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Assessment of Genetic Relationship between Six Populations of Welsh Mountain Sheep using Microsatellite Markers

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ABSTRACT: This study investigated the genetic relationship between 6 populations of Welsh Mountain sheep: 5 phenotypic breed-types within the Welsh Mountain (WM) sheep breed, which have each been bred in specific geographic areas of Wales, and the Black Welsh Mountain sheep breed. Based on DNA analysis using 8 microsatellite markers, observed heterozygosity levels were similar to those expected in livestock populations subjected to selective breeding (0.530–0.664), and all but one population showed evidence of inbreeding. Using Bayesian cluster and Neighbor-joining analyses, the Black Welsh Mountain sheep were identified as being the outlier group, and the remaining groups could be categorized into five distinct sub-populations, which reflects the geographical separation seen between these populations.

Keywords: sheep; interbreed relationship; population biology; phylogeography

INTRODUCTION

The native Welsh Mountain (WM) sheep are typically a small and hardy breed and account for the largest proportion of the estimated 4.3 million breeding ewes in Wales, kept across almost 15 000 sheep holdings (Hybu Cig Cymru 2014). They have been bred to survive in some of the harsher environments and climates in the UK, including areas of relatively poor-quality pasture. Due to the topology of Wales, with clearly defined geographical features (e.g. Snowdonia, the Cambrian Mountains, the Brecon Beacons) several different breed types of WM sheep exist across Wales, largely restricted to their respective specific local geographic areas, where the breed type was historically developed prior to more recent significant movements of livestock around the country (Carson et al. 2009). There is a belief that local breed types are best suited to the region they originate from, and that breed types will thrive less well when not in their native area. This belief, suggesting that each local breed or breed type is selectively adapted to its specific environment, is thought to have contributed to some degree of genetic isolation of breed types relative to the general WM flock. Historic reference was made to different "local varieties" of WM sheep by Wood (1937) in his report on sheep management in Great Britain, and subsequently by Duckham (1963). Wood clearly distinguishes between the "South Walien" type Welsh Mountain (likely to be the origins of the Nelson/South Wales type known today) and the WM sheep observed in other areas. He also identifies that the Welsh Mountain Pedigree flock at this time was mainly kept in the lowland areas, and suggests that these sheep are distinct from their upland relatives and no longer suited to life in the hills.

In general, most of the WM sheep are predominantly white fleeced, although one example exists of a black fleeced breed (the Black WM breed), with a conformation very similar to the other WM sheep types except for its black fleece colour. In this example, the black fleece colour is known to be caused by an allele which is dominant to the white allele (Roberts and White 1930), unlike most

other breeds where black colouration is recessive to the white one.

Detailed population data for individual WM breed types are not consistently recorded. Figures for WM sheep stated in the DEFRA report estimate the UK population at around 1.9 million breeding ewes, and this figure will consist largely of the North Wales (NW) Mountain type along with smaller local WM breed types such as the Tregaron type. Statistics on the UK sheep population available from DEFRA (2003) estimate the UK population of Nelson/South Wales (SW) Mountain type breeding ewes to be around 73 500 and the Black WM sheep to have 8060 breeding ewes. Both the Llandovery Whiteface and Black WM sheep are registered as "at risk" with the UK Rare Breed Survival Trust, meeting the criteria for an "at risk" population with less than 10 000 breeding ewes registered (DEFRA 2014).

Genetic diversity within farm livestock is needed to ensure adaption to changing production requirements and environments, and to allow continued breed improvements (Groeneveld et al. 2010). It is also essential to maintain genetic diversity to avoid the negative effects of inbreeding on production and health traits (Notter 1999; McParland et al. 2007; Carrillo and Siewerdt 2010). Human management influences selection and mating decisions in domesticated livestock, and examples exist of using animals (generally sires) from other breeds or types as a way of introducing desired traits, and in so doing, genetic diversity within the breed.

A number of studies have been conducted into the genetic diversity of farm livestock (e.g. SanCristobal et al. 2006; Granevitze et al. 2007; Medugorac et al. 2009) including European sheep breeds (e.g. Arranz et al. 2001; Dalvit et al. 2008; Ligda et al. 2009), but British breeds have generally not been included in these analyses. Examples of investigations including hill breeds, or breeds selected for relatively harsh conditions, from the UK include Lawson-Handley et al. (2007) and Bowles et al. (2014), with the former including an example of a Welsh breed – the Llanwenog. The only other example of inclusion of a Welsh breed in an investigation was the North American population of Black WM sheep (Blackburn et al. 2011), although these sheep may no longer be directly comparable to the population still found in Wales.

The current study investigates the extent of genetic diversity between the different types of WM sheep using microsatellite markers, to determine if there is any genetic basis for the suggestion that local breed types are continuing to persist in specific geographic areas, and if the different types of WM sheep identified are genetically distinct, how the populations are related to each other.

MATERIAL AND METHODS

Sample collection. In addition to the Black WM breed, five further breed types were included in this study: the Tregaron type, Llandovery Whiteface type, the NW Mountain type, the Nelson (SW Mountain) type, and the Pedigree Welsh Mountain section.

For each breed type, buccal swab DNA samples were collected from 3–5 farms and 4 sheep per farm were used for analysis. In order to obtain representative samples for each breed type, DNA was collected from different flocks, and efforts were made to avoid sample collection from closely related individuals within each flock. Sample collection was performed on-farm using Catch-All™ Sample Collection Swabs (EPICENTRE® Biotechnologies, Madison, USA) by rubbing the swab against each of the sheep's cheeks approximately 20 times. The swabs were frozen and were stored at –80°C until required.

DNA isolation. DNA extraction from buccal samples was harvested using a BuccalAmp™ DNA Extraction Kit (EPICENTRE® Biotechnologies) according to the manufacturer's instructions and samples were stored at –20°C until DNA purification. DNA was then purified using a Qiagen DNeasy® Plant Kit (Qiagen, Crawley, UK), following the Plant Tissue Mini Protocol, as per the manufacturer's instructions from the AW1 buffer incubation onwards, with an additional 60 s centrifugation step at 14 000 g of the minispin column after the AW2 wash, and 30 µl of buffer AE used for each elution. DNA was then quantified using a ThermoScientific NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and ND-1000 software (Version 3.17, 2006) on the nucleic acid setting. A DNA concentration of ≥ 4.0 ng/µl was deemed sufficient to use for PCR. Samples which fell below this were purified again. Eluted DNA from extractions was stored at –20°C until required.

Primer selection and PCR conditions. Eight microsatellite markers were identified from a ranked list of recommended markers for use in sheep popu-

lation genetics studies published by the ISAG/FAO advisory group on animal genetic diversity as part of their project Measurement of Domestic Animal Diversity (MoDAD) (Hoffmann et al. 2004), and the FAO document on Molecular Genetic Characterisation of Animal Genetic Resources (FAO 2011) which provided details of known allele ranges and primer sequences. Markers were cross-referenced to the FAO 2011 document to ensure all selected markers were on separate chromosomes and had suitable allele ranges. Oligonucleotide primers with 5' fluorescent TET labels were purchased from Sigma-Aldrich Ltd. (Gillingham, UK). Details of the sequences of the oligonucleotide primers together with annealing conditions and the number of PCR cycles are shown in Table 1. PCR conditions used were: 10 min hot start at 95°C, 30 s denaturation (95°C), 30 s annealing (temperature appropriate to primer), and 30 s elongation stage (72°C) for 35 or 40 cycles, followed by a 10 min final elongation (72°C) and infinite hold at 4°C. ImmoMix™ (Bioline, London, UK) stock solution was used in the PCR reaction mix to provide both DNA polymerase and Mg^{2+}, with a reaction concentration of 1.5mM of Mg^{2+}. Each 15 µl PCR reaction volume comprised: 7.5 µl Immomix™ (Bioline), 2 µl DNA elution, both primers at 0.5µM concentration, and molecular grade H_2O as required to reach the required volume.

The DNA was amplified in a G-Storm GS1 Thermal Cycler (Gene Technologies Ltd., Braintree, UK). Successful amplification of DNA was validated by electrophoresis on agarose gels.

Analysis of amplicons. Amplicons were diluted 100-fold with molecular grade water before being analyzed with an ABI 3730 sequencer (Applied Biosystems, Foster City, USA). Genemapper software (Version 3.7, 2004) was used to determine fragment sizes, and traces were rounded to the nearest single nucleotide size prior to allele calling. Structure analysis for Bayesian cluster was run using STRUCTURE software (Version 0.6.93, 2005) to identify the levels of allele admixture between breed types (Pritchard et al. 2000). Allele calls were assessed for prior values of K, ranging from 1 to 10, with a burn in period of 100 000 and 200 000 MCMC iterations. Each value of K was repeated 5 times. ΔK (the second order rate of change in log probability between successive values of K) was determined using STRUCTURE Harvester software (Version 0.6.93, 2005). CLUMPP software (Version 1.1, 2007) was then used to generate a consensus for the optimum value of K. The allele data recorded for each individual was then imported to Power-Marker software (Version 3.25, 2006) for analysis. The expected heterozygosity (based on populations in Hardy-Weinberg equilibrium), observed

Table 1. Primers selected from MoDAD recommended list (Hoffmann et al. 2004)

Name of primer pair	Chromosome number	Direction of primer	Primer sequence (5' → 3')	Annealing temperature (°C)	Number of cycles	Range of allele size (bp)
OarCP 34	3	forward reverse	GCTGAACAATGTGATATGTTCAGG GGGACAATACTGTCTTAGATGCTGC	62	35	112–130
OarVH 72	25	forward reverse	GGCCTCTCAAGGGGCAAGAGCAGG CTCTAGAGGATCTGGAATGCAAAGCTC	58	40	121–145
BM 8125	17	forward reverse	CTCTATCTGTGGAAAAGGTGGG GGGGGTTAGACTTCAACATACG	52	40	110–130
OarFCB 193	11	forward reverse	TTCATCTCAGACTGGGATTCAGAAAGGC GCTTGGAAATAACCCTCCTGCATCCC	52	40	96–136
OarFCB 304	19	forward reverse	CCCTAGGAGCTTTCAATAAAGAATCGG CGCTGCTGTCAACTGGGTCAGGG	58	35	150–188
OarJMP 29	24	forward reverse	GTATACACGTGGACACCGCTTTGTAC GAAGTGGCAAGATTCAGAGGGGAAG	58	35	96–150
OarJMP 58	26	forward reverse	GAAGTCATTGAGGGGTCGCTAACC CTTCATGTTCACAGGACTTTCTCTG	58	35	145–169
DYMS 1	20	forward reverse	AACAACATCAAACAGTAAGAG CATAGTAACAGATCTTCCTACA	48	40	159–211

heterozygosity, polymorphic information content (PIC), and major allele frequency were generated for each marker and each breed type, along with each population's inbreeding coefficient and breed type genetic distance, based on the shared allele frequency for each locus. PowerMarker was also used to calculate AMOVA values for sources of genetic variation across the population.

Comparison of allelic frequencies between breed types was used to construct a pair-wise grid of the genetic distances between types and was used as the input file for the Neighbor program within the PHYLIP (Version 3.2, 1989) suite of programs. Data from Balkhi sheep (Ibrahim et al. 2010) were used to provide an outlier for this grid, to allow rooting of the genetic data. The resulting outtree file was then viewed using FigTree (Version 1.3.1, 2009) (http://tree.bio.ed.ac.uk/software/figtree/), and rooted with the Balkhi population branch.

RESULTS

The number of alleles, the relative frequency of the most abundant allele, the level of heterozygosity, and the PIC values for each microsatellite marker analyzed are shown in Table 2. The 8 loci were all found to be polymorphic, with between 5 (OarFCB193) and 15 (DYMS1) alleles observed for each microsatellite marker when pooled across breeds, with the most abundant allele constituting between 18 and 73% of the total. Similarly, there were between 2 and 11 alleles present within each breed at each locus (Table 3).

Summary statistics for the data generated in PowerMarker including the inbreeding coefficients and heterozygosity measures are shown in Table 2 for each marker and in Table 3 for each breed type.

Two markers had high values for major allele frequency (BM8125 and OarFCB193) and represented those with the fewest genotypes, and lower PIC values (0.493 and 0.399, respectively). The other 6 loci studied were more variable, with PIC values between 0.612 and 0.892. Only 6 discriminatory alleles (unique to one population) were observed from the data, and the Black WM breed was observed to have the least number of alleles in total across the 8 markers. The source of variation in these populations resulted from differences in allele frequency, as there is little effect of unique alleles.

Heterozygosity measures for each marker (Table 2) were close to the expected values for populations in Hardy-Weinberg equilibrium, and were similar to those predicted for domestic livestock populations under selective breeding. When compared within breeds, observed heterozygosity (Ho) measures ranged from 0.530 to 0.664 and in most (5/6) breeds expected heterozygosity (He) values of the makers were higher than Ho. Most (7/8) markers had higher He than Ho values, with marker OarCP34 being the only one to have a slightly higher Ho value (Table 2).

Inbreeding coefficients are shown in Table 3. In most of the breeds (5/6) there is some evidence of inbreeding taking place, ranging from coefficient values of 0.062 (Llandovery type) to 0.165 (pedigree sheep). The only group not showing evidence of inbreeding is the North Wales type, where there is actually evidence of a small degree of outbreeding (based on a negative coefficient value).

The results of Bayesian cluster analysis with STRUCTURE software (Pritchard et al. 2000) show a degree of admixture between breed types with the most different being the Black WM (Figure 1). This observation is true irrespective of the value

Table 2. Number of alleles, major allele frequency, heterozygosity levels and polymorphic information content (PIC) values for each microsatellite marker analyzed

Marker	Number of alleles	Major allele frequency	Heterozygosity		PIC
			expected	observed	
OarCP34	6	0.344	0.746	0.781	0.704
OarVH72	7	0.370	0.785	0.698	0.760
BM8125	6	0.682	0.513	0.458	0.493
OarFCB193	5	0.729	0.435	0.396	0.399
OarFCB304	7	0.544	0.648	0.462	0.612
OarJMP29	11	0.365	0.770	0.656	0.740
OarJMP58	10	0.489	0.713	0.532	0.688
DYMS1	15	0.182	0.900	0.698	0.892

Table 3. Heterozygosity measures, major allele frequency, and inbreeding coefficient values for each sheep population studied. Expected heterozygosity measures are based on populations in Hardy-Weinberg equilibrium. All figures are shown to 3 decimal places, with the exception of the inbreeding coefficient generated for the North Wales population, the only negative value, being shown to one significant figure

Breed type	Heterozygosity		Inbreeding coefficient
	expected	observed	
Black Welsh Mountain	0.580	0.530	0.112
Llandovery	0.634	0.622	0.062
Nelson	0.603	0.531	0.144
North Wales	0.643	0.664	−0.0003
Pedigree	0.676	0.586	0.165
Tregaron	0.656	0.621	0.096

of K being used. Here K = 3 is shown, as it is the value least likely to show differences, but similar patterns were observed with K = 4, 5, and 6.

Calculation of AMOVA within the PowerMarker programme indicated that 8.7% of variation is caused by among population differences, with the remaining variation due to within population and individual variation.

The suggestion that the Black WM sheep are an outlier, relative to the others, is re-iterated further in a dendrogram (Figure 2) which was produced to compare the genetic distance between the breeds. In this analysis the values for the Balkhi sheep population was used as an outlier with which to root the tree. It is clear from this dendrogram that the Nelson, Llandovery Whiteface, and Tregaron types form one group, the North Wales and Pedi-

gree Welsh Mountain types form another group, and Black WM sheep segregate separately.

DISCUSSION

This is the first attempt to genetically characterize the relationship between populations of WM sheep. Slightly higher levels of heterozygosity have been reported previously in Greek (Ligda et al. 2009) and Italian (Bozzi et al. 2009) sheep breeds, with Ho ranges of 0.626–0.740 and 0.658–0.741 respectively. This suggests that levels of genetic diversity within the WM populations studied are lower than has previously been observed in other European sheep breeds. Comparable values have been reported by Alvarez et al. (2012) in Cuban hair sheep populations (Ho range of 0.596–0.675).
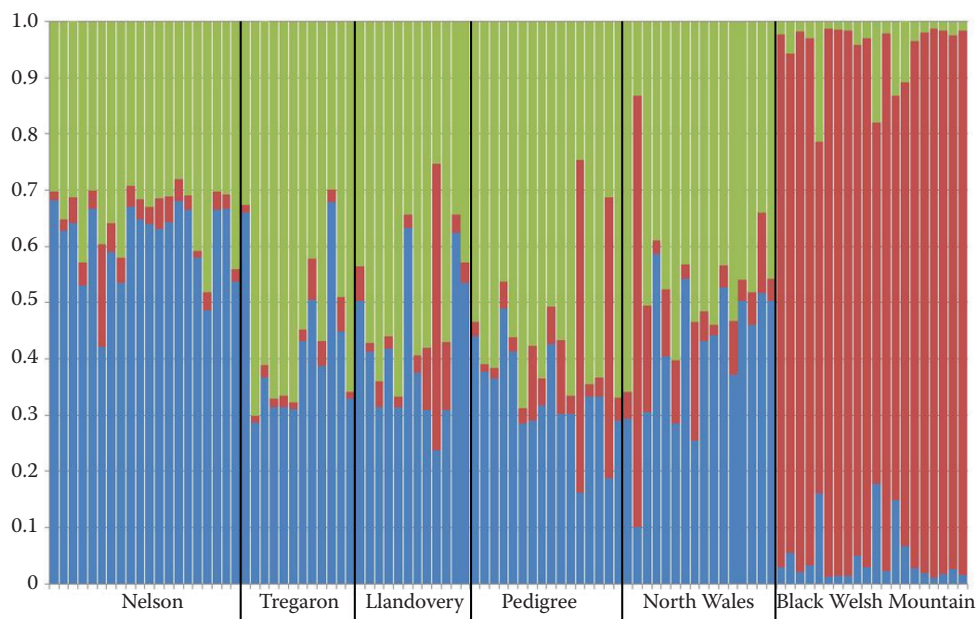

Figure 1. Bayesian cluster STRUCTURE analysis at K = 3 averaged over 5 independent runs

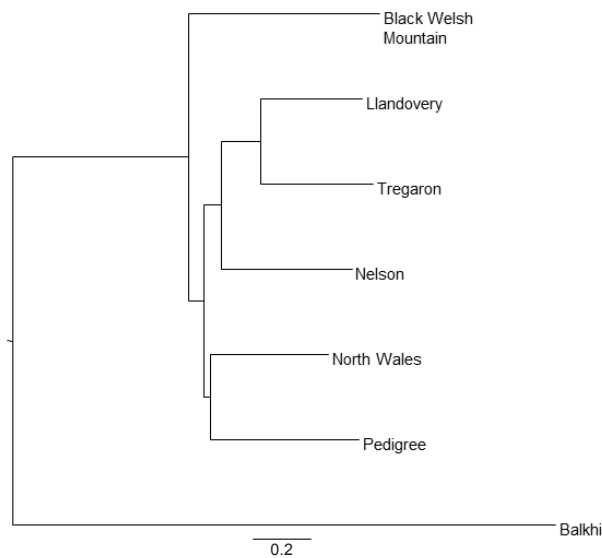*Czech J. Anim. Sci.*, 60, 2015 (5): 216–223

Original Paper

Figure 2. Neighbor-joining tree rooted with a Balkhi sheep population as the designated out-group

The only example of a negative inbreeding co-efficient was observed within the North Wales population, with a value of −0.0003 indicating that a small amount of out-breeding is taking place in this population. This suggests a positive effect of managed breeding taking place with the addition of new alleles into the population, which should reduce the risk of the negative effects to which inbred populations are prone (McParland et al. 2007). In contrast, the Pedigree WM population was the most inbred group studied, with an inbreeding coefficient of 0.165. All other breed types showed some degree of inbreeding, with coefficients ranging from 0.062 to 0.144 (Table 3). It is also worth noting that of the breeds examined the Pedigree WM type has one of the smallest population sizes. However, maintaining a breed as a pedigreed and/or registered group cannot be the sole contributing factor to its higher coefficient of inbreeding because the black WM type is also mainly kept by registered breeders but it had a lower inbreeding coefficient of 0.112.

The level of inbreeding recorded here for the Pedigree WM is an important observation, as Blackburn et al. (2011) suggest that breeds with an inbreeding coefficient greater than 0.15 warrant close monitoring. The Nelson WM type, with a coefficient of inbreeding of 0.144, is also close to this value of 0.15, and so may be another population which needs to be carefully monitored.

The use of AMOVA to examine the source of differences in heterozygosity indicated that 8.7% of the variation in allele frequency was accounted for by variation among different populations. Blackburn et al. (2011) reported 13% variation among populations and Dixit et al. (2011) found 16.5% of variation was due to between breed differences. This difference may be due to the comparatively low number of individual animals involved in the current study, or because this research has largely looked at variation between populations of different phenotypic types within the WM breed rather than difference between breeds *per se*.

The results observed from this study reflect the known history of the WM sheep and the geographical location of each population. The black WM, which have been established as a separate breed since 1920 (NSA 1998), can clearly be identified as a separate population in the dendrogram (Figure 2). The genetic differentiation seen between the other five populations of white WM sheep most likely originates from geographic isolation of populations at a time when the large-scale transport of breeding livestock around the UK was not commonplace; even today this is maintained by a strong preference for continued breeding of sub-type populations within the different regions of Wales. The closest relationship observed was between the Tregaron and Llandovery groups, which are also the closest in terms of geographical location, so have developed in a similar area. However, there is still strong support for identifiable genetic differences between them by genetic distance. The Nelson type branches separately from the Tregaron and Llandovery populations. Originating from the South Wales valleys, the Nelson type is also phenotypically distinct, being larger in body and with identifiable markings in the fleece. These south and mid Wales types are clearly split on the dendrogram from the North Wales and Pedigree groups, again with a defined split between these other two populations. This reflects similar findings made in Italian sheep breeds by Ciani et al. (2013), where along with clear interbreed differences, significant intrabreed diversity was observed within the Lecce breed of sheep.

The relationships identified between all but the Black WM types are reasonably close, which is in keeping with the findings of Agaoglu and Ertugurl (2012). When using microsatellite mark-

ers they found that genetic differences could not be established between several goat breeds using cluster analysis, except for the distinction of Angora goats. It is interesting to note that in the current study the genetic data produced correlate with the observable phenotypic differences, but this is not always possible to demonstrate (Rendo et al. 2004; Agaoglu and Ertugrul 2012).

CONCLUSION

There is a common belief among traditional hill sheep farmers that sheep populations are adapted to thrive in specific geographic areas. While the results of the current research cannot be used to identify specific phenotypes which may lend an animal to being highly adapted to a particular environment, it has been able to identify genetic differentiation between the different types of WM sheep which supports the view that they have different genetic characteristics in both the genes selected for phenotype and in selectively neutral microsatellite markers. As such, we conclude that the results observed here make a distinction between each population of WM sheep studied. While the Black WM is a distinct breed, the genetic differentiation seen between the remaining five populations of white WM sheep most likely originates from historical geographic isolation of populations which has been maintained by the continued breeding of sub-type populations within Wales.

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