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SHORT COMMUNICATION

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Genome-wide association reveals the locus responsible for four-horned ruminant

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Summary

Phenotypic variability in horn characteristics, such as their size, number and shape, offers the opportunity to elucidate the molecular basis of horn development. The objective of this study was to map the genetic determinant controlling the production of four horns in two breeds, Jacob sheep and Navajo-Churro, and examine whether an eyelid abnormality occurring in the same populations is related. Genome-wide association mapping was performed using 125 animals from the two breeds that contain two- and four-horned individuals. A case–control design analysis of 570 712 SNPs genotyped with the ovine HD SNP Beadchip revealed a strong association signal on sheep chromosome 2. The 10 most strongly associated SNPs were all located in a region spanning Mb positions 131.9–132.6, indicating the genetic architecture underpinning the production of four horns is likely to involve a single gene. The closest genes to the most strongly associated marker (*OAR2_132568092*) were *MTX2* and the HOXD cluster, located approximately 93 Kb and 251 Kb upstream respectively. The occurrence of an eyelid malformation across both breeds was restricted to polled animals and those carrying more than two horns. This suggests the eyelid abnormality may be associated with departures from the normal developmental production of two-horned animals and that the two conditions are developmentally linked. This study demonstrated the presence of separate loci responsible for the polled and four-horned phenotypes in sheep and advanced our understanding of the complexity that underpins horn morphology in ruminants.

Keywords GWAS, horn morphology, ruminant

The horns carried by ruminants are in many ways their most emblematic feature. They play an important role in sexual selection in wild and feral sheep (Johnston *et al.* 2013; Martin *et al.* 2014), and animal handlers have bred for naturally hornless animals (termed polled) since at least the 16th century. This long-standing selection for the polled condition is detectable in patterns of genomic variation, with several studies showing markedly reduced heterozygosity across a region of sheep chromosome (OAR) 10 that harbours the *RXFP2* gene (e.g. Kijas *et al.* 2012; Fariello *et al.* 2014). Recently, a 1.8-kb insertion in the 3′-UTR of *RXFP2* was shown to be associated with polledness (Wiedemar & Drögemüller 2015). The current study sought to investigate two sheep breeds that display unusual horn characteristics. The first is the Jacob sheep, an ancient breed of largely unimproved piebald animals that carry more than two horns (termed polycerates). The earliest origins of the breed are unclear; however, for many centuries, Jacob sheep were maintained in the United Kingdom where other polycerate breeds are either now extinct (e.g. Jersey sheep) or remain as heirloom breeds such as the Manx Loaghtan on the Isle of Man. Isolated introductions of Jacob sheep into the United States have occurred since the 1900s, and admixture with other breeds has likely occurred. The second breed is the Navajo-Churro, which is also polycerate but with a very different population history. The breed is descendent from Iberian Churra sheep developed by the Spanish and imported to North America, after which Native American Indians developed the animals for textiles. The origin of the polycerate trait in both populations is unclear, and it remains an open question whether it has a common origin or developed independently. Interestingly, both breeds also exhibit an eyelid abnormality that presents with variable severity (Fig. 1b–e). In Jacob sheep, it is sufficiently widespread to be considered an acceptable component of the defined breed standard. It is not clear

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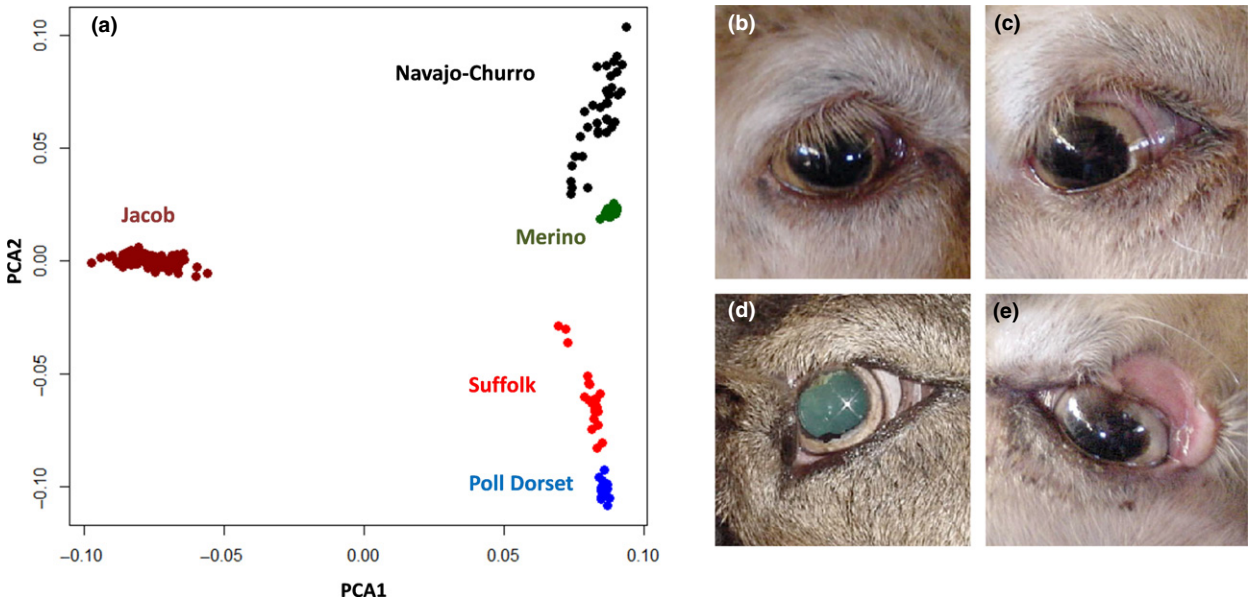

Figure 1 Genetic relationship between breeds and an eyelid abnormality. (a) Clustering of animals from five breeds using 541 363 SNPs. Individuals are plotted using the first two principal components (PCA1 and 2) and coloured to represent their breed of origin. (b–e) The Jacob and Navajo-Churro breeds were scored for an eyelid abnormality, which presented with a range of severity: (b) Normal round eyelid scored grade 1 (absent). (c) Almond-shaped eyelid with a slight break in the eyelashes scored grade 2. (d) Tuft in eyelid scored grade 3. (e) Complete eyelid split with corneal involvement scored grade 4. The association between the eyelid abnormality and horn morphology is given in Table 1.

whether the eyelid abnormality is pleiotropic to the polycerate condition or independent; however, a similar observation has been documented in cattle, whereby polled animals display an increased number of eye lashes and may also exhibit eyelid hypertrichosis (Allais-Bonnet *et al.* 2013). This raises the possibility that genes involved in skin differentiation around the eye are also involved in horn morphogenesis. The objectives of this study were to (i) investigate the co-segregation of the two traits and (ii) use the recently developed ovine HD SNP Beadchip to map the locus responsible for four horns in sheep and determine if it is separate from the *RXFP2* gene involved in polledness.

Blood samples were collected from 126 Jacob and 38 Navajo-Churro individuals maintained by four breeders in the United States (Puddleduck Farm, Brownsville, OR; Three Fates Farm, Crete, IL; Hillside Jacobs, Sparta, MI; and Belle Ridge Farms, Excelsior Springs, MO). Animals from each breeder could be considered as related, and the extent of relatedness was estimated from SNP data using D_{ST} as described below. Genomic DNA was extracted from these 164 animals and genotyped using Illumina's ovine HD BeadChip by a commercial service provider (GeneSeek). Data filtering was performed using PLINK v1.07 (Purcell *et al.* 2007). Four individuals with a SNP call rate <98% were removed from genomic analysis. A total of 35 294 loci with call rates <95% were pruned, leaving 570 712 SNPs with an average call rate of 99.7% per animal. The genotypic dataset has been submitted to the Dryad repository with DOI number doi:10.5061/dryad.1p7sf. To evaluate the level of genetic diversity within both breeds and their relationship to other breeds, genotypes were co-analysed with available HD data from Merino ($n = 36$), Suffolk ($n = 23$) and Poll Dorset industry sires ($n = 18$), described previously (Hayes *et al.* 2012; Kijas *et al.* 2014). Genotypes were merged to create a single dataset of 541 363 SNPs that was used to estimate three metrics of within-breed genetic diversity (P_N, D_{ST} and H_E; Table S1) and multidimensional scaling analysis of a D_{ST} matrix containing all 237 animals (Fig. 1a). The resulting cluster pattern revealed that Jacob sheep were strongly genetically divergent and that they displayed comparatively low within-breed diversity (Table S1). In contrast, the Navajo-Churro displayed higher diversity and clustered most closely to Merino, likely reflecting their shared Iberian ancestry (Fig. 1a, Table S1).

In preparation for mapping the polycerate phenotype, animals were assigned one of four horn classifications (Table 1, Fig. 2a–d). The majority of animals (68/164 or 41%) carried four horns without scurs, whereas the bulk of the remaining animals (35%) had two horns in common with many domestic sheep breeds and wild ovids (Table 1). A small number of Navajo-Churro ewes was polled (6%), facilitating an association analysis seeking to verify whether the polled gene in this breed is the same as recently described in other domestic sheep (Wiedemar & Drögemüller 2015). To conduct this analysis, polled and horned individuals (combining two- and four-horned animals of both breeds) were assigned as cases ($n = 10$) and controls ($n = 125$) respectively before allelic association testing was

Table 1 Frequency of horn phenotype and eyelid score in two breeds of sheep.

	Poll horn status				
Eyelid status	Polled	2 horn	4 horn	Other	Totals
Jacobs					
Normal (score 1)	0	54	53	8	115
Affected (score 2+)	0	0	9	2	11
Navajo_Churros					
Normal (score 1)	8	3	6	14	31
Affected (score 2+)	2	0	0	5	7
All animals (Totals)					
Normal (score 1)	8	57	59	22	146
Affected (score 2+)	2	0	9	7	18

The number of animals given is prior to the removal of four individuals with poor SNP quality that were excluded from the GWAS.

evaluated using the –ASSOC function in PLINK and a chi-square test. Animals with horn classifications other than those depicted in Fig. 2a,b were excluded ($n = 29$; Table 1). When ranked by *P*-value, four of the five top ranked markers were located at Mb positions 29.3–29.5 on OAR10, which contains the *RXFP2* gene. This result confirmed that the polledness trait in Navajo-Churro is associated with the same locus as other polled breeds (Table S2, Fig. S1).

A second case–control design GWAS was performed to investigate the genetic basis of four horns. For this analysis, two-horned sheep from both breeds were coded as controls ($n = 57$) and four-horned animals as cases ($n = 68$). Polled animals were excluded, along with 29 sheep displaying horn configurations other than those depicted in Fig. 2a,b. These included animals with five horns, fused horns or the presence of scurs. To control for genetic stratification arising from breed differences unrelated to the trait, the Cochran–Mantel–Haenszel test was applied using the –mh flag as part of the association testing before –log (*P*-values) were plotted in genomic order (Fig. 2e,f). Additional information describing this method for correcting stratification and other aspects of the GWAS methodology are given elsewhere (Purcell *et al.* 2007; Kijas *et al.* 2013). A single strong association signal was observed on sheep chromosome 2, indicating that the genetic architecture underpinning the production of four horns is likely to involve the action of a single gene. The 10 most strongly associated SNPs were all located in a region spanning Mb position 131.9–132.6 on OAR2 (Fig. 2e, Table 2). The most strongly associated marker (*OAR2_132568092*, $P = 3.72 \times 10^{-18}$) was located at Mb position 132.568. This variant, g.132568092T>C, displayed markedly different genotype frequencies between animals grouped by horn classification. Two-horned sheep had a high frequency of C/C homozygotes (>90%) compared with four-horned animals, which were mostly heterozygous (82%, Table 2). This is consistent with anecdotal information suggesting the polycerate trait is dominant. To identify genes in the associated region, SNP locations on genome assembly version OARv3.1 were compared with the available protein-coding gene annotation (Jiang *et al.* 2014). The closest gene to the peak SNP is *metaxin 2* (*MTX2*), located approximately 93 kb upstream at Mb position 132.6–132.7. The second closest gene is *HOXD1*, located approximately 251 kb upstream (at Mb position 132.8), which is a member of the HoxD cluster comprising 13 members spanning a 100-kb interval. Only two other protein-coding genes are annotated within 1 Mb of the peak signal (*HNRNPA3* and *NFE2L2*), and both are located around 800 kb upstream.

In addition to mapping the genetic determinant of four horns, we sought to explore aspects of the segregation of the eyelid abnormality illustrated in Fig. 1b–e. It is possible that the two conditions are developmentally independent and appear at high frequency due to the impact of inbreeding and genetic isolation. This is particularly relevant to the Jacob sheep breed, which is genetically separated from other breeds (Fig. 1a) and has comparatively low within-breed diversity (Table S1). This prompted a search for evidence of co-segregation between the two phenotypes using an analysis of the eyelid abnormality within each horn morphology class. This analysis returned a significant result, whereby the appearance of the eyelid abnormality differed according to the horn classification ($\chi^2 = 9.28$, $P = 0.009$). The frequency of affected animals was approximately 13% within four-horned sheep (9/68, Table 1) and higher within a small sample of polled individuals (2/10; Table 1). It is important to note the number of available polled animals was small, meaning additional examples are required to more accurately estimate the frequency of the eye abnormality within polled sheep. Importantly, zero cases were detected in two-horned sheep of either breed (0/57). The incidence of the abnormality did not significantly differ between male and female sheep ($\chi^2 = 0.53$, $P = 0.467$; Table S3). Together, this suggests the eyelid abnormality may be associated with departures from the normal development of two horns and that the two conditions are developmentally linked. The frequency of the eyelid abnormality was low, meaning additional cases are needed to validate any association. Although these are the first data indicating a pleiotropic effect of horn morphology in sheep, strong evidence has emerged from other ruminant species. In goats, the polled intersex syndrome arises from a deletion upstream of *FOXL2* that misregulates the gene's ovarian-specific expression, causing a female-to-male sex reversal in homozygous polled individuals (Pailhoux *et al.* 2001). Furthermore, gene targeting in goat demonstrated that *FOXL2* loss of function severely interferes with normal eyelid development (Boulanger *et al.* 2014) and that it is down-regulated in polled horn bud (Wiedemar *et al.* 2014). In addition, polled cattle carry eye changes affecting eyelash number and in some cases also eyelid hypertrichosis (Allais-Bonnet *et al.* 2013). Taken together, these studies

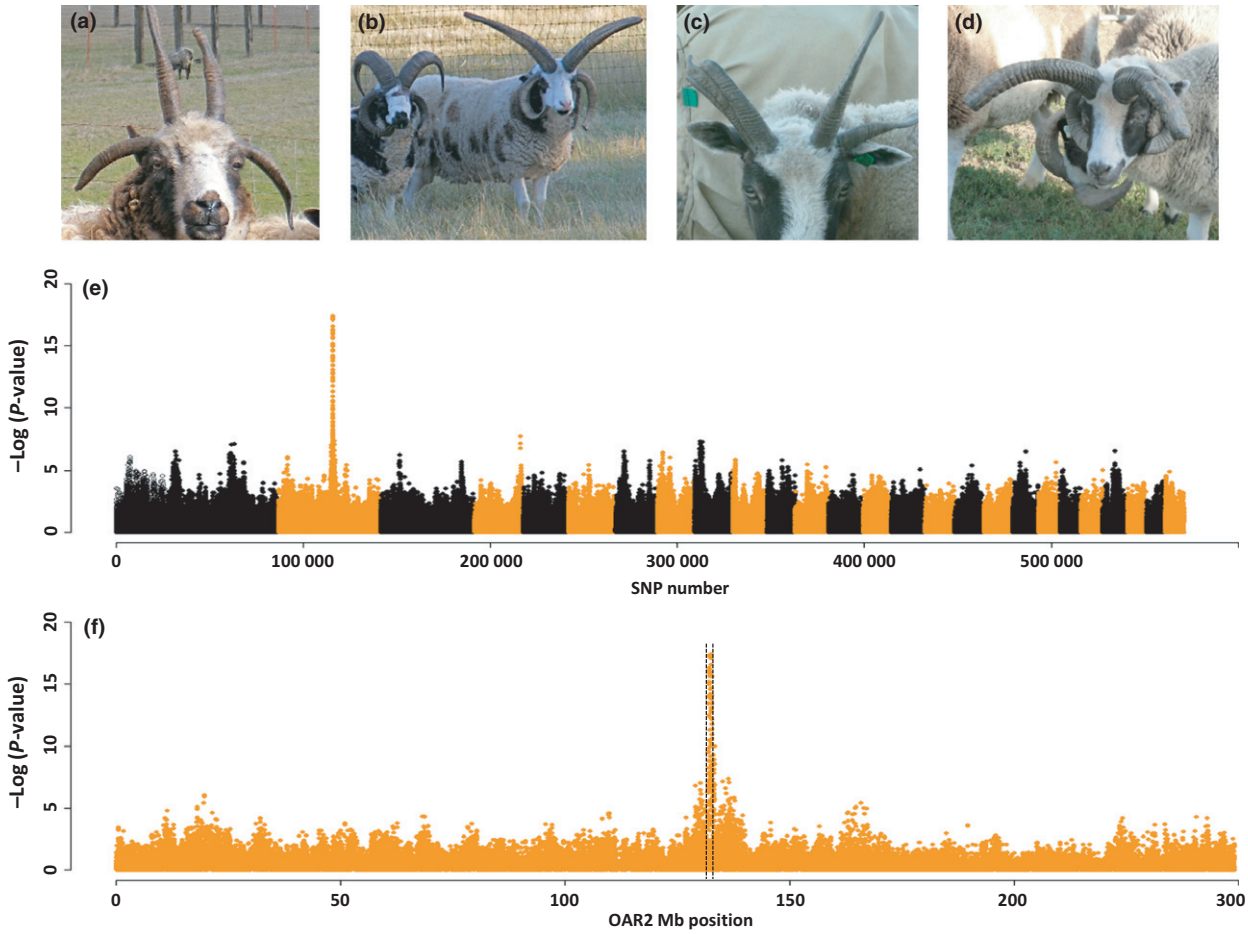

Figure 2 Examples of the polycerate trait and genome-wide association results. The polycerate phenotype presented with a range of expression. (a) Ewe with four horns positioned as two upper and two lower horns. (b) Jacob ram with two upper and two lower horns. Note the position of the upper horns displays a more forward position compared with the four-horned ewe. (c) A five-horned Jacob ram with three left-side fused horns. This animal is an example of the 29 individuals scored as 'other' in Table 1 and excluded from the GWAS. (d) Four-horned ram with pronounced forward positioning of the upper horns. (e) Genome-wide association results for all SNPs. The strength of association is given as –log (*P*-values) for loci plotted in genomic order. SNPs were coloured using black or orange to indicate odd and even numbered chromosomes respectively. (f) SNPs spanning approximately 300 Mb of sheep chromosome 2. The association peak is located at Mb position 132 and spans approximately 700 kb. The identity of the peak SNP is described in Table 2.

Table 2 Top-ranked SNPs associated for four-horned sheep.

SNP	BP	Allele	AF	AF_2H	AF_4H	*P*	log *P*	Bonf
OAR2_132568092	132 568 092	T	0.393	0.045	0.575	3.72E-18	17.4	11.7
OAR2_131989887	131 989 887	A	0.494	0.851	0.299	4.96E-18	17.3	11.6
OAR2_132002079	132 002 079	A	0.466	0.114	0.657	5.86E-18	17.2	11.5
OAR2_132555670	132 555 670	A	0.391	0.053	0.575	7.69E-18	17.1	11.4
OAR2_132574008	132 574 008	C	0.406	0.070	0.590	2.71E-17	16.6	10.8
OAR2_132018763	132 018 763	T	0.450	0.149	0.672	4.72E-17	16.3	10.6
s13300.1	132 138 166	A	0.456	0.158	0.679	6.10E-17	16.2	10.5
OAR2_132141156	132 141 156	A	0.456	0.158	0.679	6.10E-17	16.2	10.5
OAR2_132001419	132 001 419	G	0.422	0.114	0.627	8.56E-17	16.1	10.3
OAR2_132381448	132 381 448	C	0.447	0.132	0.649	1.03E-16	16.0	10.3

The 10 most strongly associated SNP for the four-horn trait are listed, ranked using the log *P*-value. The 10 SNPs span approximately 600 kb spanning Mb positions 131.9–132.6. The allele frequency (AF) across the set of Navajo-Churro and Jacobs sheep is given for the named allele and separately for the set of two-horned (AF_2H) and four-horned (AF_4H) sheep. The negative log *P*-value is given for both the unadjusted value (log *P*) and following Bonferroni single-step adjustment for multiple testing (Bonf).

demonstrate that genes involved in skin differentiation are also involved in horn morphogenesis in cattle and goats. The results presented here suggest this is also likely to be the case in sheep.

In summary, this experiment resolved the genomic location controlling the production of four horns in sheep to a small region on ovine chromosome 2. Thus, the polycerate phenotype is not due to an allele in series with the recently reported *RXFP2* variant associated with the natural absence of horns located on OAR10 (Wiedemar & Drögemüller 2015). Rather, the four-horn condition appears to be under the control of separate gene; however, no protein-coding genes are currently annotated within the associated region surrounding Mb position 132.5. This raises the likelihood that the causal mutation is regulatory in nature and acts to alter the function of a neighbouring gene or genes. Comparative data from other species indicates the HoxD cluster is the positional candidate most likely to be involved in horn ontogenesis. Much is known about the function of HoxD genes during limb development, and their role is to coordinate the twin processes of growth and patterning that influences body shape (reviewed in Zakany & Duboule 2007). Of particular relevance is the coordinated expression of four HoxD members (*HoxD10, HoxD11, HoxD12* and *HoxD13*) responsible for digit development and number (Delpretti *et al.* 2012). Any direct impact on horn bud differentiation in ruminants is currently speculative; however, the proximity of the HoxD cluster warrants additional experimentation. This study has advanced understanding of the complexity that underpins horn morphology in ruminants and serves as a prerequisite to the identification of the sequence variants responsible.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 GWAS for the polledness (hornless) trait.

Table S1 Genetic diversity compared between five sheep breeds.

Table S2 Top ranked SNP associated with polledness in Navajo Churro sheep.

Table S3 Distribution of the eyelid abnormality in either sex.