

The Genetic Inheritance of the Blue-eyed White Phenotype in Alpacas (*Vicugna pacos*)

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Abstract

White-spotting patterns in mammals can be caused by mutations in the gene *KIT*, whose protein is necessary for the normal migration and survival of melanocytes from the neural crest. The alpaca (*Vicugna pacos*) blue-eyed white (BEW) phenotype is characterized by 2 blue eyes and a solid white coat over the whole body. Breeders hypothesize that the BEW phenotype in alpacas is caused by the combination of the gene causing gray fleece and a white-spotting gene. We performed an association study using *KIT* flanking and intragenic markers with 40 unrelated alpacas, of which 17 were BEW. Two microsatellite alleles at *KIT*-related markers were significantly associated ($P < 0.0001$) with the BEW phenotype (*bew1* and *bew2*). In a larger cohort of 171 related individuals, we identify an abundance of an allele (*bew1*) in gray animals and the occurrence of *bew2* homozygotes that are solid white with pigmented eyes. Association tests accounting for population structure and familial relatedness are consistent with a proposed model where these alleles are in linkage disequilibrium with a mutation or mutations that contribute to the BEW phenotype and to individual differences in fleece color.

Keywords: association, coat color, *KIT*, pigmentation

Introduction

Alpacas (*Vicugna pacos*) were presumably domesticated and selected in large part for their highly valued fibre/fleece, which occurs in a wide range of colors and patterns. Mammalian pigmentation is controlled by the distribution of eumelanin (black/brown pigments) and pheomelanin (red/yellow pigments) and appears to be determined by a small number of genes shared among different species (Jackson 1994; Barsh 1996; Newton et al. 2000). The genetics of alpaca coat-color inheritance is poorly understood, and most information on color inheritance was, until recently, based on breeder's observations. However, breeders frequently report low predictability of fleece color outcomes in breeding programs, suggesting that coat color is inherited polygenically (Mcgregor 2006). Recent studies have begun to unravel the genetic complexity underlying fleece color determination in alpacas (Powell et al. 2008; Feeley and Munyard 2009; Cransberg and Munyard 2011; Feeley et al. 2011; Valbonesi et al. 2011). The coding region has been sequenced for *MC1R*, *ASIP*, *TYRP1*, *MATP* and *TYR* in a variety of alpaca fleece colors. Polymorphisms identified

in *MC1R* are associated with the production of pheomelanin or eumelanin and mutations within *ASIP* are associated with black fibre (Feeley and Munyard 2009; Feeley et al. 2011).

As well as the type and amount of pigment, the development, migration, and survival of melanocytes also affects final pigmentation patterns. Genes known to cause spotting patterns through influencing melanocyte development include *PAX3*, *SOX10*, *MITE*, *SLUG*, *EDN3*, *EDNRB*, and *KIT* (Tachibana et al. 2003). Mutations in these genes result in a range of white-spotting phenotypes in a variety of mammals (e.g., Geissler et al. 1988; Hofstra et al. 1996; Destefano et al. 1998; Santschi et al. 1998; Southard-Smith et al. 1999; Yajima et al. 1999; Sánchez-Martín et al. 2002). Solid white alpacas are valued by breeders because their fleece can be more easily dyed to produce any desired color during processing and manufacturing. A range of white-spotting phenotypes are observed in alpacas including “white face,” “all white,” “blue-eyed white,” and a white spot anywhere on the body. Based on pedigree analysis, it has been proposed that the alpaca white-spotting allele is dominant to solid color (Merriwether and Merriwether 2003).

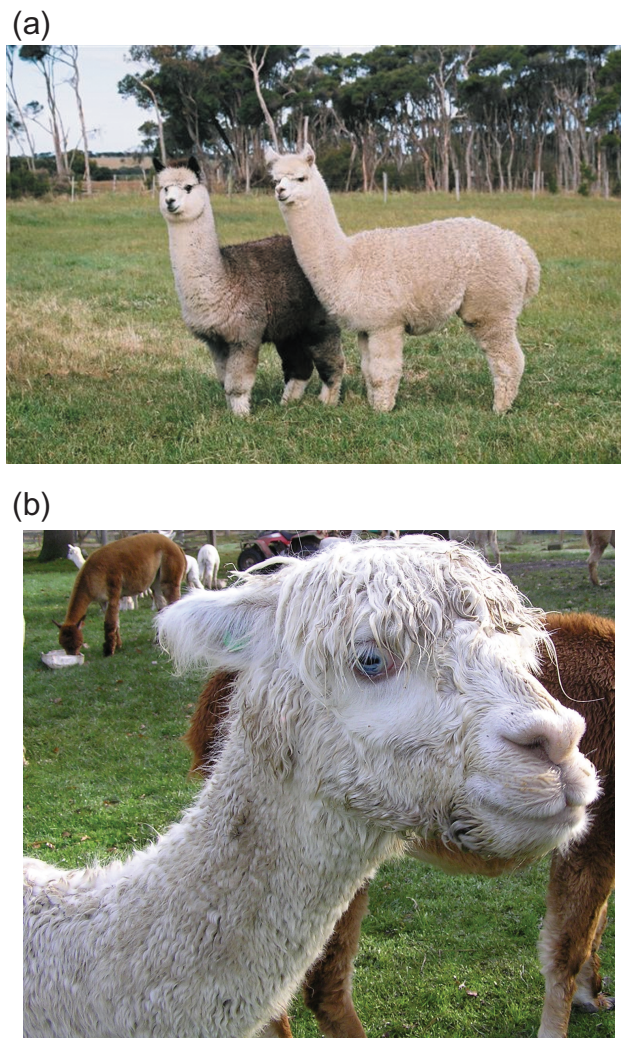


Figure 1. Photograph of an alpaca with (a) the typical gray fleece phenotype characterized by white face, chest, and feet (left) standing next to an all white alpaca (right) and (b) the BEW phenotype, which is characterized by a solid white coat, blue irides, and often deafness.

A further white-spotting pattern in alpacas occurs in gray alpacas. Gray alpacas typically have a white-spotting pattern of gray fleece separated by white fleece on the face, legs, and neck (sometimes referred to as “tuxedo”; [Figure 1a](#)). There is, however, variation in the extent of this pattern on the animal with some animals having less-extensive white patterning that doesn’t include the feet (Gregory DM of Chiverton Alpacas, personal communication). Therefore, we use the term “gray” here to refer to both a fleece color and a fleece pattern in alpacas. Based on herd book records, [Paul \(1999, 2006\)](#) proposes that gray fleece in alpacas is the result of a dominant diluting allele operating on black and brown pigment, as well as minor white-spotting alleles producing the characteristic white face, neck, and feet of gray alpacas. The mutation causing gray has been hypothesized to be homozygous lethal as matings between gray alpacas lead to a 2:1 (66%) instead of 3:1 (75%) ratio of gray to nongray offspring. In other species, this ratio can indicate homozygote lethality ([Pulos and Hutt 1969](#); [Hintz and Van Vleck 1979](#); [Russell 1979](#); [Silvers](#)

[1979](#)). It is not known whether this results from the diluting or spotting alleles associated with gray in alpacas. The observation of gray offspring from nongray parents suggests the presence of cryptic/hidden gray animals ([Paul 1999, 2006](#)). Evidence of embryonic lethality or sublethality of roan and white-spotting mutations in homozygotes has been reported in horses, rats, and mice ([Gruneberg 1936](#); [Hintz and Van Vleck 1979](#); [Geissler et al. 1981](#); [Niwa et al. 1991](#); [Hosoda et al. 1994](#); [Santschi et al. 1998](#)).

White alpacas are sometimes afflicted with the blue-eyed white (BEW) phenotype ([Figure 1b](#)), which is characterized by a solid white coat, blue irides, and often deafness ([Gauly et al. 2005](#)). The BEW trait is common in alpacas, although its prevalence is hard to measure because the International Alpaca Registry does not include eye-color information. Although the deafness does not pose a welfare concern for an alpaca in a herd situation, many breeders regard the BEW phenotype as a defect or undesirable trait. The inheritance pattern of the BEW phenotype has not been proven, but

breeders have postulated that it results from an interaction (of unknown type) between the gray gene and the white-spotting gene because BEW animals typically have many white and/or gray relatives in their pedigree (Merriwether and Merriwether 2003; Paul 2006). However, no studies have been done on a molecular level to understand the possible identity of these genes.

The *KIT* gene, which encodes the *c-kit* receptor tyrosine kinase, has been implicated in pigment variations, including white-spotting phenotypes, in a range of species (Chabot et al. 1988; Geissler et al. 1988; Fleischman et al. 1991). In humans, a heterozygous deletion of *KIT* leads to a pigmentation defect with random patches of unpigmented skin and hair called piebaldism, a condition similar to the white-spotting phenotype in mice (Fleischman et al. 1991). In pigs, the dominant white coat-color phenotype is caused by 2 mutations within the *KIT* gene, which lead to an absence of melanocytes in the hair follicles and hair bulbs (Johansson Moller et al. 1996; Marklund et al. 1998). Four depigmentation phenotypes (tabiano, roan, sabino-1, dominant white) have been mapped to a region of the equine Chromosome 3 harbouring the *KIT* locus (Marklund et al. 1999; Mau et al. 2004; Brooks and Bailey 2005). Furthermore, the cattle *KIT* locus is considered the main candidate gene for the *spotting* (S) locus (Reinsch et al. 1999; Fontanesi et al. 2010). In domestic cats (*Felis catus*), markers close to *KIT* are associated with white spotting (Cooper et al. 2006). Investigations into differential gene expression between colors of alpaca fibre found *KIT* expression was lowest in white animals and highest in bay animals (intermediate expression in black animals; Munyard 2011). Therefore, the alpaca *KIT* gene is a major candidate for white-spotting patterns.

Because of the importance of the all (solid) white phenotype and the BEW phenotype in the alpaca industry, our goal was to 1) identify genetic markers associated with these phenotypes and 2) to characterize the occurrence of these markers in known pedigrees and the general population. Here, we report the first evidence that the BEW phenotype is controlled by mutation/s at or nearby the *KIT* locus and provide diagnostic opportunities and practical guidelines for breeders.

Materials and Methods

Animals

Three data sets were used to investigate the genetic basis of the BEW phenotype (Figure 2). First, a sample of 40 unrelated individuals (17 BEWs; 23 non-BEWs: 2 black, 4 brown, 4 fawn, 6 gray, 4 white, and 3 multicolored animals; Table 1) were studied to examine association of *KIT* markers with the BEW phenotype (data set A). These individuals were determined to be unrelated according to 3 generations of pedigree data. Haplotypes were constructed manually from inspection of family relationships within a pedigree of 12 individuals (data set B) to confirm the associated haplotypes identified in data set A. To examine the phenotypic effects of individual haplotypes, a larger sample of 171 individuals

(29 BEWs, 66 black, 23 white, 13 gray, 21 brown, 8 fawn, and 11 multicolored) of mixed relatedness was studied (data set C). Blood samples were collected by collaborating veterinarians. Pedigree information was accessed through the IAR (International Alpaca Registry) database on the Australian Alpaca Association website (<http://www.alpaca.asn.au/>). Blood was stored in PaxGene™ tubes (PreAnalytiX), and genomic DNA was extracted using a commercially available kit according to the manufacturer's instructions (AxyPrep blood genomic DNA miniprep kit, Axygen Biosciences, USA). Some DNA samples were also extracted from saliva swabs (Oragene; DNAGenotek, Kanata, Ontario, Canada) according to manufacturer instructions. Eye and coat color were reported by sample collectors, and photos of animals were obtained where possible.

Microsatellite Marker and Allele Detection

Tandem repeats within the GeneScaffold_2179 of the *V. pacos* genome sequence, vicPac1, (<http://www.ensembl.org/>), which contains the *KIT* gene, were identified using an online repeat finder tool (Benson 1999). The vicPac1 genome sequence is a 2.51X assembly with sequencing reads aligned to the human genome. Comparative analysis of the human, bovine, dog, and mouse genomes (<http://www.ensembl.org/>) places the *PDGFRA* gene downstream of *KIT* and *KDR* upstream of *KIT* in these species. Repeats within and flanking *PDGFRA* and *KDR* were obtained from GeneScaffold_809 and GeneScaffold_2179 of the *V. pacos* genome sequence, respectively, to examine the possibility of either gene contributing to the BEW trait (Figure 3). Marker order in a *de novo* assembled alpaca genome sequence was identical to order in the low coverage, vicPac1 genome (Johnson WE, unpublished data). Primers were designed using Primer3 (Rozen and Skaletsky 2000). The 5' end of the forward primer from each pair was modified with a M13(-21) universal sequence tag (5'-TGTAACGACGGCCAGT-3') to enable the incorporation of the universal fluorescent-labeled M13(-21) primer (Schuelke 2000). The polymerase chain reaction (PCR) products were then analyzed on a 3730XL capillary analyser (Applied Biosystems) by Macrogen, Korea, and allele sizes were scored using GeneMapper 4.0 software (Applied Biosystems). We genotyped 9 *KIT*-associated microsatellite markers of which 4 were within *KIT* and 5 were in the adjacent regions (see Supplementary Material online).

PCR Conditions

Amplification of microsatellite loci was carried out in a 12 μ L volume consisting of approximately 50–100 ng of DNA template, 0.0885 μ L 10 μ M forward primer, 0.35 μ L 10 μ M reverse primer, 0.35 μ L 10 μ M fluorescent-labeled M13(-21) primer (FAM, VIC, NED, or PET), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 5 \times $MgCl_2$ -free buffer, and 1 U/ μ L GoTaq Flexi Taq DNA polymerase (Promega). The cycling protocol was 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at the optimized annealing temperature, 40 s at 72°C. This

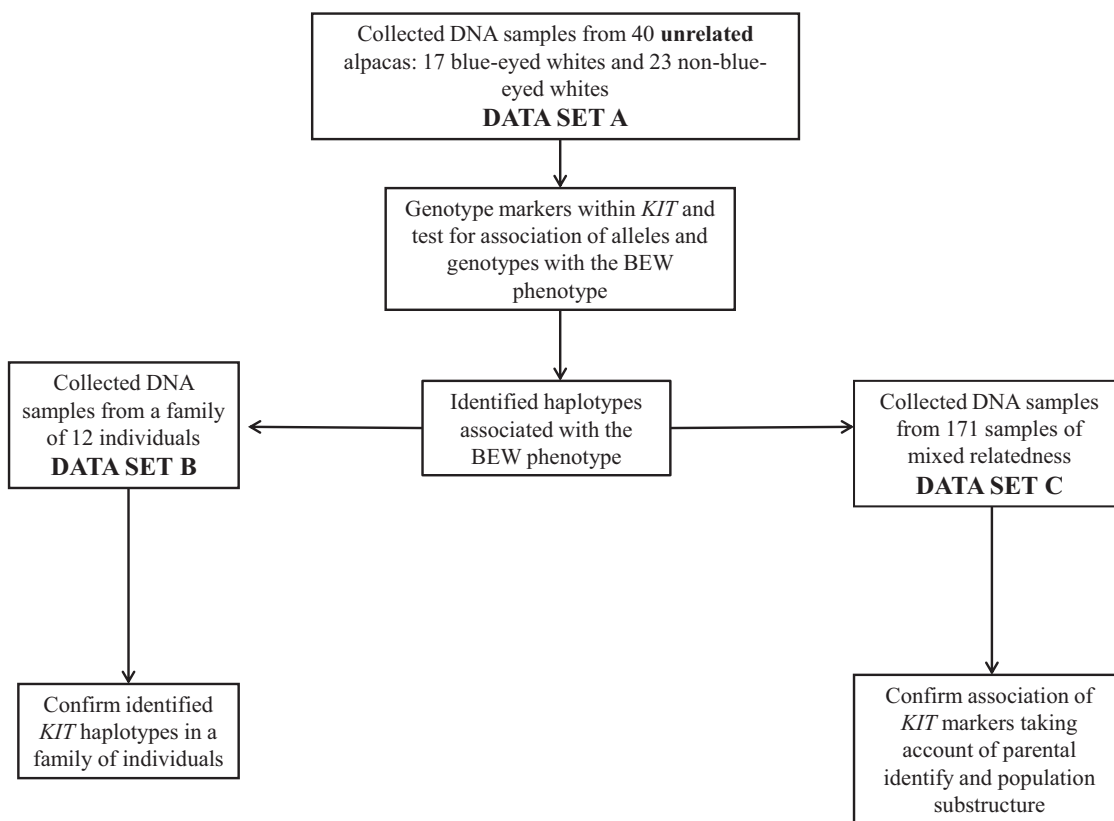


Figure 2. Flow chart diagram describing the use of 3 data sets to investigate the genetic inheritance of the BEW phenotype.

was followed by 8 cycles of 30 s at 94°C, 45 s at 53°C and 45 s at 72°C. The final extension step was at 72°C for 10 min.

Marker-Phenotype Association Analyses

SamplePower (IBM SPSS Version 3.0) was applied to estimate the power of the case–control association tests. The criterion for significance (α) was set at 0.050, and the tests were 2-tailed. Marker diversity was analyzed for data set A using GenAlex version 6.1 (Peakall and Smouse 2006). A case–control analysis based on χ^2 and Fisher's exact tests for genotypes were conducted within GraphPad Prism using 40 unrelated animals (data set A). Both test types were performed, as chi-squared tests approximate a P value, whereas Fisher's tests provide an exact P value. Chi-squared tests give an easy-to-interpret chi-squared value to indicate the strength of association. To account for multiple testing, we used the Bonferroni correction and considered significant only those markers for which raw $P < 0.05/7 = 7.14 \times 10^{-3}$.

The genotypes of a further 60 genome-wide microsatellite markers were used to measure and adjust for possible unmatched population structure in cases and controls of data set C. These microsatellite markers were from previously published studies of Camelidae species (Lang 1996; Penedo 1998; Penedo et al. 1999; Sarno et al. 2000; Mariasegaram et al. 2002; Bustamante et al. 2003; Evdotchenko 2003) or were identified in the Ensembl vicPac1 genome sequence (see Supplementary Material online). STRAT software was

used to conduct association tests while controlling for population stratification (as assessed by microsatellite markers), which can cause spurious associations (Pritchard et al. 2000). STRAT works with the output of the STRUCTURE program (Pritchard and Rosenberg 1999; Pritchard et al. 2000; Falush et al. 2003). We used the default settings assuming correlated allele frequencies and the admixture model for one population. We ran STRUCTURE for 50 000 burn-in steps followed by 20 000 replications. We used 10 000 permutations to calculate P values within STRAT. Another possible cause of spurious association is family structure. Therefore, tests for association were also performed using the SAS *surveyrreg* procedure (version 9.1, SAS Institute). This procedure was used to fit a linear regression model, which controlled for parent identity. Tests for association of alleles with gray fleece and white fleece were conducted using data set C.

Results

The Blue-eyed White Phenotype Is Associated with Two *KIT* Alleles

About 7 of the 9 markers tested were polymorphic and used in subsequent analyses. The number of alleles at each marker ranged between 5 and 9 (see Supplementary Material online). Shannon's Information Index (Sherwin et al. 2006) was between 1.800 (*KIT3*) and 1.296 (*KKDR4*) and observed heterozygosity ranged from 0.676 (*PDGFRA1*)

Table 1 Details of coat-color phenotype, eye-color phenotype, sex, and *KIT* marker genotypes of unrelated alpacas used in the current study. Dam and sire fleece color are also provided. Fleece color is denoted for animals where pedigree records of fleece color were available. Individuals with assumed recombination events are bolded (recombinant for *beu1* haplotype)

Sex	Fleece color	Eye color	Dam fleece color	Sire fleece color	49774 genotype	KIT10 genotype	KIT3 genotype
Female	White	Blue	White	White	236/241	266/278	-/-
Female	White	Blue	White	White	234/236	266/275	255/255
Female	White	Blue	-	-	236/241	266/275	268/270
Male	White	Blue	White	White	241/241	272/278	239/270
Female	White	Blue	White	White	236/241	266/278	268/270
Male	White	Blue	White	Silver gray	236/241	266/278	268/270
Female	White	Blue	White	White	236/241	266/278	268/270
Female	White	Blue	Brown/white	White	234/236	266/275	253/253
Male	White	Blue ^a	White	Fawn	236/241	266/278	-/-
Female	White	Blue	Fawn	White	236/241	266/272	239/268
Female	White	Blue	-	-	236/241	266/278	268/270
Female	White	Blue	Fawn	Rose gray	236/241	266/278	268/270
Female	White	Blue	Silver gray	White	236/241	266/278	268/270
Female	White	Blue	Black	White	236/241	266/278	268/270
-	White	Blue	-	-	236/241	-/-	-/-
Male	White	Blue	Fawn	White	236/241	266/272	-/-
Male	White	Blue	-	-	236/241	266/278	268/270
Female	Black	Pigmented	Black	Fawn	233/236	275/278	253/253
Female	Black	Pigmented	Black	Black	247/247	239/239	257/257
Male	Brown	Pigmented	Fawn	Fawn	234/243	266/275	239/253
Male	Brown	Pigmented	-	-	241/247	239/278	257/270
Female	Brown	pigmented	Brown	Brown	234/241	275/284	253/253
Female	Brown	Pigmented	Brown	Rose gray	236/241	220/220	268/270
Female	Fawn	Pigmented	Brown	Brown	234/241	278/281	255/270
Female	Fawn	Pigmented	White	Brown	234/243	239/275	253/257
Female	Fawn	Blue	White	White	236/236	266/272	268/270
Male	Fawn	Pigmented	Brown	Fawn	234/247	239/275	253/257
Female	Rose gray	Pigmented	White	Fawn	234/236	266/275	251/268
Female	Silver gray	Pigmented	Silver gray	Brown/silver gray	234/243	266/275	251/268
Male	Silver gray	Pigmented ^b	-	-	234/236	266/278	255/268
Female	Rose gray	Pigmented	Rose gray	Rose gray	234/236	266/275	251/268
Female	Silver gray	Pigmented	-	-	236/247	239/266	-/-
Female	Silver gray	Pigmented ^b	Silver gray	Silver gray	236/243	239/266	257/268
Female	Black/white	Pigmented	Silver gray	White	236/241	263/278	255/270
Female	Brown/white	Pigmented	Brown	Rose gray	236/247	239/266	257/268
Female	Black/white	Blue	White	Black	234/236	266/278	-/-
Female	White	Pigmented	White	Fawn	234/247	239/275	251/257
Female	White	Pigmented	-	-	234/241	275/278	253/270
Female	White	Pigmented	-	-	236/243	272/278	268/270
Female	White	Pigmented	White	White	236/241	275/278	270/272

-/- denotes where genotypes could not be obtained due to poor amplification.

^asome pigment bleed through; ^bwith blue speckles

to 0.949 (KIT10). The study had a power of greater than 99.9% to detect significant differences in genotype frequencies between cases and controls at 49774. The genotypes of markers within *KIT* (49774, KIT10 and KIT3) showed a significant association with the BEW phenotype (Table 2). The microsatellite 49774 showed the strongest association with the BEW phenotype ($\chi^2 = 21.56$, bonferroni corrected $P = <0.0007$) for the genotypes, whereas tests for association of individual alleles with the BEW phenotype were significant but showed weaker association than tests for association

with genotypes ($\chi^2 = 6.93$ and $\chi^2 = 13.19$). The other markers within *KIT*, KIT10, and KIT3 showed the same pattern of high association with genotypes. The genotypes 236/241, 266/278, and 268/270 at markers 49774, KIT10, and KIT3, respectively, were significantly associated with the BEW phenotype. This suggested that 2 mutations contribute to the BEW phenotype. The markers flanking *KIT* did not show a significant association (bonferroni corrected $P > 0.065$) for genotypes with the BEW phenotype. The significance of association of marker 49774 with the BEW phenotype was

Table 2 Results of chi-square tests, as well as their error probabilities (P), for associations of intragenic *KIT* marker genotypes (49774, KIT10, KIT3) and markers flanking *KIT* (KKDR4, KKDR5, PDGFRA1, PDGFRA3) with the BEW phenotype. All tests were 2-sided with an α value of 0.05 and used 40 unrelated individuals

Trait	Marker	χ^2	Chi-square P value	Fisher's exact test P value
BEW	PDGFRA1	6.728	0.0095 (0.0665)	0.0252 (0.1764)
BEW	PDGFRA3	0.02724	0.8689 (>1.00)	1.000 (>1.00)
BEW	49774	21.56	<0.0001 (<0.0007)	<0.0001 (<0.0007)
BEW	KIT10	12.82	0.0003 (0.021)	0.0009 (0.0063)
BEW	KIT3	10.61	0.0011 (0.0077)	0.0024 (0.0168)
BEW	KKDR4	2.332	0.1267 (0.8869)	0.1642 (>1.00)
BEW	KKDR5	2.692	0.1009 (0.7063)	0.1872 (>1.00)

Bonferroni corrected P values are provided in brackets () alongside raw P values.

Table 3 Patterns of allelic variation of microsatellite 49774 in various color classes of alpaca fleece in a cohort of 171 related individuals

Genotype	Blue-eyed white	Black	Brown	Fawn	Gray	Multi	White with dark eyes
234/236	2	5	5	1	7	3	6
234/234	0	16	2	0	1	0	2
241/247	0	1	2	2	0	0	0
234/241	0	6	2	2	1	7	4
247/247	0	5	2	0	0	0	0
234/247	0	24	6	0	0	0	1
236/247	1	2	1	1	4	1	0
234/249	0	1	0	0	0	0	0
236/241	22	0	0	1	0	0	1
234/243	0	2	0	0	0	0	0
241/241	2	0	0	0	0	0	8
236/243	0	1	0	0	0	0	0
243/243	0	1	0	0	0	0	0
232/234	0	2	0	0	0	0	0
236/236	2	0	1	1	0	0	1
Total	29	66	21	8	13	11	23

tested in a larger cohort of 171 individuals (data set C) and significance remained after correcting for population substructure ($\chi^2 = 77.385$, $P \leq 1.0 \times 10^{-4}$) and parental identity ($P = < 0.0001$; Table 3). Two individuals with colored fleece and blue eyes were included in this study and did not have the BEW-associated *KIT* genotype.

Haplotype Analysis

A family of 12 individuals was genotyped for the *KIT* markers associated with the BEW phenotype, and no Mendelian errors were identified in microsatellite genotypes (Figure 4). The association of 2 haplotypes with the BEW phenotype was also evident in the pedigree data. Indeed it can be seen that all affected individuals in the pedigree possessed the same *KIT* haplotypes as affected individuals in the unrelated animals in data set A. Examination of microsatellite genotypes within a pedigree allowed the identification of 2 haplotypes *beW1* (236 at 49774, 266 at KIT10, 268 at KIT3) and *beW2* (241 at 49774, 278 at KIT10, 270 at KIT10). The haplotypes *beW1* and *beW2* were present in affected (BEW) offspring, whereas unaffected parents carried either haplotype in homozygous or heterozygous form. The *beW1* haplotype of I-2 was inherited by all her offspring with blue eyes, and

none of her offspring with dark eyes. All offspring of I-2 also inherited the *beW2* allele from their sire; however, only the offspring that also inherited *beW1* from their dam are BEW. There was complete cosegregation of the *beW1/beW2* genotype and the BEW phenotype within this pedigree.

Once the BEW-associated haplotypes had been confirmed in a family, individuals within data set A were examined for recombination events that might suggest a smaller critical region. As only 3 markers showed significant association with the BEW phenotype, a conservative approach was taken when identifying recombination events, and only individuals who carried at least 2 consecutive alleles of haplotype *beW1* or *beW2* were considered. If one pertains to the assumption that the mutation causing the gray fleece phenotype is homozygous lethal and that the *beW1* haplotype is in linkage disequilibrium with this mutation, then 2 individuals in Table 1 (bolded) suggest that this mutation is 3' of 49774.

The Distribution of *KIT* Alleles in Alpacas of Different Fleece Colors

To explore the phenotypic significance of the individual alleles associating with the BEW phenotype, we extended our study to examine a larger cohort of individuals of mixed

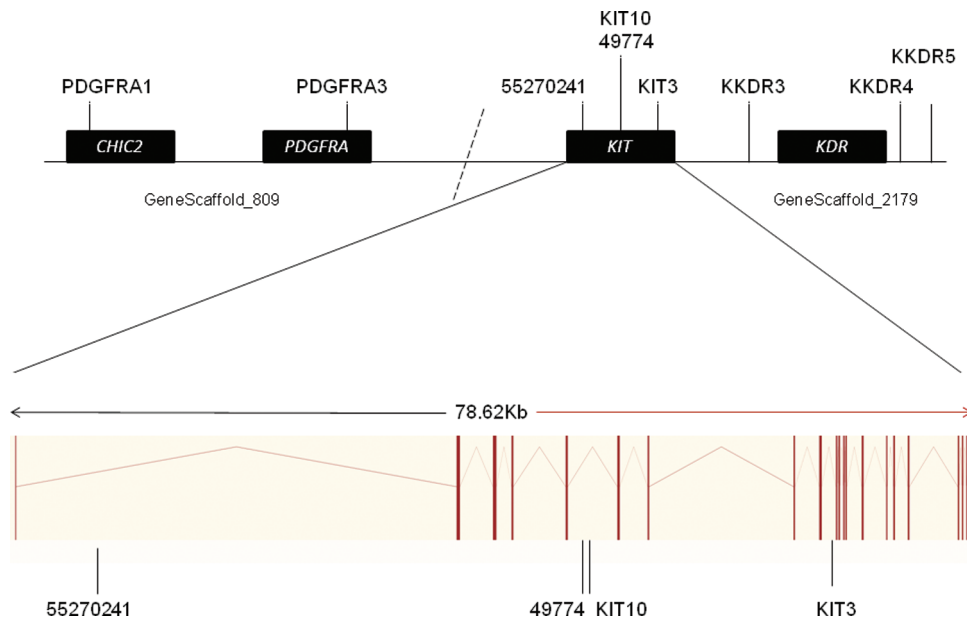


Figure 3. The positions of microsatellite markers used in this study, relative to gene positions. Markers were derived from 2 scaffolds from the *Vicugna pacos* Ensembl genome (GeneScaffold_809 and GeneScaffold_2179). The dotted line represents the hypothetical join between these scaffolds based on conservation of this gene order in human, bovine, murine, and canine genomes (<http://www.ensembl.org/>). The lower part of the figure shows the 29 exons of the alpaca *KIT* gene as vertical bars separated by intronic sequences.

relatedness (data set C). The *bev1* haplotype was present in animals of all color classes but in high abundance in gray animals. The test for association with gray fleece had a power of 90.1% to detect significance using the frequency distribution of the 236 allele at 49774. There was evidence for association of 49774 with gray fleece color even when taking into account possible population structure using STRAT software ($\chi^2 = 11.771$, $P = 2.0 \times 10^{-3}$). The level of significance for marker 49774 (with gray fleece) was reduced for regression analysis after accounting for shared parentage ($P = 0.0872$). There were 13 gray individuals in the 171 samples studied. Eleven of 13 (84.61%) gray individuals (including rose and silver grays) possessed the *bev1* allele at marker 49774, 10 at KIT10 and 11 at KIT3 (Table 3). No *bev1* homozygotes were identified in the 13 gray individuals. Although 5 of 171 (3.21%) individuals were homozygous for the 236 allele at 49774 (Table 3), no individuals of any color had the *bev1* haplotype at all 3 markers. This haplotype appears to be frequent in the alpaca population with 13 of 23 (56.52%) non-BEW unrelated individuals possessing the *bev1* allele at marker 49774.

The 241 allele at marker 49774 was present in animals of all fleece colors. Nine individuals (8 solid white and 1 BEW) were homozygous at all 3 markers for the *bev2* haplotype. The test for association with white fleece had a power of greater than 99.9% to detect significance using the frequency distribution of the 241/241 genotype at 49774. Evidence for association was detected for 49774 with white fleece after correcting for population structure ($\chi^2 = 33.844$, $P \leq 0.01 \times 10^{-3}$) and shared parents ($P = 0.0002$). Eight of 23 (34.78%)

white animals with pigmented eyes were homozygous for the *bev2* allele (Table 3). This allele appears to be common in the alpaca population with 7 of 23 (30.43%) non-BEW unrelated animals carrying the allele.

Discussion

Mammalian coat and skin color appear to be determined by a similar set of genes across different species (Jackson 1994; Barsh 1996; Newton et al. 2000). Most notably, these include melanocortin-receptor 1 (*MC1R*), Agouti signaling protein (*ASIP*), tyrosinase (*TYR*), microphthalmia-associated transcription factor (*MITF*), membrane-associated transporter protein (*MATP*), mast cell growth factor (*MGF*), α -melanocyte stimulating hormone (α -*MSH*), melanophilin (*MLPH*), tyrosinase-related protein 1 (*TYRP1*), oculocutaneous albinism type 2 (*OCA2*), and c-kit (*KIT*). The *KIT* gene, which encodes the c-kit receptor tyrosine kinase, has been implicated in pigment variations (primarily white-spotting phenotypes) in a range of species. The swine dominant white phenotype is caused by 2 mutations in the *KIT* gene: one is a gene duplication, and the other is a splice-site mutation in intron 17 leading to skipping of exon 17 of one of the copies (Marklund et al. 1998). Characterization of the equine *KIT* locus has found multiple independent mutations that are presumed to be responsible for dominant white-spotting (Haase et al. 2007). Furthermore, cattle breeds carrying different putative alleles at the spotting locus show genetic heterogeneity within the *KIT* gene (Fontanesi et al. 2010). *KIT* has been implicated in white-spotting patterns in many species

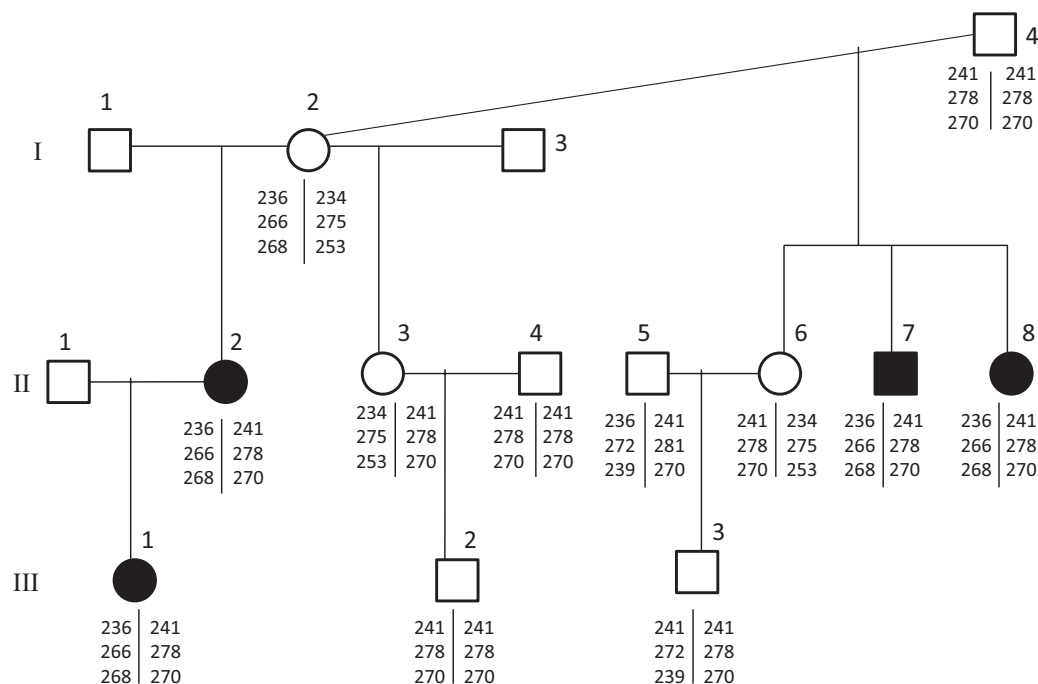


Figure 4. Mendelian inheritance of alpaca *KIT* alleles within a family segregating the BEW phenotype. Individuals shaded black have the BEW phenotype, whereas unshaded individuals are animals with white fleece and pigmented irides. For each individual, the genotypes are given at markers 49774, *KIT10*, and *KIT3* in this order.

but also in roan coat color in horses and pigs (Marklund et al. 1999; Cho et al. 2011).

In alpacas, the only molecular genetic studies of coat color have been to screen candidate genes for coat color dilution, absence of dark fibre, black fleece, and lightness of fibre (Feeley and Munyard 2009; Cransberg and Munyard 2011; Feeley et al. 2011; Guridi et al. 2011). *KIT* is one of the major mammalian coat color loci and affects pigmentation through influencing the migration of melanocytes from the neural crest during embryogenesis (Chabot et al. 1988; Geissler et al. 1988), and is therefore a reasonable candidate gene for white-spotting patterns in alpacas, as well as the rarer BEW phenotype.

Genetic case–control studies such as this must be conducted mindful of the possibility of false positive results (Lohmueller et al. 2003; Price et al. 2010). To decrease the likelihood of spurious results, we first tested for association of microsatellite markers with the BEW phenotype using a set of individuals known to be unrelated for at least 3 generations (data set A), and a strong association was identified between *KIT* genotype and the BEW phenotype. We then confirmed the presence of haplotypes identified in data set A, in a family (data set B), and indeed the same two haplotypes *bev1* and *bev2* were present in affected (BEW) individuals. We also tested a larger cohort containing some related individuals for association of *KIT* markers with the BEW phenotype (data set C). To address the possibility that our results were caused by spurious associations due to unknown population structure, we genotyped 171 cases and controls with 60 genome-wide microsatellite markers. We also used a second association test that controlled for one

generation of pedigree relatedness. We were, however, only able to access pedigree information for animals postimportation to Australia, and it is therefore possible that related animals were inadvertently included but not controlled for. This larger data set also showed strong association of the BEW phenotype with *KIT* marker genotypes. The 3 data sets (A, B, and C) including a group of unrelated individuals, a pedigree, and a larger cohort of samples all supported the involvement of 2 *KIT* alleles with the BEW phenotype.

The analysis presented here demonstrates a strong association but not unequivocal relationship between the BEW phenotype and *KIT* genotype. We therefore postulate that these microsatellites are in linkage disequilibrium with the causative mutation, which may be in *KIT* or a nearby promoter or enhancer region. The finding of no significant association of markers in the genes neighboring *KIT* (*PDGFRA* and *KDR*) supports the conclusion that the mutations associated with the *bev1* and *bev2* haplotypes lie close to the *KIT* gene. It is also possible that the BEW phenotype is caused by a single mutation that has multiple *KIT* haplotypes, that is, that recombination may have placed the causative mutation on a second haplotype. However, under these circumstances we would expect to see a class of BEW animals that are homozygous for the *bev1* allele, as well as a class of BEW animals that are *bev2* homozygotes. However, of the 5 animals that have the 236/236 genotype at 49774, only 2 are BEW and only 2 of 10 animals with the genotype 241/241 are BEW. The finding of strong association of *KIT* genotype with the BEW phenotype at 3 different markers within *KIT* suggested the presence of 2 haplotypes that contribute to the

BEW phenotype. This is in agreement with breeder's suggestion that 2 alleles are required for the expression of the BEW trait. The findings in this study suggest that 2 haplotypes representing 2 mutations contribute to the BEW phenotype. We propose that the BEW phenotype is the result of the cumulative hypopigmentation effects of 2 *KIT* mutations.

The involvement of a common allele with the gray and BEW phenotype has been proposed previously (Paul 1999; Merriwether and Merriwether 2003; Paul 2006) although this study is the first to use molecular genetics to investigate this. Upon examination of a larger cohort of individuals, it was evident that an association exists between the *bew1* haplotype and the gray fleece phenotype. Most gray individuals (11 of 13) carried 1 copy of the *bew1* haplotype. The association of the *bew1* haplotype with gray fleece was not as strong as the association of *bew1/bew2* with the BEW phenotype. This is not surprising as the *bew1* haplotype is present in many nongray individuals. This finding is in agreement with suggestions by Paul (1999, 2006) that there are hidden gray animals that carry a gray allele but do not have a base fleece coat color which allows its visualization, that is, white-spotting patterns maybe hard to see in animals whose base coat color is light such as fawn or white. The findings in this study are in agreement with findings by Paul (1999, 2006) regarding the dominance of the gray phenotype and the involvement of a white-spotting gene, such as *KIT* with the gray phenotype. Based on its function in other species, *KIT* is not a likely candidate for pigment dilution; however, the *bew1* allele at *KIT* may control the patterning of white fleece on a gray alpaca. No individuals were found to be homozygous for the *bew1* haplotype. Five individuals had the *bew1* allele at 49774 but not the *bew1* alleles at *KIT10* or *KIT3*. It is likely that these individuals carry a recombination event between marker 49774 and the *bew1* mutation or genotyping error. Therefore, it is probable that these 5 exceptions do not carry the mutation associated with the *bew1* haplotype. The genotypic distributions of marker genotypes at *KIT* add further support to the hypothesis of Paul (1999, 2006) that a mutation associated with gray fleece is homozygous lethal. It is not known whether this allele is homozygous lethal only in a gray genetic background; however, our data suggests that the *bew1* mutation is homozygous lethal in most, if not all genetic backgrounds, as no individuals with the full *bew1* haplotype were identified in the 171 individuals studied. This is not uncommon in other species where mutations in the *KIT* locus often produce lethal or sublethal phenotypes in homozygous condition (Russell 1979; Silvers 1979; Nocka 1990).

The phenotypic effect of the mutation associated with the *bew2* haplotype is less clear. There was a tendency for *bew2/bew2* animals to have white fleece; however, none of these animals had blue eyes. The data in Table 3 demonstrate that the *bew2* allele is present in a wide variety of color classes. The *bew2/bew2* genotype did not correlate with all individuals with white fleece, suggesting that white fleece has multiple genetic origins. This may also, in part, be a function of errors in the alpaca pedigree database records where owners are misassigning individuals with subtle white-spotting patterns as solid white. The phenotypic effect of the *bew2* haplotype and associated mutation

requires further investigation such as functional studies of melanocyte distribution in *bew2/bew2* animals.

There is considerable debate among breeders regarding the defectiveness of the BEW phenotype. Many believe the BEW phenotype to be a genetic flaw requiring purging from the national stock. Others believe that the deafness of a BEW alpaca does not lead to any animal welfare issues as the animals quickly compensate with their other senses and by following the cues of other animals in the herd. Given that the haplotypes *bew1* and *bew2* are relatively common in the alpaca population, alpaca breeders who wish to avoid mating BEWs may inadvertently do so by mating individuals carrying *bew1* and *bew2*. This study is the first to report on the genetic inheritance of this trait and provides breeders with insight into its genetic determinants to enable increased efficiency in breed management.

In conclusion, we have identified markers within *KIT*, which are likely in linkage disequilibrium with the mutation/s causative of the BEW trait in alpacas. Our study provides a genetic testing tool to enable breeders to select either for or against the haplotypes associated with the BEW phenotype. We will continue the search for the specific mutation/s responsible for the BEW phenotype using next-generation sequencing technologies.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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