MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 2555–2566 doi: 10.1111/j.1365-294X.2011.05076.x

Genome-wide association mapping identifies the genetic basis of discrete and quantitative variation in sexual weaponry in a wild sheep population

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Abstract

Understanding the genetic architecture of phenotypic variation in natural populations is a fundamental goal of evolutionary genetics. Wild Soay sheep (*Ovis aries*) have an inherited polymorphism for horn morphology in both sexes, controlled by a single autosomal locus, *Horns*. The majority of males have large normal horns, but a small number have vestigial, deformed horns, known as scurs; females have either normal horns, scurs or no horns (polled). Given that scurred males and polled females have reduced fitness within each sex, it is counterintuitive that the polymorphism persists within the population. Therefore, identifying the genetic basis of horn type will provide a vital foundation for understanding why the different morphs are maintained in the face of natural selection. We conducted a genome-wide association study using ~36 000 single nucleotide polymorphisms (SNPs) and determined the main candidate for *Horns* as *RXFP2*, an autosomal gene with a known involvement in determining primary sex characters in humans and mice. Evidence from additional SNPs in and around *RXFP2* supports a new model of horn-type inheritance in Soay sheep, and for the first time, sheep with the same horn phenotype but different underlying genotypes can be identified. In addition, *RXFP2* was shown to be an additive quantitative trait locus (QTL) for horn size in normal-horned males, accounting for up to 76% of additive genetic variation in this trait. This finding contrasts markedly from genome-wide association studies of quantitative traits in humans and some model species, where it is often observed that mapped loci only explain a modest proportion of the overall genetic variation.

Keywords: genome-wide association study, horn morphology, quantitative trait locus mapping, *RXFP2*, sexual selection, wild population

Received 14 December 2010; revision received 13 February 2011; accepted 17 February 2011

Introduction

Revealing genes and genomic regions contributing to trait variation in wild populations creates the opportunity to understand why genetic variation is often maintained despite apparent directional selection (Merilä *et al.* 2001; Kruuk *et al.* 2008). This is particularly true in cases where different genotypes can cause similar phenotypes with unequal fitness (e.g. Gratten *et al.* 2008). Until recently, such studies were limited by a paucity of genetic resources and high costs of marker development and genotyping. However, the advent of next generation sequencing technology has led to a rapid expansion in the scale and affordability of genomic studies of wild populations (Ellegren & Sheldon

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2008; Dalziel *et al.* 2009; Slate *et al.* 2010). In particular, the detection of large numbers of single nucleotide polymorphisms (SNPs) provides a critical resource for identifying specific loci contributing to trait variation. The following study utilized newly available genetic resources to identify the genetic architecture of an inherited polymorphism for sexual weaponry in a wild vertebrate population, where low fitness phenotypes persist despite apparent directional selection. Mapping genetic variation to a specific candidate locus offers an unparalleled opportunity to estimate the effects of specific alleles on phenotype and provides a vital foundation for future studies examining the relationship between genotype and fitness.

Horns are a form of sexual weaponry present in all wild species of sheep and play an important role in competition between males for access to mates. The Soay sheep of St Kilda are a feral population of primitive domestic sheep, which have an unusual inherited polymorphism for horn type. The majority of males have large, normal horns with heritable variation in horn size (h^2 = 0.327; Johnston *et al.* 2010), whereas a small percentage (~12%) of males develop deformed, vestigial horns, known as scurs, which cannot be used in conflict for access to mates. Females have smaller horns and are either normal-horned (33%), scurred (39%) or polled (absence of any horn, 28%). Previous studies suggest that the horn-type polymorphism could be maintained by antagonistic selection between the sexes, with heritable variation in horn size maintained by the strength and direction of selection varying with fluctuating environmental conditions (Clutton-Brock *et al.* 1997; Preston *et al.* 2003; Robinson *et al.* 2006, 2008). However, scurred males and polled females have reduced fitness within each sex, so explaining why these phenotypes persist within the population remains an unresolved challenge.

The horn-type polymorphism is controlled by a single autosomal locus, *Horns*, which has been mapped to a ~7.4-cM region of chromosome 10 in Soay sheep (Johnston *et al.* 2010) and a 200-kbp region in domestic sheep (Montgomery *et al.* 1996; Pickering *et al.* 2009); *Horns* is also a strong candidate locus for a quantitative trait locus (QTL) with a large effect on horn size in normal-horned Soay sheep males, suggesting that then genotype at *Horns* may not only be responsible for discrete variation in horn type, but also for much of the quantitative genetic variation in horn size (Johnston *et al.* 2010). However, because of the low precision to which *Horns* has been mapped and the complex nature of the inheritance of *Horns* (Coltman & Pemberton 2004), a clear candidate gene has yet to be identified in Soays. Consequently, an understanding of the inheritance of horn phenotype, the effect size of the horn size QTL and the maintenance of the polymorphism has remained elusive.

In recent years, genomic studies in both domestic and wild sheep have benefitted from projects led by the International Sheep Genomics Consortium (ISGC, http://www.sheephapmap.org), which has included the preliminary assembly of the virtual sheep genome (Dalrymple *et al.* 2007) and the development of a SNP chip designed to probe more than 50 000 SNPs throughout the genome. The latter commercially available Ovine SNP50 BeadChip (Illumina Inc., San Diego, CA, USA) provides an extremely useful tool for gene mapping by genome-wide association studies (GWAS), particularly in wild populations such as Soay sheep, where such high resolution genotyping has not previously been available. The following study presents a GWAS for loci affecting discrete variation in horn type and QTL affecting quantitative variation in horn size, using the Ovine SNP50 BeadChip. We mapped *Horns* and a horn size QTL to a single candidate gene, Relaxin-like receptor 2 (*RXFP2*, UniProt accession number Q8WXD0) and confirmed that *Horns* not only controls horn type, but that it is also a QTL with a major contribution to heritable variation in horn size in normal-horned males.

Materials and methods

Study population

The Soay sheep of the St Kilda archipelago (Scotland, UK, 57°49′N, 8°34′W) are a primitive feral breed of domestic sheep, related to the Mediterranean and Asiatic mouflon, *Ovis aries orientalis* (Chessa *et al.* 2009). The study population in Village Bay, Hirta, was established in 1932 with the introduction of 107 sheep from the neighbouring island of Soay and has been studied on an individual basis since 1985 (Clutton-Brock *et al.* 2004). Each spring, at least 95% of lambs are caught, ear-tagged and sampled for genetic analysis.

Phenotype data set

Horn type, length and base circumference are recorded annually during a 2-week period in August (in which 49–67% of the study population are captured), during the rut in November and December, and/or after death. Horn types are defined as follows: normal horns are sturdy and consist of a bony core (*os cornu*) covered in a keratin sheath; scurred horns consist of keratin but lack an *os cornu* and are more loosely attached to the head, and a polled phenotype is a complete absence of visible horn growth. Occasionally, females do not develop scurs until several years after birth. Therefore, to maximize the accuracy of horn-type classification,

horn type in males was only categorized if captured during or after the rut of the year of birth (≥6 months of age), and females were only categorized if captured during or after the August catch the year after birth (≥16 months of age). Horn length was measured as the length (in mm) from the base of the horn, along the outer curvature to the tip, and base circumference was measured as the circumference (in mm) around the base of the horn at the closest point to the skull.

Soay sheep pedigree

The Soay sheep mating system is promiscuous, with individual females often consorting with multiple males. Maternal links were assigned through field observations and confirmed using molecular analysis, and paternal links were inferred through molecular analysis only (Overall *et al.* 2005). Paternity was assigned using Cervus 3.0 (Kalinowski *et al.* 2007), where males were assigned at >80% confidence, with no more than one mismatch between father and offspring or between the mother–father–offspring trio over the 14–18 microsatellite loci used. Genomic DNA was extracted from blood samples or from ear-punch tissue using DNeasy Tissue kit (Qiagen).

Modelling quantitative variation in phenotype using the Animal Model

Quantitative variation in horn size was modelled as a combination of fixed and random effects using a restricted maximum likelihood (REML) procedure known as the 'Animal Model' (Henderson 1975; Lynch & Walsh 1998; Kruuk 2004), incorporating correlations of effects between relatives to estimate additive genetic variance (and hence the heritability) of a trait. Fixed effects in the model included age (fitted as a nine-level factor, ages 0–8+) and capture period (fitted as a factor corresponding to the four main annual expeditions to St Kilda); random effects included an additive genetic effect (heritability); a permanent environment effect (animal identity effect grouping repeated measurements); birth year and capture year (accounting for variation attributed to specific environmental effects associated with these years). This model is referred to here as the 'polygenic' model. The variance components corresponding to random effects were estimated using ASReml v1.0 (Gilmour *et al.* 2002). The phenotypic variance (V_P) was calculated as the sum of the variances attributed to each random effect; the effect sizes of each random effect were then calculated as the proportion of V_P explained by the random effect, e.g. the additive genetic effect (or heritability, h^2) is calculated as the proportion of V_P explained by the additive genetic variance (V_A) i.e. $h^2 = V_A/V_P$. The significance of each random effect was tested by dropping the effect from the full model, and comparing the full and reduced models using a likelihood ratio test (LRT) distributed as chi-square with 1 d.f. Estimated breeding values (EBVs) for horn length and base circumference in normal-horned males with at least one measurement during their lifetime were calculated using best linear unbiased prediction (BLUP; Lynch & Walsh 1998). We have noted that simulation studies indicate that the estimation of EBVs though BLUP can be subject to bias and can lead to anti-conservatism in testing predictions (Hadfield *et al.* 2009). We describe why we are confident that our results are unbiased in the discussion and show in the supplementary information (Appendix S1, Supporting information) that using raw phenotypic values at a single age revealed qualitatively similar results.

GWAS for horn type and horn size

Single nucleotide polymorphism markers selected for the Ovine SNP50 BeadChip are evenly distributed throughout the genome with an intra-marker distance of mode 34.7 kb and median 44.4 kB. A total of 486 sheep were typed at 50 722 SNP markers on the Ovine SNP50 BeadChip using the Illumina Bead Array genotyping platform at Illumina Inc. (Teo *et al.* 2007). These sheep were originally selected based on the highest and lowest EBVs for hind leg length, a trait that is genetically unrelated to horn type and horn size (S. E. Johnston, unpublished data). A total of 35 831 SNPs of known position passed the quality control criteria (genotyping frequency >95%, minor allele frequency >0.05 and Hardy–Weinberg Equilibrium $P > 0.001$, calculated using PLINK v1.06; Purcell *et al.* 2007) and were included in further analysis. Performing a large number of tests for association with discrete and quantitative variation increases the chance of obtaining false-positive results. However, as many of the SNPs are in linkage disequilibrium (LD), not all tests are independent. We calculated the effective number of tests using the software K_{eff} v Sep 2007 (Moskvina & Schmidt 2008) specifying a sliding window of 50 SNPs; the threshold P-value for genome-wide association was calculated as $P = 1.859 \times 10^{-6}$. The positions of all SNPs associated with horn type and size were uploaded to the CSIRO Virtual Sheep Genome Browser v1.0 (http://www.livestockgenomics.csiro.au/) to determine their positions relative to known genes and protein coding regions in the human and cattle genomes. The r^2 statistic of LD was calculated between markers in regions highly associated with horn type using PLINK v1.06 (Purcell *et al.* 2007).

GWAS for discrete variation in horn type. Individual sheep were divided into five categories based on sex and horn type: normal males ($N = 160$), scurred males ($N = 12$), normal females ($N = 101$), scurred females ($N = 121$) and polled ($N = 51$) females. The nonrandom genotypic association between horn type and each individual SNP genotype was tested in all sheep using a chi-square test implemented in R v2.10.1. As the distribution of phenotypes between males and females is different, we also conducted a GWAS for horn type in females only. However, we did not conduct a males-only analysis, as the small number of scurred males ($N = 12$) enhanced the risk of obtaining false-positive associations between the SNP genotype and horn type in males.

GWAS for quantitative variation in horn size in normal-horned males. Individual EBVs for horn length and base circumference were extracted for normal-horned males genotyped on the Ovine SNP50 BeadChip where at least one horn measurement had been made during their lifetime ($N = 160$). Associations between SNP genotypes and EBVs were calculated using EMMAX v07Mar2010 (Beta), which uses mixed models to test association whilst accounting for population structure and relatedness between individuals (Kang *et al.* 2010), reducing potential biases and spurious associations that can be caused by the sampling of relatives or structured populations (Balding 2006).

Fine-scale association between candidate gene RXFP2 *and horn type*

Three assays (G100364, G100365 and G100366) were developed by AgResearch Ltd. (Invermay Agricultural Centre, Mosgiel, New Zealand) to genotype 56 SNPs spanning a region of ~406 kB across *RXFP2*, using a Sequenom MassARRAY® system with an iPlex amplification and extension protocol; these SNPs had not previously been included on the Ovine SNP50 BeadChip. Individuals from the Soay sheep mapping panel ($N = 564$, see Beraldi *et al.* 2006 and Johnston *et al.* 2010 for more information on the selection criteria) were genotyped, and SNPs were selected for further analysis based on the following quality control thresholds: Conservative/moderate call rate >85%; MAF >0.05; pedigree mismatches <2% per SNP, tested with PEDSTATS (Wigginton & Abecasis 2005); Hardy–Weinberg equilibrium $P > 1 \times 10^{-5}$.

The three most highly associated SNPs from the AgResearch assay (G100364-AS001072, G100365-AS001110, G100364-AS001071, named in this study as *SNP10*, *SNP11* and *SNP27* respectively) were typed in 3123-related individuals with available DNA samples using a multiplex SNP-SCALE protocol (Kenta *et al.* 2008; see Appendix S2, Supporting information for primer sequence information and PCR conditions). Pedigree mismatches were tested using PEDSTATS and were either resolved using pedigree information or by scoring problem individuals as untyped. Individual sheep were divided into five categories based on sex and horn type: normal males ($N = 1068$), scurred males ($N = 156$), normal females ($N = 221$), scurred females ($N = 281$) and polled females ($N = 168$), and genotypic association was calculated in all sheep using chi-square and Fisher's exact tests implemented in R v2.10.1.

Examining a QTL for horn size at Horns

To examine the contribution of *RXFP2* to genetic variance in horn size, an additional '*Horns*' animal model was constructed, fitting the genotype at *SNP10* (i.e. *CC*, *CT* or *TT* as predictors for *Horns* genotypes Ho^+/Ho^+, Ho^+/Ho^P and Ho^P/Ho^P respectively) as an additional random effect to the polygenic model outlined above. By comparing the likelihoods of the polygenic and *Horns* models, it was possible to determine (i) whether the *Horns* genotype had a significant effect on quantitative variation for horn size and (ii) the proportion of the phenotypic and additive genetic variance explained by *Horns*. An additional animal model was constructed with *Horns* genotype as a fixed effect rather than a random effect, to estimate the effect of each *Horns* genotype on horn size. The significance of *Horns* as a fixed effect was determined by resampling the genotype at *Horns* 1000 times.

Results

GWAS of discrete and quantitative horn phenotypes

The most significant association between SNP genotype and discrete horn type was with the marker *OAR10_29448537.1* on chromosome 10 (all sheep: $P = 2.05 \times 10^{-37}$; female sheep: $P = 2.98 \times 10^{-38}$; Fig. 1). The same locus also had the highest association with EBVs for horn length and base circumference in normal-horned males only ($P = 1.19 \times 10^{-8}$ and 4.53×10^{-10}, respectively; Fig. 2). This marker does not fall within any known or predicted gene sequences, but is ~43.3 kB downstream from the 3′ end of the closest gene, *RXFP2*, and ~175.2 kB upstream from the 5′ end of the gene *FRY* (*Drosophila* Furry). The second and third most highly associated SNPs in both discrete and quantitative analyses, *OAR10_29538398.1* and *OAR10_29511510.1*, were in LD with *OAR10_29448537.1* ($r^2 > 0.3$; Fig. 1b) and were immediately adjacent to *RXFP2*

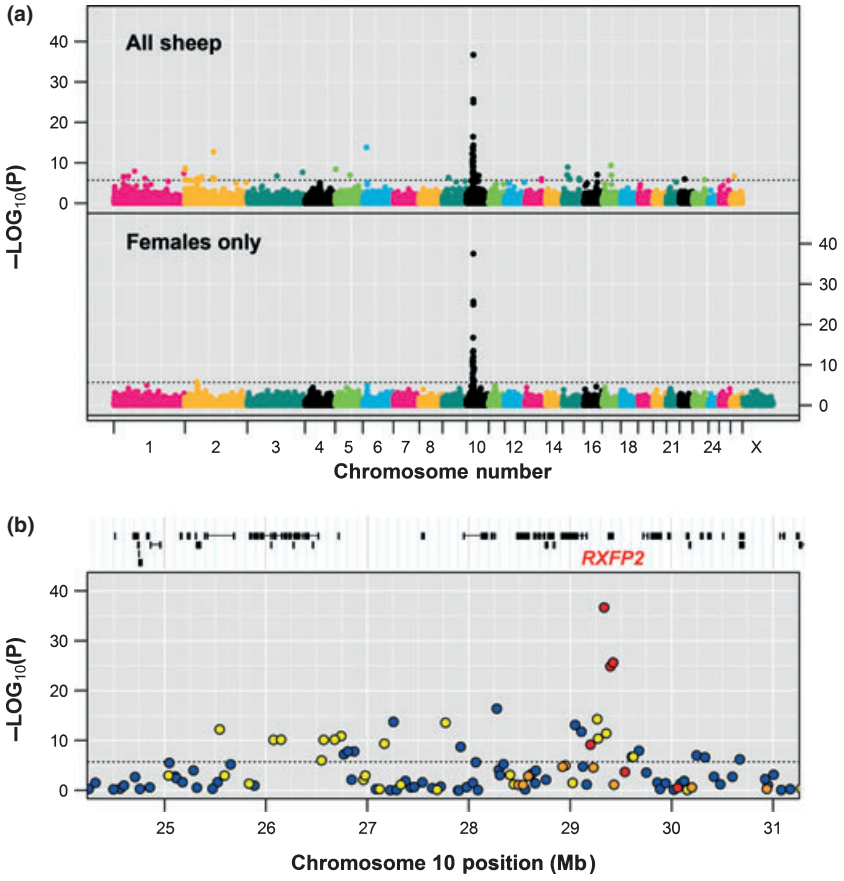

Fig. 1 Genome-wide association for horn type and linkage disequilibrium (LD) for chromosome 10 region of high association. (a) Manhattan plot of genome-wide association between Ovine SNP50 BeadChip single nucleotide polymorphisms (SNPs) and discrete horn type in all sheep and in females only. *P*-values were estimated using a chi-square statistic. Dotted lines indicate the significance threshold at $P = 1.859 \times 10^{-6}$ (equivalent to an experiment-wide threshold of $P = 0.05$), and all positions are relative to the Real Sheep Genome Assembly v1.0. Points are colour coded by chromosome. (b) The chromosome 10 genomic region with the highest association with horn type in both sexes combined. SNPs are colour coded based on LD with the most highly associated SNP, OAR10_29448537 (red: $r^2 > 0.3$, orange: $r^2 > 0.2$, yellow: $r^2 > 0.1$, blue: $r^2 < 0.1$). Known cattle protein coding regions and SNP positions are as displayed in the CSIRO Virtual Sheep Genome Browser v2.0.

(266 bases from the 5′ end) and within *RXFP2*, respectively. This indicates that *RXFP2* is the most likely candidate for *Horns* and for a QTL for horn length previously characterized within this region (Johnston *et al.* 2010).

Smaller but significant associations with discrete horn type were found at additional loci throughout the genome (Figs 1 and 2; a full list is given in the Appendix S3, Supporting information). In the analysis of all sheep, 65 further SNP loci were significantly associated with horn type; however, further examination found that 55 of these loci had a minor allele frequency of <0.1 in phenotyped sheep, and so we could not rule out a false-positive association because of chance over-representation of rare genotypes among scurred males ($N = 12$). In females, where sampling of all three horn phenotype classes was higher (the smallest class, polled, being $N = 51$), significant associations were only found within a 7.2-Mb region spanning *RXFP2*, with the exception of one marker on chromosome 2, *OAR2_49295397.1*, occurring ~126 kB upstream from the nearest gene, Contactin Associated Protein-like 3 (*CNTNAP3*; $P = 1.6 \times 10^{-6}$). This marker again had a low minor allele frequency within phenotyped females (MAF = 0.078), where 19 of 22 heterozygous females had normal horns and so was not pursued further in the current study.

In normal-horned males only, there was a significant association between base circumference and a SNP on chromosome 22, *OAR22_9161453.1*, which occurs within an intron of protein kinase type 1 (*PRKG1*; $P = 1.12 \times 10^{-6}$). This locus could be a further contributor to genetic variation in horn morphology among normal-horned males, although given its small effect relative to

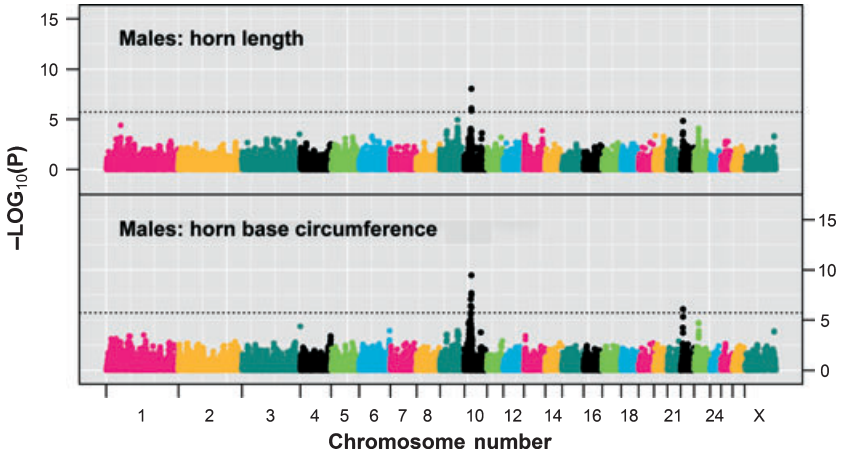

Fig. 2 Genome-wide association results for quantitative variation in horn size in normal-horned males. Results are based on association with single nucleotide polymorphisms (SNPs) and estimated breeding values for horn length and horn base circumference in normal-horned males ($N = 160$). *P*-values were estimated using EMMAX v07Mar2010 (Kang *et al.* 2010). Points are colour coded by chromosome. Dotted lines indicate the significance threshold at $P = 1.859 \times 10^{-6}$ (equivalent to an experiment-wide threshold of $P = 0.05$), and all positions are relative to the Real Sheep Genome Assembly v1.0.

RXFP2, it was not pursued further in the current study. With the obvious exception of the *RXFP2* region in domestic sheep (Montgomery *et al.* 1996; Pickering *et al.* 2009), there was no evidence of overlap between any significant loci identified in this study and loci known to influence horn development in other Bovid species, including goats and cattle (see Discussion).

Fine-scale association between RXFP2 *and horn type in all sheep*

Seventeen SNPs within and around *RXFP2* were associated with horn type in the Soay sheep mapping pedigree ($P < 0.05$, $N = 569$, Fig. 3). Three SNPs showed particularly high association in analyses of all sheep, females only and males only; these were *SNP10* (occurring within the flanking sequence at the 3′ end of *RXFP2*), *SNP11* (occurring within an intron of *RXFP2*, 11.4 kB upstream from *SNP10*) and *SNP27* (occurring within the 5′ flanking sequence of *RXFP2*, 46.4 kB upstream from *SNP11*; Fig. 3). *SNP10* showed the strongest association in all sheep ($P = 6.89 \times 10^{-44}$; Fig. 3), in females only ($P = 5.56 \times 10^{-33}$) and in males only ($P = 4.26 \times 10^{-15}$). *SNP11* was also strongly associated with horn type in females ($P = 4.51 \times 10^{-29}$), and *SNP27* was almost as strongly associated with horn type in males ($P = 8.41 \times 10^{-15}$). When typed in the full Soay sheep pedigree ($N = 3123$), *SNP10* showed the strongest association with horn type in all analyses (all sheep: $P = 1.402 \times 10^{-231}$; female sheep only: $P = 2.147 \times 10^{-164}$; male sheep only: $P = 1.043 \times 10^{-66}$; Table 1). The association observed between male horn

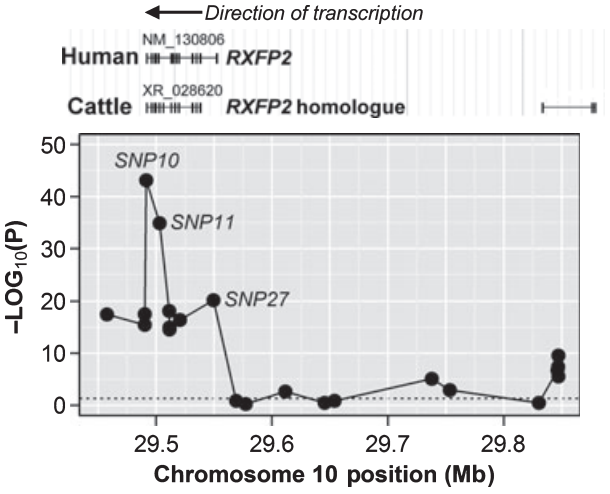

Fig. 3 Association between horn type and single nucleotide polymorphisms (SNPs) around *RXFP2* in the Soay sheep mapping panel. Association was tested between horn phenotype and 21 polymorphic SNP markers in the Soay sheep mapping panel. Homologous human and cattle protein coding regions are given above the graph and were obtained from the CSIRO Virtual Sheep Genome Browser v2.0. The relative SNP positions were estimated using the Real Sheep Genome Assembly v1.0. *P*-values were estimated using a chi-square statistic, and the dotted line indicates the significance threshold of $P = 0.05$.

type and *SNP27* was significant but lower than that of *SNP10* ($P = 5.931 \times 10^{-22}$).

Our findings, combined with evidence in domestic sheep (Pickering *et al.* 2009), imply that the *C* and *T* alleles at *SNP10* are in strong LD with the putative alleles Ho^{+} and Ho^{P} described in previous gene mapping papers (Dolling 1961; Coltman & Pemberton 2004), where Ho^{+} is a wild-type allele conferring normal

Table 1 Distribution of horn types for each *Horns* genotype in male and female Soay sheep

Sex	Horn type	Predicted *Horns* genotype		
		Ho^+/Ho^+	Ho^+/Ho^P	Ho^P/Ho^P
Males	Normal	279	542	141
	Scurred	3	26	116
Females	Normal	165	23	2
	Scurred	2	218	25
	Polled	2	25	127

horns, and Ho^P is a mutant allele conferring a scurred or polled phenotype in males and females, respectively. In females, *Horns* acts as an additive locus, where the genotypes Ho^+/Ho^+, Ho^+/Ho^P and Ho^P/Ho^P confer normal, scurred and polled phenotypes, respectively (Table 1). As there is some difficulty distinguishing between scurred and polled females in early life, it may be the case that many of the polled females with the genotype Ho^+/Ho^P are actually scurred females who did not, or have not yet developed scurs during their lifetime. In males, *Horns* appears to act as a dominant locus, where 97% of sheep with Ho^+/Ho^+ and Ho^+/Ho^P genotypes have normal horns, whereas Ho^P/Ho^P males develop horns or scurs at a ratio of ~1:1 (Table 1).

Association between Horns *and horn size in normal-horned males*

Length and base circumference were heritable (h^2 = 0.366 and 0.297, $P < 0.001$; Table 2). Fitting the *Horns* genotype as an additional random effect improved the models ($P < 0.001$, LOD = 55.22 and 53.39 for horn length and base circumference, respectively) and explained a significant proportion of the phenotypic variance in horn length and base circumference ($horns^2$ = 0.406 and 0.378, respectively), with the remaining heritability decreasing to h^2 = 0.142 and 0.119, respectively. Therefore, the genotype at *Horns* explains ~76% of the heritable variation in horn length and base circumference, confirming that *Horns* is a QTL with a major effect on horn size. Further models found that including maternal identity as an additional random effect did not improve the polygenic model (P = 0.09) and that there was no effect of *Horns* genotype on body weight (kg); relative to the intercept Ho^+/Ho^+, the effect sizes of Ho^+/Ho^P and Ho^P/Ho^P were 0.141 mm (SE = 0.218, t = 0.65) and −0.101 mm (SE = 0.303, t = 0.33). This confirms our finding in a previous study (Johnston *et al.* 2010) that there is no evidence that the *Horns* locus region contributes to overall body size in the Soay sheep population.

Table 2 Estimated random effect sizes for horn size in normal-horned males for polygenic and *Horns* models

Trait	Model	Mean (SD)	V_{obs}	V_P	Additive genetic (h^2)	*Horns* ($horns^2$)	Permanent environment (c^2)	Year of birth (b^2)	Year of growth (y^2)	Residual (ε^2)
Horn length	Polygenic	258.6 (130.1)	16 916	2715 (143.3)	0.366 (0.076)	NF	0.327 (0.074)	0.08 (0.032)	0.03 (0.012)	0.198 (0.013)
	Polygenic +*Horns*			3640 (1492)	0.142 (0.072)	0.406 (0.243)	0.213 (0.096)	0.069 (0.039)	0.022 (0.012)	0.148 (0.061)
Horn base circumference	Polygenic	137.0 (27.89)	777.98	321.8 (16.41)	0.297 (0.076)	NF	0.322 (0.073)	0.061 (0.026)	0.066 (0.024)	0.255 (0.017)
	Polygenic +Horns			414.5 (157.9)	0.119 (0.063)	0.378 (0.236)	0.209 (0.09)	0.048 (0.027)	0.048 (0.026)	0.198 (0.076)

Variance components, effects and standard errors were estimated using the program ASReml v1.0 (Gilmour *et al.* 2002). V_{obs} is the observed phenotypic variance, and V_P is the phenotypic variance defined as the sum of the variance components in the animal model. Each random effect is reported as effect size (i^2) for additive genetic (heritability), *Horns* genotype, permanent environment, birth year, capture year and residual effects, respectively; the effect size was calculated as the proportion of V_P attributed to each effect. Horn measurements are in millimetres (mm). Numbers in parenthesis are the standard error unless otherwise stated. Variance components were calculated from 1998 records in 937 genotyped individuals for horn length and 1979 records in 934 genotyped individuals for horn base circumference for which age and birth year were known. NF, random effect not fitted.

Effect of SNP genotype on horn dimensions in normal-horned males

In normal-horned males, the genotype at *Horns* had a significant effect on horn size ($P < 0.001$, Fig. 4). Relative to the intercept Ho^+/Ho^+, the effect sizes of Ho^+/Ho^P and Ho^P/Ho^P for horn length were −28.74 mm (SE = 3.049, $t = 9.41$) and −76.42 mm (SE = 4.326, $t = 17.66$), respectively; for horn base circumference, these were −10.74 mm (SE = 1.035, $t = 10.37$) and −25.06 mm (SE = 1.459, $t = 17.18$), respectively, indicating that the substitution of a Ho^P to a Ho^+ allele had an approximately additive effect on horn size, with the Ho^+ allele exhibiting slight dominance over the Ho^P allele. Separate analyses of lambs (age 0), yearlings (age 1) and adults only (age 2+) found the effect of the *Horns* locus on horn size was similar in all age classes. The *t*-statistics obtained from our animal model were higher than any statistic obtained from resampling the genotype at *Horns* 1000 times; even the most significant resampled data sets had a much smaller *t*-statistic (e.g. maximum of 3.46 for horn length) than the one we observed in our true data set. Therefore, differences in quantitative variation in horn size appear to be explained by *Horns*, rather than polygenic effects that are associated with *Horns* genotype because of other causes, such as cryptic genetic structure in the population.

Discussion

In this study, we have fine-mapped a locus underlying a Mendelian trait to a single candidate gene, *RXFP2*, and for the first time in Soay sheep, we can distinguish animals with identical horn phenotypes but different underlying genotypes. Furthermore, we have also shown that an associated quantitative trait is likely to have a surprisingly simple genetic basis, where the same locus explains a large proportion of the additive genetic variation in horn size in normal-horned males. This study provides a rare example of using GWAS to identify a strong candidate gene likely to be responsible for variation in a heritable trait in a wild nonhuman population. In this discussion, we propose a new inheritance model of horn phenotype in Soay sheep, examine the contribution of *Horns* locus to phenotypic and genetic variation in the population and discuss how a lack of perfect association may give an insight into the action of *RXFP2* upon horn phenotype.

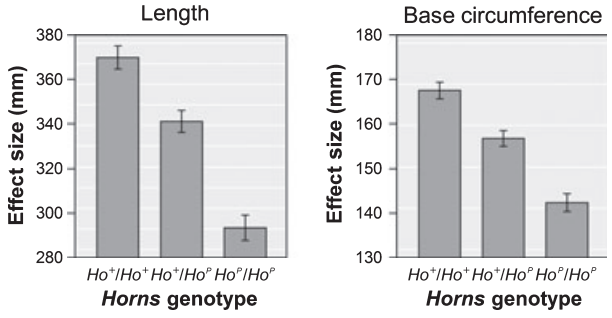

Fig. 4 Effect sizes of each *Horns* genotype on horn length and base circumference in normal-horned males. Effect sizes are given for each *Horns* genotype when fitted as a fixed effect in an animal model implemented in ASReml v1.0 (Gilmour *et al.* 2002). Effect sizes are given relative to the model intercept and are estimated based on 1998 records in 937 genotyped individuals for horn length and 1979 records in 934 genotyped individuals for horn base circumference. Fixed effects in the animal model included capture age, capture period and *Horns* genotype, and random effects included additive genetic, permanent environment, birth and capture year effects. Standard errors are given for each *Horns* genotype when fitted as the model intercept. The intercept of the model is for individuals captured during the August expedition at age 2 in the year 2008.

RXFP2 *as a candidate gene for* Horns

Our results show a strong association between horn phenotype and SNPs in and around *RXFP2*, and there are several reasons why we are confident that *RXFP2* is the principle candidate locus for *Horns*. First, associations between horn type and SNPs in the immediately adjacent genes are dramatically weaker (i.e. a difference in the *P*-value of at least 10^{-14}) in comparison to associations observed at *RXFP2* (Fig. 1, Appendix S3, Supporting information). Second, in male humans and mice, *RXFP2* expression is positively correlated with the blood concentration of testosterone, and mutations in *RXFP2* are associated with impaired testicular descent, reduced bone mass density and osteoporosis (Ferlin *et al.* 2008; Feng *et al.* 2009; Yuan *et al.* 2010). In Soay sheep, castration of ram lambs within 1 day of birth is known to have a profound effect on horn development, where horn phenotypes develop similarly to those of females, including polled phenotypes that have never been observed in noncastrated rams (Jewell 1997). This suggests that sex hormones play an important role in horn development. Therefore, it is feasible that a mutation affecting the function of a gene associated with male hormone levels and bone development, such as *RXFP2*, has an effect on horn type and size. Finally, the *Horns* locus in domestic sheep has been mapped to a 200-kb region of chromosome 10 in domestic sheep (Pickering *et al.* 2009), and a causal mutation at the *RXFP2* locus has been identified (N. K. Pickering and J. C. McEwan, unpublished data).

Lack of synteny with loci affecting horn morphology in other Bovid species

In this study, we found no overlap between loci associated with horn morphology in Soay sheep and those

known to control horn type in other Bovid species. For example, the *polled* locus in cattle has been mapped to a ~3-Mb region at the proximal end of cattle chromosome 1 in a number of breeds (Brenneman *et al.* 1996; Harlizius *et al.* 1997; Drögemüller *et al.* 2005; Wunderlich *et al.* 2006; Cargill *et al.* 2008), and a locus affecting scur development, *Scurs*, has been mapped to chromosome 19 in the Canadian Beef Cattle Reference Herd (Asai *et al.* 2004), although the same locus was not responsible for scurs in a French Charolais pedigree (Capitan *et al.* 2009). In the domestic goat (*Capra aegagrus hircus*), the polled condition mapped to the distal end of goat chromosome 1 and is closely linked to a gene associated with abnormalities in sex determination (Vaiman *et al.* 1996; Pailhoux *et al.* 2001). None of these regions are homologous to sheep chromosome 10 or any other region with a significant association with horn phenotype in this study. Therefore, the genetic control of horn morphology is likely to be controlled by different genes within the Bovidae.

Inheritance of horn type in Soay sheep

Our data suggest a new model for horn inheritance in the Soay sheep population, which is simpler than the three-allele model previously hypothesized (Coltman & Pemberton 2004). The new model is based on the assumption that the genotype at *Horns* is predicted by the observed genotype at *SNP10*. In females, *Horns* is likely to act as an additive biallelic locus, with Ho^{+}/Ho^{+} conferring normal horns, Ho^{+}/Ho^{P} conferring scurred horns and Ho^{P}/Ho^{P} conferring the polled phenotype, with some overlap in phenotypes occurring, particularly in Ho^{+}/Ho^{P} and Ho^{P}/Ho^{P} females. In males, *Horns* is likely to be a dominant locus, where Ho^{+}/Ho^{+} and Ho^{+}/Ho^{P} confer normal horns, but Ho^{P}/Ho^{P} confers either normal or scurred horns at a ratio of roughly 50:50. The presence of normal or scurred horns in Ho^{P}/Ho^{P} individuals may be a threshold trait determined by an underlying liability, which could be affected by additional genetic, prenatal and/or environmental factors (Falconer & Mackay 1996; Hartl & Clark 2007). Therefore, *Horns* explains discrete and quantitative variation in horn type to a large degree, but we cannot rule out significant associations observed in other parts of the genome that may explain the remaining polygenic variation in horn phenotype.

Does the lack of perfect association indicate that horn type is a threshold trait?

The SNPs most strongly associated with variation in horn phenotype are in LD and occur at the 3′ end of the gene, suggesting that the causal mutation occurs towards the end of the transcribed sequence. None of our SNPs occur with an exon or show a perfect association with horn type; therefore, it is unlikely that we have identified the causal mutation. This may be because of ancestral recombination (and therefore imperfect LD) between any causative mutation and the most highly associated SNPs. However, there may be other explanations as to why a perfect association does not occur. For example, given that the candidate locus *RXFP2* is a receptor for hormones associated with male primary sex characters (Feng *et al.* 2009), levels of *RXFP2* expression may explain most, but not all of an individual's liability for a particular horn phenotype (Falconer & Mackay 1996), indicating that horn type is a threshold trait. This is consistent with the observations that having more copies of the Ho^{+} allele results in larger horns in normal-horned males, and males with genotype Ho^{P}/Ho^{P} can express two distinct phenotypes; it may be that Ho^{P}/Ho^{P} males have a liability near to the threshold between scurs and normal horns. Nonetheless, a full understanding of the mechanism by which different alleles of *RXFP2* affect horn morphology will require further investigation.

Contribution of Horns *to quantitative genetic variance in horn morphology*

In this study, we refined our previous mapping work by increasing the confidence of a horn morphology QTL (from LOD 2.51 to >50) and by decreasing the confidence interval from 34 cM (Johnston *et al.* 2010) to a single gene. In addition, we can now understand the effect of each allele at the QTL, by showing that having more copies of Ho^{+} confers larger horns. QTL studies in wild populations often have low sample sizes and reduced power to estimate QTL effects, resulting in upwardly biased estimates of QTL effect size (Beavis 1998). Here, we increased the sample size from the previous QTL study more than fourfold (from $N = 217$ to 937), yet obtained similar estimates of the effect size of the *Horns* locus (horn length: $horns^2 = 0.406$, previous $QTL^2 = 0.392$; base circumference: $horns^2 = 0.378$, previous $QTL^2 = 0.237$). Therefore, we are confident that as much as 76% of the additive genetic variation in both horn length and horn circumference is attributable to the genotype at *Horns*. This finding is striking, as recent studies of quantitative traits in humans have found the largest QTL effect sizes to be small; in addition, much of the polygenic variation in human studies remains unmapped, contributing to the notion of missing heritability (Flint & Mackay 2009; Manolio *et al.* 2009; Ehrenreich *et al.* 2010; Slate *et al.* 2010). Our data form an interesting counter-example of this phenomenon. Therefore, it may be difficult to generalize about the number

and magnitude of genes affecting any quantitative trait, even for morphometric traits (such as horn length and circumference) that are typically regarded as being truly polygenic.

As repeated measures of horn length were available for many males, we used EBVs from animal models, rather than raw phenotypic data, in the GWAS analysis of horn quantitative traits. Recent simulation studies have indicated that estimation of EBVs through best linear unbiased prediction (BLUP) can be subject to bias and can lead to anti-conservatism in testing predictions (Hadfield *et al.* 2009). However, there is good reason to be confident that our results are unbiased. First, we only included EBVs of males who had actually been measured, and so the EBVs used here should be relatively robust. Second, our major gene for horn size was at the same SNP as the horn-type locus, and the probability of obtaining a false-positive result for horn size at this SNP rather than at one of the other 36 033 SNPs is very low. Finally, and most importantly, a GWAS study of horn size traits using raw phenotypic values at a single age revealed qualitatively similar results (see Appendix S1, Supporting information).

Future directions

As our evidence suggests that *Horns* may be a threshold trait, further fine mapping of this trait may fail to reveal any position that is perfectly associated with horn type in Soay sheep. Future data from the Ovine SNP50 BeadChip could indicate whether the same set of genes explain polymorphisms in horn phenotype in different breeds and species, as well as determine whether *Horns* contributes to genetic variation in other wild sheep species, especially those for which fitness data in the wild have been measured (Coltman *et al.* 2003; Poissant *et al.* 2008). Most importantly, this study has laid the foundation for investigating the relationship between *Horns* genotype and traits associated with fitness, providing a critical advance towards understanding the evolution and maintenance of the horn-type polymorphism within the Soay sheep population.

Acknowledgements

We thank Jake Gratten, Peter Visscher, Alastair Wilson, Philine Feulner and Benoit Auvray for valuable discussions regarding data analysis, some of which was performed on the Iceberg High Performance Computing server at the University of Sheffield. Research staff at AgResearch Ltd and Maria Elena Mannarelli provided guidance with laboratory work. Comments from the associate editor and two anonymous reviewers improved this manuscript. Permission to work on St Kilda was granted by National Trust for Scotland, and logistical support was provided by QinetiQ. Data from the OvineSNP50 BeadChip and draft genome were provided by the International Sheep Genomics Consortium and obtained from http://www.sheephapmap.org in agreement with the ISGC Terms of Access. S.E.J. was supported by a BBSRC CASE studentship.

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Supporting information

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

Appendix S1 GWAS study of horn size in normal-horned male traits using best unbiased linear prediction (BLUP) and raw phenotypic values.

Appendix S2 SNP-SCALE protocol for typing SNPs around *RXFP2*.

Appendix S3 GWAS Results: A list of all SNP markers significantly associated with discrete and quantitative variation in horn morphology.

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