Use of microsatellite markers to include or exclude individuals as Barbados Blackbelly sheep

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Abstract

Barbados Blackbelly sheep are present in many islands of the Caribbean and other countries of the world. This study was undertaken to ascertain the level of genetic variability within the Barbados Blackbelly population through the use of microsatellite loci to include or exclude individuals as belonging to this population. Using phenotypic characteristics, fifty individuals randomly selected from Barbados constituted the reference Blackbelly population. Other Barbadian selected “Blackbelly” sheep, described as offtypes or mixtures, along with sheep belonging to populations of West African, Virgin Island White and Blackbelly from the United States of America (USA) were also included for comparison. Polymerase chain reaction genotype analysis using dinucleotide microsatellite markers were used to examine the genetic diversity present in the reference population. Eleven ovine microsatellite markers were amplified to generate allele frequencies. The results suggest that the observed heterozygosity prevailing in the Barbados Blackbelly population is well below the expected levels. Additionally, only 80% of reference population was correctly classified as Barbados Blackbelly. None of the “Blackbelly” offtypes, mixtures or sheep from the USA were classified as belonging to the reference group.

Key words: Barbados Blackbelly sheep, assignment tests, genetic diversity, microsatellites, Gene Class.

Introduction

Sheep are an important resource in the Caribbean largely for meat; though use is also made of the manure and hides. The Barbados Blackbelly sheep is a unique breed exhibiting light brown to dark reddish brown hair and a black belly and the females are highly prolific, producing two to four lambs per lambing (Thomas, 1997). At maturity a ram can weigh between 68-90 kg, while mature ewes average 40-59 kg. The population over time has become well adapted to Barbadian biotic and abiotic stresses and to the traditional husbandry systems in the island. Over the years individuals have been exported from the island to many countries of the Caribbean and extra regionally to USA, Malaysia and Taiwan. It is estimated that there are currently approximately 24,000 Blackbelly sheep in Barbados.

Awareness of the value of this animal genetic resource is currently prompting deliberate efforts at optimum utilization and conservation of the species. This unique germplasm resource can be threatened by loss of genetic diversity, if deliberate breeding programs are not...
formulated and implemented in the future. Registration of the breed would provide accurate and definitive descriptions based on information obtained from farmers and scientists.

Microsatellites have been used successfully recently to characterize the genetic polymorphism among livestock including sheep (Mugai et al. 2000, Farid et al. 2000, Hughes et al. 1998, Arranz et al. 1998). Polymerase chain reaction (PCR) amplification of the variable dinucleotide sequence repeats followed by electrophoresis can allow for rapid microsatellite genotyping. It has been reported that allele frequency differences between breeds generated from microsatellite variation at different loci, can be used to identify the breed of an individual sheep (Farid et al. 2000, Buchanan et al. 1994). This preliminary study is primarily intended to ascertain the level of genetic variability within the Barbados Blackbelly population. It is also intended to show that microsatellite loci can be used to include or exclude individuals as belonging to this population.

Materials and Methods

Selection

The reference population for this study consisted of 50 Barbados Blackbelly sheep, a minimum of 4 from each of the 11 parishes in Barbados, selected at random by staff of the Ministry of Agriculture. The animals were true to Blackbelly phenotype as defined by the Livestock Division, Ministry of Agriculture, but not closely related. For assignment testing to the local Barbados Blackbelly sheep population, five Blackbelly sheep from Oklahoma, USA, plus another 17 taken from Barbadian farmers were analyzed. The local selections (Figure 1) included two breeds (West African, and Virgin Island White), and a number of Barbados Blackbelly “offtypes or mixtures” (Table 1). Approximately 2-5 ml of blood was drawn from each of the selected animals into EDTA containing vacutainer tubes and

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbados Blackbelly</td>
<td>50</td>
</tr>
<tr>
<td>Virgin Island White</td>
<td>2</td>
</tr>
<tr>
<td>West African</td>
<td>3</td>
</tr>
<tr>
<td>*Blackbelly offtypes and mixtures</td>
<td>9</td>
</tr>
<tr>
<td>“Black” Blackbelly</td>
<td>3</td>
</tr>
<tr>
<td>USA Blackbelly</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
</tr>
</tbody>
</table>

*animals with horns, coloration suggesting presence of other breeds in the individual, etc.

later used for DNA extraction.

Microsatellites

Eleven pairs of bovine dinucleotide microsatellite markers previously evaluated against the ovine species (de Gortari et al., 1997) were used. The chromosomal locations, sequences, annealing temperatures and expected size range of amplified products (Table 2) were obtained from the International Livestock Research Institute, Kenya. The primers were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA).
### Table 2: Autosomal microsatellite loci used in the study

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome</th>
<th>Allele size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAF65</td>
<td>15</td>
<td>111 – 139</td>
</tr>
<tr>
<td>McM527</td>
<td>5</td>
<td>155 - 191</td>
</tr>
<tr>
<td>MAF214</td>
<td>16</td>
<td>186 - 204</td>
</tr>
<tr>
<td>HSC</td>
<td>unknown</td>
<td>267 - 301</td>
</tr>
<tr>
<td>OarFCB20</td>
<td>2</td>
<td>88 - 158</td>
</tr>
<tr>
<td>ILSTS056</td>
<td>10</td>
<td>145 – 175</td>
</tr>
<tr>
<td>ILSTS005</td>
<td>7</td>
<td>186 - 218</td>
</tr>
<tr>
<td>SR-CRSP-5</td>
<td>18</td>
<td>139 - 153</td>
</tr>
<tr>
<td>TGLA53</td>
<td>12</td>
<td>117 - 165</td>
</tr>
<tr>
<td>OarJMP29</td>
<td>24</td>
<td>120 - 150</td>
</tr>
<tr>
<td>MAF035</td>
<td>23</td>
<td>90 - 118</td>
</tr>
</tbody>
</table>
Figure 1. Some of the animals used in the study

- 'True' blackbelly
- West African
- "black" Blackbelly
- Blackbelly crossed with West African
- Blackbelly with horns
- Blackbelly mixed with other breed
Laboratory Procedures

DNA was extracted from 400 µl of whole blood using a Qiagen FlexiGene DNA isolation kit (QIAGEN Inc., Valencia, California) according to the manufacturer’s recommendations. Quantification of DNA yield was done by comparison with a molecular weight standard on 1% agarose gel stained with ethidium bromide (Sambrook et al, 1989). Typically DNA yields of 5-10 µg were obtained. The DNA was amplified via PCR in a Technne GENIUS thermocycler using the microsatellite primer pairs described above. The 15 µl amplification reactions contained 50 ng template DNA, 1.0 µM of each primer, and final concentrations of 2.0 mM MgCl₂, 0.2 mM dNTPs (Promega), 1x PCR buffer without MgCl₂ (Promega) and 0.125 units of *Taq* polymerase (Promega). PCR reaction annealing temperatures were as shown in Table 2. 4 µl of the PCR product plus 2 µl of loading buffer (0.25 % Bromophenol, 45 % sucrose) was loaded on to a 1.5 mm thick 25 x 16 cm square 8% nondenaturing polyacrylamide gel in 0.5X TBE (0.09M Tris-Borate, 0.002M EDTA, pH 8.0). Electrophoresis was carried out at room temperature for 6 hours at 5 mA constant current using a Bio-Rad Protean II electrophoresis apparatus. The resulting amplified bands were visualized following staining in 0.5 µg/ml ethidium bromide in 0.5X TBE for 15 minutes. DNA bands were observed with UV light and photographed using the Kodak Digital Science electrophoresis documentation and analysis system were scored using 25 bp DNA ladder (Promega) and semi log graph paper.

Data Analyses

Genetic polymorphism was determined by counting the mean number of alleles at each locus. Counting was also used to compute observed heterozygosity for each locus. Expected unbiased heterozygosity was evaluated using FSTAT version 2.9.3. (Goudet, 2001). Assignation or exclusion of individuals using molecular marker genotyping was effected using GeneClass (Cornuet et al, 1999).

Results

The 11 microsatellite primers used detected a total of 93 alleles with an average of 8.45 ± 2.58 per locus from the 50 sheep analyzed in the reference population. Figure 2 show alleles generated with microsatellite OarFCB20 on some of the Barbados Blackbelly sheep. All loci were polymorphic, (Figure 3) with the lowest number of alleles being 3 (MAF214) and the highest being 11 (HSC, ILSTS056 and TGLA53).

![Figure 2. Gel picture showing alleles generated when microsatellite OarFCB20 was amplified. Lane 7 from the left is a marker lane (sizes to right). The other lanes represent Barbados Black Belly individuals. The first three lanes from the left represent homozygous individuals while the fourth and fifth are heterozygotes.](image-url)
Figure 3. Number of alleles per locus

Figure 4 shows that the observed heterozygosity, $H_O$, ranged from 0.14 (SR-CRSP-5) to 0.42 (OarFCB20) with an average over all loci for the reference population of $0.29 \pm 0.02$. Five (MAF65, OarFCB20, TGLA53, MAF035 and ILSTS005) of the 11 loci had heterozygosity values greater than 35%. Three loci (HSC, SR-CRSP-5 and MAF214) had observed heterozygosity of less than 20%. The unbiased estimate of heterozygosity, $H_E$, of $0.74 \pm 0.04$ was much greater than the average $H_O$.

Figure 4. Expected and observed heterozygosity (Hz) at each locus
Of the 50 individuals phenotypically selected as representing the Barbados Blackbelly sheep population 80% were assigned correctly. These tests were done using the leave-one-out procedure, i.e. the individual was excluded when performing its assignment to the breed. The frequency method was used with a threshold of 0.0100. Both of the Virgin Island White individuals and two of the three sheep of West African origin were excluded from the Barbados Blackbelly population. Similarly none of the five animals belonging to “Blackbelly” of Oklahoma, USA were assigned to the Barbados Blackbelly population. Interestingly, none of the three “black” Blackbelly sampled or eight of the nine “offtype or mixture” Blackbelly animals selected locally were assigned to the Barbados Blackbelly population. The only exception that was included was the “offtype” represented by a four inch white-tipped tail.

Discussion

Successful genotyping analysis using nondenaturing PAGE can be tedious due to the presence of false or pseudo bands. Lahiri et al. (1997) detailed a number of guidelines among which was the suggestion that under such conditions one needs to know the expected size of products. The appearance and intensity of bands are also important in identifying the true bands. Adherence to many of these guidelines along with careful PCR allowed for correct interpretation of PAGE results.

The average number of alleles per locus of 8.45 present in the reference population suggests a high level of genetic variability in the Barbados Blackbelly sheep. This is not surprising bearing in mind that the breed is thought to have evolved from crosses of African hair sheep and European wool breeds; combining the hair coat, tasty flesh, extended breeding season and tropical adaptability from West Africa with the prolificacy, greater size and hornlessness of an original wooled European breed (Thomas 1997).

Nevertheless, the significant difference between $H_e$ (0.74) and $H_o$ (0.29) show departure of allele frequency distributions from the Hardy-Weinberg equilibrium. The degree of homozygosity in the Barbados Blackbelly may be due to the Wahlund effect (Hartl and Clark, 1989). If the local population is thought to be comprised of many genetically variable lines, then individual animals may be fairly inbred while the population as a whole is genetically diverse.

Buchanan et al. (1994) and Farid et al. (1999) have shown that the assignment test can be a powerful tool for identifying individual sheep as members of a specific population. Cornuet et al. (1999) have described likelihood-based methods, using allele frequency whereby individuals are assigned to specific populations if the probability is above some threshold. That only 80% of the individuals of the reference population were correctly assigned as compared to the greater than 90% obtained by others (Buchanan et al. 1994, Farid et al. 1999) is most intriguing. One possible explanation for the high exclusion percentage may be that the likelihood-based method of Cornuet et al. (1999) had an exclusion threshold of 0.0100. In addition, the high genetic diversity (average of 8.45 alleles per locus) and low levels (0.29) of observed heterozygosity suggest significant genetic differences among individuals of the reference population in spite of the fact that the reference population was carefully selected to reflect defined phenotypic characteristics of the Barbados Blackbelly.

The exclusion of “Blackbelly” sheep from Oklahoma, USA may be explained as due to genetic drift resulting from crossbreeding and selection pressure within that population. It must also be noted that the founder animals for the USA population were exported from Barbados more than one hundred years ago.
All of the offtypes and mixtures were excluded from the Barbados Blackbelly reference population except for one. This study suggests that reliance on phenotypic characteristics can in most instances assign individuals to the Barbados Blackbelly population. Given the genetic variability present among the reference population it is not surprising that the offtypes and mixtures were excluded. The sole individual included as part of the reference population, looks exactly like any of its members except for a four inch instead of two inch white at the tip of the tail.

Acknowledgements

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Bibliography


LAHIRI, D.K., ZHANG, and A., NURNBERGER, J.I. 1997. High-resolution detection of PCR products from a microsatellite marker using a Nonradioisotopic technique. *Biochemical and Molecular Medicine* 60:70-75

MUGAI, A.W., WATTS, P.C., HIRBO, J., IMBUGA, M., INIGUEZ, L., KEMP, S., HANOTTE, O., and REGE, J.E.O. 2000. Assessment of the genetic diversity and relationships among African fat-

Cold Spring Harbor, NY. Cold Spring Harbor Laboratory.

THOMAS, G. 1997. Review of literature of Barbados Blackbelly sheep – Its history and
performance characteristics. A publication of CARDI and the CIDA funded Caribbean
Sheep Production and Marketing Project.