Characterisation of major histocompatibility complex class IIa haplotypes in an island sheep population

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Abstract

The ovine MHC class IIa is known to consist of six to eight loci located in close proximity on chromosome 20, forming haplotypes that are typically inherited without recombination. Here, we characterise the class IIa haplotypes within the Soay sheep (Ovis aries) on St. Kilda to assess the diversity present within this unmanaged island population. We used a stepwise sequence-based genotyping strategy to identify alleles at seven polymorphic MHC class IIa loci in a sample of 118 Soay sheep from four cohorts spanning 15 years of the long-term study on St. Kilda. DRB1, the most polymorphic MHC class II locus, was characterised first in all 118 sheep and identified six alleles. Using DRB1 homozygous animals, the DQA (DQA1, DQA2 and DQA2-like) and DQB (DQB1, DQB2 and DQB2-like) loci were sequenced, revealing eight haplotypes. Both DQ1/DQ2 and DQ2/DQ2-like haplotype configurations were identified and a single haplotype carrying three DQB alleles. A test sample of 94 further individuals typed at the DRB1 and DQA loci found no exceptions to the eight identified haplotypes and a haplotype homozygosity of 21.3%. We found evidence of historic positive selection at DRB1, DQA and DQB. The limited variation at MHC class IIa loci in Soay sheep enabled haplotype characterisation but showed that no single locus could capture the full extent of the expressed variation in the region.

Keywords Major histocompatibility complex · Soay sheep · Haplotype · Balancing selection

Introduction

The major histocompatibility complex (MHC) is a genomic region containing highly polymorphic genes which encode cell surface proteins involved in the presentation of pathogen-derived peptides to T cells, enabling an immune response (Klein 1986). The highly complex and polymorphic nature of the MHC region has made it a focus of many studies in immunology and evolution; however, these features make it difficult to develop locus-specific assays to genotype individual loci. Over evolutionary time, mammalian MHC loci can be viewed as going through a birth and death process (Nei et al. 1997). New MHC loci are thought to be created through gene duplication events, resulting in multiple loci harbouring similar alleles. Some are lost through decay, producing pseudogenes and gene fragments, which are characteristic of mammalian MHC regions. Additionally, allelic diversity within many MHC loci is high, and selection may favour the maintenance of numerous and divergent alleles within a population (Wakeland et al. 1990; Lenz 2011). Genotyping the MHC region is therefore often particularly challenging, and locus-specific assays can difficult to develop, as multiple loci and pseudogenes may co-amplify with primers that are too generic, whilst allelic dropout may occur with primers that are too specific (Babik et al. 2009).

The MHC class II loci exhibit high linkage disequilibrium (LD) (Lee et al. 2012), and many studies take advantage of this by genotyping a single locus, assuming that it is representative of the full haplotypic diversity (e.g. Babik et al. 2005; Harf and Sommer 2005; Bollmer et al. 2007; Biedrzycka et al. 2011; Gelasakis et al. 2013; Kamath et al. 2014). However, despite high LD, selection favouring particular alleles could mean that some alleles at specific loci are identical across
different haplotypes (de Bakker et al. 2006; Traherne et al. 2006). For example, in sheep, an allele at one locus can be found in combination with multiple alleles at other loci (Hickford et al. 2007; Ballingall et al. 2015; Ali et al. 2016). Genotyping only a single locus may disguise variation at other loci, reducing the power to detect differing selection pressures. This highlights the need to characterise haplotypic variation when studying the MHC.

The unmanaged but intensively studied Soay sheep (O. aries) population on the island of Hirta, in the St. Kilda archipelago, Scotland, presents an excellent opportunity to study MHC selection in a large mammal. The Hirta population of Soay sheep originated from 107 animals which were translocated from the neighbouring smaller island of Soay in 1932 (Clutton-Brock et al. 2004), and the population has remained closed ever since. MHC variation within the study population was therefore expected to be limited compared with larger populations experiencing immigration. Since 1985, the long-term study of the Soay sheep has collected DNA samples, as well as life history and phenotype data, for many thousands of individuals (Clutton-Brock et al. 2004). Fully characterising the MHC class IIa haplotype variation within the Soay population might, therefore, be feasible.

The two major families of antigen-presenting MHC molecules are class I, which present endogenous antigens (primarily from intracellular pathogens) to CD8+ T cells, and class II which present exogenous antigens (primarily from extracellular pathogens) to CD4+ T cells (Klein 1986). Unusually, the class II region within ruminants is split into two distinct subregions, class Ia and Ib (Andersson et al. 1988; van Eijk et al. 1995), with the classical class II loci, which have previously been associated with parasite resistance (see Lee et al. 2011), clustered in the class IIa region. The class IIa loci include the highly polymorphic DRB1, DQA and DQB loci, as well as the less polymorphic DRA (Ballingall et al. 2010). Duplicated pairs of DQA and DQB loci have been identified in domestic sheep, Ovis aries (Scott et al. 1987; Wright and Ballingall 1994; Ballingall et al. 2015, 2018b). Three types of DQA alleles (DQA1, DQA2 and DQA2-like; Ballingall et al. 2015) and DQB alleles (DQB1, DQB2 and DQB2-like; Ballingall et al. 2018a, b) have been identified. DQA1 and DQA2 are known to be different loci, as are DQB1 and DQB2; however, the origins of the DQA2-like and DQB2-like alleles are less well defined. Typically, DQA2-like and DQB2-like alleles are found on haplotypes in conjunction with DQA2 and DQB2, and the DQA1 and DQB1 loci are absent. Therefore, the two typical haplotype configurations are DQA1 + DQA2 with DQB1 + DQB2 and DQA2 + DQA2-like with DQB2 + DQB2-like. Whether the DQA2-like and DQB2-like alleles represent independent loci or are simply divergent alleles at the DQA1 and DQB1 loci remains unclear (Ballingall et al. 2015, 2018b). A recent study by Ali et al. (2016) identified haplotypes with all three allele types, which would suggest that the DQA2-like and DQB2-like alleles are derived from independent loci.

A previous study of MHC variation in Soay sheep using the OLADRBI microsatellite located in the second intron of the class Ia locus DRB1 found evidence for selection acting on this region (Paterson et al. 1998). However, as outlined above, how well the single OLADRBI microsatellite locus represents diversity across the MHC class Ia region of the Soay sheep is unknown. Since the Paterson et al. (1998) study, single locus genotyping methods targeting the polymorphic regions of the classical class Ia loci have been developed for domestic sheep. These include DRB1 (Ballingall and Tassi 2010), DQA (Ballingall et al. 2015) and DQB (Ballingall et al. 2018b). In this study, we aim to (1) genotype a sample of Soay sheep at the classical DRB1 and DQ loci using sequence-based genotyping and (2) define the MHC class Ia haplotypes in the Soay sheep study population using animals identified as homozygous for each of the DRB1 alleles. Additionally, we aim to (3) look for evidence of positive selection acting on ovine MHC alleles during their evolutionary history. Characterising the MHC class Ia loci in this population will facilitate the development of a method to determine haplotypes for large numbers of individuals, enabling subsequent investigation of the evolutionary mechanism maintaining diversity within this region.

Methods

Study system

Monitoring of Soay sheep in the Village Bay area on Hirta has been carried out intensively since 1985 (Clutton-Brock et al. 2004), including catching lambs in spring for weighing, ear-tagging and sampling for genetic analysis. Most sheep are also caught in August, when phenotypic measurements, faecal samples for strongyle egg counts and blood samples are taken. During the August catches of 2012 to 2014, aliquots of blood were collected into Tempus™ Blood RNA Tubes (ThermoFisher Scientific).

Genomic DNA preparation

Genomic DNA (gDNA) was previously extracted from either peripheral blood or ear punch tissues using either the phenol/chloroform method (Bancroft et al. 1995) or QIAGEN DNeasy or QIAamp DNA Mini kits (QIAGEN, Dusseldorf, Germany) following the manufacturer’s protocol.
Sequence-based genotyping

DRB1 genotyping

The DRB1 locus is the best characterised MHC class II locus in O. aries, and the Immuno Polymorphism Database (IPD-MHC - https://www.ebi.ac.uk/ipd/mhc) contains over 100 ovine DRB1 alleles and corresponding allelic nomenclature. Locus-specific primers and a sequence based genotyping method, which targets the polymorphic second exon, have previously been developed (Ballingall and Tassi 2010). The primer pair 330_F and 329_R (Ballingall and Tassi 2010) was tested initially as it generates full DRB1 exon 2 sequences (Table 1). However, a 1-bp deletion in the DRB1*13:01 allele generated a mixed sequence from which the alleles could not be unambiguously identified. Therefore, the forward primer 455_F, a modification described in Corbishley et al. (2016) which sits downstream of the deletion, was used in preference (Table 1). Between 27 and 31 sheep were randomly selected from each of four cohorts (1993, 1998, 2003, 2008) and genotyped to identify DRB1 homozygous individuals for subsequent analysis of DQ diversity, with the expectation that DRB1 homozygotes were more likely to be homozygous at DQ loci due to linkage disequilibrium.

DQA genotyping

For each DRB1 allele identified, four DRB1 homozygous individuals were genotyped at the DQA loci (DQA1, DQA2 and DQA2-like). Individuals homozygous for the rare allele DRB1*10:01 were not identified from the initial DRB1 screen, and thus, DQA haplotypes were determined from heterozygous individuals. Primers DQA1_F and DQA1_R were used to amplify DQA1 and primers DQA2_F and DQA2_R were used to amplify DQA2 and DQA2-like (Table 1). For DQA2/DQA2-like homozygous individuals, the DQA1 primers were not expected to generate a PCR product and the DQA2 primers were expected to amplify both DQA2 and DQA2-like loci and therefore generate a product that was heterozygous in appearance.

DQB genotyping

DQB loci (DQB1, DQB2 and DQB2-like) were characterised in individuals which were both DRB1 and DQA homozygous. Primers DQB-F and DQB1-R or DQB2-R were used to amplify the DQB loci (Table 1). The DQB primers were not completely locus-specific and some cross-amplification was expected, depending upon the alleles present (Ballingall et al. 2018b). Due to the cross-amplification of loci, it was not possible to unambiguously identify DQB alleles from DRB1*10:01 heterozygotes. A subsequent screen of a much larger number of individuals (data not included here) was necessary to find individuals homozygous for the DRB1*10:01 allele, and the corresponding DQB alleles were identified from these homozygotes.

Table 1 Details of the primers used and their PCR conditions

| Target loci | DNA target | Primer name | Primer sequence | T_A | PCR cycle times (s) | Primer reference |
|-------------|------------|-------------|----------------|-----|---------------------|-----------------|
| DRB1        | gDNA       | 330_F       | ATTAGCCTCYCCCAGGAGKC | 55  | 30, 30, 30          | (Ballingall and Tassi 2010) |
|             | 455_F      | TATCCCCGTCTCTGCAGCAGCACATTTC | 58  |                  | (Corbishley et al. 2016) |
|             | 329_R      | CACCCCCGCGCTACCTCAGGCGCCGC | 55–58 |                | (Ballingall and Tassi 2010) |
| DQA1        | gDNA       | DQA1_F      | ACCTGACTCACCTGACCACA | 55  | 60, 60, 60          | (Ballingall et al. 2015) |
|             | DQA1_R     | AACACATACTGTTGGTAGCAGCA | 55  |                  |                  |
| DQA2 and 2-like | gDNA   | DQA2_F      | ACTACCAATCTCATGTGCTCCTCT | 58  |                  |                  |
|             | DQA2_R     | GGAGTAGAATGGTGGACACTTACC | 58  |                  |                  |
| DQA1 and 2  | cDNA       | 244_F       | GCTGAGMCCACACTTTGGAAGAG | 55  | 60, 60, 60          | (Ballingall et al. 2015) |
|             | 241_R      | GAGTAGTATCACGACTTTAAGTCC | 55  |                  |                  |
| DQA1, 2 and 2-like | cDNA | 348_F       | GAGGATGTTGCTCTACAACAGAGC | 55  |                  |                  |
|             | 357_R      | GAGGGAGCGCAAGAAGAAGAAGAAGA | 55  |                  |                  |
| DQB         | gDNA       | DQB_F       | CCCCGCAGAGGATTTCTCG | 58–60 | 30, 30, 30     | (Ballingall et al. 2018b) |
|             | DQB1_R     | CCGGCACCTCACCTCGGCGCTGC | 60  |                  |                  |
| DQB2 and 2-like | gDNA | DQB2_R      | ACGCTCACTCGCGCTGCC | 58  |                  |                  |
| DQB1 and 2  | cDNA       | 245_F       | TGGGTGTGACTACCATATTAST | 55  | 60, 60, 60       | (Ballingall et al. 2018b) |
|             | 248_F      | ACGCASSYATTAYAGAAGACG | 55  |                  |                  |
| DQB2-like   | cDNA       | 392_F       | ATTAGTGTTGCTTTTTTCT | 55  |                  |                  |
|             | 395_R      | AAAATATCTCGAGGCTGACG | 55  |                  |                  |
|             | 401_R      | CAAGAACACGCAGCTATTACA | 55  |                  |                  |
PCR amplification

PCR reactions were carried out in a final volume of 25 μL and contained 12.5 μL Promega GoTaq Green mastermix, 0.5 μM of each primer, approximately 25 ng genomic DNA and water. Cycling conditions were 94 °C for 5 min, then 35 cycles of 94 °C for 30 or 60 s, 55–58 °C for 30 or 60 s and 72 °C for 30 or 60 s (see Table 1) and a final extension of 72 °C for 5 min. PCR products were checked using gel electrophoresis on a 1% agarose gel.

RT-PCR amplification of full length DQ transcripts

Full-length transcripts were amplified from RNA to validate the previously unidentified sequences from genomic DNA. For individuals for which Tempus™ Blood RNA Tubes were available (see Study system), DRB1 and DQA loci were amplified from genomic DNA to identify homozygous individuals for RNA extraction. Total RNA was extracted from 3 mL of Tempus tube blood stored at −20 °C using the Tempus™ Spin RNA Isolation Kit (ThermoFisher Scientific) at half the recommended volumes. Reverse Transcription was carried out using the ImProm-II™ kit (Promega) with oligo(dT)15 primers. RT-PCR reactions were carried out using the primer combinations described in Table 1. No single primer set amplified all alleles. As RNA was not available for any individuals homozygous for haplotype H, F/H heterozygous individuals were used instead.

Cloning of multi-allelic PCR products

In order to phase novel alleles from heterozygous or multi-locus amplifications, PCR fragments were cloned into the pGEM-T easy vector (Promega). The presence of the correct insert was confirmed using colony PCR and plasmid DNA from 12 to 20 colonies was purified for sequencing using QIAprep Spin Miniprep Kit (Qiagen).

Sequencing and sequence analysis

PCR products were purified using exonuclease I and Antarctic phosphatase, except DQB2 PCR products which were gel-purified using the Macherey-Nagel Nucleospin Gel and PCR Clean Up kit, prior to Sanger sequencing. Alleles were sequenced using BigDye 3.1 chemistries on an AB 3730 genetic analyser.

Sequence analysis was carried out in Geneious 7.1.9. For DRB1 sequences, heterozygous peaks were called using the Heterozygote Plugin and checked by eye. DRB1 sequences were then compared to alleles from the IPD-MHC database using a custom BLAST. DRB1 genotypes were called when known alleles accounted for all variants within the sequence.

DQA and DQB sequences were also analysed using the Heterozygote Plugin within Geneious 7.1.9 to call heterozygous peaks. Due to multi-locus amplification, some sites contained three or four peaks, which were called by eye. A custom BLAST was used to compare sequences to an appropriate DQA database (Ballingall et al. 2015) or DQB database (Ballingall et al. 2018b). Genotypes were only called when all variants were accounted for by known alleles. Following cloning and RT-PCR of unknown alleles, the custom BLAST was updated to include the new alleles, and uncalled sequences were then compared to the updated database.

Nomenclature

The novel full-length DQA and DQB alleles identified here were named according to the nomenclature system described in Ballingall et al. (2015, 2018), were submitted to the European Nucleotide Archive (ENA; accession numbers LR025203-LR025213) and were included in the IPD-MHC database (Ballingall et al. 2018a). Genomic fragments that were considered too short to receive official nomenclature, i.e. those for which full-length transcripts could not be obtained, fell into one of two categories. Either, they matched an existing allele in GenBank that is also too short to receive official nomenclature, in which case, they were named after the accession number of the existing allele. Alternatively, the allele does not have a match in GenBank and was named according to the locus and haplotype designation and submitted to the ENA (accession numbers LR025788–LR025790).

Phylogeny of DQB

Due to the lack of locus-specific DQB primers, loci could not be determined for all amplified alleles, and thus, a phylogeny of the DQB sequences was generated to help clarify their DQB locus of origin. The DQB alleles sequenced were aligned using Clustal Omega (Sievers et al. 2011), along with previously identified DQB sequences from O. aries (Ballingall et al. 2018b), with domestic pig (accession NM_001113694.1.1) and human (accession M24364.1) DQB sequences as outgroups. Model selection for MrBayes, implemented in Topali v2 (Milne et al. 2009), selected the K80 model of DNA substitution (Kimura 1980) with gamma distribution. The phylogeny was generated in Geneious v7.1.9 using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) using 1,000,000 generations, with a burn-in of 25,000 generations.

Validation of haplotypes

To confirm that the haplotypes characterised in homozygous individuals were consistent, DRB1 and DQA loci were genotyped in an additional 95 sheep previously not genotyped by this study. These sheep were not first order relatives of each
other and were born between 1984 and 2010. These individuals were selected as they were genotyped on the Ovine Infinium HD SNP BeadChip for a previous study (Johnston et al. 2016). These individuals represent the maximum genetic variation within the population for the given number of individuals (Johnston et al. 2016).

**Positive selection analyses**

Positive selection acting over the evolutionary history of MHC alleles can be detected by comparing the ratio of non-synonymous (dN) to synonymous (dS) substitutions across sites (dN/dS) (Hill and Hastie 1987; Hughes and Nei 1988) and was assessed using CODEML implemented within the PAML4 package (Yang 2007). Analyses were carried out for DRB1 and for the duplicated DQ loci as all DQA loci combined (DQA1, DQA2, DQA2-like) or all DQB loci combined (DQB1, DQB2, DQB2-like) in order to maximise allelic sample sizes. As selection may differ between the duplicated loci, analyses were carried out separately for DQA1, DQA2, DQB1 and DQB2, although not for DQA2-like and DQB2-like because allele sample sizes were too small (n = 3 and n = 4, respectively). Alleles included in positive selection analyses are listed in Supplementary Table 2.

CodeML input neighbour-joining trees for each locus as described above were produced in Geneious v7.1.9 using HKY model and 5000 bootstrap replicates using O. aries alleles with assigned nomenclature (https://www.ebi.ac.uk/ipd/mhc; Ballingall et al. 2011, 2018a). The null hypothesis that neutrality is operating was first modelled for each locus using M1a in PAML, which assumes two possible classes of selection, ω₁ < 1 as estimated from the data and ω₁ = 1. This was then compared to the alternative hypothesis using model M2a in which positive selection is assessed using the two site classes considered under M1a as well as a third, ω₂ > 1 as estimated from the data. The model which best fit the data was inferred by calculating the likelihood ratio test (LRT) p value calculated as two times the difference between the models, compared to the chi-squared distribution with two degrees of freedom (Yang 2007; Posada 2009). Type I errors can be inflated when using the LRT with high levels of recombination, so the models M7 and M8 were also tested in PAML as they have previously been shown to be more robust to recombination (Anisimova et al. 2003). Model M7 is the null model of neutrality with a β distribution, and M8 as the alternative hypothesis, with β and ω. Codon frequency model 2 (F3x4) was used throughout. Additionally, positive site selection analyses were carried out within CodeML (Bayes Empirical Bayes (BEB) within model M2a) and within HyPhy using FEL and MEME methods.

**Results**

**Sequence-based genotyping**

**DRB1**

Six DRB1 alleles were identified among the 118 individuals from the four cohorts born in 1993, 1998, 2003 and 2008. All six alleles were represented in the IPD-MHC database and each allele was assigned to an individual haplotype (A–F; Table 2). Novel alleles identified by this study are highlighted in italics and accession numbers are shown in brackets.
Table 2). Allele frequencies are shown in Supplementary Fig. 1. In total, 33 (27.5%) \( \text{DRB1} \) homozygous sheep were identified, with five of the six \( \text{DRB1} \) alleles represented by a minimum of three homozygous individuals. Only \( \text{DRB1}*10:01 \) was not represented in homozygote form within the 118 sheep tested.

**DQA loci**

\( DQA \) genotyping of \( \text{DRB1} \) homozygous individuals and \( \text{DRB1}*10:01 \) heterozygotes provided evidence for an additional two haplotypes (G and H) (Table 2). The \( \text{DRB1}*01:01 \) and \( \text{DRB1}*22:01 \) alleles were each associated with two different \( DQA \) haplotypes. \( DQA1 \) primers failed to amplify a product in three haplotypes, (\( DQA1 \) null haplotypes, A, E and G), and a \( DQA2 \)-like allele was identified in each of these haplotypes.

Six \( DQA \) alleles did not match full length sequences described in (Ballingall et al. 2015), and only one of these (\( DQA1*Z28420 \) on haplotype C) matched a sequence on GenBank (accession number Z28420). Full-length transcripts (768 bp) were generated for six of these \( DQA \) alleles (Table 2).

\( DQA1 \)-null haplotypes (A, E and G) were not amplified. A single allele at each of the loci matched alleles in Ballingall et al. (2015). A further two alleles at each of these loci exhibited varying degrees of cross-locus amplification, dependent upon the alleles present (Supplementary Table 1). The \( DQB2 \)-like cDNA primers did not amplify any alleles from haplotypes known to carry a \( DQB2 \)-like allele, likely due to polymorphisms at the primer binding regions but perhaps due to poor quality RNA samples.

A single allele at each of the loci matched alleles in Ballingall et al. (2018b), and a further two alleles at each of these loci exhibited varying degrees of cross-locus amplification, dependent upon the alleles present (Supplementary Table 1). The \( DQB2 \)-like cDNA primers did not amplify any alleles from haplotypes known to carry a \( DQB2 \)-like allele, likely due to polymorphisms at the primer binding regions but perhaps due to poor quality RNA samples.
$DQB1$ and $DQB2$ matched alleles in GenBank (Table 2). The remaining alleles were novel and were assigned temporary nomenclature (Table 2). Full-length sequences for the two novel alleles on haplotypes E and H could not be generated, and full-length transcripts failed to amplify for the other five alleles in haplotypes C, D, F and H as shown in Supplementary Table 1. Three $DQB$ alleles were identified from haplotype G, two from gDNA only and one from cDNA only (Supplementary Table 1). Full exon 2 sequences were obtained for all $DQB$ alleles (Supplementary Fig. 3).

**Phylogeny of $DQB$ loci**

Phylogenetic analysis of the $DQB$ loci revealed three distinct clusters, corresponding to the three loci $DQB1$, $DQB2$ and $DQB2$-like (Fig. 1). All Soay $DQB$ alleles are located within one of the three clusters. Alleles from each haplotype fell into two different clusters, with the exceptions that haplotype G carried two alleles that clustered within $DQB2$, and a third that clustered most closely with $DQB2$-like. Haplotype C alleles were both located within the $DQB2$ cluster; however, it should be noted that both alleles only include exon 2 and not the 3′ UTR where the phylogenetic signal is strongest. Haplotype C alleles were therefore designated according to the primer set with which they were amplified.

**Validation of haplotypes**

Sequence-based genotyping across $DRB1$, $DQA$ and $DQB$ loci revealed eight haplotypes (Table 2). Direct sequencing of $DRB1$ and $DQA$ loci in 94 individuals did not reveal any deviations from the haplotypes identified using homozygous individuals. With the addition of the novel alleles on haplotypes C, F and H to the custom BLAST, alleles from all direct sequencing products could be determined, even from $DQA2/DQA2$-like heterozygous products from which four alleles co-amplified. Within these 94 individuals, 21.3% were homozygous.

**Positive selection and recombination analyses**

For all loci, the null model of neutrality was rejected and the models incorporating positive selection were selected (Supplementary Table 3). Models M2a and M8 (positive selection) both fit the data significantly better than M1a and M7 (neutrality). However, after applying Bonferroni correction for multiple tests at $DQA$ and $DQB$, ($\alpha$-threshold becomes 0.017), the M1a and M7 models of neutrality are a better fit at $DQA2$. Additionally, positively selected sites were identified at all loci (Fig. 2), with 12 sites identified at $DRB1$, 14 at $DQA$ and 13 at $DQB$, although only 10, 5 and 6 sites, respectively, were detected by more than one method. The majority of PSS at all three regions, $DRB1$, $DQA$ and $DQB$, have also been identified as antigen binding sites in human homologues. Results of separate PSS analyses for $DQA1$ and $DQA2$, as well as $DQB1$ and $DQB2$, are shown in Supplementary Fig. 4 and found fewer PSS than when loci were combined with fewer overlapping PSS at $DQA1/DQA2$ than for $DQB1/DQB2$.

**Discussion**

Six $DRB1$ alleles in Soay sheep were identified, which matched sequences previously identified in commercial Scottish sheep breeds and held in the IPD-MHC database. The level of homozygosity at the $DRB1$ locus was 27.1% of 118 individuals, which is higher than previously observed in other breeds of sheep (Stear et al. 2005; Hermann-Hoesing et al. 2008). However, at the MHC class IIa haplotype level, this is an over estimate of homozygosity as some individuals homozygous for $DRB1*01:01$ or $DRB1*22:01$ are heterozygous at $DQA$ and $DQB$ loci. The level of MHC class IIa haplotype homozygosity reduced to 21.3% in the 94
validation samples genotyped across the DRB1 and DQ loci. Note that these samples were originally selected to maximise representation of Soay sheep diversity, so it is possible they are not totally representative of the population as a whole. No dramatic change in DRB1 allele frequencies was observed over time (Supplementary Fig. 1), but sample sizes were too small to analyse temporal trends statistically. Further investigation at the population level will provide better estimates of the level of homozgyosity and whether haplotype frequencies have changed over time within the Soay sheep population.

DQA and DQB genotyping revealed a total of eight MHC class IIa haplotypes in the Soay sheep. DRB1 and DQA genotyping from MHC class IIa homozygous animals was relatively straightforward due to locus-specific primers (although the DQA2 primer pair did co-amplify DQA2-like alleles). DQB primer pairs, however, showed varying levels of cross-amplification depending upon the alleles carried by the haplotype, which made phasing of alleles challenging without extensive cloning. The limited amount of MHC class IIa haplotype diversity has made identifying variation at the DQA and DQB loci from homozygous animals possible. Furthermore, assigning DQB alleles to loci was only possible using phylogenetic analysis. DQB genotyping would be improved by further development of locus-specific primers, although this is challenging due to limited genomic sequence in this region and the lack of locus-specific characteristics in exon 2 (van Oorschot et al. 1994; Wright and Ballingall 1994). Both known DQ haplotype configurations (DQ1 + DQ2 and DQ2 + DQ2-like) were identified, as well as a novel configuration (two DQB2 alleles with DQA2 + DQA2-like + DQB2-like on haplotype G) in Soay sheep. Both DQB2 alleles on haplotype G were phylogenetically clustered within the DQB2 group (Fig. 1) and feature DQB2 locus-specific 3’ UTR sequences. The two DQB2 alleles were amplified from either cDNA (DQB2*12:01) or gDNA (DQB2*09:01). Whilst this might suggest that the DQB2*09:01 allele is not transcribed, many of the DQB alleles identified from Soay sheep failed to amplify from cDNA, including both DQB2-like alleles. Previous work has shown that all DQA and DQB loci can be expressed (Ballingall et al. 2018b), including DQB2-like*01:01 which could not be recovered from cDNA here. It is possible that the alleles we were unable to retrieve from cDNA were not expressed or were expressed at undetectable levels within the peripheral blood samples tested or in the haploptic combinations analysed, although it perhaps more likely reflects a combination of low cDNA quality and diversity in the primer binding sites. Whether the DQB2*09:01 allele is transcribed or not remains uncertain, but it cannot be ruled out that haplotype G carries three DQB loci. A previous study of MHC class IIa haplotypes using genomic DNA in Texel sheep identified a haplotype with three DQA and three DQB loci and another with two DQA and three DQB loci (Ali et al. 2016). Non-specific transcript analysis, such as RNAseq, would be valuable in assessing if all loci and alleles are transcribed. However, variation in the number of DQ loci and DQ haplotype configurations may be more common than previously thought in O. aries.

An unusual allele was identified on haplotype A which was identical to the DQB-E1 sequence described by Herrmann-Hoessing (Unpublished, Accession number HQ728697.1) and described here as DQB2-like*03:01. This sequence, clustered with DQB2-like alleles, did not carry the single codon deletion within the second exon described in other DQB2-like alleles (Ballingall et al. 2018a, b) but does feature the typical DQB2-like*03:01 sequence (see Ballingall et al. 2018a). As haplotype A carries the DQA2 + DQA2-like configuration, it would be expected that it also carries the DQB2 + DQB2-like configuration. Allele DQB2-like*03:01 appears, therefore, to be a divergent DQB2-like allele. Recent developments in long read sequencing technologies, such as Oxford Nanopore or PacBio, would be valuable in determining which alleles are separate loci and which are divergent alleles, though high-quality, long-stranded DNA might be more easily obtained from domestic sheep not living on an isolated island.

Numerous alleles were shared among the MHC class IIa haplotypes within the Soay sheep. Pairwise alignment of the predicted amino acid sequences of second exons of the DRB1, DQA and DQB loci within each haplotype identified the greatest overall amino acid similarity between haplotypes A, G and A, E. However, the functional similarity of alleles is difficult to assess without detailed information on the diversity of peptides presented by each MHC molecule.

Variation in MHC class II DQ molecules is formed from the combination of α and β chains. DQ molecules are therefore the products of polymorphic DQA1 + DQB1 and DQA2 + DQB2 genes (Ballingall et al. 2018b). DQA2-like + DQB2-like combinations are likely to provide additional functional diversity as this combination has been shown to express at the cell surface following co-transfection (Ballingall et al. 2018b). Haplotypes E and G share the same DQA2*01:02:01 + DQB2-like*01:01:01 + DQB2-like*01:01:01 allelic combination, so both haplotypes will generate the same DQ2-like molecule. Therefore, an individual homozygous or heterozygous for haplotypes E and G might have fewer DQ molecules compared to other haplotype combinations. On the other hand, haplotype G carries two DQB2 genes. If both are expressed and capable of forming functional molecules in combination with DQA2*01:02:01, it would have increased DQ molecule diversity. Intra-haplotype pairing of different DQA and DQB gene combinations may also provide additional class II molecules; however, not all allelic combinations are necessarily capable of generating functional molecules (Ballingall et al. 2018b).

The eight haplotypes characterised here are likely to be representative of all MHC class IIa variation in the Soay sheep. The DRA locus, which shows only limited allelic
diversity in *O. aries* (Ballingall et al. 2010), was not genotyped here. It is unlikely that genotyping the DRA locus would have further subdivided any haplotypes. Whilst the haplotypes from only a small number of primarily homozygous animals were characterised, the haplotypes were validated at the DRB1 and DQA loci in an additional 95 animals, and no new allelic combinations were identified. The DQB loci remain to be validated, and potential variation in the number of loci means that there is an unknown fraction of missing variation (Babik 2010). It is not certain that every haplotype has been detected; however, the extensive sequencing of multiple loci carried out throughout this study means that only alleles that occur at very low frequency will have been missed from our analysis.

Across all loci tested here, positive selection was found to be a more likely explanation for the observed diversity than neutrality, even comparing models M7 and M8 which are considered to be relatively robust to recombination (Anisimova et al. 2003). The strength of the signal was weakest at DQA2 (M2a and M8 p = 0.027), which is no longer significant after applying Bonferroni correction. Positively selected sites were also identified at all loci. Multiple PSS algorithms were tested due to their differing abilities to detect PSS under various selection scenarios, and as predicted, sites were not consistently detected by the three methodologies. The greatest variability was found at DQA and DQB (Fig. 2), which was not resolved by analysing the duplicated loci separately (Supplementary Fig. 4). This may be due to the complex nature of selection acting on the MHC, which could vary in space or time according to the parasite communities a population is exposed to, and therefore, different antigen-binding sites could be expected to experience different levels of continuous or episodic positive selection which are best detected using different algorithms. Recombination is unlikely to have a strong effect on PSS analyses (Anisimova et al. 2003). Nevertheless, there was a high level of congruence between PSS and antigen-binding sites in human homologues. This strongly suggests that there is a strong signal of positive selection within ovine MHC class IIA exon 2 loci, which is known to contain the antigen binding sites.

Whilst the DRB1 locus remains the most polymorphic ovine MHC class II locus (106 DRB1 alleles, compared to 27 DQA and 34 DQB alleles), by characterising the haplotypes within the Soay sheep, we are able to identify extensive variation at each of the DQ loci. The limited number of known alleles at the DQ loci compared to DRB1 is due to the development of effective locus-specific primers for DRB1 (Ballingall and Tassi 2010), whilst genotyping DQ allelic diversity has proved more challenging due to gene duplication and haplotype diversity. In contrast to the ovine DR molecule which is formed from the highly polymorphic DRB1 and the relatively non-polymorphic DRA (Ballingall et al. 2010), at least two ovine DQ molecules are formed from combinations of the polymorphic and duplicated DQA and DQB loci (Scott et al. 1987; Wright and Ballingall 1994; Ballingall et al. 2015, 2018b). Additionally, diversity within the DQ molecules may be achieved by combining the products of two DQA loci with two DQB loci, although not all combinations were able to produce detectable MHC class II molecules on the surface of transfected cells (Ballingall et al. 2018b). The allelic diversity observed at the ovine DQ loci suggests that it is likely to be important in immune recognition of pathogens and the loci subject to corresponding selection pressures. Without haplotype characterisation, the full complement of MHC class IIA variation cannot be detected, which may limit the ability of subsequent evolutionary analyses to detect relationships between genotypes, phenotypes and fitness and therefore elucidate the evolutionarily mechanisms maintaining the high levels of diversity observed at the ovine MHC class IIA region.

Eight MHC class IIA haplotypes have been identified in the Soay sheep population through characterisation of allelic diversity at the DRB1, DQA1 and DQB1 loci. Additionally, PSS were identified at all three gene regions, which are likely to be involved in antigen-binding given their correspondence to antigen-binding sites in human and murine homologues. The diversity observed within the Soay sheep population is reduced compared to studies of domestic sheep (Ali et al. 2016; Koutsogiannouli et al. 2016), and, using this in-depth knowledge of the MHC class IIA haplotype diversity within the Soay sheep population, it should now be possible to develop a rapid SNP-based genotyping system in order to generate large-scale population data across the more than 30-year study of Soay sheep on St. Kilda. This would greatly facilitate analyses of evolutionary processes underlying the maintenance of the variation in the MHC class IIA region.

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