsatellite loci in the total population from Peruvian alpacas sampled in the Pacomarca experimental ranch.

Locus	Alleles (*n*)	H_O	H_E	HW	PIC	F_{IS} (f)[a]	F_{IT} ($\ominus$)[a]	F_{ST} (F)[a]
CVRL01	11	0.466	0.631	NS	0.604	0.15989	0.17890	0.02263
CVRL05	5	0.529	0.547	NS	0.467	0.16272	0.18163	0.02259
CVRL06	9	0.261	0.343	NS	0.331	0.16073	0.17947	0.02232
CVRL08	6	0.629	0.649	NS	0.608	0.16300	0.18199	0.02269
GLM2	3	0.014	0.056	NS	0.055	0.16051	0.17935	0.02244
GLM4	12	0.783	0.831	NS	0.806	0.16350	0.18166	0.02171
GLM5	10	0.340	0.861	NS	0.841	0.15306	0.17230	0.02271
GLM6	8	0.650	0.771	NS	0.731	0.16138	0.18024	0.02249
GLM7	8	0.754	0.781	NS	0.749	0.16337	0.18228	0.02261
LAB1	12	0.588	0.776	NS	0.754	0.16008	0.17880	0.02229
LAB13	4	0.266	0.533	**[a]	0.421	0.15778	0.17604	0.02169
LAB3	20	0.341	0.929	NS	0.919	0.15193	0.17106	0.02255
LAB7	9	0.654	0.792	NS	0.761	0.16119	0.17987	0.02226
LCA19	10	0.564	0.756	NS	0.717	0.15992	0.17861	0.02224
LCA22	6	0.591	0.610	NS	0.541	0.16299	0.18178	0.02244
LCA23	11	0.598	0.852	NS	0.830	0.15919	0.17742	0.02168
LCA24	8	0.568	0.571	NS	0.541	0.16307	0.18209	0.02273
LCA33	2	0.179	0.163	NS	0.149	0.16218	0.18082	0.02225
LCA36	3	0.485	0.540	NS	0.444	0.16186	0.18089	0.02270
LCA37	11	0.746	0.863	***	0.845	0.16171	0.18076	0.02273
LCA5	10	0.638	0.762	NS	0.723	0.16136	0.18006	0.02230
LCA54	6	0.580	0.736	NS	0.689	0.16042	0.17947	0.02269
LCA56	9	0.693	0.705	NS	0.670	0.16348	0.18225	0.02245
LCA63	8	0.730	0.722	NS	0.673	0.16390	0.18282	0.02263
LCA65	13	0.850	0.879	NS	0.864	0.16394	0.18235	0.02201
LCA66	16	0.833	0.865	NS	0.849	0.16365	0.18243	0.02246
LCA68	9	0.531	0.817	**	0.791	0.15798	0.17699	0.02258
LCA71	5	0.504	0.516	NS	0.443	0.16273	0.18163	0.02257
LCA77	11	0.639	0.815	**	0.787	0.16062	0.17903	0.02194
LCA8	8	0.862	0.840	NS	0.816	0.16451	0.18362	0.02286
LCA82	6	0.602	0.575	NS	0.528	0.16372	0.18268	0.02268
LCA83	7	0.692	0.740	NS	0.695	0.16270	0.18180	0.02281
LCA85	9	0.669	0.843	**	0.819	0.16073	0.17921	0.02203
LCA86	3	0.607	0.589	NS	0.514	0.16383	0.18235	0.02214
LCA90	9	0.410	0.763	***	0.728	0.15645	0.17534	0.02239
LCA94	8	0.813	0.810	NS	0.779	0.16411	0.18301	0.02262
LGU49	12	0.801	0.851	NS	0.831	0.16323	0.18200	0.02243
LGU50	6	0.662	0.676	NS	0.614	0.16352	0.18192	0.02200
LGU51	8	0.761	0.764	NS	0.728	0.16374	0.18279	0.02279
LGU52	6	0.562	0.607	NS	0.566	0.16238	0.18128	0.02257
LGU56	6	0.664	0.659	NS	0.615	0.16374	0.18241	0.02233
LGU68	9	0.710	0.699	NS	0.663	0.16413	0.18256	0.02205
LGU75	9	0.504	0.643	NS	0.618	0.16047	0.17951	0.02268
LGU76	12	0.533	0.758	**	0.729	0.15914	0.17802	0.02246
LGU79	12	0.861	0.882	NS	0.867	0.16394	0.18272	0.02246
LGU93	13	0.827	0.848	NS	0.828	0.16379	0.18261	0.02250
VIAS A1	3	0.329	0.375	NS	0.330	0.16190	0.18005	0.02166
VIAS A2	4	0.427	0.575	NS	0.489	0.16012	0.17901	0.02249
VOLP01	8	0.358	0.778	***	0.742	0.15527	0.17382	0.02196
VOLP03	6	0.765	0.775	NS	0.736	0.16374	0.18259	0.02253
VOLP04	15	0.707	0.857	NS	0.842	0.16101	0.18000	0.02264
VOLP10	3	0.294	0.538	***	0.445	0.15781	0.17697	0.02275
VOLP12	6	0.669	0.750	NS	0.707	0.16232	0.18085	0.02211
VOLP32	21	0.630	0.859	NS	0.842	0.15932	0.17836	0.02265
VOLP33	4	0.457	0.581	NS	0.511	0.16060	0.17952	0.02254
VOLP59	6	0.564	0.602	NS	0.536	0.16252	0.18140	0.02255
VOLP67	6	0.391	0.641	***	0.584	0.15821	0.17710	0.02243
VOLP68	7	0.415	0.547	NS	0.513	0.16024	0.17931	0.02271
VOLP72	6	0.404	0.821	***	0.792	0.15509	0.17444	0.02290
VOLP77	10	0.763	0.858	NS	0.839	0.16240	0.18099	0.02220
YWLL08	25	0.540	0.926	NS	0.918	0.15623	0.17532	0.02262
YWLL29	7	0.613	0.624	NS	0.595	0.16313	0.18209	0.02266
YWLL36	12	0.766	0.853	NS	0.833	0.16233	0.18129	0.02263
YWLL38	4	0.727	0.722	NS	0.669	0.16397	0.18260	0.02228
YWLL40	7	0.629	0.666	NS	0.603	0.16285	0.18155	0.02234
YWLL43	6	0.300	0.630	***	0.570	0.15636	0.17547	0.02265
YWLL44	14	0.778	0.868	NS	0.850	0.16247	0.18118	0.02234
YWLL46	7	0.380	0.434	NS	0.401	0.16153	0.18047	0.02260
YWLL59	14	0.708	0.884	NS	0.870	0.16064	0.17948	0.02245

Table 2 (*continued*)

Locus	Alleles (*n*)	H_O	H_E	HW	PIC	F_{IS} (f)[a]	F_{IT} ($\ominus$)[a]	F_{ST} (F)[a]
Total	**599**							
Average	**8.68**	**0.582**	**0.701**		**0.663**	**0.16127**	**0.18008**	**0.02243**

(n) Total number of alleles detected per locus; (H_O): observed heterozygosity; (H_E): expected heterozygosity; (PIC): polymorphic information content; (HW) significance results of Hardy–Weinberg equilibrium tests; (F_{IS}, F_{IT}, F_{ST}): F-statistic.

* $P \leq 0.05$.

[a] Jacknifing estimates over the loci.

*** $P \leq 0.01$.

** $P \leq 0.00$ and NS, $P \geq 0.05$ non-significant deviation.

Table 3
Variability parameters for 69 microsatellite loci in two alpaca breeds sampled in the Pacomarca experimental ranch.

Breed	Sample size	Total no. of alleles	MNA	H_O	H_E	PIC	F_{IS}
Huacaya	74	556	8.06	0.583	0.695	0.655	0.162
Suri	66	534	7.74	0.581	0.686	0.642	0.154

(MNA): average number of alleles; (H_E): average expected heterozygosity; (H_O): observed heterozygosity; (PIC): polymorphic information content; (F_{IS}): inbreeding coefficient.

Most of the loci contributed in similar extent to the low genetic differentiation observed between both breeds. The value of genetic differentiation (F_{ST}) between the two breeds Huacaya and Suri was 0.026, with a gene flow (Nm) between both breeds of 9.31. Likewise, the hierarchical AMOVA revealed a low level of genetic differentiation between Huacaya breed and Suri breed ($F_{ST}=0.025$), with a higher differentiation value within populations (78%) than between populations (19%).

3.2. Association analyses with fiber diameter trait

When using a larger number of microsatellite markers in the association analysis of quantitative trait loci, the false positives probability (spurious associations) is high. To solve this, different criteria to filter the microsatellite markers analyzed were used. Chan et al. (2009) take into account the linkage disequilibrium ($r^2 > 0.2$) and minor allele frequency (MAF) < 0.05 for randomly removing one of each pair of SNP marker in linkage disequilibrium. This study used a strategy that considered the MAF < 0.05 and the interrelationships between the microsatellite loci with the high and low probability values for a hypothetical gene, that increases or decreases the dependent variable (fiber diameter or comfort factor). Then, in a preliminary step the genetic distance relationships between the 69 microsatellite markers with the hypothetical major gene was obtained. A dendogram was built by the UPGMA method that provided a best resolution of the relationships between the 69 microsatellite markers with this major gen. The phylogenetic tree showed a main cluster between the major gene with six microsatellite markers: LCA68, GLM6, LGU50, VOLP59, LCA85 and LCA90 (Fig. 1).

The second step was to find significant associations of these six microsatellite markers, which were previously clustered by the phylogenetic tree, with the FD EBV. The associations were performed by means of the general linear model, in which the most significant findings were the association of the LCA68, VOLP59, LCA90 and GLM6 loci ($P < 0.0001$, $P=0.0175$, $P=0.0258$ and $P=0.0318$, respectively); see Table 4. The LCA85 and LGU50 microsatellite markers showed no significant differences ($P \geq 0.05$). Subsequently, a DUNCAN multiple-stage test was employed to detect the significantly different effects of the alleles for the four significant microsatellites loci, in this case the genotypes and alleles with frequencies minor to five were eliminated. Seven alleles (199, 189, 201, 197, 203, 205 and 195) from the LCA68 showed statistical differences, being the 195 allele the most unfavorable allele increasing the fiber diameter (with an average EBV of 4.43 μm for the Huacaya breed and 3.28 μm for the Suri breed). VOLP59 locus also showed significantly different associations for the alleles 112 (Huacaya breed) and 110 (Suri breed) decreasing the fiber diameter (EBV of −1.09 μm and −0.81 μm, respectively). LCA90 locus reported significantly different associations for the alleles group 243, 229 and 227 with the 231 and 249 alleles, being the EBV for the 231 and 249 alleles of 0.32 μm and 1.27 μm (Huacaya breed) and 0.24 μm and 0.94 μm (Suri breed), respectively. The GLM6 locus presented no statistically significant contrast; however its EBV are also shown in Table 4.

4. Discussion

4.1. Genetic variability and population structure

The Food and Agriculture Organization of the United Nation (FAO) (2011) recommend the use of microsatellite markers to study the genetic diversity in livestock species, due to their proven value in the studies of variation within and among breeds. At present, the microsatellites are also, one of the genetic markers most widely used in genetic distance studies. A relatively large number of microsatellites (a panel of 69 microsatellite markers) were used in this study, in order to analyze the genetic variability, and also to investigate their association with the fiber diameter trait in Peruvian alpaca populations (both Huacaya and Suri breeds).

The genetic diversity levels based on alleles number reported herein were slightly lower than the results reported

Fig. 1. Dendrogram using UPGMA method, based on 69 microsatellite markers and the hypothetical major gene related to the fiber diameter (mic) and comfort factor (conf), in alpaca populations from Peru.

by Paredes et al.(2013), who used a panel of twenty microsatellite markers in other Peruvian alpaca populations (average number of 11.5 alleles). This low variability detected is probably due to the implementation of a genetic selection program in the Pacomarca experimental ranch (Gutiérrez et al., 2009), having as a principal objective a continuous

Table 4
Associations of four microsatellite markers alleles with the breeding value for fiber diameter in two breeds (Huacaya and Suri), from Peruvian alpacas.

Loci	Allele	Allele frequency[a]	FD (μm)	Expected breeding values[b]	Indexed genetic values (μm)[c]	
					Huacaya	Suri
LCA68	199	13	22.11	84.560[A]	−1.81	−1.34
	189	9	21.51	89.130[A]	−1.27	−0.54
	201	41	22.49	92.536[A]	−0.87	−0.65
	197	8	22.86	92.761[A]	−0.85	−0.63
	203	6	21.68	93.868[A]	−0.74	−0.55
	205	14	24.09	96.220[A]	−0.44	−0.33
	195	5	30.60	137.843[B]	4.43	3.28
VOLP59	112	68	22.14	90.668[A]	−1.09	−0.81
	110	66	23.52	99.037[A]	−0.11	−0.08
LCA90	243	5	21.89	90.059[A]	−1.16	−0.86
	229	55	22.50	89.934[A]	−1.18	−0.87
	227	30	22.78	94.796[A]	−0.61	−0.45
	231	9	24.64	102.766[B]	0.32	0.24
	249	15	25.98	110.814[B]	1.27	0.94
GLM6	143	5	23.19	73.514[A]	−3.10	−2.30
	155	39	22.19	91.073[B]	−1.04	−0.78
	151	8	22.80	92.805[B]	−0.84	−0.63
	157	67	22.93	95.256[B]	−0.55	−0.41
	153	16	24.37	106.263[B]	0.73	0.54

FD: Fiber diameter measures (μm).
[a] Number of alleles analyses at each locus.
[b] The DUNCAN test values: the different letters A and B are significantly different for the breeding values (fiber diameter), following Benjamini and Hochberg's FDR methodology (FDR=5%).
[c] Positive genetic values: unfavourable alleles that increase the fiber diameter measures.

selection for thin fiber during the last twenty years, could have caused erosion of the variability throughout the successive generations. Others alpaca populations previously analyzed (Paredes et al., 2013) have maintained their original variability, due to that they were not subjected to any selection process.

The sampling selection strategy does not seem to have influenced this variability. On the other hand, the pedigree information of these alpaca populations were analyzed, in order to know if there were relatives. The results showed that the relationship levels of each group (alpacas with the highest and the lowest probability) were not higher than the average levels in all samples.

The important results obtained by means of the microsatellite markers used in this work, would suggest that these markers could be used for estimating genetic diversity levels in the alpaca populations. The results showed relatively high number of alleles per locus. On the other hand, the genetic variability was the lowest in contrast to the results exhibited in others Peruvian alpaca populations: 14.5 (Agapito et al., 2008) and 9.6 (La Manna et al., 2011); in alpacas from different origins: 9.3 from USA (Mariasegaram et al., 2002) and 11.7 from Bolivia (Barreta et al., 2012). Takezaki and Nei, (1996) indicated that markers for measuring genetic variation in a population should have an average heterozygosity ranging from 0.3 to 0.8. In the present study, the gene diversity indicated a high level (H_E=0.70).

The genetic diversity estimates in the two alpaca breeds were relatively similar. The total number of alleles for the Huacaya breed (556) was slightly higher than the Suri breed (534), with average values of 8.06 (Huacaya) and 7.74 (Suri). The observed (0.583 vs. 0.581) and expected (0.69 vs. 0.68) heterozygosity values were similar in both breeds Huacaya and Suri, respectively. With regards to these results, La Manna et al. (2011) found similar values on the total number of alleles but higher values from observed and expected heterozygosity 0.75 vs 0.74 (Huacaya breeds) and 0.78 vs 0.77 (Suri breed), respectively. A decrease of the observed heterozygosity (H_O=0.58 vs. the H_E=0.70) was found in the total population (both alpaca breeds). This moderate unbalance between observed and expected heterozygosity may possibly be also explained by the selection practiced (continuous selection of genotypes) in the Pacomarca experimental ranch.

Very few microsatellite markers have exhibited deviation from HWE in both breeds, Huacaya (LCA90, LGU76 and VOLP01) and Suri (LAB13, VOLP01, VOLP10 and YWLL43), mainly due to the deficit from heterozygosity, and a high value of F_{IS} in both alpaca breeds. A previous study performed by Barreta et al. (2012) showed no significant deviations from HWE in the VOLP01 locus. Meanwhile, Paredes et al. (2013) indicated significant deviation at the same locus. On the other hand, La Manna et al. (2011) and Agapito et al. (2008) reported significant deviations from HWE in the locus LCA37.

Deviation from HWE can reveal several possible causes, such as, selective mating, population sub-structuring, shortage of samples, low levels of polymorphism, the presence of non-amplified alleles (Sun et al., 2012) and the lack of neutrality relative to selection (selection favouring homozygotes) (Maudet et al., 2002), which may also causes a reduction in the heterozygosity. In this work, inbreeding was the most likely cause of reducing from the heterozygosity in both breeds Huacaya (F_{IS}=0.16) and Suri (F_{IS}=0.15).

The overall F_{IS} value (0.16) in the total populations was moderate, suggesting a deficit of heterozygote, which may be attributed to the inbreeding, being the inbreeding coefficient value moderate, and similar values were also obtained for both alpaca breeds Huacaya and Suri. As well as, the previous study performed on alpaca populations from the Peruvian Andean communities (Paredes et al., 2013) showed a similar value (F_{IS}=0.15), to the described herein. However, Barreta et al. (2012) reported the lowest inbreeding levels (F_{IS}=0.019 to 0.114) in Bolivian alpacas.

The genetic differentiation coefficients revealed for both alpaca breeds (Huacaya and Suri) are very similar (F_{ST}=0.02) and presented a high gene flow (Nm=9.31). These results were supported by previous studies performed in Peruvian alpaca populations, which reported low levels from genetic differentiation (F_{ST}): 0.02 (Paredes et al., 2013) and 0.0002 (La Manna et al., 2011). Meanwhile, Barreta et al. (2012) showed in Bolivian alpacas highest levels of F_{ST} ranging from 0.053 to 0.077. The analysis of molecular variance showed that the highest genetic variation was found within populations (78%), whereas, the genetic variation between populations was only 2.5%.

4.2. Association analyses with fiber diameter trait

The studies for the identification of genes, associated with the production traits in the alpaca are limited, due to the incomplete saturation of the genome map and the non-chromosomal location of the microsatellite markers. Around one hundred and fifty microsatellite markers from alpaca are published (Munyard et al., 2009), but about 4000 microsatellite loci have been needed for creating a high density of genome coverage in others species (Ihara et al., 2004).

Some studies relating to QTL have been published, as QTL for mohair traits in goats (Visser et al., 2011), and the identification of three chromosomal regions, which would be influencing the fiber diameter from the sheep wool (Parsons et al., 1994; Ponz et al., 2001). Furthermore, previous studies also indicated that the differential expression from the Keratin genes might have an important role in regulating the fiber diameter, staple strength, and colour and brightness wool from the goat cashmere and sheep wool (Jin et al., 2011; Adelson et al., 2004; Gong et al., 2011; Itenge-Mweza et al., 2007).

This is the first paper showing a possible association from microsatellite markers with a fiber diameter trait using breeding values as reference. The previous study reported by Paredes et al. (2013) suggested genetic associations of three microsatellite loci (CVRL07, LGU68 and LCA65) with the phenotypic fiber diameter trait. These results should be considered as a first approximation of association in South American camelids (especially the alpaca), wherein few studies using the molecular tools have been made, given that the sample size and the number of microsatellite markers are also limited. Added to these facts, most of the microsatellite markers isolated from South American camelids have not been located in their karyotype, and this situation complicates the association studies greatly.

The more significant finding from this work was the association of four microsatellite loci (LCA68, VOLP59, LCA90 and GLM6) with EBV for the fiber diameter. However the GLM6 locus presented statistically no significant contrast, according to the Benjamini–Hochberg procedure (Table 4). A total of eleven out of fourteen alleles showed associations with negative genetic values (decreasing the fiber diameter), wherein only three alleles gave positive genetic values (increasing the fiber diameter).

These findings are important, given that greatly can contribute to that alpacas with the best breeding values can be selected, in order to improve alpaca herds (thin fiber). Once determined the association between a microsatellite marker with a quantitative trait, the marker-assisted selection might greatly increase the efficiency and effectiveness for the breeding compared to conventional breeding based on phenotypic record. The selection of the best genotypes in function to few microsatellite markers would allow that specific genotypes can be easily identified and selected. In addition the selection could be performed at birth, and the selection from both sexes for the sex-linked traits. These characteristics accelerate the breeding programs response.

According to our results, one selection criteria for improving (decrease) the fiber diameter in these alpaca populations would be removing the unfavorable alleles: 195 (4.43 μm and 3.28 μm), LCA68 locus; 231 (0.32 μm and 0.24 μm) and 249 (1.27 μm and 0.94 μm) from LCA90 locus, for the Huacaya and Suri breeds, respectively.

The relative high number of alleles for decreasing the fiber diameter (eleven alleles with negative genetic values), could be due to the breeding program implemented by Pacomarca since 1992. A goal of this breeding program was decrease fiber diameter in its alpaca herds. Nevertheless, even in this experimental ranch with elite alpacas, some unfavorable alleles (positive genetic values, μm) were found. Selection against these alleles which are associated with an increase in the fiber diameter could be carried out in this alpaca population. It might be interesting to select negative alleles or to go against the positive alleles, depending of the frequencies in the population to be selected (whenever, the selection does not affect the variability of the population, mainly if this population is endangered).

Further studies are needed to confirm the associations found in this work. Other alpaca populations should be selected, likewise greater sample sizes (the few sampling size might cause the lower frequencies of some alleles, as well as a possible overestimate from the alleles effects, which were found as significant).

5. Conclusions

This work identified four microsatellite loci (LCA68, VOLP59, LCA90 and GLM6), which were significantly associated with the breeding values for the fiber diameter trait. A total of eleven out of fourteen alleles showed negative genetic values (decreasing the fiber diameter, μm), in which the most significant association was the 195 allele (LCA68). Nevertheless, these findings should be considered as a first approximation of the association between microsatellite markers and fiber diameter trait in Peruvian alpacas. Future studies with greater number of validated populations and new alpaca populations would be required to confirm the effect of these alleles. The genetic structure results from this alpaca populations revealed considerable levels of genetic variability, low coefficients of inbreeding, and a high percentage of genetic variance within populations (78%), suggesting that, these populations contains a rich genetic resources for continuing with the development of further breeding strategies, as well as for alpacas selection with desirables alleles for improving the phenotypic traits.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no financial support for this work that could have influenced its outcome.

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