Advances in Fibre Production Science in South American Camelids and other Fibre Animals

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Universitätsverlag Göttingen

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Published in 2019 by Universitätsverlag Göttingen

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2019

Bibliographic information published by the Deutsche Nationalbibliothek

The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at http://dnb.dnb.de.

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Suggested citation:
M. Gerken, C. Renieri, D. Allain, H. Galbraith, J. P. Gutiérrez, L. McKenna, R. Niznikowski, M. Wurzinger (eds.) (2019): Advances in Fibre Production Science in South American Camelids and other Fibre Animals. Universitätsverlag Göttingen, Göttingen

Revision of English language: Lisa McKenna
Revision of Spanish language and abstracts: Juan Pablo Gutiérrez
Typesetting and layout: Lisa McKenna, Marvin Heuduck, Martina Gerken
Fotos (cover, session titles): Alexander Riek and Martina Gerken
Cover design: Margo Bargheer and Jutta Pabst

https://univerlag.uni-goettingen.de
ISBN: 978-3-86395-408-6
DOI: https://doi.org/10.17875/gup2019-1158

Preface

Animal fibres from South American camelids and other fibre or wool bearing species provide important products for use by the human population. The contemporary context includes the competition with petrocarbon-based artificial fibres and concern about excessive persistence of these in the natural environment. Animal fibres present highly valuable characteristics for sustainable production and processing as they are both natural and renewable. On the other hand, their use is recognised to depend on availability of appropriate quality and quantity, the production of which is underpinned by a range of sciences and processes which support development to meet market requirements.

Such support includes the efforts of the Animal Fibre Working Group (AFWG) of the European Federation of Animal Sciences (EAAP) which was instituted in 2007 and tasked with creating a network for investigation and dissemination of information in Europe and internationally. One task has been the organisation of scientific meetings, and continuing the tradition of previous European Symposia on South American camelids. These include the recent 5th Meeting in Sevilla (Spain: 2010) and 6th Meeting at EAAP, Nantes (France: 2013). References to these and other meetings, workshops and publications may be found on the AFWG website: http://www.eaap.org/presentation/scientific-structure/commissions-working-groups/animal-fiber-working-group/.

The present publication derives from the 7th European Symposium on South American Camelids and 3rd European Meeting on Fibre Animals (http://www.sympcam.org/). This meeting was held in the conference facility of the Domus Pacis Hotel, Assisi, Italy, on 12 to 14 June 2017. It was organised by Prof Dr Carlo Renieri and his colleagues Dr Attilio De Cosmo, Dr Francesco Fantuz, Dr Antonietta La Terza, Prof Alessandro Valbonesi (University of Camerino), Dr Marco Antonini (ENEA), and Maurizio Gubbiotti (University Marconi, Roma) with support from the scientific board comprising AFWG colleagues. We wish to thank Dario Pediconi, Cristina Nocelli, Irene Pazzaglia, Stefano Pallotti (University of Camerino) who helped us during the symposium. We also thank all participants who readily agreed to chair sessions or to participate in the Round Table.

We are very grateful to Loro Piana (http://www.sympcam.org/loropiana/) for generous funding support which enabled the attendance of international speakers and provided scholarships for three young scientists from Latin American countries.

Individual papers and abstracts, where full papers were not available, were printed from the manuscripts supplied by the authors. The assistance of Marvin Heuduck (Göttingen University) and the editors of Göttingen University Press in the editorial process is acknowledged.

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The editors

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PCR-RFLP Method for Testing ASIP EXON 4 Mutations in Llamas

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Abstract. The basic coat colors of mammals are determined by the relative proportion of two types of pigments, eumelanin and pheomelanin. The agouti signaling protein (ASIP) plays a crucial role in melanogenesis, by increasing the production of pheomelanin and decreasing the eumelanin synthesis by blocking the signaling pathway of the melanocortin 1 receptor (MC1R). In several species, loss of function mutations of the ASIP gene is responsible for the black coat color. The polymorphisms of ASIP exon 4, c.325_381del and c.292C> T, have been previously associated with eumelanic phenotypes in llamas (Daverio et al., 2016) and also in alpacas (Chandramohan et al., 2013). The objective of this work was to develop an alternative method to DNA sequencing for genotyping both polymorphisms. Fifty one llama DNA samples, including controls of known sequence were analyzed. PCR products spanning ASIP exon 4 were digested with the enzyme BveI, that recognizes the c.292C>T mutation, followed by electrophoresis in 8% polyacrylamide gels to simultaneously detect the SNP and the deletion. Analysis of the band patterns presented complete concordance between DNA sequencing and PCR-RFLP genotypes. Genotyping results from the samples showed that all dark llamas (n=13) were homozygous for the deletion, homozygous for the c.292C> T polymorphism or heterozygous for both, while none of these combinations were observed in the pheomelanic animals here analyzed (n=20). The results of this work support the findings of previous studies and also show the usefulness of the PCR-RFLP technique as a relatively fast, simple and cost-effective method to determine the ASIP exon 4 variants in llamas.

Resumen. Los colores de capa básicos de los mamíferos están determinados por la proporción relativa de dos tipos de pigmentos, eumelanina y feomelanina. La proteína de señalización agouti (ASIP) juega un rol crucial en la melanogénesis, incrementando la producción de feomelanina y disminuyendo la síntesis de eumelanina por bloqueo de la vía de señalización del receptor 1 de melanocortina (MC1R). En varias especies, mutaciones con pérdidas de función del gen ASIP son responsables del color de capa negro. Los polimorfismos del exón 4 de ASIP, c.325_381del y c.292C> T, han sido asociados previamente con fenotipos eumelánicos en llamas (Daverio et al., 2016) y también en alpacas (Chandramohan et al., 2013). El objetivo de este trabajo fue desarrollar un método alternativo a la

secuenciación de ADN para genotipar ambos polimorfismos. Se analizaron 51 muestras de ADN de llamas, incluyendo controles de secuencia conocida. Los productos de PCR que abarcan el exón 4 de ASIP fueron digeridos con la enzima Bvel, que reconoce la mutación c.292C> T, seguida por electroforesis en geles de poliacrilamida al 8 % para detectar simultáneamente el SNP y la deleción. El análisis de los patrones de banda presentó concordancia completa entre los genotipos de secuenciación y los de PCR-RFLP. Los resultados de genotipado de las muestras mostraron que todas las llamas oscuras (n=13) eran homocigotas para la deleción, homocigotas para el polimorfismo c.292C> T o heterocigotas para ambos, mientras que ninguna de esas combinaciones se observó en los animales feomelánicos aquí analizados (n=20).Los resultados de este trabajo apoyan los hallazgos de estudios previos y también muestran la utilidad de la técnica de PCR-RFLP como un método relativamente rápido, sencillo y de bajo costo para determinar las variantes del exón 4 de ASIP en llamas.

Keywords: llamas, PCR-RFLP, ASIP-genotyping, eumelanic

Introduction

Coat color in animals has important functions in camouflage, protection against UV radiation and communication, among others. Compared to the wild species, domestic animals present a fascinating diversity of colors product of artificial selection (Reissmann & Ludwig, 2013). During the last decade, molecular techniques have allowed the identification of genes and alleles underlying this variation in a large number of species. The color of the coat basically depends on the proportions of the pigments eumelanin (black or dark brown) and pheomelanin (reddish-yellow). The production of these pigments is mainly controlled by the melanocortin-1 receptor (MC1R) and agouti signaling peptide (ASIP) (Bultman et al., 1992). MC1R interaction with its antagonist ASIP produces pheomelanic pigments by blocking the interaction with its natural ligand alpha-MSH (Lu et al., 1994). Dominant black is usually produced by variations in the coding sequence of the MC1R gene (Klungland et al., 1995; Marklund et al., 1996; Kijas et al., 1998). Gain of function mutations that produce constitutive activation of MC1R lead to the synthesis of eumelanin and a dark coat. On the other hand, in species where the black color is recessive, non-functional alleles of the ASIP gene are responsible for the dark coloration. Loss-of-function ASIP mutations have been described in species such as dog (Kerns et al., 2004), horse (Rieder et al., 2001), sheep (Royo et al., 2008). In alpaca, three mutations in ASIP exon 4 c.325_381del, c.292C>T and c.353G>A have been also associated to black coat (Feeley et al., 2011; Chandramohan et al., 2013). In llama, the mutations c.325_381del and c.292C>T have been also showed association with black color but the c.353G>A has not been identified so far (Daverio et al., 2016).

In Argentina, the llama is the most abundant domestic camelid and exhibits a wide variety of coat colors that meet different commercial interests. Although the

value of camelids fiber greatly depends on its diameter, the color is also important for the textile industry (Frank et al., 2006, Mueller et al., 2010). In this context, black, reddish brown and golden colors are usually some of the most required (Frank et al., 2006). However, the color of offspring based only on the parents' phenotype is hard to predict in camelids. Thus, the use of molecular tests to determine the genotype of the breeding animals could be useful in selection programs. Nevertheless, if the number of animals is high, sequencing may be expensive and additionally it requires specialized equipment, not always available in small laboratories. The main objective of this work was to develop a simple technique based on Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) to detect c.325_381del and c.292C>T polymorphisms, as an alternative to DNA sequencing.

Materials and Methods

Thirty three blood llama samples (20 pheomelanic and 13 eumelanic) from diverse herds from Argentina were collected by jugular puncture. Total genomic DNA was isolated following the LiCl protocol described in Daverio MS (2015).

Using the PCR technique, exon 4 of the ASIP gene was amplified in the 33 samples and 18 controls of known sequence. Amplification reactions were carried out as described in Daverio et al., (2016), using as template 60-70 ng of genomic DNA. The PCR products were digested using the restriction enzyme Bvel (BspMI). The enzyme cuts the amplicon into two fragments, one of 83 bp and the other one of 374 bp or 317 bp depending on whether the allele carries or not the c.325_381del. If the c.292C> T polymorphism is present, it creates a new cutting site for Bvel generating a different banding profile.

Digestion mix consisted of 6 µl of PCR product, 6.5 U of Bvel (Thermo Fisher Scientific Inc.), 0.3 µl of Oligos 50X, 1 µl of Buffer O 10X and 6 µl of bidistilled water that was incubated at 37 ºC overnight. Restriction fragments were analyzed by electrophoresis on an 8 % polyacrylamide gel for 90 min at 200 V stained with GelRedTM and visualized using an UV trans illuminator. Fragment length determination was done by comparison with a 50 bp commercial ladder (Embiotec SRL).

Results and Discussion

We adjusted amount of PCR product, digestion time and enzyme quantity to standardize PCR-RFLP conditions. Digestion of control samples yielded band patterns in all cases concordant with genotypes previously obtained by ASIP exon 4 sequencing. Homozygous genotypes for c.292T that did not have the c.325_381del mutation (T/T, -/-) showed two fragments of 192 and 182 bp, in addition to the 83 bp band present in all digestions. Homozygous deletion genotypes (C/C, del/del), generated a 317 bp fragment while controls without the

deletion (C/C, -/-) showed a 374 bp band. Heterozygous individuals for one or both polymorphisms also had the expected combined patterns: 374 bp and 317 bp (C/C,-/del); 317 bp, 192 and 182 (C/T, -/del); 374 bp, 192 and 182 bp (C/T, -/-) (Fig. 1.)

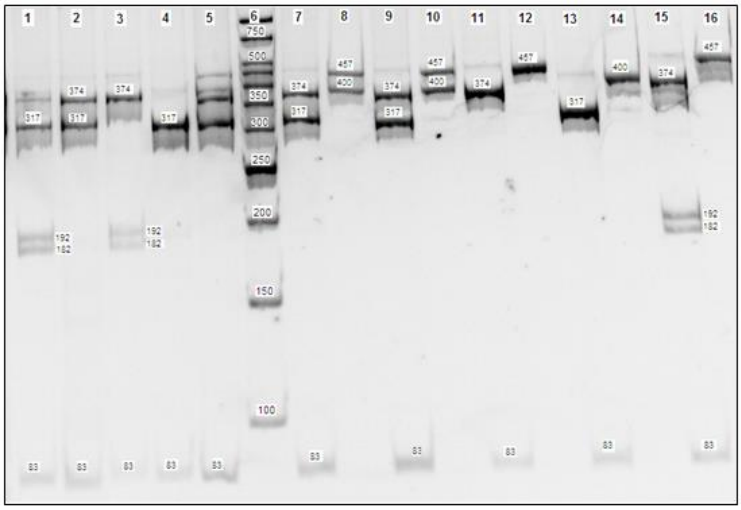

Figure 1: Electrophoresis pattern of ASIP exon 4. Lines 1 to 5 and 7, 9, 11, 13, 15: digested PCR products. Lines 8, 10, 12, 14 and 16: PCR products before digestion. Line 6: molecular size marker of 50 bp. Line 5: incomplete digestion.

After confirming that digestion patterns were as expected, unknown samples were genotyped. PCR-RFLP analysis revealed 6 different genotypes (Table 1). No shared genotypes were found between the two color groups; three were exclusive of eumelanic samples and the remaining three of pheomelanic animals.

The two mutations here studied have been associated to the recessive black coat in alpacas (Feeley et al., 2011; Chandramohan et al., 2013) and llamas (Daverio et al., 2016). Substitution c.292C> T, causes a p.R98C change that introduces an additional cysteine into the region of ASIP which makes contact with MC1R (Kiefer et al., 1997). The other mutation, c.325_381del, leads to the loss of 19 amino acids (p.C109_R127del) within the same domain. Both mutations are predicted to be deleterious and to alter normal protein function. Therefore, the mutated ASIP protein is unable to bind MC1R to induce pheomelanin production and only eumelanin is synthesized, resulting in a black coat. In agreement with that, we found that black llamas were all homozygous for c.292T (T/T), homozygous c.325_381del (del/del), or in a lower frequency (3/13), compound heterozygotes for both mutations, whereas these genotypes were not found in pheomelanic individuals. Thus, our results provide additional support to the previously reported

association between ASIP variants and coat color in domestic camelids (Feeley et al., 2011; Chandramohan et al., 2013, Daverio et al., 2016)

Table 1: ASIP exon 4 genotypes in eumelanic and pheomelanic llamas

Genotype		Phenotype	
c.292C>T	c.325_381del	Pheomelanic	Eumelanic
C/C	del/del	0	6
T/T	-/-	0	4
C/T	-/del	0	3
C/C	-/-	9	0
C/T	-/-	2	0
C/C	-/del	9	0

del: deletion -: non deleted variant

Conclusion

The PCR-RFLP technique proved to be useful for genotyping c.325_381del and c.292C> T polymorphisms. It offers the advantage of being based on a single PCR reaction and is suitable for reading on polyacrylamide gels.

The confirmation of the association between these variants and the black coat still requires the analysis in a larger number of animals and further family based segregation studies. The methodology presented here would be adequate for this purpose, since it allows to genotype the two variants simultaneously, without needing of DNA sequencing.

Acknowledgments. We would like to thank Miriam B. Silbestro for her technical assistance. This work was supported by grants from The National Scientific and Technical Research Council (CONICET) and from the Commission for Scientific Research of the Province of Buenos Aires (CIC) from Argentina.

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Animal fibres from South American camelids and other fibre or wool bearing species provide important products for use by the human population. The contemporary context includes the competition with petrocarbon-based artificial fibres and concern about excessive persistence of these in the natural environment. Animal fibres present highly valuable characteristics for sustainable production and processing as they are both natural and renewable. On the other hand, their use is recognised to depend on availability of appropriate quality and quantity, the production of which is underpinned by a range of sciences and processes which support development to meet market requirements. This collection of papers combines international experience from South and North America, China and Europe. The focus lies on domestic South American camelids (alpacas, llamas) and also includes research on sheep and goats. It considers latest advances in sustainable development under climate change, breeding and genetics, reproduction and pathology, nutrition, meat and fibre production and fibre metrology.

Publication of this book is supported by the Animal Fibre Working Group of the European Federation of Animal Science (EAAP). 'Advances in Fibre Production Science in South American Camelids and other Fibre Animals' addresses issues of importance to scientists and animal breeders, textile processors and manufacturers, specialised governmental policy makers and students studying veterinary, animal and applied biological sciences.

GEORG-AUGUST-UNIVERSITÄT GÖTTINGEN

ISBN: 978-3-86395-408-6

Universitätsverlag Göttingen