THE GENETICS OF VARIANT RED AND CHANGELING COAT COLOUR PHENOTYPES IN HOLSTEIN CATTLE

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ABSTRACT

Although most Holstein cattle are either black with white spots or red with white spots, two new coat colour traits called Variant Red (VR) and changeling, have recently been recognized by Holstein Canada. Variant red cattle look the same as red and white Holsteins but VR appears to be inherited as a dominant trait. Changeling cattle are born red and turn black by adulthood. Candidate genes for changeling included *melanocortin 1 receptor* (*MC1R)*, *agouti signalling protein* (*ASIP)*, *attractin* (*ATRN)*, and *melatonin receptor 1A* (*MTNR1A)*. Candidate genes for variant red included these same genes and *β-defensin300 (*putative *K* locus*)*. Polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) and sequencing were used to genotype single nucleotide polymorphisms (SNPs), which were recently discovered in our lab, for use in determining co-segregation in appropriate families. Co-segregation analysis excluded *MC1R*, *ASIP*, *ATRN*, or *MTNR1A* for changeling and VR, assuming single gene inheritance. Microsatellites and SNPs were used to map VR to a region on BTA27 surrounding and including *β-defensin300* (LOD = 3.255). Cattle *β-defensin300* was characterized from genomic DNA and mRNA from skin and appears to be homologous to canine *β-defensin103* which is involved in black/red coat colour differentiation in many dog breeds. Although no mutations were found in the coding sequence, several were found in the 5', putative promoter region. No polymorphisms consistently and exclusively occurred in VR and not in changeling and/or black and white Holstein cattle. Common relatives occurred in the pedigrees of several variant red and changeling cattle. A hypothesis was developed which suggests that a common mutation is required for the occurrence of either phenotype. A dominant mutation in a second gene would be required for VR, potentially a promoter mutation in *β-defensin300*. Two recessive alleles at a yet unidentified second gene, potentially a promoter mutation in *ASIP*, would be required for the changeling phenotype to occur.

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LIST OF ABBREVIATIONS

α-MSH	Alpha melanocyte stimulating hormone
°C	Degrees Celsius
μL	Microlitre
ASIP	*Agouti signaling protein*
ATRN	*Attractin*
bp	Base pair
BTA	*Bos taurus* autosome
cAMP	Cyclic adenosine phosphate
cDNA	Complimentary deoxyribonucleic acid
cM	CentiMorgan
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
LOD	Log of the odds
MC1R	*Melanocortin 1 receptor*
$MgCl_2$	Magnesium chloride
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MTNR1A	*Melatonin receptor 1A*
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
pM	Picomole
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
UTR	Untranslated region
VR	Variant red

1.0 GENERAL INTRODUCTION

Coat colour has long been a popular area of genetic research, likely due to its highly visible nature. Coat colour is a trait that is easily observable and often serves to rapidly distinguish individuals, strains, and breeds in many mammalian species. In some species like cats and horses, coat colour is a critical feature distinguishing breeds.

Mammals are capable of producing two forms of pigment. Eumelanin is black, brown, or grey in colour, while phaeomelanin is red, yellow or cream in colour (Searle, 1968). Despite this simplistic starting point, mammals are capable of producing a wide variety of coat colour shades and patterns that extend beyond simply black or red. Different pigments can be expressed in different areas of the body, alternating along individual hair shafts, or changing throughout the life of the animal. These complexities rely on interaction of numerous genes that dictate when and where certain pigments are expressed, as well as the specific shade of each pigment. A small change in one aspect of the pigmentation pathway can significantly alter the final colour.

Adding to the intricacies of coat colour genetics is the ability of a similar colour phenotype to be created through various mechanisms. In dogs, the phaeomelanin colour can be produced through a homozygous recessive mutation at *melanocortin 1 receptor* (*MC1R*), *e*/*e* (Newton et al. 2000), or through the presence of the dominant allele at *agouti signaling protein*, a^y/_ (Berryere et al. 2005). Recently, in Holstein cattle, two new coat colour phenotypes have been recognized. Variant red (VR) appears similar to the traditional red phenotype produced by the recessive allele at *MC1R* (*e*/*e*) but follows a dominant inheritance pattern and does not rely on the presence of the *e MC1R* allele. Changeling is a colour phenotype that begins as red with white spots but changes to black with white spots as the cow matures. Traditional colours for Holstein cattle include black or red, both with varying amounts of white spotting.

The focus of this study is to understand the genetic mechanisms underlying the VR and changeling phenotypes and determine whether they are related. The main approach involves investigating candidate genes chosen based on similar colours in other species.

2.0 LITERATURE REVIEW

2.1 Cattle Coat Colour Genetics

Like other mammals, cattle are capable of producing two forms of pigment, phaeomelanin and eumelanin. Phaeomelanin encompasses the red/yellow/cream spectrum and eumelanin encompasses the black/brown/grey spectrum. These pigments are produced in pigment cells called melanocytes and then transported up the hair shaft resulting in pigmented hair (Willier, 1942). Whether eumelanin or phaeomelanin is produced depends on the activity of various genes. Some alter the intracellular levels of cyclic adenosine monophosphate (cAMP). Increased levels of cAMP result in increased activity of the tyrosinase enzyme and increased intracellular levels of tyrosinase bring about the expression of eumelanin (Burchill et al. 1989). As reviewed by Ito (1989), in addition to increasing expression of eumelanin in the melanocytes through an increase in environmental concentrations, tyrosinase is a vital enzyme in the production of both eumelanin and phaeomelanin. The amino acid, tyrosine, is converted to 3,4-dihydroxyphenalalanine (DOPA) and then to dopaquinone through the enzymatic activity of tyrosinase. In the presence of cysteine or glutathione, dopaquinone is transformed into phaeomelanin. In the absence of cysteine or glutathione, dopaquinone is converted to eumelanin.

2.1.1 *Melanocortin 1 Receptor*

Melanocortin 1 receptor (*MC1R*) encodes a member of a subfamily of G protein-coupled receptors. *MC1R* is specifically expressed in melanocytes (Mountjoy et al. 1992). Binding of α-Melanocyte Stimulating Hormone (α-MSH) to MC1R (Fig. 2.1) results in a two- to three fold rise of intracellular cAMP (Mountjoy et al. 1992). This increase of cAMP brings about an increase in tyrosinase, and this escalated intracellular activity of tyrosinase results in production of eumelanin (Burchill et al. 1989).

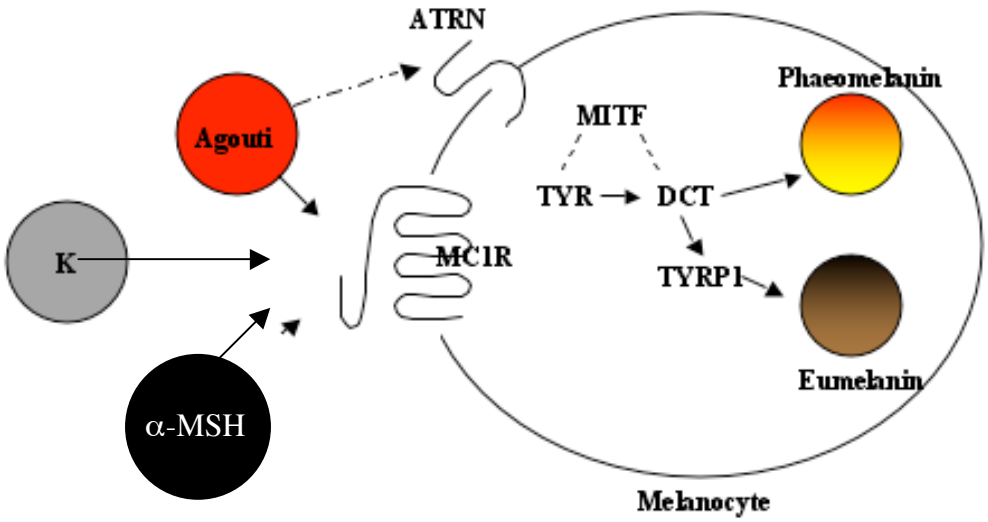
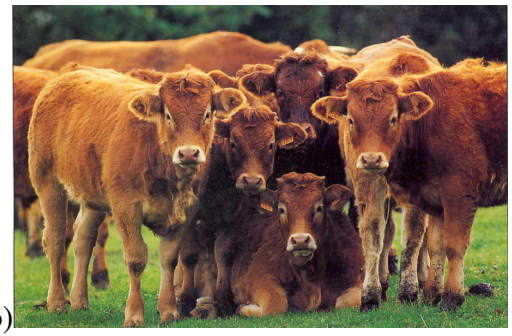


Figure 2.1 The melanocyte pigment pathway. Melanocortin 1 Receptor (MC1R) interacts with extracellular ligands α-Melanocyte Stimulating Hormone (α-MSH), K, and Agouti to produce eumelanin or phaeomelanin. Attractin (ATRN) acts as a mediator in binding of Agouti to MC1R.

Bovine *MC1R* is located on BTA 18 (Werth et al. 1996) and encodes a 317 amino acid product (GenBank NM_174108). Three alleles have been described: E^+, E^D and *e*. Klungland et al. (1995) described an amino acid substitution, L99P, which causes the dominant E^D allele. They report the E^D allele is constitutively expressed with one E^D allele being sufficient to dictate that the animal only produces eumelanin, as shown by the bull in Fig. 2.2a. A deletion of a guanine at nucleotide 310 results in a frameshift, producing a premature stop codon and truncated *MC1R* product. This mutation is associated with the recessive *e* allele. It is predicted that the truncated MC1R protein cannot bind α-MSH, therefore cattle that have two copies of the *e* allele (Fig. 2.2c) are not capable of producing eumelanin (Joerg et al. 1996), and the wild-type *MC1R* allele, E^+, allows for the expression of both black and red pigments, as shown by the cow in Fig. 2.2c.

a)  b) 

c) 

Figure 2.2 The phenotypes resulting from *MC1R* genotypes a) dominant E^D black b) traditional *e/e* red c) wild-type E^+.

Others have postulated the presence of additional *MC1R* variants. Rouzaud et al. (2000) described an E^l allele of *MC1R* as being a 12 bp duplication at nucleotide 669, inserting four additional amino acids. They found this allele in both the heterozygous and homozygous states in the Aubrac and Gasconne cattle breeds but did not suggest that this mutation was associated with a specific coat colour. Graphodatskaya et al. (2002) discovered additional *MC1R* variants: E^{d1}, E^{d3}, and e^f. E^{d1} is a 667C>T, changing an arginine to a tryptophan. They also described E^{d2}, the same 12 bp duplication described previously by Rouzaud et al. (2000). E^{d1} and E^{d2} were found in Brown Swiss cattle, with an E^{d3} animal being postulated as having a C allele at E^{d1} and not having the duplication of $E^{d2,}$ which appears to be the same as the E^+ wild-type allele. A T297I mutation they termed e^f, was reported in a red Holstein bull that was heterozygous for *e*.

2.1.2 *Agouti Signaling Protein*

Bovine *agouti signaling protein* (*ASIP)* is comprised of three exons and encodes a 133 amino acid protein, sharing 78% homology to mouse *ASIP* (Girardot et al. 2004). The ASIP protein binds MC1R and acts as a competitive antagonist to α-MSH, preventing the production of eumelanin (Ollman et al. 1998) (Fig 2.1).

While in other species, such as dogs and mice, multiple alleles are recognized at *ASIP*, cattle do not show any mutations within the *ASIP* coding region (Royo et al. 2005). In dogs, four *ASIP* alleles are recognized: a^y, a^w, a^t, and *a*. Each allele codes for a particular pattern of eumelanin and phaeomelanin distribution over the entire body or on each individual hair (Fig. 2.3). Two mutations affecting adjacent amino acids in exon 4 of dog *ASIP*, A82S and R83H, occur in dogs with a^y (Berryere et al. 2005). S82 H83 is associated with the sable or fawn coat colour of a^y. The *a* allele, producing a recessive black coat colour, is associated with a R96C missense mutation (Kerns et al. 2004).

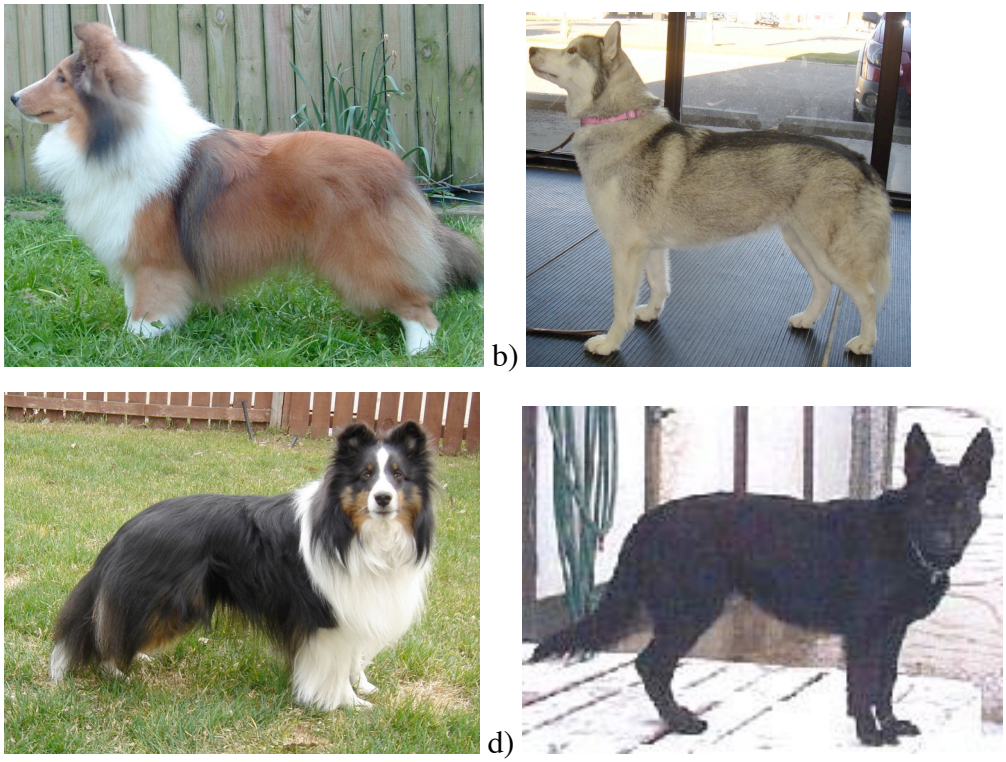


Figure 2.3 Phenotypes resulting from the canine *ASIP* alleles a) a^y sable or fawn, b) a^w wolf sable, c) a^t black and tan, and d) *a* recessive black.

Two inheritance models have been attributed to variation of *ASIP* in cattle. Searle (1968) outlines a model described by Lauvergne (1966) that postulated four *ASIP* alleles; A^+ producing dun, a^w as seen in Brown Swiss, a^y as seen in Jersey, and a^s non-agouti. Olson (1998) postulated a six allele model for *ASIP* in cattle. The genetic mutations corresponding to those alleles have not been found. Olson (1998) postulated an a^i allele, light undersides, and an a^w allele, taupe brown. The presence of a recessive black allele, *a*, and a wild type A^+ allele, allowing for expression of both black and red pigment, was postulated by Adalsteinsson (1995). Majeskie (1970) described a coat colour pattern postulated to be associated with *ASIP* termed patterned blackish which he referred to as A^{bp}. None of these mutations have been identified in *ASIP*. Dun in Highland and Galloway cattle has been shown to be caused by a mutation in *SILV* (Schmutz 2008) and by a mutation in *TYRP1* in Dexter cattle (Berryere et al. 2003).

Girardot et al. (2006) reported a mutation in *ASIP* resulting in the brindle pattern, A^{br}. A long interspersed nuclear element (LINE) insertion between the skin specific *ASIP* promoter and the first coding exon of *ASIP*, when present with the E^+ wild-type allele of *MC1R*, results in the brindle coat colour pattern in Normande cattle (Fig. 2.4).

Figure 2.4 The brindle coat colour pattern in cattle

Morgan et al. (1999) found evidence that *ASIP* was imprinted in mice. The A^y allele of murine *ASIP* resulted in a gradient of phenotypes, including yellow, mottled, and pseudoagouti, a phenotype that appears similar to the agouti banded hair pattern, when mice were genotyped as A^y/a. Homozygous A^y is lethal. It was found that when A^y/a dams of each phenotype variant were mated to *a*/*a* sires, the dam's phenotypic variation was more common among the A^y/a offspring.

This effect was not seen when *a*/*a* dams and A^y/*a* sires were used, indicating that inheriting the A^y allele from the dam had an influence on the A^y phenotypic variants expressed in the offspring.

2.1.3 *Attractin*

Attractin (*ATRN*) encodes a single pass transmembrane protein that assists in regulating the switch from eumelanin to phaeomelanin synthesis, based on studies in mice (Miller et al. 1997). Bovine *ATRN* is located on BTA 13 (Edeal et al. 2000) and codes for an 1415 amino acid protein (GenBank NP_776420). No alleles have yet been postulated or found in cattle *ATRN*.

ATRN has been most extensively characterized in mice, in which the phenotype is termed *mahogany*. The mahogany mutations suppress the *Agouti* phenotypes. ATRN, present on melanocytes, specifically binds ASIP and functions as a checkpoint required for ASIP signal transduction mediated through MC1R (He et al. 2001) (Fig 2.1). Three mutant alleles have been discovered in murine *ATRN*: *mg*, mg^{3J}, and mg^{L}, with mg^{3J} having a stronger effect than *mg* and mg^{L}, producing a darker phenotype (Gunn et al. 1999). The murine *ATRN* phenotypes are all present on an agouti background, with the *mg* allele producing a mouse with eumelanin on the dorsal surface and phaeomelanin on the ventral surface. The mg^{3J} allele decreases the expression of phaeomelanin resulting in a darker phenotype, and the mg^{L} allele produces a eumelanin dorsal stripe (Gunn et al. 2001).

2.1.4 *Melatonin Receptor 1A*

Melatonin receptor 1A (*MTNR1A*) encodes a high affinity G protein coupled receptor for melatonin (Ebisawa et al. 1994). Melatonin plays a significant role in pigmentation switching in various vertebrates, including the swamp eel (*Synbranchus marmoratus*), Northern Leopard Frog (*Rana pipiens*), Yellow Cururu Toad (*Bufo ictericus*), and Green Anole (*Anolis carolinensis*; Fig. 2.5; Castrucci et al. 1997). In each case, addition of melatonin to cultured cells was able to lighten α-MSH darkened skin as well as antagonize α-MSH darkening. Addition of melatonin to Siberian hamster hair follicle cultured cells inhibited melanogenesis triggered by α-MSH (Logan et al. 1980). Logan et al. (1980) found that this occurred without affecting tyrosinase enzyme

levels, suggesting that melatonin exerts its affect on the pigmentation pathway after tyrosinase is produced.

Figure 2.5 Melatonin is responsible for the colour change seen in the Green Anole. (Photo copyrighted to John Pickering, 2006 / www.discoverlife.org)

Two forms of melatonin receptors are present in mammals (Ebiwasa et al. 1994), MTNR1A and MTNR1B, though the tissues in which they are expressed vary significantly between species. MTNR1A is readily found in human skin, while MTNR1B is rarely found (Slominski et al. 2003) and MTNR1A is not present in skin from mice while MTNR1B is (Slominski et al. 2004). *MTNR1A* has been mapped in cattle to the distal portion of BTA 27 (Messer et al. 1997). No other information about cattle *MTNR1A* is yet known.

2.1.5 *β-Defensin300*

β-Defensin300 is a member of the large *β-Defensin* family of genes that code for proteins with moderately conserved consensus sequence including six conserved cysteine amino acids that form three disulphide bonds resulting in similar secondary structure (Lehrer et al. 2002). Selsted et al. (1993) first isolated 13 β-Defensin peptides from bovine neutrophils and described the new family of proteins that is capable of antibacterial activity against *Escherichia coli* and

Staphylococcus aureus. These 13 peptides were comprised of between 38 and 42 amino acids and shared a high similarity in primary structure. Goldammer et al. (2004) has shown that *β-Defensin5* mRNA is increased in mastitic udder quadrants of cattle compared to uninfected quadrants of the same cow. Roosen et al. (2004) found various forms of *β-Defensin* (*BNBD9*, *BNBD12*, *DEFB401*, *BNBD1*, and *BNBD3*) in cattle udders infected with *Actinomyces pyogenes* mastitis as well as in healthy udder halves (*BNBD3*). Selsted et al. (1993) predicted that there is a high possibility for *β-Defensins* to be expressed in many other tissues, due to their presence in myeloid elements and tracheal epithelium.

Research in the years following the discovery of β-Defensins by Selsted et al. (1993) has shown that *β-Defensins* are expressed in many tissues from multiple species. Various *β-Defensin* genes are expressed in human (Ali et al. 2001), mouse (Dorschner et al. 2003), rat (Hartmann, et al. 2006), and dog (Candille et al. 2007) skin. Of particular note is the discovery that *CBD103*, a gene coding for *β-Defensin 103* in dogs and referred to as the *K* locus, is responsible for dominant black coat colour (Candille et al. 2007). Candille et al. (2007) have shown that CBD103 shares structural similarity to Agouti and is found in high levels in skin, allowing it to function as a competitive ligand for MC1R (Fig 2.1). Three alleles are recognized at the *K* locus in dogs: $K^B > k^{br} > k^y$. The dominant K^B allele competitively binds to MC1R, initiating a response similar to α-MSH, resulting in the production of eumelanin. The recessive k^y allele does not bind to MC1R, allowing Agouti to bind and produce any of the accepted agouti phenotypes (Fig. 2.6). The k^y allele acts as the wild-type allele, distinguished from K^B which has a deletion of glycine residue 23. *CBD103* corresponds best to predicted bovine *β-Defensin300* (GenBank XM_001256004).

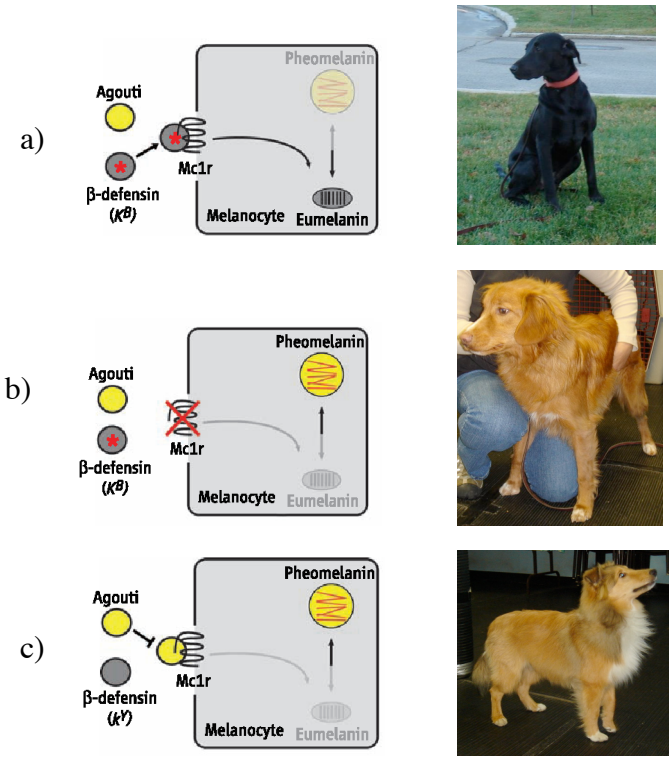


Figure 2.6 *K* alleles act with MC1R and Agouti to produce canine coat colour. a) The dominant K^B allele preferentially binds MC1R, producing eumelanin, as seen in most black dogs. b) A non-functional MC1R cannot bind either Agouti or β-defensin, resulting in phaeomelanin production, as seen in Nova Scotia Duck Tolling Retrievers. c) The recessive k^y allele does not bind MC1R, allowing binding of agouti and the production of phaeomelanin, as seen in sable Shetland Sheepdogs. (Adapted from Candille et al. 2007)

2.2 Holstein Cattle

2.2.1 Breed History

Holstein cattle are a breed distinguished by their recognizable colour patterns and high milk yield (www.holstein.ca). The first Holstein was imported to North America in 1881 from Holland, where the breed originated. Later that same year, the first Holstein was imported from the United States to Canada (www.holstein.ca). Since that time, the Holstein breed has grown in popularity. Over 92% of the dairy cattle registered in Canada in 2007 were purebred Holsteins.

The remaining 8% of dairy animal registrations are comprised of Ayrshire, Brown Swiss, Canadienne, Guernsey, Jersey, Milking Shorthorn, and various goats (Table 2.1) (Canadian Dairy Information Centre, 2008).

Table 2.1 Number of dairy animals, by breed, registered in Canada in 2007. (Canadian Dairy Information Centre, 2008).

Breed	2007 Registrations	Percentage of Total
Ayrshire	6,407	2.56
Brown Swiss	1,615	0.65
Canadienne	230	0.09
Guernsey	232	0.09
Holstein	230,283	92.19
Jersey	6,689	2.68
Milking Shorthorn	245	0.10
Goats	4,098	1.64
Total	249,799	100.00

The Holstein breed is registered and monitored through Holstein Canada, a member owned organization, comprised of approximately 12,000 members across Canada. Holstein Canada strives to improve the Holstein breed through animal identification and registration, assessing conformational stability of individual cattle, organizing and providing performance data including complete genealogical information, and by supporting research into areas important to the expansion and maintenance of the breed (www.holstein.ca).

2.2.2 Traditional Colours

The majority of Holsteins in North America express a phenotype that is black with white spotting. There is no preference for the amount of black or white on an animal, and the phenotype can vary from nearly all white to nearly all black. Various other colors make up the remaining registered Holstein cattle in Canada (Table 2.2). It has been estimated that 25% of the Holsteins originally imported from Holland were carrying the recessive red allele, though

significant effort was put into eliminating the red phenotype in the early developmental years of the breed in North America (Leduc 2006). A.B.C. Reflection Sovereign (Ex-Extra) *RC, a bull born in 1946, lead to the eventual acceptance of red Holstein cattle as purebred. He carried the recessive red allele and many of his sons, half of which also carried red, became very influential sires in the Holstein breeding program (Leduc 2006). In 1969, red and white cattle were registered through a separate herdbook, with the combination of the red and white and black and white herdbooks occurring in 1976 (www.holstein.ca).

Table 2.2 Proportion of Holstein cattle of each color registered with Holstein Canada in 2007.

Black & White	Red & White	Variant Red	Changeling	All Black	All Red	Other
98.18%	1.56%	0.06%	0.11%	0.01%	0.01%	0.08%

The traditional black and white and red and white colour patterns in Holsteins follow the Mendelian inheritance pattern attributed to the two common alleles of *MC1R*. Black is dominantly inherited with the E^D allele, and red is recessively inherited as *e*/*e*. The wild-type E^+ allele produces a colour dependent on the other allele present. When present with E^D, the coat colour will be black, and when present with *e* will be red.

2.2.3 Recently Recognized Colours

Recently two new colour patterns have been recognized in Canadian Holstein cattle. They have been termed variant red (VR) and changeling for the purposes of this research.

VR appears phenotypically similar to traditional e/e red (Fig. 2.7), but follows dominant inheritance relative to black, rather than the recessive pattern of traditional red. According to Leduc (2006) the VR phenotype can been traced back to a cow by the name of Surinam Sheik Rosabel. She was born in 1980 and sired by a well-known bull, Puget-Sound Sheik, who was homozygous black with white spotting, as was the dam. Rosabel produced a large number of red calves, regardless of whether she was mated to black and white bulls, red and white bulls, or black bulls heterozygous for red. Additionally, Rosabel, a red and white cow, was able to produce black and white calves when mated to a traditional red bull, a feat that should not be

possible if Rosabel were assumed to be red due to e/e at MC1R. Two VR calves from Rosabel went on to be popular sires, Surinam Treasure and Surinam Trazarra.

Though Holstein Canada has termed this phenotype variant red, it is also commonly referred to as dominant red, due to its mode of inheritance, or the Rosabel Effect, from the dam that first expressed the phenotype.

a) 

b) 

Figure 2.7 The VR phenotype appears similar to traditional red. a) a VR cow b) a traditional red cow

According to Leduc (2006) changeling is a trait first recognized in offspring of a bull named Roybrook Telstar. Telstar, a black and white bull, was born in 1963. Many of his offspring were born red and white but changed to black and white by the time they were mature (Fig. 2.8). Since two black and white cattle can produce changeling offspring, the trait appears to

be inherited recessively. Leduc (2006) reported a situation in 1972 where a young red and white bull was purchased at a high price, only to show up at stud as a black and white animal. Telstar produced many very good bull calves, assisting in the spread of the changeling trait throughout the Holstein population.

Figure 2.8 A changeling calf part of the way through a change from red and white to black and white.

It should be noted that nutritional deficiency of copper or excess of molybdemum can also result in a loss of hair pigmentation in cattle (Humphries et al. 1983). Though also associated with these nutritional factors are a number of other symptoms including poor growth, gait changes, and skin lesions (Humphries et al. 1983), all of which are absent from changeling cattle.

2.2.4 Holstein Cattle Colour Nomenclature

Part of the Holstein Canada registration process includes recording the colour of the cattle, including whether or not they carry any of the recessive colour traits based on pedigree information. Initially, only black and white cattle could be registered, so not as much emphasis was placed on recording the colour, though the addition of red and white cattle to the herdbook in the 1976 (www.holstein.ca) brought the need to record potential red carriers.

Holstein Canada has devised a nomenclature system for recording coat colour information for individual cattle. Black and white cattle are recorded as B&W, while red and white cattle are recorded as R&W. If a black and white animal carries a recessive red allele as determined by parental or offspring data or genetic testing, it is designated with *RC for "red carrier" (www.holstein.ca).

The recognition of VR and changeling colour traits has required a naming system for them as well. Changeling cattle are recorded as B/R, for Black/Red, while carriers of changeling are designated with *BRC. All B/R cattle are also designated as *BRC, but not all *BRC cattle are B/R, a black and white or a red and white animal can be designated as *BRC if they pass on changeling without expressing it (www.holstein.ca).

The nomenclature for VR is somewhat redundant. A VR cow will be recorded as R&W, in addition to *BC, black carrier, and *VRC, variant red carrier (www.holstein.ca), since a VR cow can appear red but still produce black calves. Since the causative mutation for VR has yet to be discovered, we are unable to determine if an individual is heterozygous for VR or homozygous. If the cow is homozygous for VR, then they are only able to produce VR calves, never a black calf. As such, the designation of *BC for black carrier is misleading. Additionally, since VR cattle can carry the allele for traditional red, some VR cattle also have the designation of *RC for red carrier.

3.0 ANALYSIS OF CANDIDATE GENES FOR VARIANT RED

3.1 Introduction

The objective of this study was to investigate candidate genes for the Variant Red (VR) phenotype in Holstein cattle. The candidate genes included some that were already known to be involved in the pigment pathway: *MC1R*, *ASIP*, and *ATRN*. *β-Defensin300* was chosen due to its function in dogs (Candille et al. 2007). *MTNR1A* was chosen as a positional candidate gene. *β-Defensin300* and *MTNR1A* were previously uncharacterized in cattle, so in order to fully assess their potential involvement in the VR phenotype, their genomic sequence was determined in several cattle and polymorphisms identified. Partial cDNA sequence was also determined.

Based on families including both VR and black cattle, VR appears to be dominantly inherited (Leduc 2006). Segregating sires or dams that are able to produce both VR and black offspring are VR themselves.

I hypothesize that the causative gene for VR is *β-Defensin300*. *CBD103* in dogs functions in determining whether *MC1R* or *ASIP* dictate the overall coat colour (Candille et al. 2007). I predict that the red of the VR phenotype is created by the *ASIP* gene, but a mutation in *β-Defensin300* distinguishes VR cattle from black cattle in that VR cattle express phaeomelanin while black cattle express a solid eumelanin phenotype commonly associated with the dominant *MC1R* allele. In black cattle, the phaeomelanin produced by *ASIP* is masked by a combination of two recessive *β-Defensin300* alleles and a dominant *MC1R* allele.

3.2 Material and Methods

3.2.1 Animals

DNA samples from four families segregating for variant red (Fig. 3.1) and three individual variant red cattle, for a total of 64 cattle, were obtained from a private farm. Of these 64 cattle, 34 were variant red, three were traditional red, and 27 were black, all with white spotting. Both sexes were represented in all colors. All cattle were purebred Holsteins, registered

with Holstein Canada. Families M and D1 were analyzed for parentage by GenServe (Saskatoon, Canada). Twenty-three cattle of different breeds and crosses were used for comparison purposes.

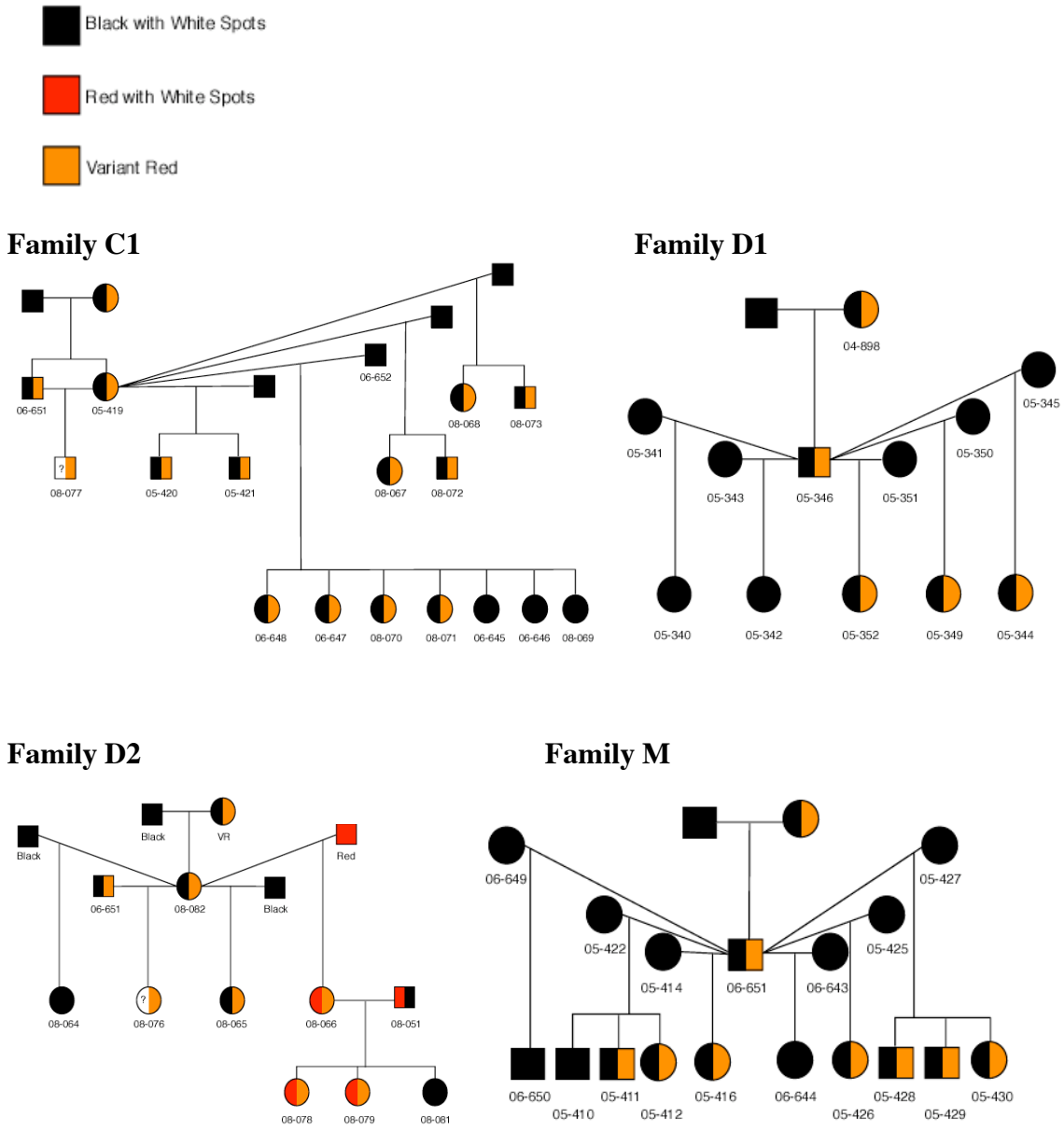


Figure 3.1 Pedigrees of the four segregating families used in the study of VR. Samples from individuals without identification numbers were not available.

3.2.2 DNA Preparation

Genomic DNA was extracted from hair, blood, and semen as previously described (Schmutz et al. 1995). RNA and cDNA were synthesized from the skin samples. Genomic DNA was initially extracted from hair using a protocol provided by GenServe (Saskatoon, Canada). High levels of degradation were noticed in a number of samples, so an alternate extraction protocol (Epicentre, Madison, WI) was used on later samples. Extraction protocols are outlined in Appendix A.

3.2.3 DNA Analysis

3.2.3.1 *Melanocortin 1 Receptor*

3.2.3.1.1 Sequencing

Although *MC1R* is composed of a single exon, it was sequenced in three overlapping parts due to its size. The primers used (Table 3.1) were either taken from the work of Klungland et al. (1995) and Joerg et al. (1996) or were designed from published *MC1R* mRNA sequence (AF445642).

All PCR reactions consisted of 1 μl (50-100 ng) of template DNA, 1.5 μl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 μl of 10 mM dNTP, 0.9 μl of 25 mM $MgCl_2$, 0.1 μl of *Taq* polymerase (5 U/μl; Fermentas, Burlington, ON), 1 μl of each primer (10 pM/μl) and 9.2 μl of ddH_2O to a total volume of 15 μl. DNA was denatured for 4 minutes at 94°C, followed by 35 cycles at 94°C for 50 seconds, the appropriate annealing temperature for 50 seconds, and 72°C for 50 seconds. This was followed by a 4 minute dwell at 72 °C.

Table 3.1 Characterization of primers used to sequence *MC1R*.

Primer Name	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
MC1R 5'UTR	F: 5'-CTACCGCGGCCCGGTAAGGC-3'	553	70
E6	R: 5'-GCCTGGGTGGCCAGGACA-3'		
P6	F: 5'-GGAGGTGTCCATCTCTGACGG-3'	676	63
E4	R: 5'-GAAGTTCTTGAAGATGCAGCC-3'		
MC1R3Stop	F: 5'-TACGTCCACATGCTGGCCCGG-3'	427	62
MC1R3Stop	R: 5'-CCTCCTCATTCTGCACAGCCTCC-3'		

Resultant PCR fragments were excised from an agarose gel and purified with QIAquick gel extraction protocol (Qiagen, Mississauga, ON). Purified product was sequenced at the National Research Council of Canada Plant Biotechnology Institute. Sequence analysis and alignment was conducted using the Sequencher 4.1 software (Gene Codes Corporation, Ann Arbor, MI). Primers used for sequencing were the same as were used for PCR amplification.

3.2.3.1.2 Genotyping

All cattle were genotyped prior to further study to determine E^D, E^+, and *e* alleles of *MC1R*. Since the variant red phenotype cannot be differentiated from traditional red, all *e*/*e* cattle were excluded from the study. Cattle were genotyped using primers E5 and E6 (Klungland et al. 1995) and primers P6 and P7 (Joerg et al. 1996) (Table 3.2) using the PCR protocol detailed in secion 3.2.3.1.1. The resultant product was then digested overnight at 37°C with 0.75 µl of *AciI* (10 U/µl) (Fermentas, Burlington, ON), 1.5 µl Fermentas Buffer O, and 0.25 µl ddH_2O. Primers E5 and E6 yield a 138 bp product that, when digested with *AciI* and run on a 4% agarose gel stained with ethidium bromide, results in an uncut 138 bp band for the E^+ and *e* alleles, and cut bands of 8, 33, and 97 bp for the E^D allele.

Table 3.2 Characterization of primers used to genotype *MC1R* alleles.

Primer Name	Orientation	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
P6	F	5'-GGAGGTGTCCATCTCTGACGG-3'	531	59
P7	R	5'-CCGGGCCAGCATGTGGACGTA-3'		
E5	F	5'-CAAGAACCGCAACCTGCACT-3'	138	63
E6	R	5'-GCCTGGGTGGCCAGGACA-3'		
Cow*MC1R*MM	F	5'-CCTGGAGGTGTCCATCCCTGG-3'	226	63

The PCR reaction for primers P6 and P7 was the same as that for primers E5 and E6 except that the annealing temperature was 63°C. The product was digested overnight at 35°C with 0.75 µl *MspI* (10 U/µl) (Fermentas, Burlington, ON), 1.5 µl Fermentas Tango Buffer, and 0.25 µl ddH_2O. Primers E5 and E6, when digested with *MspI* and run on a 1.5% agarose gel, yield an uncut 531 bp band for the *e* allele, and cut bands of 29, 201 and 301 bp for the E^+ and E^D alleles.

Segregating family D1 was genotyped at a *MC1R* 126 C>T SNP (GenBank AAO21702). The 15 µl reaction consisted of 1 µl (50-100 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.45 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl each of primers Cow*MC1R*MM and E6 (10 pM/µl) and 9.65 µl of ddH_2O. DNA was denatured for 4 minutes at 94°C, followed by 35 cycles at 94°C for 50 seconds, 63°C for 50 seconds, and 72°C for 50 seconds. This was followed by a 4 minute dwell at 72 °C. The forward primer introduces a purposeful mismatch so that when the 226 bp product is incubated with *AciI* (Fermentas, Burlington, ON) at 37°C for 6.5 hours the T allele cuts into bands of 21, 73 and 132 bp. A natural cut site produces constant bands of 94 and 132 bp for both the cut (T) and uncut (C) alleles. The enzyme reaction consisted of 0.75 µl *AciI* (10 U/µl) and 1.5 µl Fermentas Buffer O and was run on a 3% agarose gel with ethidium bromide staining.

3.2.3.2 *Agouti Signaling Protein*

The dams and sires of the variant red families that segregate for VR were genotyped at an *ASIP* 2684C>T SNP identified previously in our lab by Tom Berryere (unpublished data). Segregating parents of families D1 and C1 were found to be heterozygous at the SNP, so the offspring in those families were subsequently genotyped to analyze co-segregation of this SNP with the VR phenotype. The primers used were Cow Agouti int3mm#2 and Cow Agouti int3#3 (Table 3.3) and the PCR protocol was as outlined in section 3.2.3.1.1. The product was then digested for three hours at 65°C with 0.75 µl *BsrI* (10 U/µl) (New England BioLabs, Ipswich, MA), 1.5 µl NE Buffer 3, and 0.25 µl ddH_2O and run on a 4% agarose gel stained with ethidium bromide. There is a natural cut site producing a constant band of 149 bp. The remaining 286 bp fragment is uncut with the T allele, but cuts into bands of 22 and 264 bp with the C allele.

Table 3.3 Characterization of primers used to genotype *ASIP* 2684C>T SNP.

Primer Name	Orientation	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
CowAgouti int3mm#2	F	5'-GTTGAAATGCACAGTGTTCCTATTC-3'	435	59
CowAgouti int3#3	R	5'-CCATTGAACACTGTGGCTC-3'		

3.2.3.3 *Attractin*

The parents segregating for VR were genotyped at an *ATRN* 2375G>A SNP identified by Tom Berryere in our lab (unpublished data). The segregating dam of family C1 was found to be heterozygous at the SNP so that family was genotyped as well. Primers CowAtrn int19-for and CowAtrn exint20-rev (10 pM/µl) (Table 3.4) were used with a PCR protocol as outlined in section 3.2.3.1.1. The product was then digested overnight at 37°C with 0.75 µl *MvaI* (10 U/µl) (Fermentas, Burlington, ON), 1.5 µl

Fermentas R Buffer, and 0.25 µl ddH_2O, then run on a 4% agarose gel stained with ethidium bromide. During digestion, the G allele cuts to bands of 53 and 134 bp. The A allele remains uncut, producing a band of 187 bp.

Table 3.4 Characterization of primers used to genotype *ATRN* 2375G>A SNP.

Primer Name	Orientation	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
CowAtrnint19	F	5'-GATTTCCAGTGACTCAAAGTGG-3'	187	59
CowAtrnexint20	R	5'-CTCACCATGCTCTGTGATGACTGC-3'		

3.2.3.4 *Melatonin Receptor 1A*

3.2.3.4.1 Genotyping and Linkage

While the previous candidate genes investigated had previously published sequence and documented SNPs, *MTNR1A* did not. In order to determine whether *MTNR1A* was a valid candidate gene for VR, segregating families C1, D1 and M were genotyped at five microsatellites surrounding *MTNR1A*, then analyzed to determine if the VR trait mapped to this location on BTA 27. If so, *MTNR1A* would be a viable candidate gene.

The microsatellites that were genotyped were chosen based on location relative to *MTNR1A*, number of alleles, and percentage of heterozygosity. Microsatellites were chosen that were located approximately 5 cM and 10 cM either side of *MTNR1A*, had a large number of alleles and a heterozygosity of at least 60%. Based on these requirements, microsatellites *BMS1001*, *BM871*, *INRA016* and *DIK4084* from the US Meat Animal Research Center (http://www.ars.usda.gov/Main/docs.htm?docid=2340), and a microsattelite discovered in intron 1 of *MTNR1A* of whole genomic shotgun sequence of BTA27 (GenBank NW_930548), were chosen for genotyping (Table 3.5).

Table 3.5 Characterization of primers used to genotype five microsatellite markers surrounding *MTNR1A*.

Microsatellite	Primer Sequence	No. of alleles	Heterozygosity	Temp (°C)
BMS1001	F: 5'-GAGCCAATTCCTACAATTCTCTT-3' R: 5'-AGACATGGCTGAAATGACTGA-3'	9	63%	58
BM871	F: 5'-TTCCTCAAACTGTGAACACACC-3' R: 5'-CCATGAGGTCACAAAGGGTC-3'	10	74%	58
MTNR1A Int2	F: 5'-GTGATTACTCTTATCTGGTAG-3' R: 5'-CTCAGCTAGAGGCTTTGCC-3'	≥5	unknown	53
INRA016	F: 5'-ACGCAGACCTTAGCATAGGAGA-3' R: 5'-GTCGCAATGAGTTGGACACAAC-3'	8	75%	60
DIK4084	F: 5'-TTGCCAGGAATGTCATCAAG-3' R: 5'-GCATTGGCCTCAGAGAACAT-3'	7	61%	55

PCR was carried out in 15 µl reactions consisting of 1 µl (50-100 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl of each primer (10 pM/µl) and 9.2 µl of ddH_2O. Forward primers were end labelled with P^{32} and T4 Polynucleotide Kinase (New England BioLabs, Ipswich, MA). The PCR temperature protocol consisted of 4 minutes at 94°C, followed by 35 cycles at 94°C for 50 seconds, the appropriate annealing temperature for 50 seconds, and 72°C for 50 seconds. This was followed by a 4 minute dwell at 72°C. Bands were resolved on a 6% denaturing polyacrylamide gel and exposed on an x-ray film.

3.2.3.4.2 Sequencing

Whole genome shotgun sequence of BTA 27 (GenBank NW_930548), predicted *MTNR1A* sequence (GenBank XM_614283), and partial genomic *MTNR1A* sequence (GenBank BTU73327) were used to design primers for sequencing of genomic DNA and cDNA (Table 3.6).

Genomic DNA from exon 1 was obtained from three cattle using primers MTNR1A5'#1-F and MTNR1AInt2#2-R. Reaction volumes of 15 µl were used, consisting of 1 µl (50-100 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl of each primer (10 pM/µl), 3.9 µl of Betaine and 5.3 µl of ddH_2O. DNA was denatured for 4 minutes at 94°C, followed by 37 cycles at 94°C for 50 seconds, 57°C for 50 seconds, and 72°C for 50 seconds, followed by a 4 minute dwell at 72 °C.

Genomic DNA was obtained for exon 2 from four cattle, using primers MTNR1A Int2-F with MTNR1A 3'-R (Table 3.6) and the PCR protocol outlined in section 3.2.3.1.1.

Partial cDNA, consisting of a portion of exon 2, was obtained from the skin of a Hereford steer using primers MTNR1A Ex2-F and MTNR1A Ex3-R (Table 3.6). The previously described reaction protocol was used, with an annealing temperature of 57°C.

Resultant PCR fragments were excised from an agarose gel and purified with QIAquick gel extraction protocol (Qiagen, Mississauga, ON). Purified product was sequenced at the National Research Council of Canada Plant Biotechnology Institute. Sequence analysis and alignment was conducted using the Sequencher 4.1 software (Gene Codes Corporation, Ann Arbor, MI).

Table 3.6 Characterization of primers used to sequence *MTNR1A*.

Primer Name	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
MTNR1A Ex2 –F	5'-GGAATTGCCATCAACCGCTATTG-3'	661	57
MTNR1A Ex3-R	5'-CTATTAACGGAGAGGGTTTGC-3'		
MTNR1A5'#1-F	5'-TGTCCGCACAGGCAGCGGCT-3'	439	57
MTNR1AInt2#2-R	5'-TGCGTGCTTGCAAAGGTGGCGGAACA-3'		
MTNR1A5'-F	5'-TGAGCCAGTGTTCTCAGTGGAA-3'	313	57
MTNR1AInt1-R	5'-ACCTTAGATAACCATCATGCG-3'		
MTNR1A Int2-F	5'-TAACTGTGATATCGGGTCAT-3'	1082	53
MTNR1A 3'-R	5'-TTCCTAGGCATCTACCAAGA-3'		

A region 5' of *MTNR1A* containing a -742C>T SNP was sequenced at the National Research Council of Canada Plant Biotechnology Institute from genomic DNA from 26 cattle obtained using primers MTNR1A5'-F and MTNR1A Int1-R. A modified booster PCR protocol was used (Kidd et al. 1995). Initial 15 μl reaction volume consisted of 2 μl (100-200 ng) of template DNA, 1.5 μl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 μl of 10 mM dNTP, 0.9 μl of 25 mM $MgCl_2$, 0.1 μl of *Taq* polumerase (5 U/μl; Fermentas, Burlington, ON), 0.2 μl of each primer (10 pM/μl), 3.9 μl of Betaine, and 6.9 μl ddH_2O. Reactions were denatured for 4 minutes at 94°C, followed by 10 cycles of 50 seconds at 94°C, 50 seconds at 57°C, and 50 seconds at 72°C. The PCR was then stopped and 1 μl of each of the initial primers were added to every reaction. PCR was resumed for 25 cycles of 50 seconds at 94°C, 50 seconds at 57°C, and 50 seconds at 72°C, followed by a 4 minute dwell at 72°C.

3.2.3.5 *Beta-Defensin*

3.2.3.5.1 Sequencing

Cattle sequence for *β-Defensin300* was not available in GenBank. Sequencing was accomplished through the design and implementation of a nested primer technique. Primers KNest1-F and KNest1-R located approximately 500 bp outside of the estimated coding sequence of *β-Defensin300* were designed from whole genome shotgun sequence of BTA 27(GenBank NW_001494395). This resulted in a product of approximately 2500 bp which was extracted from a 1% agarose gel (Qiagen, Mississauga, ON) and used as a template for reactions with other internal primers (Table 3.7) in order to obtain sequence for the entire coding, intronic and 5'/3' flanking regions of *β-Defensin300*. The nested design allowed sequencing of roughly 500 bp 5' of the start codon of *β-Defensin300*. An additional 619 bp 5' of that was sequenced using primers KDef5'#1-F and KDef5'#1-R on non-nested product and the sequence protocol outlined in section 3.2.3.1.1. Between six and 17 cattle of varying breeds and coat colours were sequenced for each region (Table 3.8). cDNA from pigmented skin was sequenced in 13 cattle of varying breeds and coat colours.

Table 3.7 Characterization of primers used to sequence *β-Defensin300*.

Primer Name	Region	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
KNest1-F	Entire Coding	5'- GCGTATCCATGCAGGTTCAG-3'	2500	63
KNest1-R		5'- AGGCCTGAGGTCAGTCAGAACA-3'		
KDef5'#1-F	-429bp to -725bp	5'- ATCTGTGGTAGGAGTTCAGAG-3'	725	59
KDef5'#1-R		5'- AAGTCCTGAGAGAGCCTGAGC-3'		
KNest1-F	5' region	5'- GCGTATCCATGCAGGTTCAG-3'	600	61
KwiEx1-R		5'- CCTGGAACAGGCAGCAAGAACA-3'		
KEx1a-F	Exon 1	5'- GGAATTGCAGCTGCTGAATGCTT-3'	435	57
KBlastInt-R		5'- CCTGTCTCATGAACAGGTATA-3'		
KIntron-F	Intron 1	5'- TGTTCTTGCTGCCTGTTCCAGG-3'	760	61
KIntron-R		5'- AATCAGAGCACACTGGCCGCT-3'		
Kdefensin-F	Exon 2	5'- GGCACCAGATAACTTATTAGAGGG-3'	405	57
Kdefensin-R		5'- CTAAGTCACCTGTTTGGTAAC-3'		
Kdefensin-F	3' region	5'- GGCACCAGATAACTTATTAGAGGG-3'	700	61
KNest1-R		5'- AGGCCTGAGGTCAGTCAGAACA-3'		
KEx1Start-F	cDNA	5'- ATGGCTGCAGACCTGGAGC-3'	291	59
KEx2Stp-R		5'- TCATTTCTTCTTCCGGCAGCAT-3'		

Table 3.8 The list of individual cattle and the *β-defensin300* sequence obtained from their genomic DNA and/or cDNA.

			Regions Sequenced						
ID #	Breed	Colour	-429 bp to –725 bp	-428bp to -1bp	Ex 1	Int 1	Ex 2	3'	cDNA
05-419	Holstein	VR	*						
04-898	Holstein	VR		*					*
08-069	Holstein	VR		*					
08-071	Holstein	VR		*					
06-651	Holstein	VR		*	*	*	*	*	
08-082	Holstein	VR		*	*	*	*	*	*
08-066	Holstein	VR		*	*	*	*	*	
08-078	Holstein	VR		*	*	*	*	*	
08-076	Holstein	VR		*	*	*	*	*	
08-079	Holstein	VR		*	*	*	*	*	
08-065	Holstein	VR		*	*	*	*	*	
08-052	Holstein	Red	*	*	*	*	*	*	
08-053	Holstein	Red	*	*	*	*	*	*	
02-494	Guernsey	Red		*					
02-496	Guernsey	Red		*					
02-497	Guernsey	Red		*					
03-011	Hereford	Red with White							*
02-2293	Charolais	Dilute Red							*
00-307	Simmental	Red							*
08-081	Holstein	Black		*					
08-064	Holstein	Black		*					
06-1078	Holstein	Black	*	*	*	*	*	*	
06-1082	Holstein	Black	*	*	*	*	*	*	
08-051	Holstein	Black	*	*	*	*	*	*	
02-685	Galloway	Black							*
06-795	Holstein x Galloway	Black							*
04-560	Galloway x Brown Swiss	Black							*
31J	Galloway	White with Black Points							*

Table 3.8 Continued

			Regions Sequenced						
ID #	Breed	Colour	-429 bp to –725 bp	-428bp to -1bp	Ex 1	Int 1	Ex 2	3’	cDNA
SP	Speckled Park	White with Black Points							*
04-561	Galloway	Dun							*
82	Galloway	Dun with White Belt							*
02-514	Brown Swiss	Taupe Brown		*					
02-517	Brown Swiss	Taupe Brown		*					
02-699	Brown Swiss	Taupe Brown							*
02-479	Jersey	Fawn		*					
02-480	Jersey	Fawn		*					
02-481	Jersey	Fawn		*					

VR = variant red; Ex1 = exon 1 of *β-defensin300;* Ex2 = exon 2 of *β-defensin300;*
Int1 = intron 1 of *β-defensin300*

PCR protocol for purifying the original large product used a 15 µl reaction consisting of 2 µl (100-200 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl each of KNest1-F and KNest1-R (10 pM/µl) and 8.2 µl of ddH_2O. The reaction was denatured for 4 minutes at 94°C, followed by 35 cycles of 94°C for 1 minute, 63 for 1 minute, and 72°C for 1 minute 50 seconds. It was then extended for 4 minutes at 72 and run on a 0.8% agarose gel. All other PCR reactions used protocol as detailed in section 3.2.3.1.1

3.2.3.5.2 Genotyping and Linkage

The location of *β-Defensin300* in cattle was predicted to be on BTA 27 by using known dog sequence for *CBD103* (Candille et al. 2007) and NCBI’s BLAST tool. One microsatellite located near this predicted *β-Defensin300* was identified using MARC (http://www.ars.usda.gov/Main/docs.htm?docid=2340). I used whole genome shotgun sequence

of BTA 27 (GenBank NW_001494395) to identify two additional microsatellites, *DLD1* and *DLD2*, located 22011 bp and 156848 bp from *β-Defensin300* respectively (Table 3.9). Family M was genotyped at each of the three microsatellites in order to determine if VR mapped to a region near or including *β-Defensin300*.

PCR was accomplished with 15 µl reactions consisting of 1 µl (50-100 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl of each primer (10 pM/µl) and 9.2 µl of ddH_2O. Forward primers were end labelled with P^{32} and T4 Polynucleotide Kinase (New England BioLabs, Ipswich, MA). The PCR protocol consisted of 4 minutes at 94°C, followed by 35 cycles at 94°C for 50 seconds, the appropriate annealing temperature for 50 seconds, and 72°C for 50 seconds. This was followed by a 4 minute dwell at 72°C. Bands were resolved on a 6% polyacrylamide gel and exposed on x-ray film.

Table 3.9 Characterization of primers used to genotype three microsatellites surrounding *β-Defensin300*.

Microsatellite	Primer Sequence	No. of alleles	Heterozygosity	Temp (°C)
BMS1001	F: 5'-GAGCCAATTCCTACAATTCTCTT-3' R: 5'-AGACATGGCTGAAATGACTGA-3'	9	63%	58
DLD1	F: 5'- CATGATGCTCTCCATGCTTCAG-3' R: 5'- TGTGAGGACTCTCAGAGGTCT-3'	≥2	86%	59
DLD2	F: 5'- CATTCTGACACCAACAGGT-3' R: 5'- TGTCTCACTCAGGTATGTCAATC-3'	≥4	56%	59

3.3 Results

3.3.1 *Melanocortin 1 Receptor*

3.3.1.1 Sequence Analysis

MC1R mRNA consists of 1772 nt (GenBank AF445642) and codes for a 317 amino acid protein. No new mutations were found in the complete coding sequence for *MC1R*. The sequence that I obtained included the complete coding sequence from one VR bull, including 211 bp 5' of the start codon and 149 bp 3' of the stop codon. Whole or partial *MC1R* sequence was obtained from 15 additional cattle, representing Holsteins (three black, four VR, and two changeling), five Brown Swiss (taupe brown), and one Jersey (fawn).

3.3.1.2 Genotyping

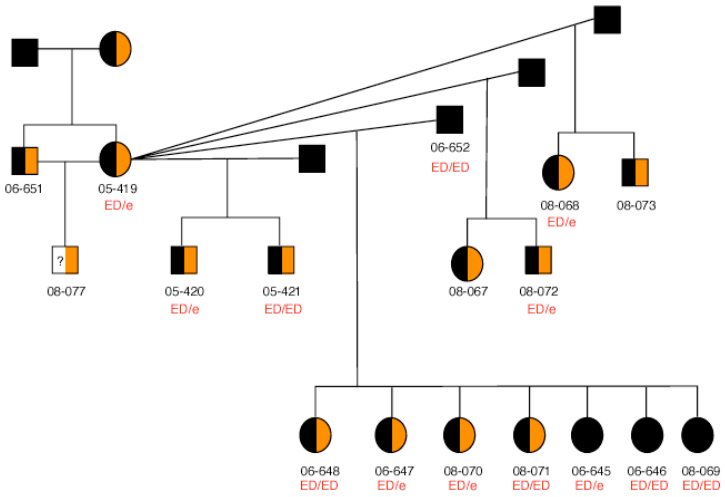
Twenty eight red Holsteins and seventeen black Holsteins from segregating families M, C1, D1 and D2 were genotyped for the three *MC1R* alleles traditionally considered as coat colour alleles (Fig. 3.2). Of the 28 red Holsteins genotyped, only one (08-080) was found to be *e/e* red, the rest were genotyped as either E^D/e, E^D/E^D, or E^D/E^+ (Table 3.11). This *e/e* animal was therefore excluded from further analysis since it could not be determined if it was traditional red or variant red in colour.

MC1R did not co-segregate with the VR phenotype (Fig. 3.3), based on the 126C>T *MC1R* SNP. Likewise, no genotype of the traditional *MC1R* alleles affecting colour consistently occurred in all VR cattle (Table 3.10). Therefore *MC1R* alone is insufficient to cause the VR phenotype.

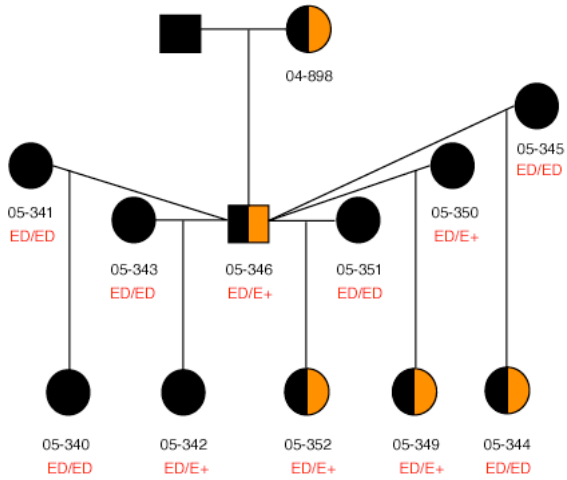
Table 3.10 *MC1R* genotypes of 28 red Holsteins belonging to one of four VR families.

Genotype	E^+/E^+	E^+/E^D	E^+/e	E^D/e	E^D/E^D	e/e
# of Animals	0	5	0	15	7	1

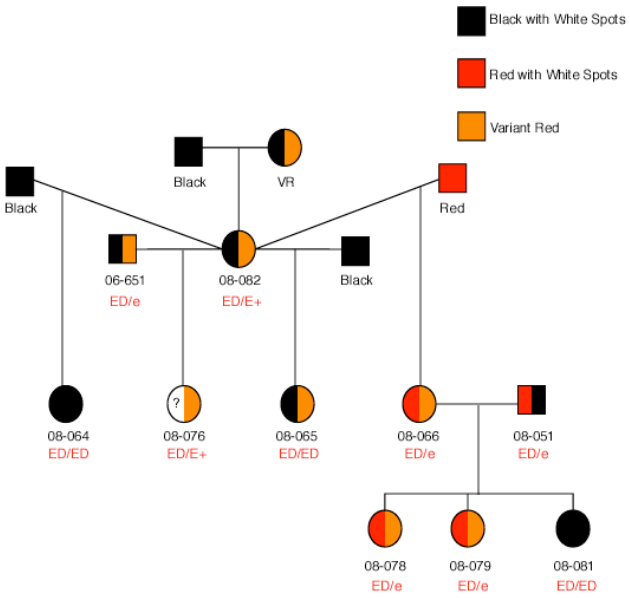
Family C1



Family D1



Family D2



Family M

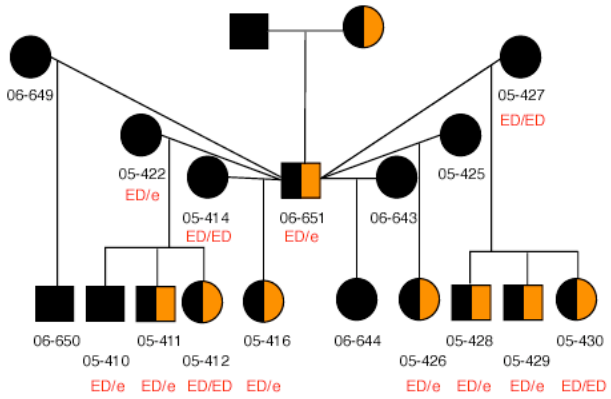


Figure 3.2 *MC1R* genotypes (shown in red) for families C1, D1, D2, and M.

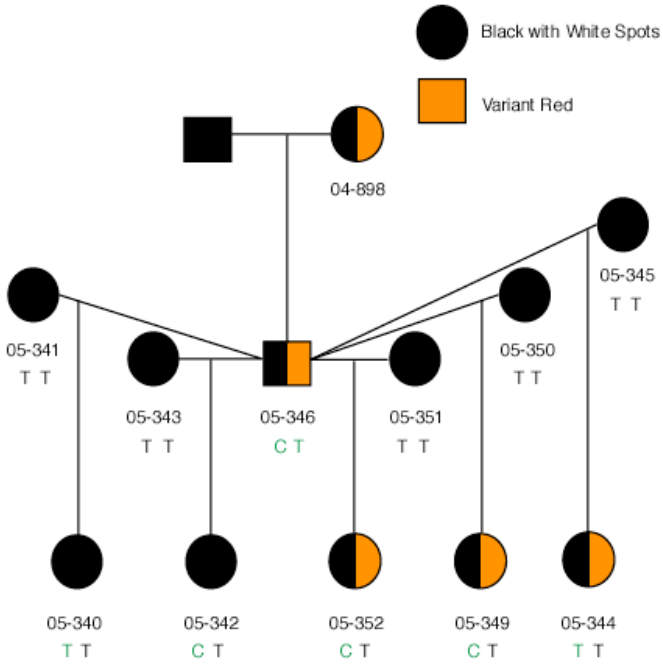


Figure 3.3 126C>T *MC1R* genotypes for family D1. Green alleles indicate that they were inherited from the segregating sire.

3.3.2 *Agouti Signaling Protein*

The segregating parents for families M, C1, D1, and D2 were genotyped at the 2684C>T SNP in intron 3 of *ASIP*. The sire of D1 (05-346) and the dam of C1 (05-419) were found to be heterozygous, so their respective families, totaling sixteen individuals, were also genotyped (Fig. 3.4). In both families, the VR offspring inherited either the C allele or the T allele from the segregating parent. Within each family, no one allele was consistently inherited with the VR phenotype.

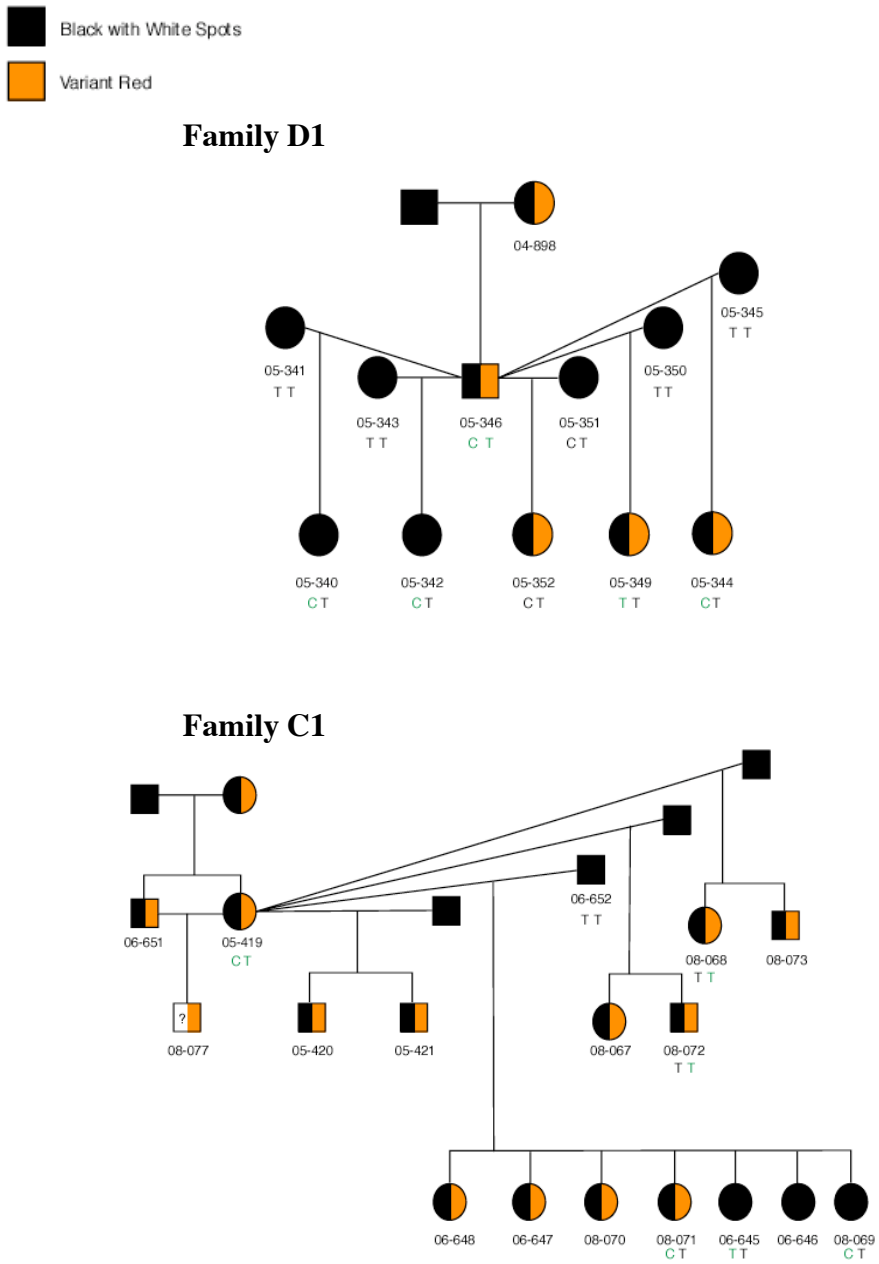


Figure 3.4 Genotypes at *ASIP* 2684C>T. Offspring alleles highlighted in green were inherited from the segregating parent.

3.3.3 *Attractin*

The segregating parents for families D1, M, and C1 were genotyped at the 2375G>A *ATRN* SNP. The dam of family C1 was found to be heterozygous, so that family, consisting of eight individuals, was also genotyped at the SNP (Fig. 3.5). Four of the offspring were uninformative, as they were each A/G, as were the dam and sire. But the remaining two offspring were A/A and G/G. Both calves were VR, one inherited the dam's A allele and one inherited the dam's G allele, so no one allele was consistently inherited with the VR phenotype.

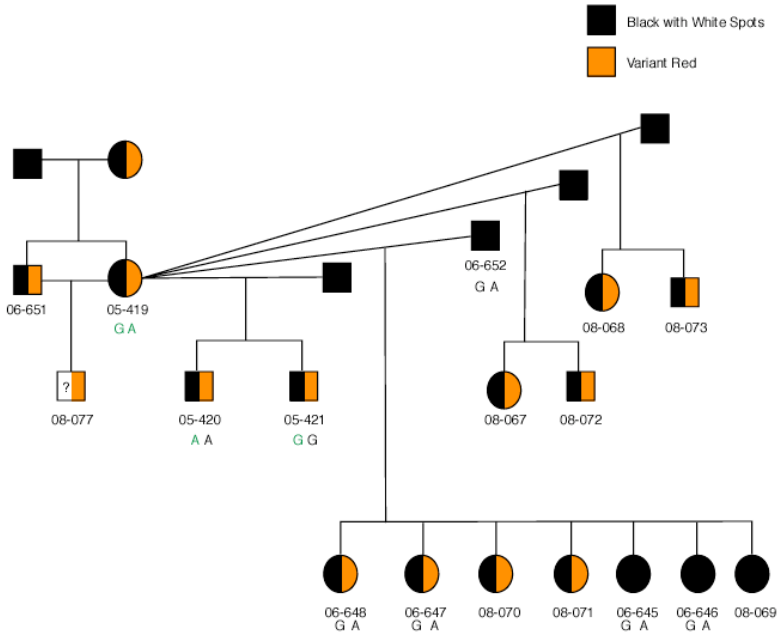


Figure 3.5 Genotypes for 2375G>A *ATRN* SNP in Family C1. Offspring alleles highlighted in green indicate that they were inherited from the segregating dam.

3.3.4 *Melatonin Receptor 1A*

3.3.4.1 Genotyping and Linkage

The two largest families with sufficient quantity of remaining sample, families D1 and M, were genotyped at five microsatellites surrounding *MTNR1A* and the genotypes were used in assessing linkage between the markers and the VR phenotype. The parental genotypes were

taken into consideration to determine which of the offspring's alleles were inherited from the sire or the dam. Each offspring was then categorized as either non-recombinant, recombinant, or uninformative if both the dam and sire were the same genotype as the calf. By arranging the microsatellites in the order in which they occur on the chromosome (Fig. 3.6) and plotting the recombinant status of each offspring, it was possible to follow a string of recombinants through various regions of the chromosome. A designation of recombinant (R) implies that a recombination event has to have occurred between the particular marker and the gene responsible for the VR phenotype. It then follows that if two or more adjacent markers show recombination, that the causative gene cannot be located between those markers unless a double crossover occurred. It was noted that two calves in family M (05-412 and 05-416) genotyped as recombinant at markers *DIK4084* and *BMS1001* located on either side of *MTNR1A*, effectively ruling out *MTNR1A* as the causative gene for VR.

Family	Calves	*DLD2*	*DLD1*	*β-defensin 300*	*BMS 1001*	*BM871*	*Int2 of MTNR1A*	*INRA016*	*DIK4084*
(Mbp)		4.02	4.16	4.18	8.62	9.78	14.38	15.43	29.1
D1	05-344		I		N	N	N	N	N
	05-349		I		N	N	N	N	N
	05-352		Sire homo		N	N	N	N	N
	05-340		I		N	N	N	U	N
	05-342		I		N	N	N	R	R
M	06-650	N	N		N	I	I	U	-
	05-410	U	N		U	I	I	N	N
	05-411	U	U		N	I	Family	N	N
	05-412	U	U		R	I	Not	R	R
	05-416	U	N		R	Sire homo	Genotyped	?	R
	06-644	?	N		N	I	I	-	N
	05-426	N	?		N	I	I	N	N
	05-430	N	N		N	I	I	U	N
	05-429	N	N		N	I	I	U	U
	05-428	-	-		-	I	I	U	U

Figure 3.6 Seven microsatellites surrounding *MTNR1A* and *β-defensin300* were genotyped in offspring of variant red families D1 and M. Offspring designated as N are non-recombinant at that marker. R indicates recombination. U designates offspring that are uninformative and ? indicates samples that did not work after multiple analyses.

3.3.4.2 Sequencing

Partial coding sequence for *MTNR1A* was obtained from genomic DNA of 28 cattle of various coat colours, including VR, changeling, traditional red, and black. Regions sequenced include exon 1 with 162 bp 5' flanking sequence and 46 bp of intron 1 for a total of 439 bp, exon 2 with 85 bp of intron 1 and 58 bp of 3' flanking sequence for a total of 1013 bp (GenBank EU716174) (Appendix B), a 5' region from -637 bp to -991 bp including a -742C>T SNP. Partial cDNA sequence was also obtained from skin of a Hereford steer (GenBank EU716172) (Appendix C) and includes 622 bp of exon 2, not including the stop codon.

Genotyping of 24 cattle at the -742C>T SNP, potentially in the promoter region, was done through sequence analysis. Neither allele consistently segregated with any of the coat colour phenotypes tested (Table 3.11).

3.3.5 *β-Defensin300*

3.3.5.1 Microsatellite Analysis

Families M and D1 were genotyped at three microsatellites surrounding *β-Defensin300*. When the markers were organized in the orientation in which they occur on the chromosome, a pattern of recombination can be viewed (Fig. 3.6). Two calves (05-412 and 05-416) were recombinant at *BMS1001*, located approximately 4.5 cM from *β-Defensin300*. When these same two calves were genotyped at *DLD1*, located 0.02 cM or roughly 20,000 bp on the opposite end of *β-Defensin300* as *BMS1001*, one calf was deemed to be non-recombinant and the other calf was uninformative. *β-Defensin300* would therefore fall within the critical region in which the gene causing variant red could be located. It therefore was studied further as a positional candidate gene.

3.3.5.2 Sequencing and SNP Analysis

The *β-Defensin* family is composed of many genes. These genes were typically assigned numbers as they were discovered in each species. This made isolating the particular gene in cattle, that is homologous to *CDB103* at the *K* locus in dogs, difficult.
I designed a nested primer test that allowed me to obtain clean sequence of *β-Defensin300* from cattle, eliminating contamination from other homologous products. I designed primers located approximately 500 bp flanking either end of the coding sequence, purified that product from genomic DNA of six cattle, then ran primers for various overlapping regions of the *β-Defensin300* coding sequence using that purified smaller product as a template. When this final product was sequenced, it yielded clean sequence data.

The entire coding sequence of *β-Defensin300*, along with flanking and intronic sequence, was obtained from genomic DNA from VR, black, and traditional red Holstein cattle (GenBank EU715240) (Appendix D). Partial sequence was also obtained from Jersey, Guernsey, and Brown Swiss cattle. To confirm that this sequence was the correct *β-Defensin*, amino acid homology was studied among species. *β-Defensin300* sequence from cattle shares 84% homology with horse (GenBank NW_00179901.1), 81% homology with pig (GenBank NP_000609), 79% homology with dog wild type (k^y) (GenBank AAY59710), and 75% homology with human (GenBank NP_061131). While the homology between species appears relatively low, the six conserved cysteines characteristic to *β-Defensins* (Lehrer et al. 2002) are found in sequence from all five species (Fig. 3.7) proving that it is a *β-Defensin*.

Table 3.11 Genotypes of 24 Holstein cattle at the -742C>T *MTNR1A* SNP.

ID #	Colour	-742C>T
06-888	Black with White	C/T
06-896	Black with White	T/T
06-874	Black with White	C/T
06-892	Black with White	C/C
06-867	Black with White	C/C
06-865	Black with White	C/C
06-898	Black with White	C/T
08-051	Black with White	T/T
06-866	Black with White	C/C
06-873	Black with White	T/T
08-052	Red with White	C/T
05-346	VR	C/T
06-651	VR	C/C
05-419	VR	C/C
08-071	VR	C/C
08-069	VR	C/C
06-891	Changeling	C/T
06-893	Changeling	C/C
06-897	Changeling	C/T
06-895	Changeling	C/T
06-917	Changeling	C/C
06-889	Changeling	T/T
06-834	Changeling	C/C
06-844	Changeling	C/C

VR = variant red

Figure 3.7 Amino acid alignment comparing the protein sequence of *β-defensin300* in cattle to two alleles of dog (k^y allele GenBank AAY59710), pig (GenBank NP_999609), human (GenBank NP_061131), and horse (GenBank NW_001799701.1). Grey blocks represent regions where the amino acid sequence differs from cattle. Periods (.) signify the stop codons. Numbers indicate the start codon in exons 1 and the start of exon 2. Red asterisks designate the six conserved cysteines.

There are five *β-Defensins* located within 1.5 Mbp of each other on BTA27 (Fig. 3.8). None of these would have been excluded as a candidate gene based on the recombinant at *BMS1001* (Fig. 3.6). To ensure that I was investigating the correct *β-Defensin*, I compiled an amino acid alignment of each *β-Defensin* (Fig. 3.9). Of the five *β-Defensins*, three share high homology (i.e. > 70%), with a common region of several amino acids in the middle of the peptides. Whereas two, including *β-Defensin300*, have low homology to the others. *β-Defensin300* shares 45% amino acid homology with *β-Defensin7* (*DEFB7*) (GenBank NP_001095832), 44% with *β-Defensin8* (*DEFB8*) (GenBank NP_001095833), 41% with *β-Defensin1* (*DEFB1*) (GenBank NP_783634), and 10% with *β-Defensin* (*DEFB*) (GenBank NP_001071601).

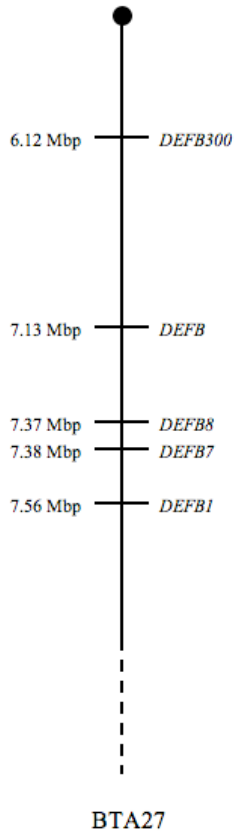


Figure 3.8 Five *β-Defensins* are located within 1.5 Mb of each other on BTA27.

```
                                                        *       *    *
DEFB300  MRLYYLLFALLFLFLLPVPGNGGIISGLQRYYCKI:RSGRCALIGCLPK
DEFB7    MRLHHLLLALLFLVLSAGSGFTQGVRNFVT::CRINR:GFCVPIRC:PG
DEFB8    MRLHHLLLALLFLVLSAGSGFTQGVRNFVT::CRINR:GFCVPIRC:PG
DEFB1    MRLHHLLLTLLFLVLSAGSGFTQGISNPLS::CRLNR:GICVPIRC:PG
DEFB     MRVFHLLLLALGLLLSQLGPGASQLTALGQ

                  *         **
DEFB300  EE::QIG:CSLSGR:KCCRKK
DEFB7    HRRRQIGTC:LGPRIKCCR:::
DEFB8    HRR:QIGTC:LGPQIKCCR:::
DEFB1    NLR:QIGTC:FTPSVKCCRWR:
```

Figure 3.9 Amino acid alignment of five *β-Defensin* located on BTA27. Grey blocks represent regions where the amino acid sequence differs from *β-Defensin300*. Red asterisks designate the six conserved cysteines.

The structure of cattle *β-Defensin300* (Fig 3.10) and canine *β-Defensin103* are similar. Cattle *β-Defensin300* is comprised of two exons. Exon one consists of 58 bp from the start codon. Partial cDNA sequence (GenBank EU715239) analysis shows that there are at least 77 bp of sequence in the 5' UTR. The single intron is 660 bp in length. Exon 2 is 146 bp to the stop codon. The exact length of the 5' and 3' UTRs is not known. I attempted to amplify product from genomic DNA and cDNA using primers located gradually further away from the known coding sequence. If I was able to amplify a product from both genomic DNA and cDNA, then I concluded that the cDNA extended at least that far. If at any time I was able to amplify a product from genomic DNA but not from cDNA, then I was able to conclude that the location of the primer was outside of the cDNA sequence. Using this method I determined that the 5' UTR extends at least as far as 187 bp before the start codon, but not as far as 495 bp before the start codon. Likewise, the 3' UTR extends at least as far as 2 bp after the stop codon, but not as far as 43 bp after the stop codon.

The six cattle that were initially sequenced using the nested approach included one VR Holstein bull, three black and white Holsteins, and two red and white Holsteins.
While no SNPs were detected in the coding or intronic sequence, five SNPs were located in the 5' UTR in close proximity to the start codon, -34A>G, -42A>G, -69A>G, -264C>T, and -319A>G.

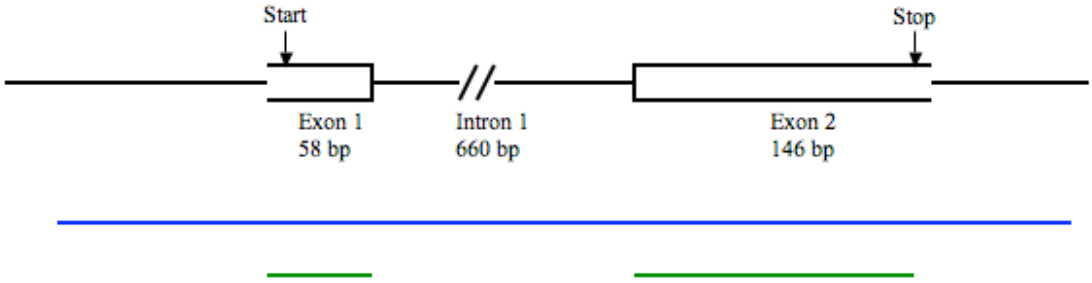


Figure 3.10 *β-Defensin300* was sequenced from genomic (blue) and cDNA (green).

The coding region was then sequenced using genomic DNA from 6 additional cattle, and in cDNA prepared from skin samples of 13 cattle. All or part of the 5' region containing the five SNPs was sequenced in a total of 35 cattle (Table 3.12). These additional cattle were of various breeds or crosses and included examples of variant red, traditional red, black, dun, and cream

coat colours, in addition to traditional Jersey, Guernsey, and Brown Swiss colourations. The genotypes at these 5 SNPs forms a haplotype. At least four haplotypes were observed (Table 3.13), three in variant red cattle.

One variant red cow (08-082) was heterozygous at the -34A>G SNP and was a segregating dam with both black and variant red offspring (Fig. 3.11). The offspring and available sires for this family were therefore also sequenced. Haplotypes of the alleles at the five SNPs identified in the 5' region of *β-Defensin300* were evident in this family (Table 3.12).

This small two-generation family consisted of four calves per generation (Fig. 3.7). Of the four offspring of 08-062, three were VR but one could have inherited this phenotype from the sire. Two others had an unavailable sire and were genotyped as A/G like the dam, so were also uninformative. Unfortunately, only one calf in each generation was informative (08-065 and 08-079). Both of these calves had to have inherited the VR phenotype from 08-082 and both also inherited her chromosome with haplotype 1. While this is not conclusive proof that *β-Defensin300* is the gene causing VR, it is suggestive.

Table 3.12 Genotypes of 35 cattle at five SNPs in *β-defensin300*.

ID #	Breed	Colour	*MC1R* Genotype	Sequence Source	Genotype -319 A>G	-264 C>T	-69 A>G	-42 A>G	-34 A>G	Haplotype
06-651	Holstein	VR	E^D/e	genomic	A/A	C/C	A/A	A/A	G/G	1/1
08-076	Holstein	VR	$E^{D/}/E^+$	genomic	A/A	C/C	A/A	A/A	G/G	1/1
08-079	Holstein	VR	$E^{D/}/e$	genomic	A/A	C/C	A/A	A/A	G/G	1/1
08-065	Holstein	VR	$E^{D/}/E^D$	genomic	A/A	C/C	A/A	A/A	G/G	1/1
08-069	Holstein	VR	$E^{D/}/E^D$	genomic			A/G	A/G	A/G	1/2
08-082	Holstein	VR	$E^{D/}/E^+$	genomic	A/G	C/T	A/G	A/G	A/G	1/2
08-066	Holstein	VR	$E^{D/}/e$	genomic	A/G	C/T	A/G	A/G	A/G	1/2
08-078	Holstein	VR	$E^{D/}/e$	genomic	A/G	C/T	A/G	A/G	A/G	1/2
08-071	Holstein	VR	$E^{D/}/E^D$	genomic			G/G	G/G	A/A	2/2
04-898	Holstein	VR	$E^{D/}/E^+$	genomic			A/A	G/G	A/A	3/3
08-052	Holstein	Red	e/e	genomic	A/A	C/C	A/A	A/A	G/G	1/1
08-053	Holstein	Red	e/e	genomic	A/A	C/C	A/A	A/A	G/G	1/1
08-080	Holstein	Red	e/e	genomic	A/G	C/T	A/G	A/G	A/G	1/2
06-1082	Holstein	Black		genomic	A/A	C/C	A/A	A/A	G/G	1/1
06-1078	Holstein	Black		genomic	A/G	C/T	A/G	A/G	A/G	1/2
08-051	Holstein	Black	$E^{D/}/e$	genomic	A/G	C/T	A/G	A/G	A/G	1/2

Table 3.12 Continued

ID #	Breed	Colour	*MC1R* Genotype	Sequence Source	Genotype -319 A>G	-264 C>T	-69 A>G	-42 A>G	-34 A>G	Haplotype
08-064	Holstein	Black	$E^{D/}E^{D}$	genomic	A/G	C/T	A/G	A/G	A/G	1/2
08-081	Holstein	Black	$E^{D/}E^{D}$	genomic	A/G	C/T	A/G	A/G	A/G	1/2
02-494	Guernsey	Red	E^{+}/e	genomic	A/G	C/T	A/G	A/G	A/G	1/2
02-497	Guernsey	Red	E^{+}/e	genomic	A/G	C/T	A/G	A/G	A/G	1/2
04-496	Guernsey	Red	E^{+}/E^{+}	genomic	G/G	T/T	G/G	G/G	A/A	2/2
02-517	Brown Swiss	Taupe Brown	E^{+}/E^{+}	genomic	A/G	C/T	A/G	A/G	A/G	1/2
02-699	Brown Swiss	Taupe Brown		cDNA			A/A	A/G	A/G	1/3
02-514	Brown Swiss	Taupe Brown	E^{+}/E^{+}	genomic	G/G	T/T	G/G	G/G	A/A	2/2
02-479	Jersey	Fawn	E^{+}/E^{+}	genomic	G/G	T/T	G/G	G/G	A/A	2/2
02-480	Jersey	Fawn	E^{+}/E^{+}	genomic	G/G	T/T	G/G	G/G	A/A	2/2
02-481	Jersey	Fawn	E^{+}/E^{+}	genomic	G/G	T/T	G/G	G/G	A/A	2/2
31J	Galloway	White/ Black Points		cDNA			A/A	A/A	G/G	1/1
04-561	Galloway	Dun	$E^{D/}E^{D}$	cDNA			A/A	A/G	A/G	1/3
82	Galloway	Dun /White Belt	$E^{D/}$e	cDNA			A/A	A/G	A/G	1/3
06-795	Holstein x Galloway	Black	$E^{D/}E^{D}$	cDNA			A/A	A/G	A/G	1/3
00-307	Simmental	Red		cDNA			A/A	G/G	A/G	3/4
02-2293	Charolais	Dilute Red		cDNA			A/A	G/G	A/A	3/3
03-011	Hereford	Red		cDNA			A/A	G/G	A/A	3/3
SP	Speckled Park	White/ Black Points		cDNA			A/A	G/G	A/A	3/3

VR = variant red

Table 3.13 *β-Defensin300* haplotypes of SNPs in the 5' region derived from family analysis or inferred from sequence data.

Haplotype	-319	-264	-69	-42	-34
1	A	C	A	A	G
2	G	T	G	G	A
3	?	?	A	G	A
4	?	?	A	G	G

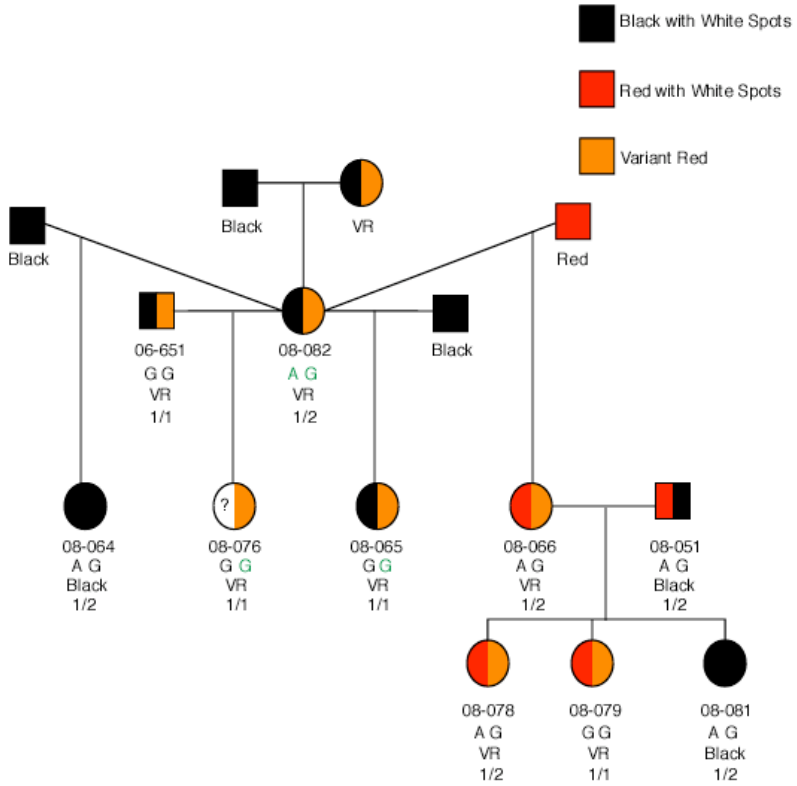


Figure 3.11 *β-Defensin300* -34A>G SNP genotypes for VR family. Offspring alleles inherited from the dam are highlighted in green. Haplotypes are represented by either 1/1 or 1/2.

3.3.5.3. Linkage Analysis

By combining the family data from the microsatellites near *β-Defensin300* and the SNPs in the 5' UTR, linkage analysis to variant red could be conducted. A LOD score of 3.255 was obtained (Table 3.14) with a LOD of ≥3.0 indicating linkage. This also suggests that *β-Defensin300* could be the causative gene for this phenotype.

Table 3.14 Linkage mapping of variant red to a region including *β-Defensin300*.

				LODs			
				0.01	0.05	0.10	0.15
Family	Marker	Phase	Recombinance	cM	cM	cM	cM
D1	*BMS1001*	Unknown	5:0	1.1823	1.0927	0.9753	0.8513
D2	*β-Defensin300* SNPs	Known	1:0+2u	0.2967	0.2788	0.2553	0.2304
M	DLD1/DLD2	Unknown	7:0+3u	1.7756	1.6503	1.4859	1.3121
				3.2546	3.0218	2.7165	2.3938

3.4 Discussion

Genetic determination of black or red coat colour has been extensively researched in many species and has been primarily linked to the function of *MC1R*. Klungland et al. (1995) proposed the presence of three *MC1R* alleles in cattle: E^D, *e*, and E^+. They described E^D as being constitutively dominant, where the presence of only one E^D allele is sufficient to dictate that the overall colour of the animal is black. My data suggests that this is not completely true. I genotyped 35 related Holstein cattle and, of the 28 red individuals, found 27 of those to have at least one E^D allele (Table 3.11). This suggests that there is some other gene capable of altering the *MC1R* pathway to the extent that E^D is not constitutively expressed.

The *e* allele of *MC1R* is associated with a deletion of one of two G residues at position 771 (Joerg et al. 1996) which then introduces a premature stop codon and produces a truncated *MC1R* product (Klungland et al. 1995). An individual homozygous for the *e* allele is not capable of producing a functional MC1R, which prevents binding of α-MSH and therefore the melanocyte produces its default pigment, phaeomelanin. In order for an individual to have a functional copy of *MC1R* (E^D), yet produce only phaeomelanin, it would suggest that an

additional gene product is present and capable of interfering with the ability of α-MSH to bind to the MC1R. Or conversely, that α-MSH still binds to MC1R, but the intercellular cascade of events that is triggered by the binding is halted before eumelanin is produced. None of the additional *MC1R* variants described by Rouzaud et al. (2000) or Graphodatskaya et al. (2002) were consistently found in sequences from VR cattle. Likewise, *MC1R* genotyping conducted by several commercial laboratories is insufficient to explain red versus black coat colour in Holstein cattle. Although all *e*/*e* cattle are red (with the exception of some changeling cattle that can be *e*/*e* and change from red to black with age), not all red cattle are *e*/*e*. This may have an important impact on Holstein breeders that are striving for red cattle. Current industry understanding is that all red cattle are *e*/*e* at *MC1R*, so will always produce red calves when mated to an *e*/*e* bull. But if one of those cattle are VR instead of *e*/*e* red, then resultant offspring may be black.

ASIP was chosen as a likely candidate for VR primarily through observation of its function in dogs. When looking at dogs, the dominant mutant a^y allele of *ASIP* creates a phenotype that is often similar in appearance to *e*/*e* red. It would then fit if this pattern mirrored itself in cattle, producing a phaeomelanin-based phenotype through the binding of a mutant ASIP to MC1R, effectively blocking the production of eumelanin. Likewise, dogs can exhibit a recessive black phenotype with homozygous inheritance of the *a* allele of *ASIP* (Kerns et al. 2004). A similar recessive black allele has been postulated in cattle (Adalsteinsson 1995). The pattern that this implies would fit what is seen with the dominant VR phenotype.

While mutations have been found in the coding region of dog *ASIP*, to date, no coding region mutations have been discovered in cattle (Royo et al. 2005; Girardot et al. 2005). A 2684C>T SNP in intron 3, discovered by Tom Berryere (unpublished data), was therefore used to determine whether there was co-segregation between *ASIP* and the VR phenotype. A segregating VR family was genotyped at the 2684C>T *ASIP* SNP (Fig. 3.4) but no co-segregation was observed, suggesting a coding mutation in *ASIP* is unlikely to cause VR, including the action of a mutation similar to that of canine a^y or a recessive black *a* allele.

Morgan et al. (1999) found evidence that *ASIP* is maternally imprinted in mice. To rule out the possibility that *ASIP* is also imprinted in cattle, a family out of a segregating dam (family C1) and a family out of a segregating sire (family D1) were used in the analysis of co-segregation. No co-segregation was seen between *ASIP* 2684C>T SNP and the VR phenotype in either family (Fig. 3.4).

ASIP held substantial promise as a candidate gene for VR, so while it was discounted through segregation analysis, focus was directed towards another candidate that is suspected to play a role in the functioning of *ASIP*, *ATRN*. Mutated murine *ATRN* (*mahogany*) alleles incompletely prevent binding of ASIP to MC1R. The wildtype allele allows for normal binding of ASIP and the production of a normal agouti phenotype (Gunn et al. 1999). Gunn et al. (1999) proposed two possible modes of action for murine *ATRN*. One being that ATRN acts as a low-affinity receptor for ASIP and the other that ATRN is required for desensitization of MC1R. In both cases, ATRN would act to regulate binding of ASIP to MC1R. To apply this to the case of VR in Holsteins, the VR cattle would possess the wildtype allele of *ATRN*, allowing them to express a red *ASIP* phenotype. Black cattle would then have a mutated *ATRN* allele, preventing binding of ASIP to MC1R and resulting in production of eumelanin through decreased binding competition for α-MSH. One segregating VR parent was found to be heterozygous at the 2375A>G *ATRN* SNP but no co-segregation of the SNP with the VR phenotype was seen (Fig. 3.5). This effectively discounts *ATRN* as the causative gene for VR.

Melatonin is reported to affect pigmentation in many species, including amphibians, reptiles and fish (Filadelfi and Castrucci, 1994) and also murine melanoma cells (Castrucci et al. 1997). As a result, *MTNR1A* was chosen as candidate under the reasoning that a mutation in the receptor may allow the *in utero* environment to affect a prepartum alteration in coat colour. Since *MTNR1A* was not previously described in cattle, the first step in determining whether it was a viable candidate gene for VR was linkage mapping.

MTNR1A was mapped to BTA 27, approximately 10 cM from the final candidate gene, *β-Defensin300*. *CBD103*, or the *K* locus, plays a significant role in production of coat colour in dogs by determining whether *ASIP* or *MC1R* dictate the overall colour of the dog (Candille et al. 2007). The dominant mutant *K* allele, K^B, binds to MC1R produces a solid black or brown colour. The recessive wild-type *K* allele, k^y, cannot bind to MC1R and allows *ASIP* to determine colour, producing any of the *Agouti* phenotypes such as fawn/sable, black and tan, wolf sable, or recessive black.

Because of its close proximity to *MTNR1A*, both it and *β-Defensin300* could be analyzed for linkage with VR simultaneously. Two segregating VR families were genotyped at seven microsatellites surrounding *β-Defensin300* and *MTNR1A* (Fig. 3.6). A pattern of recombination in two calves from family M stretched over *MTNR1A* but stopped short of *β-Defensin300*. These

results effectively rule out *MTNR1A* as a causative gene for VR, but *β-Defensin300* remained a promising possibility.

As with *MTNR1A*, *β-Defensin300* was not previously characterized fully in cattle. Although Selsted et al. (1993) had found this gene and others in the *β-Defensin* family, they did not report any polymorphisms or the promoter sequence. There are five *β-Defensins* located on BTA27. In order to determine which *β-Defensin* was homologous to *CBD103* in dogs, the canine *K* animo acid sequence was compared to the available *β-Defensin* sequences. The amino acid homology between canine *β-Defensin103* and cattle *β-Defensin300* was 79% (Fig. 3.6). This level of homology is slightly lower than average for most gene comparisons between these two species (Schmutz et al. 2002). Since *MTNR1A* is on the same chromosomes in both dogs and cattle as these two *β-Defensins*, comparative genomics lends support that these two genes are homologous. However, there were four other *β-Defensin* genes in the same region of cattle chromosome 27. None of these other *β-Defensin* matched the canine *β-Defensin103* amino acid sequence better than cattle *β-Defensin300*.

Sequencing of *β-Defensin300* proved to be difficult. Through standard PCR techniques, I was able to obtain a product that appeared clean, but when sequenced, yielded inconsistent data, indicating that multiple products were present. As the *β-Defensin* family is quite large, with five identified *β-Defensins* located on BTA27 alone, I suspect that the primers I designed were picking up multiple family members in addition to *β-Defensin300*. I was able to get around this by using a nested PCR design, amplifying a large region of DNA surrounding *β-Defensin300*, purifying the product and using that as a template for amplification of segments of *β-Defensin300* appropriate for sequencing.

Neither the microsatellite analysis (Fig. 3.6) nor the family co-segregation analysis (Fig. 3.10) excluded *β-Defensin300* as a candidate gene for VR. However no variant was found in the coding region of this gene that could be the causative mutation.

Five variants were found in the 5' region which is commonly considered the promoter region. No TATA box was located in the 5' region of *β-Defensin300*. Prediction programs such as AliBaba2.1, would predict that SNPs -42A>G, -69A>G, and -264C>T would be the most likely to affect expression of this protein. SNP -42A>G and -264C>T alter Sp1 transcription factor binding sites, with the A and C alleles producing two Sp1 sites and alleles G and T

eliminating two Sp1 sites. SNP -69A>G alters an AP-2alpha with the A allele eliminating the AP-2alpha binding site and the G allele producing the binding site. -319A>G does alter a GATA-1 transcription binding site, but since GATA-1 is highly erythroid specific (Gong et al. 1991), there is no evidence that it is expressed in skin. Sp1 is present in human keratinocytes (You et al. 2007) and AP-2alpha is present in mouse skin (Mitchell et al. 1991) so either binding site may be required for *β-Defensin300* expression. Furthermore, Sp1 has been found to drive transcription from TATA-less promoters (Faber et al. 1993; Boisclair et al. 1993). However the ten VR cattle, none of which were red due to *e/e* at *MC1R*, did not all share a consistent derived or predicted haplotype. All of the VR cattle were both
-319A and -264C, but so were the three traditional red cattle and the five black and white Holstein cattle. No mutation was identified in *β-Defensin300* that could account for VR as a single gene dominant trait.

I was able to obtain cDNA sequence from skin samples from 13 cattle, including two VR cows, indicating that *β-Defensin300* is present in cattle skin. While this fact alone does not prove that *β-Defensin300* is *K*, it continues to support the postulation that *β-Defensin300* could be involved in pigmentation.

Numerous *ASIP* alleles have been postulated in cattle, but what remains to be answered is what determines whether an individual cow will express eumelanin through *MC1R* or phaeomelanin through *ASIP*. *K* solves this problem in dogs and is an intriguing possibility in cattle as well. While preliminary research did not find any association between *ASIP* and variant red, the involvement of *β-Defensin300* may play a key role in this scenario. If all Holsteins are fixed at *ASIP* for a particular allele, presumably wild-type, we would not expect to see any co-segregation of *ASIP* with either VR or black cattle. But if the causative mutation is in *β-Defensin300* the result may be that, in VR cattle, *β-Defensin300* allows the binding of ASIP to a functional MC1R, while the black cattle possess an alternate *β-Defensin300* allele that prevents binding of ASIP to MC1R, allowing instead for α-MSH to bind MC1R and produce eumelanin (Fig 3.12).

β-Defensin300 remains the most promising candidate for VR. As such, future research should be directed primarily towards *β-Defensin300*.

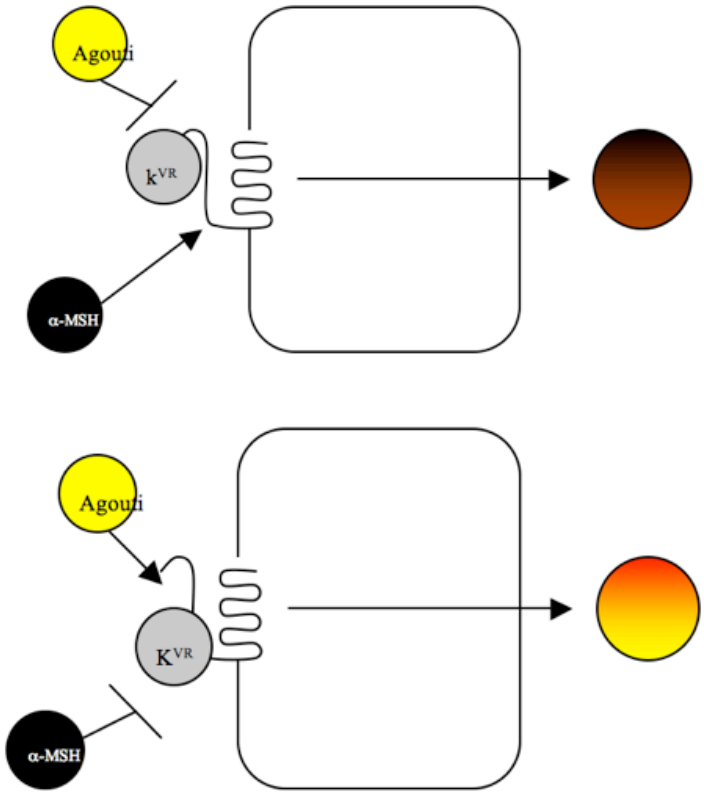


Figure 3.12 Hypothetical interaction of *β-Defensin300* (K^{VR}), agouti and α-MSH to produce the VR phenotype in E^{D}/_ cattle.

3.5 Conclusion

Variant red was linkage mapped to *β-Defensin300*, and VR co-segregated with 5' UTR variants in *β-Defensin300* in one segregating family. However, no obvious single variant in *β-Defensin300* was present in all, and only, VR cattle. As such, *β-Defensin300* remains the best candidate gene for VR of those studied, though a causative mutation has not yet been discovered.

4.0 ANALYSIS OF CANDIDATE GENES FOR CHANGELING

4.1 Introduction

The objective of this portion of the study was to determine the causative gene responsible for the changeling phenotype in Holstein cattle. *MC1R*, *ASIP*, and *ATRN* were chosen as candidate genes due to their involvement in the pigmentation pathway of other species. *MTNR1A* was chosen as a positional candidate gene.

By taking into consideration families that are capable of producing changeling and black offspring, it appears as if changeling is recessively inherited. Changeling offspring can arise from the mating of two non-changeling cattle.

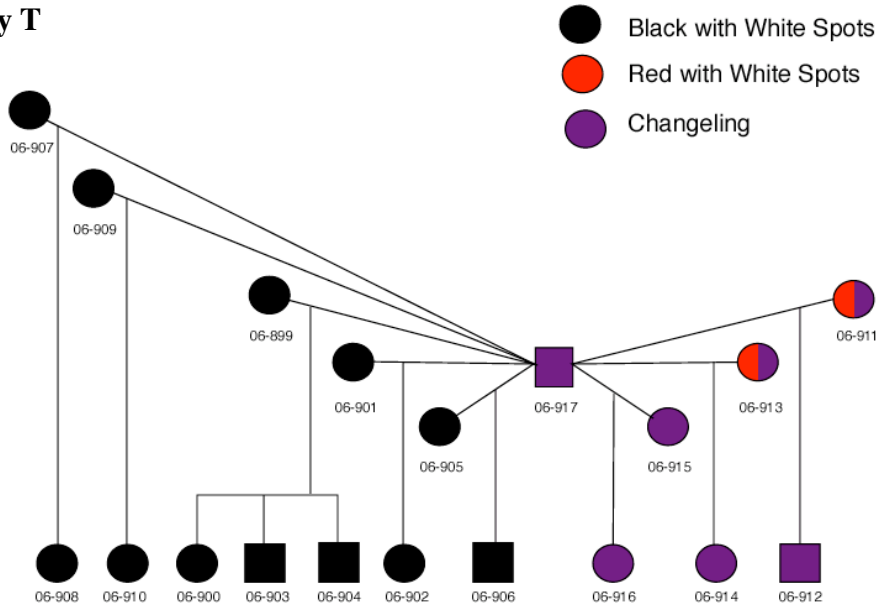
I hypothesize that the causative gene for changeling will be related to the function of *ASIP*. In other species *ASIP* is known to regulate the production of eumelanin and phaeomelanin, temporally and spatially. It is a commonly held belief that age dependent promoters of *ASIP* are responsible for the changing phenotypes of wild boar piglets and fawns. The proportion of eumelanin to phaeomelanin in dogs with *ASIP* phenotypes often appears to change as the pup matures into its adult coat. Since changeling cattle exhibit a phenotype that changes from phaeomelanin to eumelanin beginning at a couple weeks of age and is complete by the time they are a year of age, it would appear to fit with the postulated mode of action for *ASIP*.

4.2 Materials and Methods

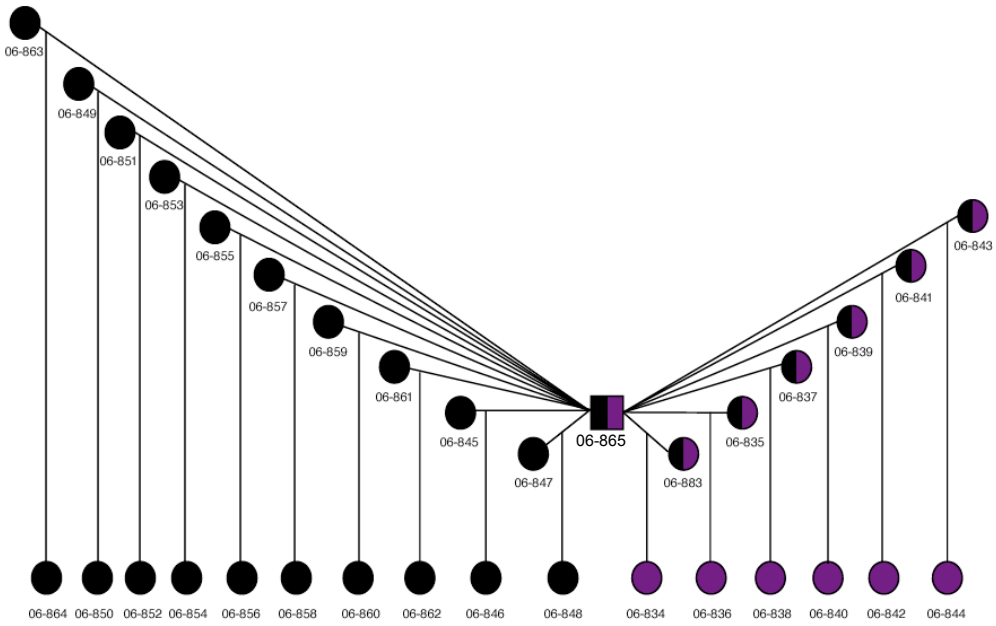
4.2.1 Animals

Previously extracted DNA samples for two families segregating for the recessive changeling phenotype (families C2 and S) and one family sired by a homozygous changeling bull (family T) (Fig. 4.1), for a total of 85 cattle, were obtained from GenServe Laboratories in 2007. These families were identified with the help of Holstein Canada and parentage was confirmed by GenServe Laboratories. One additional changeling cow, and her black aunt and grand-dam were obtained from the Greenbrae diary herd at the University of Saskatchewan. (Fig. 4.1)

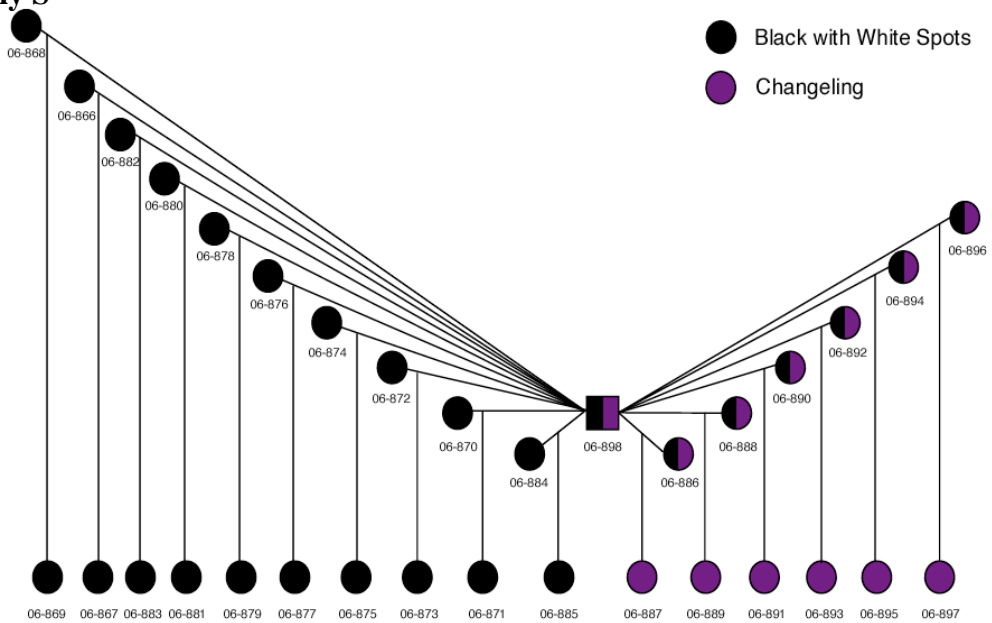
Family T



Family C2



Family S



Greenbrae Family

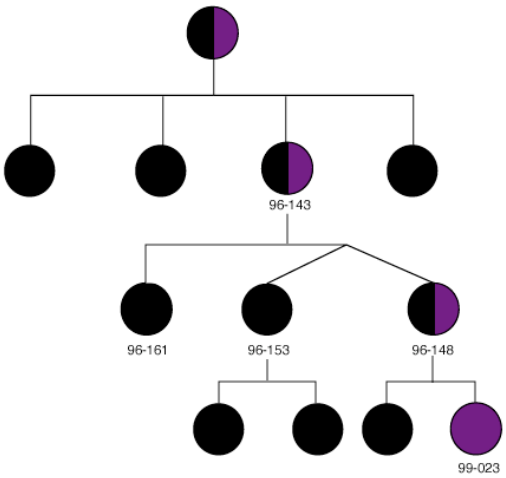


Figure 4.1 Four families of Holstein cattle used for studying the changeling phenotype.

4.2.2 DNA Preparation

DNA samples from families T, S, and C2 were received as extracted DNA from GenServe Laboratories. Additional DNA samples were received in the form of hair, blood, semen, or skin. RNA and cDNA were synthesized from the skin samples. Genomic DNA was extracted from blood and semen as previously described (Schmutz et al. 1995). Initially, genomic DNA was extracted using a protocol outlined by BovaCan Laboratories. An alternate extraction protocol (Epicentre, Madison WI) was used on later samples as high levels of degradation were noticed with the previous extraction protocol. Extraction protocols are outlined in Appendix A.

4.2.3 DNA Analysis

4.2.3.1 *Melanocortin 1 Receptor*

4.2.3.1.1 Sequencing

Product for *MC1R* sequence was obtained in three overlapping segments by utilizing primers E4 and E6 from Klungland et al. (1995) and P6 Joerg et al. (1996), and by designing primers based off of published *MC1R* mRNA sequence (GenBank AF445642) (Table 3.1) The PCR and sequence protocol used is outlined in section 3.2.3.1.1.

4.2.3.1.2 Genotyping

The three Greenbrae herd cows, the sires of families S, C2 and T, and their respective changeling offspring were genotyped for the *MC1R* alleles E^D, E^+ and *e* that are associated with coat colour using protocols previously described by Klungland et al. (1995) and Joerg et al. (1996) and in Chapter 3.

4.2.3.2 *Agouti Signaling Protein*

Changeling families C2, S, and T were genotyped at microsatellite marker *BMS995*, which was mapped to 0 cM from *ASIP* with a LOD of 3.551 using *ASIP* SNP 2684C>T (Appendix E), with primers from MARC (http://www.ars.usda.gov/Main/docs.htm?docid=12539) (Table 4.1). PCR was carried out in 15 µl reactions consisting of 1 µl (50-100 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl of each primer (10 pM/µl) and 9.2 µl of ddH_2O. Forward primers were end labelled with P^{32} and T4 Polynucleotide Kinase (New England BioLabs, Ipswich, MA). The PCR temperature protocol consisted of 4 minutes at 94°C, followed by 35 cycles at 94°C for 50 seconds, 58°C for 50 seconds, and 72°C for 50 seconds. This was followed by a 4 minute dwell at 72°C. Bands were resolved on a 6% polyacrylamide gel and exposed on an x-ray film.

Table 4.1 Characterization of primers used to genotype microsatellite marker *BMS995*.

Microsatellite	Primer Sequence	No. of alleles	Heterozygosity	Temp (°C)
BMS995	F: 5'-AATTCTTCCAACCTCCAGTGC-3' R: 5'-ACTTTTCAAGCAGGGCTCAC-3'	6	77%	58

4.2.3.3 *Attractin*

The segregating parents of families C2, S, and T were genotyped at an unpublished 2790C>T *ATRN* unpublished SNP discovered in our lab by Tom Berryere. The sires of C2 and S were found to be heterozygous at the SNP, so the changeling offspring and their respective dams were also genotyped. The protocol used is detailed in section 3.2.3.1.1 with primers and annealing temperatures listed in Table 4.2. The product was then digested for 3 hours at 65°C with 0.75 µl *BsrI* (10 U/µl) (New England BioLabs, Ipswich,

MA), 1.5 µl B+ Buffer, and 0.25 µl ddH_2O, then run on a 4% agarose gel stained with ethidium bromide. The 221 bp product cuts into bands of 57 and 164 with the T allele. The C allele does not cut with *BsrI*.

Table 4.2 Characterization of primers used to genotype *ATRN* 2790C>T SNP.

Primer Name	Orientation	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
CowATRNInt21	F	5'-CATGACACATTAGTGGGTCAC-3'	221	59
CowATRNInt22	R	5'-GGCATTGGAGTCCACGCACTG-3'		

4.2.3.4 *Melatonin Receptor 1A*

4.2.3.4.1 Sequencing

Primers to amplify both exons of *MTNR1A* were designed using whole genome shotgun sequence of BTA 27 (GenBank NW_930548), predicted *MTNR1A* sequence (GenBank XM_614283), and partial genomic *MTNR1A* sequence (GenBank BTU73327) (Table 4.3). Several primer sets were designed to attempt to amplify genomic DNA and cDNA from *MTNR1A* from cattle of several coat colours.

Primers MTNR1A5'#1-F and MTNR1AInt2#2-R were used to amplify genomic DNA from exon 1. Reaction volumes of 15 µl were used, consisting of 1 µl (50-100 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 1 µl of each primer (10 pM/µl), 3.9 µl of Betaine and 5.3 µl of ddH_2O. DNA was denatured for 4 minutes at 94°C, followed by 37 cycles at 94°C for 50 seconds, 57°C for 50 seconds, and 72°C for 50 seconds, followed by a 4 minute dwell at 72 °C.

Table 4.3 Characterization of primers used to sequence *MTNR1A*.

Primer Name	Primer Sequence	Product Size (bp)	Anneal Temp. (°C)
MTNR1A Ex2 –F	5'-GGAATTGCCATCAACCGCTATTG-3'	661	57
MTNR1A Ex3-R	5'-CTATTAACGGAGAGGGTTTGC-3'		
MTNR1A5'#1-F	5'-TGTCCGCACAGGCAGCGGCT-3'	439	57
MTNR1AInt2#2-R	5'-TGCGTGCTTGCAAAGGTGGCGGAACA-3'		
MTNR1A Int2-F	5'-TAACTGTGATATCGGGTCAT-3'	1082	53
MTNR1A 3'-R	5'-TTCCTAGGCATCTACCAAGA-3'		

MTNR1A Int2-F with MTNR1A 3'-R were used as primers to attempt to amplify genomic DNA from exon 2. Reaction volumes were 15 µl and followed the PCR and sequencing protocols detailed in section 3.2.3.1.1 and the primers and annealing temperatures listed in Table 4.3

In attempts to amplify MTNR1A cDNA from cattle skin, primers MTNR1A Ex2-F and MTNR1A Ex3-R were used (Table 4.4). The previously described reaction protocol was used(Section 3.2.3.1.1), with annealing temperature of 57°C.

4.2.3.4.2 Genotyping

Twenty-four Holstein cattle, including eight changeling, five VR, one traditional red, and ten black, were genotyped at a –724C>T SNP through sequence analysis. These cattle include changeling family S. Primers (Table 4.4) were designed from BTA27 whole genome shotgun sequence (GenBank NW_903548). A modified booster PCR protocol was used (Kidd et al. 1995). The initial 15 µl reaction volume consisted of 2 µl (100-200 ng) of template DNA, 1.5 µl of 10 X PCR reaction buffer (Fermentas, Burlington, ON), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 0.1 µl of *Taq* polymerase (5 U/µl; Fermentas, Burlington, ON), 0.2 µl of each primer (10 pM/µl), 3.9 µl of Betaine, and 6.9 µl ddH_2O. Reactions were denatured for 4 minutes at 94°C, followed by 10 cycles of 50 seconds at 94°C, 50 seconds at 57°C, and 50 seconds at

72°C. The PCR was then stopped and 1 μl of each of the initial primers were added to every reaction. PCR was resumed for 25 cycles of 50 seconds at 94°C, 50 seconds at 57°C, and 50 seconds at 72°C, followed by a 4 minute dwell at 72°C. The PCR product was then resolved on a 1% agarose gel stained with ethidium bromide, then purified and sequenced using the protocol outlined in 3.2.3.1.1.

Table 4.4 Characterization of primers used to genotype *MTNR1A* –742C>T SNP.

Primer Name	Orientation	Primer Sequence	Product Size (bp)	Annealing Temp. (°C)
MTNR1A5'	F	5'- TGAGCCAGTGTTCTCAGTGGAA-3'	355	57
MTNR1AInt1	R	5'- ACCTTAGATAACCATCATGCG-3'		

4.3 Results

4.3.1 *Melanocortin 1 Receptor*

4.3.1.1 Sequence Analysis

MC1R sequence was obtained from genomic DNA of one changeling cow and one obligate heterozygote. No new mutations were found.

Using previously described *MC1R* mRNA (GenBank AF445642) as a guide, I was able to sequence the complete coding sequence of *MC1R* including 2111 bp 5' of the start codon and 149 bp 3' of the stop codon. Whole or partial *MC1R* sequence was obtained from 17 cattle. These cattle include five black Holsteins, six variant red Holsteins, one changeling Holstein, three taupe brown Brown Swiss, and three fawn Jerseys.

4.3.1.2 Genotyping

Eighteen changeling Holsteins from families C2, S, T and the Greenbrae family were genotyped at the *MC1R* alleles commonly considered to affect coat colour (Fig. 4.2). Of the 18 cattle genotyped, five of the six possible genotypes were observed (Table 4.5).

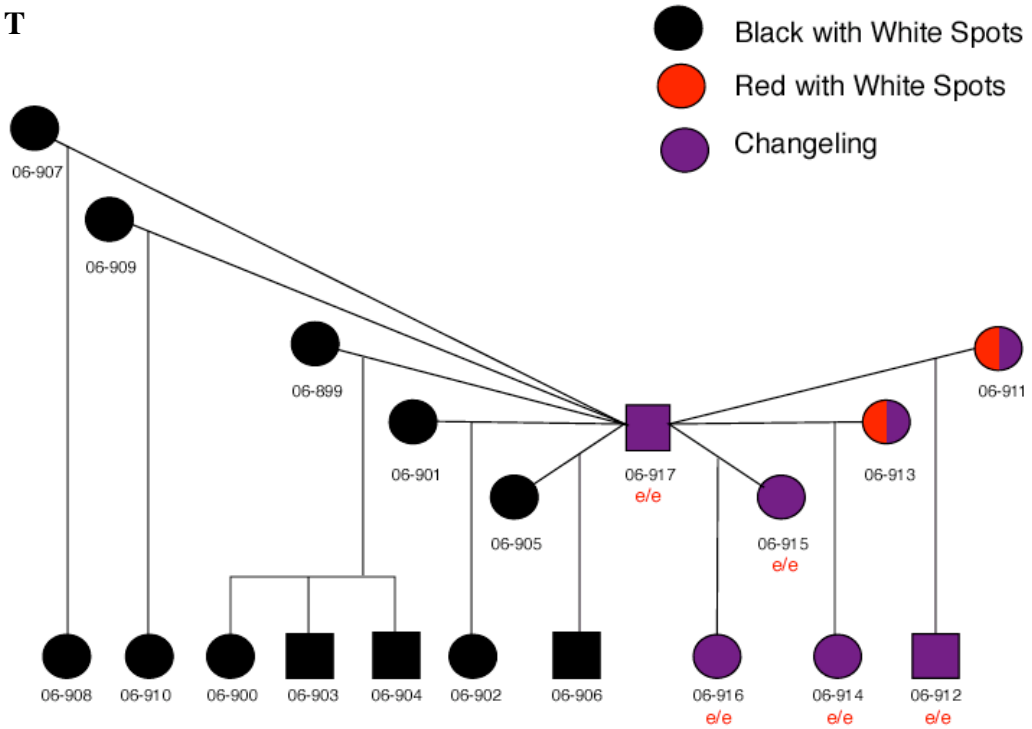
Table 4.5 *MC1R* genotypes of 18 Holstein cattle belonging to one of four changeling families.

Genotype	E^+/E^+	E^+/E^D	E^+/e	E^D/e	E^D/E^D	e/e
# of Animals	1	1	6	1	0	9

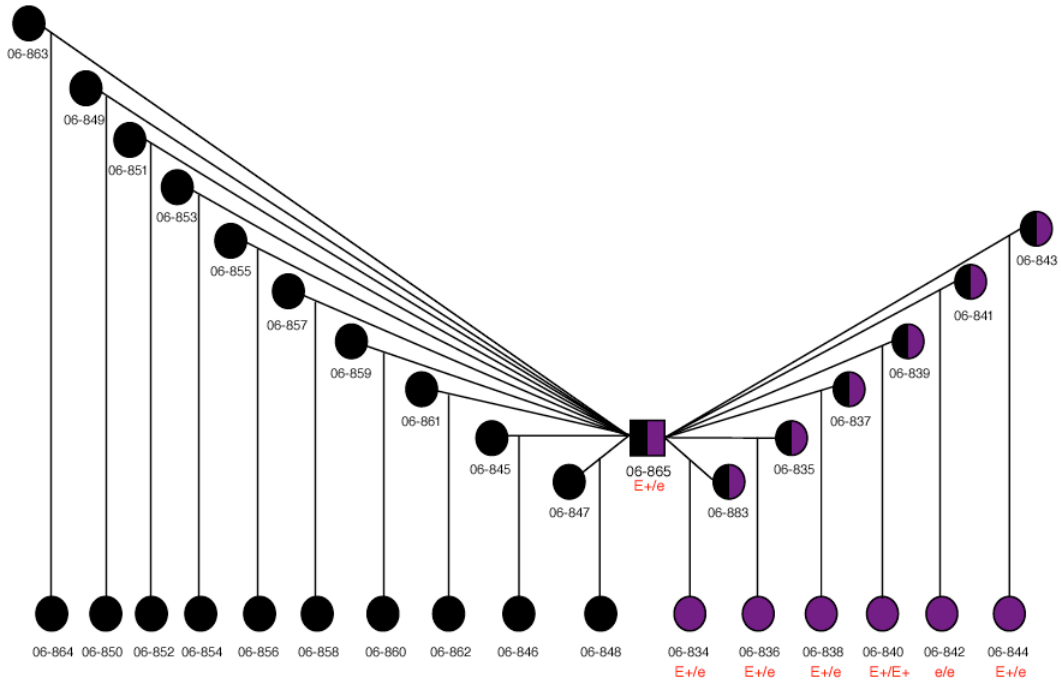
4.3.2 *Agouti Signaling Protein*

ASIP is credited with the regulation of the expression of either eumelanin or phaeomelanin, both temporally through an animal's life or at particular stages of hair growth and spatially over the individual animal's body (Silvers 1979). This made *ASIP* a primary candidate for changeling, as the phenotype is created through the switch in production of phaeomelanin to eumelanin as the animal ages. There were no known coding region mutations in cattle *ASIP*, but a 2684C>T SNP was discovered in intron 3 by Tom Berryere (unpublished data). Genotyping of segregating parents revealed that all were homozygous at this SNP, hence rendering it uninformative in testing for co-segregation of the alleles with the changeling phenotype. However, a microsatellite, *BMS995*, was mapped to 0 cM from *ASIP* with a LOD of 3.551 using this SNP (Tom Berryere, unpublished data) (Appendix E), so *BMS995* could be used to judge co-segregation. The sire of family S is heterozygous at *BMS995*, and through genotyping of the changeling offspring, five were found to inherit one of the sire's alleles, while one changeling calf inherited the other allele (Fig. 4.3). Due to the close proximity of *BMS995* with *ASIP*, since the alleles of the microsatellite did not co-segregate with the changeling phenotype, it also holds that a mutation in *ASIP* itself is not responsible for the changeling phenotype.

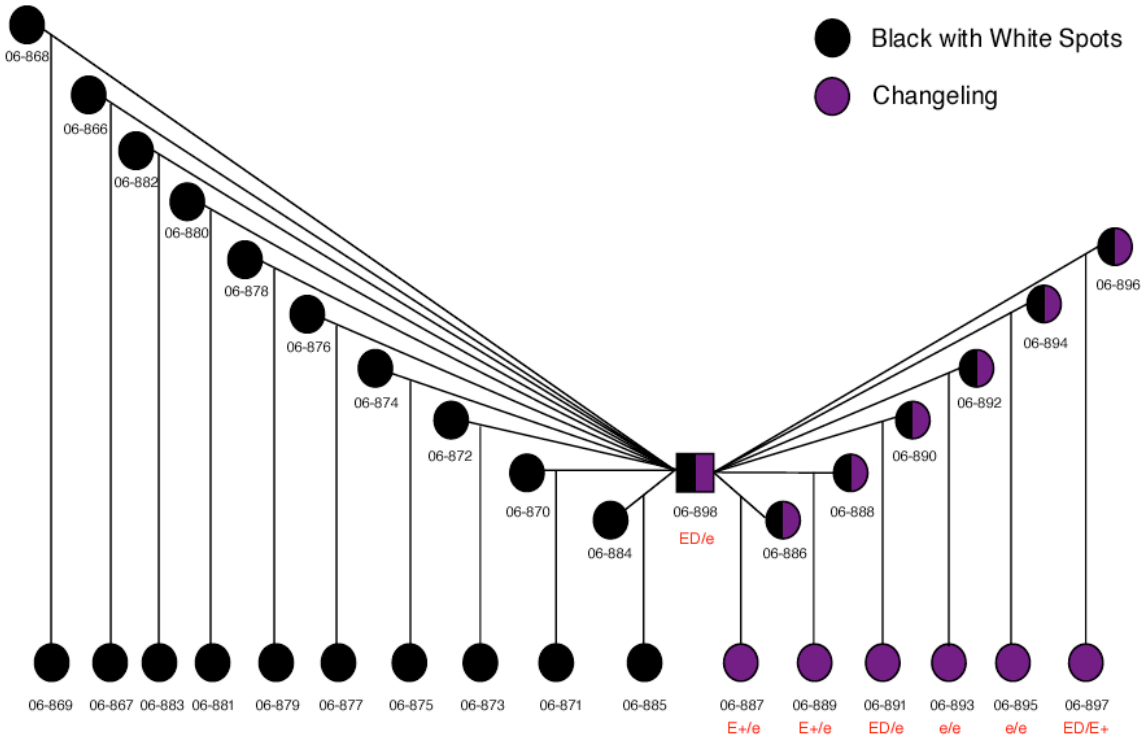
Family T



Family C2



Family S



Greenbrae Family

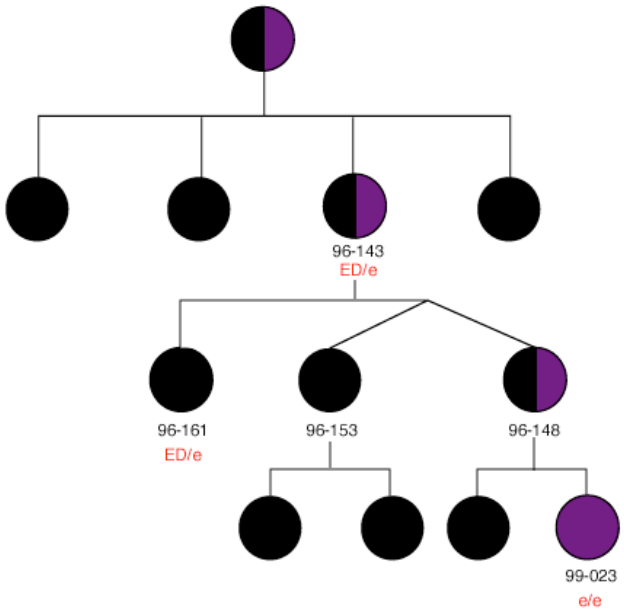


Figure 4.2 Changeling cattle from four families were genotyped for the three *MC1R* coat colour alleles.

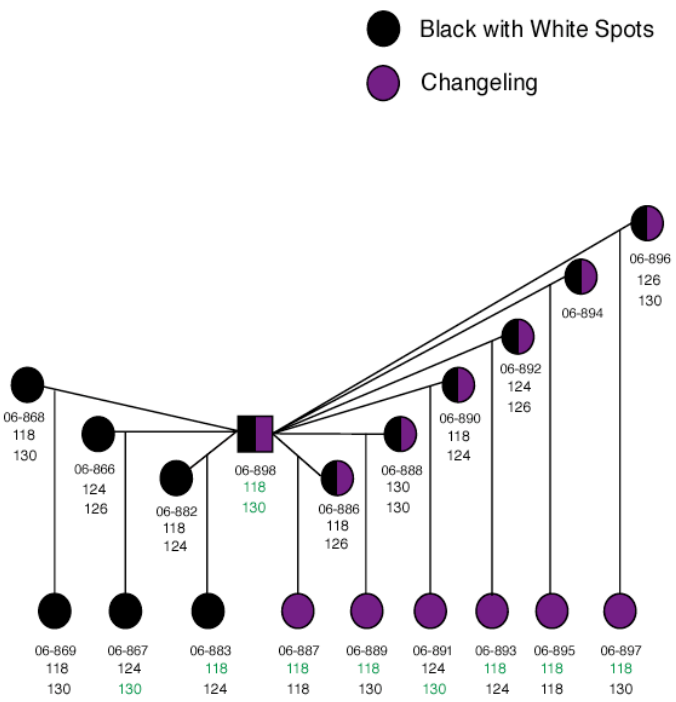
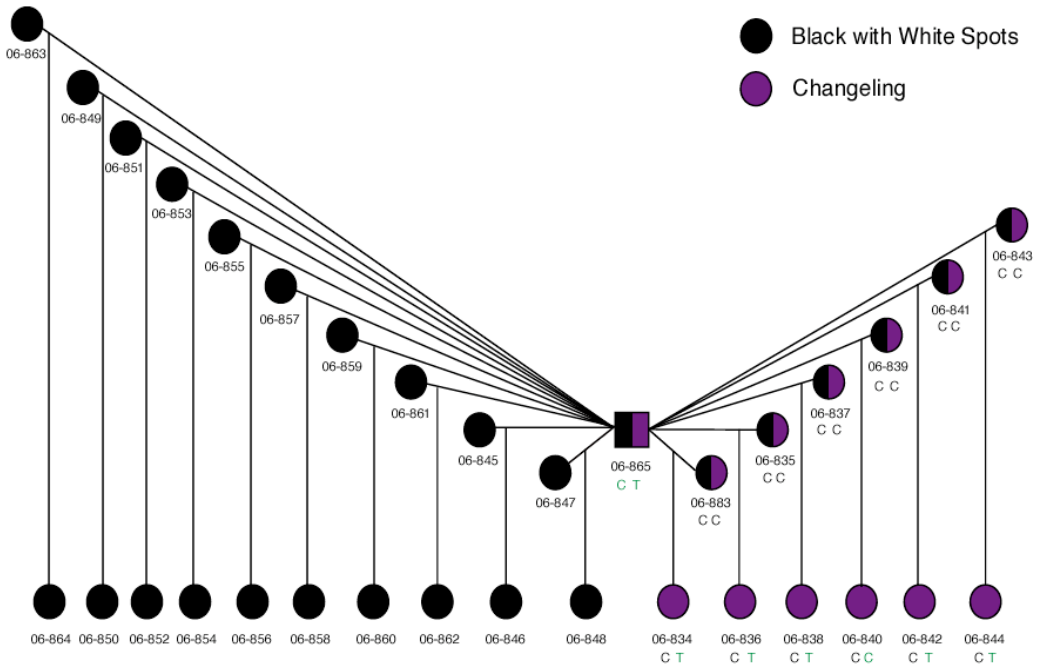


Figure 4.3 To determine co-segregation with *ASIP*, *BMS995* genotypes for Family S are shown. Alleles in green are inherited from the sire.

4.3.3 *Attractin*

The sires of families C2 and S were found to be heterozygous at *ATRN* SNP 2790C>T, so their changeling offspring and the respective dams were also genotyped at the SNP (Fig. 4.4). From family C2, five changeling offspring inherited the sire's T allele, while one inherited his C allele. In family S, three changeling offspring inherited the sire's T allele, and the remaining three changeling offspring inherited his C allele. Therefore neither allele was consistently inherited with the changeling phenotype.

Family C2



Family S

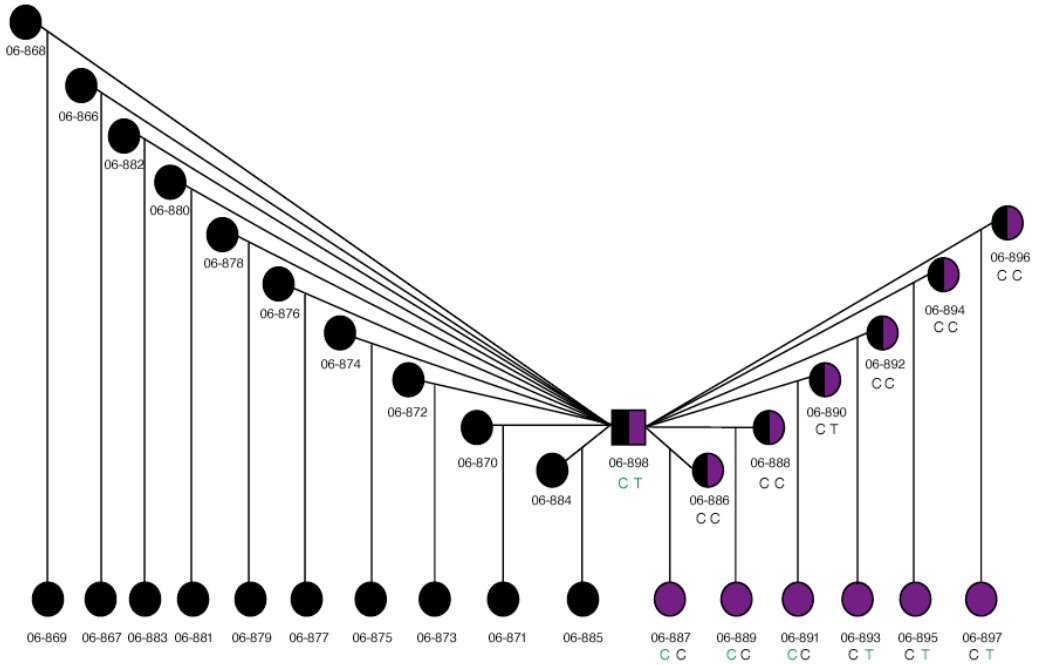


Figure 4.4 *ATRN* 2790C>T genotypes for Changeling families C2 and S. Alleles in green are inherited from the sire.

4.3.4. *Melatonin Receptor 1A*

4.3.4.1 Sequencing

Since *MTNR1A* had not previously been characterized in cattle, this was done first. *MTNR1A* consists of two coding exons in other species. Genomic sequence was obtained for exon 1 and flanking regions from three cattle, one traditional red and two black Holsteins. The exon 1 flanking region included 162 bp of 5' sequence and 46 bp of intron 1, for 439 bp in all. Exon 2 and flanking regions were obtained from four cattle, two black, one changeling and one variant red Holstein. The exon 2 flanking region included 85 bp of intron 1 and 58 bp of 3' sequence, for a total of 1013 bp (GenBank EU716174).

A PCR product was obtained that yielded partial cDNA sequence from the red skin of a Hereford steer, including 622 bp of exon 2, but did not include the stop codon (GenBank EU716172) (Fig. 4.5). cDNA from other cattle could not be obtained, although several primer sets were used.

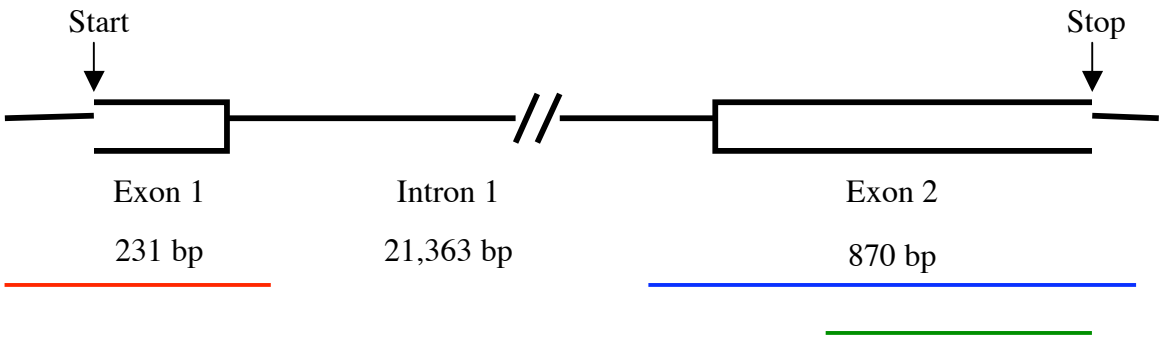


Figure 4.5 Three regions of *MTNR1A* were sequenced. The exon 1 segment from genomic DNA is designated in red, the exon 2 segment from genomic DNA in blue, and the cDNA segment in green.

The only SNP identified in the coding sequence was a 22093A>G SNP, which was found in exon 2 in a black Holstein bull in a heterozygous state. This SNP does not change an amino acid. Although one changeling was A/A, so was an unrelated black and an unrelated VR animal, suggesting this SNP is not associated with any of these phenotypes.

Twenty-four Holstein cattle were sequenced at a region 5' of *MTNR1A*, from -637 bp to -991 bp, including a -742C>T SNP. Changeling, VR, traditional red and black cattle were included in those sequenced. No genotype consistently appeared within any phenotype group (Table 3.13).

4.3.4.2 Family Studies

Included in those 24 cattle for which 5' sequence was obtained, were 13 members of family S. This family was genotyped and analyzed for co-segregation with the SNP alleles and the changeling phenotype (Fig. 4.6). Five changeling offspring out of a segregating sire were genotyped. Two of the changeling calves inherited the sire's C allele, one changeling calf inherited the sire's T allele, and two changeling calves were uninformative. Since both the C and the T allele were inherited with the changeling phenotype, *MTNR1A* can be discounted as the causative gene for changeling.

4.4 Discussion

Genotyping 18 changeling Holstein cattle at *MC1R* revealed an intriguing piece of data. Of the 18 cattle genotyped at *MC1R*, half were *e/e* (Table 4.6). The *e* mutation is a deletion of a G residue at position 771 of *MC1R* (Joerg et al. 1996) resulting in a frameshift producing a truncated MC1R protein (Klungland et al. 1995). The truncated MC1R protein produced by the *e* allele cannot bind α-MSH, therefore eumelanin is not produced. If melanocytes from these nine cattle with two non-functional copies of *MC1R* are capable of producing eumelanin, even later in the animal's life, it would then lead to reason that an alternate receptor is capable of reacting to α-MSH and triggering the melanocyte to produce eumelanin in a similar way to MC1R. An alternate receptor capable of increasing intracellular cAMP as MC1R does, would explain how

the melanocyte from an *e/e* cow could bypass the non-functional MC1R and successfully produce eumelanin.

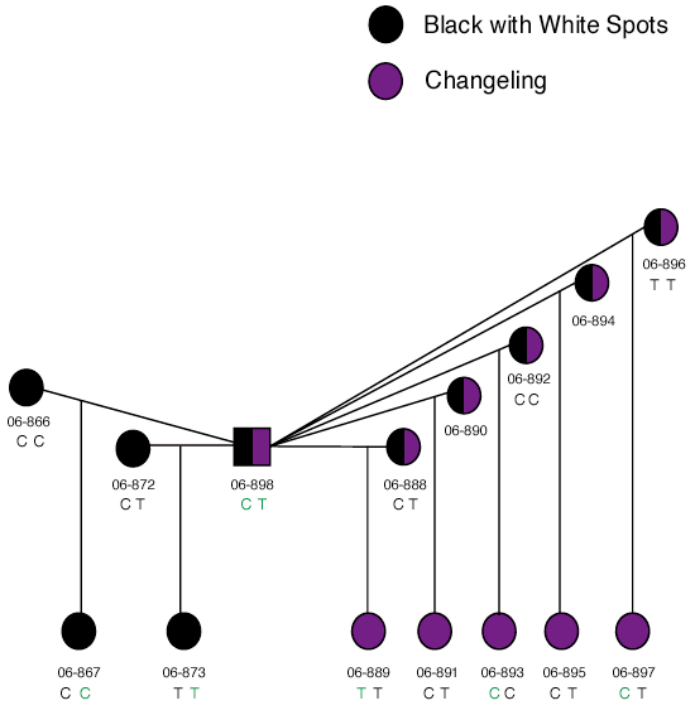


Figure 4.6 *MTNR1A* -742C>T genotypes for changeling family S. Green alleles were inherited from the segregating sire.

So how were these nine other cows, with at least one functional copy of *MC1R,* prevented from producing eumelanin early in life? This phenomenon would imply action of an antagonist, preventing the binding of α-MSH to MC1R, or preventing the proper functioning of MC1R after binding. A hypothetical explanation encompassing how *e/e* cattle could later be black or how cattle with an E^D allele could be red early in life would include an alternate receptor for α-MSH that jointly serves to prevent binding of α-MSH to MC1R, either through blocking the binding or strong competitive binding, as well as serving to function as a transient trigger for the eumelanin cascade. Transient, since it does not trigger eumelanin production early in life, but still prevents binding of α-MSH to MC1R, resulting in the expression of phaeomelanin. As the animal matures, this hypothetical alternate receptor would gain the ability to act in place of MC1R. In this manner, even cattle with a functional copy of *MC1R* would not

be producing eumelanin in adulthood due to *MC1R*, but rather due to the alternate receptor (Fig. 4.7).

No changeling cattle were E^D/E^D (Table 4.6). While it could be a chance occurrence that none of the changeling cattle that I genotyped were E^D/E^D, another possibility to consider is that E^D/E^D may be epistatic to changeling. Where one E^D allele may allow for the expression of changeling, it is possible that two E^D alleles masks the expression of the changeling phenotype. To determine if this is the case, I could genotype at least 50 changeling cattle at the *MC1R* alleles, then compare the resulting proportions of genotypes to the expected genotype frequencies in the Holstein population. Not finding any E^D/E^D changelings in the larger population would suggest that E^D/E^D may prevent the expression of the changeling phenotype, indicating an interaction between *MC1R* and the causative gene for changeling.

From a practical perspective, having Holstein calves genotyped at the three alleles known to affect coat colour would not be sufficient to separate traditional red calves that would remain red from changeling calves since the e/e genotype occurs in both.

When analyzing co-segregation of *ASIP* with the changeling phenotype, it was necessary that microsatellite *BMS995* be used as a marker, since the only SNP previously described in *ASIP* was homozygous in the sires or dams of the families segregating for changeling. Use of *BMS995*, which has been mapped 0 cM from *ASIP* with a LOD of 3.551 using the 2684C>T SNP in intron 3 (Appendix E), is sufficient to determine whether a mutation in *ASIP* is the cause of the changeling phenotype. However, if a mutation in an age dependent promoter is the cause of the phenotype, then co-segregation analysis with *BMS995* may not be sufficient. A loss of function mutation in a juvenile promoter or a gain of function mutation in an adult promoter could result in the changing phenotype from a young animal to an adult. In this case, the mutation resulting in the change would always be present in changeling cattle, but may not be active dependant on the age of the animal.

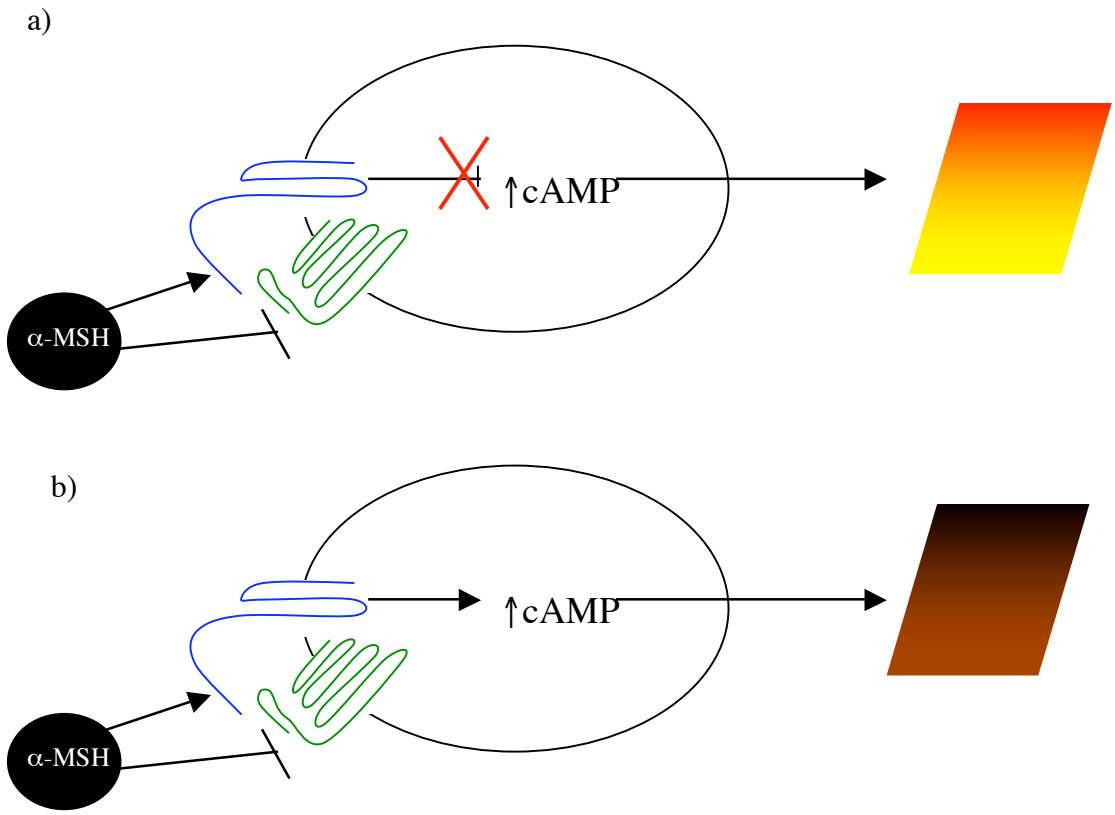


Figure 4.7 A hypothetical receptor (in blue) could prevent the binding of α-MSH to MC1R (in green). a) In a young animal, binding would not increase intracellular cAMP, resulting in phaeomelanin production. b) In an adult animal, binding would increase intracellular cAMP, resulting in eumelanin production.

Girardot et al. (2005) reported that cattle *ASIP* consists of three coding exons (exon 2, 3, and 4) and is controlled by at least three promoters (exon 1A, 1B, and 1C). These promoters drive transcription of *ASIP* isoforms specific to skin, heart, brain, spleen, lung, liver, and kidney. While the amino acid sequence of bovine *ASIP* is 78% identical to mouse and 75% identical to human *ASIP* (Girardot et al. 2005), the promoter regions do not appear to be conserved among cattle, pigs (Leeb et al. 2000), and dog (Kerns et al. 2004) based on alignments I performed. These non-conserved promoter regions among species make identifying promoter mutations difficult. As we cannot use
information obtained through research in other species as a guideline to begin searching for mutations of note in cattle, it is essentially a new task to predict relevant promoter sites.

While, in the scope of this present research, a coding region mutation in *ASIP* was determined not to be the causative gene for changeling since there was no co-segregation between it and the changeling phenotype (Fig. 4.3), it is still possible that *ASIP* plays some role in producing the phenotype. Under this assumption, focus was shifted to *ATRN* due to a link of *ATRN* and *ASIP* in relation to mouse coat colour. Gunn et al. (1999) propose two methods by which *ATRN* may influence murine coat colour. One being that ATRN is required for the desensitization of MC1R while the other points to ATRN as being a low affinity receptor for ASIP. In the case of *ATRN* in mice, the wild type *ATRN* allele allows for normal binding of ASIP to MC1R, producing a phaeomelanin *Agouti* phenotype. Mutated *ATRN* alleles serve to incompletely prevent binding of ASIP to MC1R, allowing binding of α-MSH and the production of a eumelanin phenotype.

ATRN could explain the changeling phenotype through the action of age-dependent promotors, allowing the expression of the wild type *ATRN* allele at birth resulting in a red coat colour. As the calf ages, promoter activity would be switched to allow expression of a mutated form of *ATRN*, preventing the binding of ASIP to MC1R and resulting in the production of a black coat colour. Gunn et al. (2001) reported that mutant *ATRN* mice changed color from black to reddish-brown as they approached maturity. This colour change acted independently of *ATRN*'s effect on hair banding caused by *ASIP*, making *ATRN* an even more attractive candidate gene for changeling. It was then unexpected that, upon genotyping of families C2 and S at a 2790C>T *ATRN* SNP, neither family showed an *ATRN* allele consistently co-segregated with the changeling phenotype (Fig. 4.4).

ATRN has also been linked with the presence of large vacuoles in brain and spinal cord tissue of $Atrn^{mg\text{-}3J}/Atrn^{mg\text{-}3J}$ mice (He et al. 2001) and the zitter phenotype, characterized by hypomyelination and vacuolization of the central nervous system resulting in tremors and flaccid paresis, in *zi/zi* mutant mice (Kuramoto et al. 2001). Because of the association of normal *ATRN* with nervous system development, it would be predicted that, if changeling were caused by *ATRN*, mutant *ATRN* cattle would also express symptoms of nervous system degradation. No such symptoms have been reported in changeling cattle. Rather many changeling bulls have become proven sires producing many offspring and having a great impact on the Holstein breed. As such, the fact that *ATRN* is not responsible for changeling may not be such a surprise as only one of the characteristics associated with *ATRN* has been reported in changeling cattle.

As the changeling phenotype involves the change of pigment from phaeomelanin to eumelanin over time, the search for candidate genes lead towards *MTNR1A*, a gene coding for a receptor for melatonin, a protein known to regulate pigment switching in various other species (Filadelfi et al, 1994; Castrucci et al. 1997). Filadelfi and Castrucci (1994) report that application of melatonin to skin preparations from eel and various reptiles and amphibians resulted in inhibition of darkening due to α-MSH. Castrucci et al. (1997) also reported the ability of melatonin to lighten α-MSH darkened skin and predicted that this action occurs through inhibition of cAMP production through interaction with a G protein-coupled receptor. Additionally, in murine melanoma cells, melatonin was able to stimulate tyrosinase activity by mimicking α-MSH (Castrucci et al. 1997). Melatonin is hence able to either increase α-MSH darkening or decrease α-MSH darkening, seemingly different in various species with amphibians following the former pattern and mammals following the latter. Slominski et al. (2003) found expression of human *MTNR1A* (termed *MT1*) in skin, epidermal keratinocytes, and epidermal melanocytes. Applying this to changeling cattle, it could then be predicted that a mutation is present that allows *MTNR1A* to effect coat colour expression where its function is normally masked in non-changeling cattle.

Obtaining both genomic and cDNA sequence of *MTNR1A* was difficult, even with the use of multiple primer sets. The whole genome shotgun sequence (GenBank NW_930548) and predicted *MTNR1A* sequence (GenBank XM_614283) used to design primers showed that portions of the sequence of *MTNR1A* have a high GC content. Templates that have a GC content over 65% are typically difficult to amplify, giving weak products and multiple spurious bands (Sahdev et al. 2007). The region surrounding and including exon 1 of *MTNR1A* has a 75% CG content, while the region surrounding and including exon 2 of *MTNR1A* has a 49% GC content. A modified booster PCR protocol and the inclusion of Betaine (Henke et al. 1997) in the reaction cocktail were required to overcome the high GC content. Even with the use of these additional efforts, only partial cDNA sequence was obtained. It is interesting to note is that the cDNA sequence that I was able to obtain was partial sequence including a region of exon 2 that has a GC content closer to the expected 50%. Exon 1, with the higher GC content was not amplified clearly enough to sequence. An increased GC content often leads to the formation of hairpins in the secondary structure of the chromosome (Lewin 2000) altering the ability to amplify the particular region.

Co-segregation analysis of *MTNR1A* in relation to the changeling phenotype excluded *MTNR1A* as the causative gene for changeling. In other mammalian species such as sheep (Notter et al. 2003) and goats (Chu et al. 2007), *MTNR1A* has been associated with reproductive cycles and circadian rhythms. A *MTNR1A* mutation has been preliminarily linked to seasonal versus year round estrus in goats (Chu et al. 2007). *MTNR1A* was found to account for 23.8% of the total additive genetic variance in fertility in adult sheep matings through association with two genetic variants (Notter et al. 2003). Since reproductive ability is of vital importance to the dairy industry, it can be assumed that any mutation having an adverse effect on reproductive ability or fertility would have been selected against. Therefore, if the changeling phenotype was caused by a mutation in *MTNR1A*, it would also likely correspond to altered reproductive ability and would have inadvertently been selected against. Conversely, we see that multiple changeling sires have had a large influence on the genetics of the Holstein breed based on daughter performance.

4.5 Conclusion

The relationships between the changeling phenotype and four genes were explored during this research. The four candidate genes, *MC1R*, *ASIP*, *ATRN*, and *MTNR1A*, were not found to have any positive association with the changeling phenotype, assuming a recessive single gene inheritance pattern.

Investigating the potential for an *ASIP* promoter to cause the changeling phenotype is a valuable consideration for future work. Sequencing the three promoter regions identified by Girardot et al. (2005) in changeling, variant red and black and white cattle would be a valid starting point. Reverse transcriptase PCR (RT-PCR) may yield additional promoters and would be beneficial to conduct on mRNA of changeling and black and white calves, and then later on the same mature cattle. If a juvenile promoter is driving expression of *ASIP* in a young animal beginning with a juvenile specific exon 1, then the mRNA from a young calf would potentially be different than the mRNA from the same animal when they are matured and expression of *ASIP* is driven by an adult specific promoter. It would be important to isolate mRNA from both changeling and non-changeling cattle in this manner, as it may be that a mutation in changeling cattle has caused the production of a new promoter recognition site in changeling cattle that is not present in black and white cattle.

5.0 GENERAL DISCUSSION

An understanding of the inheritance of coat colour in Holstein cattle is of great importance to the efforts of breed organizations that promote the protection and development of the breed. The recording of accurate pedigrees is vital to attempts to improve the breed and select for individual traits. When those pedigrees are questioned due to not understanding the intricacies of coat colour inheritance within the breed, the backlash can affect individual producers and the efforts of the industry as a whole. Clearly the inheritance of black as dominant due to an E^D allele and red due to *e*/*e* is insufficient, although most Holstein owners have assumed that DNA colour testing for these *MC1R* alleles has given them the complete coat colour genotype of their cattle.

When considering the VR and changeling phenotypes in the context of Holstein cattle, the phenotypes appear to deviate from the commonly expected coat colour patterns seen in cattle breeds. Despite this, similar patterns are present in other species. Two forms of inheritance for red coat colour are present in dogs, with phaeomelanin being produced by a recessive loss of function mutation in *MC1R* (Newton et al. 2000) or a dominant gain of function mutation in *ASIP* (Berryere et al. 2005). Additionally, a changing phenotype from juvenile to mature animals is seen in species such as wild boar, where the young piglets exhibit darkened stripes that fade with age (Legault, 1998), tapirs where the young exhibit white spotting on a dark background and the adults lose the white spotting and develop a white belt on a black background, and puma where cubs exhibit a spotted pattern that fades to an even color as an adult (Searle, 1968). A possible explanation for these changes in coat colour is the action of an age-dependent promoter that, while the animal is young, drives the expression of one gene, but as the animal ages expression is switched off. The mutation resulting in this changing coat colour or pattern from young to adult individuals could be a result of a loss of function mutation in an adult promoter that prevents the production of the juvenile colour pattern when active, or a gain of function mutation in a juvenile promoter that causes the production of a phenotype that differs from the adult pattern. In order to test this hypothesis, I would require pigmented skin samples from changeling calves under one month of age, 6 months of age and 1 year of age. I would then quantify *ASIP* from mRNA from each of the calves in attempts to determine differences in expression level (i.e. quantitative PCR) between young and mature individuals. If the quantity of

ASIP varies with age, then it would also be important to sequence the 5' promoter region of *ASIP* in changeling and black and white cattle in attempts to locate any promoter mutations.

My research into VR and changeling has lead to a better understanding of these two previously unrecognized coat colours and their individual inheritance patterns. There is now a clear candidate gene for variant red, *Beta-defensin 300* or *K*. More work must be done to locate a causative mutation outside the coding region, and subsequently develop a genetic test for the trait assuming single gene inheritance. Also assuming single gene inheritance, the causative gene for changeling is not one of the candidate coat colour genes that I tested, but rather a previously undefined gene or a transcription factor that impacts the expression of the familiar genes that affect coat colour.

When researching some Holstein family histories, several pedigrees had overlapping occurrences of VR and changeling. The two traits often occur simultaneously in certain families. Adding to the dilemma, is an assumption that must be made when considering the segregating families for changeling. If we consider changeling to be a single gene recessive trait, then we must also accept that there are a large number of black cows that carry the mutation for changeling that, when mated to the correct bull, produce changeling calves. If the mutation were that simple and that common, it would be anticipated that the changeling phenotype would be more prevalent in the Holstein population than it currently is, unless there is an epistatic relationship between changeling and an E^D/E^D genotype at *MC1R*. Recognizing these features, a two gene complex interaction may more effectively explain the inheritance of VR and changeling.

Under a two-gene inheritance model, the recognized inheritance patterns of dominant for VR and recessive for changeling can still hold true, though the expression of either of these phenotypes may depend on the presence of an additional mutation at a separate locus. For example, if locus B determines VR with B/B or B/b expressing VR, and locus C determines changeling with c/c expressing changeling, then the expression of either of these phenotypes may then depend on a particular genotype at a third locus, locus A (Fig. 5.1). In that case, a black cow would carry the ability to pass on VR or changeling, but would not be VR or changeling herself if she were also a/a. As such, the VR and changeling mutations may be common, though the actual phenotypes rare if the mutation that allows for the expression of those phenotypes is low in the population. I believe that this research has eliminated the possibility that one of the known

MC1R colour alleles is the required mutation in the hypothetical genes B or C to allow for the expression of VR or changeling. Each phenotype occurred in combination with any of the three *MC1R* alleles, not reliant on one particular allele for expression of VR or changeling.

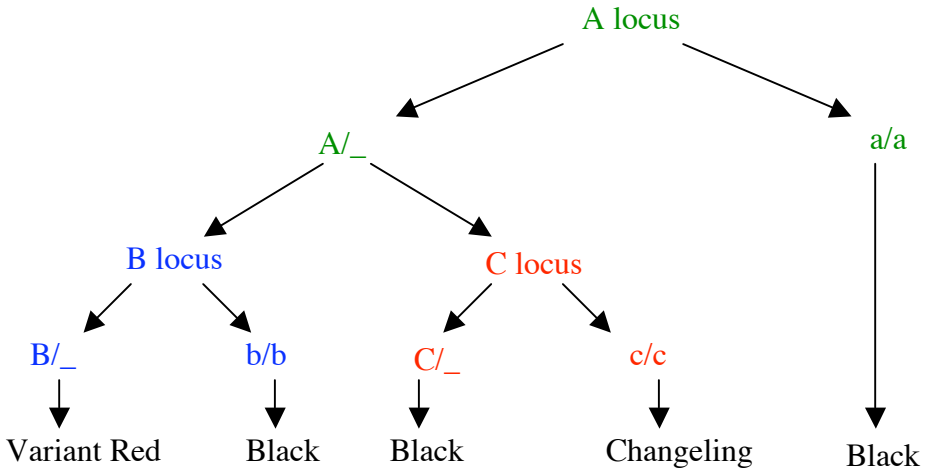


Figure 5.1 A hypothetical two-gene explanation for VR and changeling.

The use of a SNP Chip to conduct a genome scan may be beneficial in testing for either a single gene inheritance of changeling and VR or a two gene inheritance pattern. In the case of a single gene inheritance pattern, approximately 10 cattle with the VR and changeling phenotypes would be run on a SNP Chip for the whole cattle genome along with 10 black and white Holsteins as controls. If genome scans for both changeling and VR indicate the same region, different only from the black and white cattle, then it may point toward the prediction of a two gene inheritance model, with one essential mutation in common. Differing results based on the VR and changeling phenotypes would indicate the regions in the genome that are likely to co-segregate with each phenotype.

Karlsson et al. (2007) state that genome-wide association mapping of traits that follow a simple Mendelian inheritance pattern, in this case the single gene model, can be accurately completed with using only 20 samples, 10 of each phenotype. Mapping of complex traits may need up to 100-300 samples of each phenotype. The genome scan method is best done with animals that are "unrelated" within 4 generations (Karlsson et al. 2007). Ideally, I would need more samples than I currently have. This may prove difficult as the known VR and changeling cattle can be traced back to two cows, VR to Surinam Sheik Rosabel and changeling to Roybrook Telstar. Tracing the pedigrees back a couple more generations, Roybrook Telstar and

Surinam Sheik Rosabel are both related to a bull by the name of Lakefield Fond Hope (Fig. 5.2). So even before VR and changeling were recognized, the lines that produced the individual phenotypes were related. Since that time, many more crosses between lines with the VR phenotype and the changeling phenotype have occurred. As this supports the possibility of a two gene inheritance pattern, it also makes finding unrelated individuals of each phenotype difficult.

We must be aware that some of the genes that were eliminated in the course of this research based on a single gene inheritance pattern may still be candidates when considering a two gene inheritance pattern. Considering this, I believe strong candidates to include *ASIP* and *β-Defensin300*, forming a relationship similar to that seen in dogs with the interaction of *β-Defensin300*, *ASIP* and *MC1R* to produce Agouti and dominant black phenotypes (Candille et al. 2007).

Another candidate to consider for single gene inheritance as well as multiple gene inheritance is *pro-opiomelanocortin* (*POMC*). Millington (2006) reviews how POMC is cleaved to form the melanocortins, including α-MSH. Since α-MSH is involved in the signaling of MC1R prior to the production of eumelanin, a mutation resulting in altered expression of α-MSH may effect the production of eumelanin. Mutations in *POMC* have been discovered recently (Thue et al. 2003, Deobald unpublished) which could be used to study co-segregation.

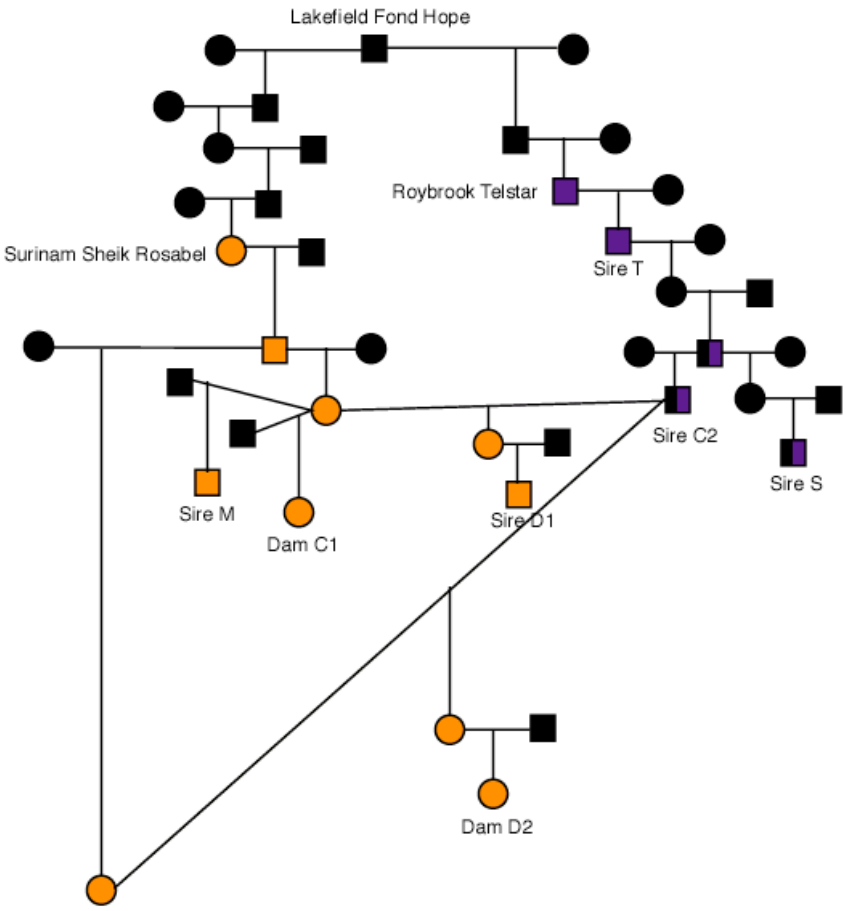


Figure 5.2 The pedigrees of the VR and changeling families are related within 4 generations.

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7.0 APPENDICES

Appendix A DNA extraction protocols

Blood Extraction Protocol

500 µl of whole blood is mixed with 500 µl of lysis buffer and spun at 10,000 rpm for 5 minutes. The supernatant is removed and the pellet is resuspended in another 500 µl of lysis buffer and spun at 100,000 rpm for 5 minutes. This step is repeated until the supernatant becomes clear. The supernatant is removed for the final time and the pellet is resuspended in 500 µl of PCR extraction buffer. 10 µl of 20 mg/ml Proteinase K is added and the solution is incubated over night at 65 °C. After incubation, 50 µl of 3M NaAcetate with a pH of 5.5 is added, along with 1 mL of 95% Ethanol. This solution is incubated overnight at -20 °C. After incubation at -20 °C, the solution is spun at 13,000 rpm for 15 minutes and the supernatant is removed. 500 µl of 70% Ethanol is added. It is then spun at 13,000 rpm for 10 minutes and the supernatant is removed. The pellet is resuspended in 400 µl of 1xTE and incubated over night at 55 °C. After incubation, the solution can be stored at 4 °C short term or -80 °C long term. 1 µl of this final solution is used as a template for PCR reactions.

Lysis Buffer (250mL final volume)

Sucrose 0.32M	27.38g
$MgCl_2$ 5mM	0.25g
1% Triton X	2.5mL
Tris 10mM pH7.5	1.25ml

PCR Extraction Buffer (250mL final volume)

KCl 50mM	0.93g
Tris-HCl 10mM pH8.3	1.25ml
$MgCl_2$ 2.5mM	0.12g
Gelatin 0.1mg/mL	0.25g
Tween 20 0.45%	1.125ml
Nonident P40 0.45%	1.125ml

Semen Extraction Protocol

One straw of semen is emptied into a 15 mL centrifuge tube. Semen buffer is added up to the 10 mL mark and the solution is vortexed until the pellet is suspended. The solution is spun at 3000 rpm for 5 minutes and the supernatant is removed. Semen buffer is added and centrifuged as before two more times. The final supernatant is removed and the pellet is resuspended in 500 µl of 1xTE and transferred to a 1.5 mL microcentrifuge tube. 6 µl of 10-20 mg/ml Proteinase K and 10 µl of 20% SDS are added and the solution is incubated for one hour at 65 °C. After incubation, 500 µl of phenol chloroform is added and the solution spun at 12,000 rpm for 5 minutes. The supernatant is removed and saved in a 1.5 mL microcentrifuge tube. 500 µl of chloroform is added and the solution is spun again at 12,000 rpm for 5 minutes. The supernatant is removed and saved in a clean 1.5 mL microcentrifuge tube. The sample is precipitated with 50 µl of 3M NaAc and 1 mL of 95% Ethanol and incubated over night at -20 °C. The solution is then spun at 13,000 rpm for 15 minutes and the supernatant is removed. The pellet is resuspended with 500 µl of 70% Ethanol and spun at 13,000 rpm for 10 minutes. The supernatant is removed and the pellet is resuspended in 200 µl of sterile 1xTE buffer and incubated over night at 37 °C to 55 °C. 1 µl of this final solution is used as a template in PCR reactions.

Semen Extraction Buffer (10ml final volume)

100mM Tris	1ml 1M Tris
10mM EDTA	0.2ml 0.5M EDTA
500mM NaCl	1ml 5M NaCl
1% SDS	0.5ml 20% SDS
2% Mercapto	0.2ml Mercaptoethanol

Semen Wash Buffer (500ml final volume)

1x SSC	25ml 20x SSC
10mM EDTA	10ml 0.5M EDTA

Hair Extraction Protocol

The follicle ends of 5-6 hairs are cut and placed in a 1.5 mL microcentrifuge tube. 250 μl of QuickExtract DNA Extraction Solution (Epicentre, Madison, WI) is added and vortexed for 10 seconds. The samples are incubated at 65 °C for 6 minutes and vortexed for another 10 seconds. The samples are then incubated at 98 °C for 2 minutes. The final solution can be stored at 4 °C for short term or -80 °C for long term. 1 μl of this solution is used as a template in PCR reactions.

Appendix B *Bos taurus* partial *MTNR1A* genomic GenBank sequence submission

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LOCUS       EU716174               1015 bp    DNA     linear   MAM 01-SEP-2008
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VERSION     EU716174.1  GI:190410786
KEYWORDS    .
SOURCE      Bos taurus (cattle)
  ORGANISM  Bos taurus
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            Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;
            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 1015)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Characterization of MTNR1A in cattle
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1015)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAY-2008) Animal and Poultry Science, University of
            Saskatchewan, 51 Campus Dr., Saskatoon, SK S7N 5A8, Canada
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Appendix C *Bos taurus* partial *MTNR1A* cDNA GenBank sequence submission

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            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 622)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Characterization of MTNR1A in cattle
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 622)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAY-2008) Animal and Poultry Science, University of
            Saskatchewan, 51 Campus Dr., Saskatoon, SK S7N 5A8, Canada
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Appendix D *Bos taurus K* genomic and mRNA GenBank sequence submissions

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            Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;
            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 1990)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Characterization of K in Cattle
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1990)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAY-2008) Animal and Poultry Science, University of
            Saskatchewan, 51 Campus Dr, Saskatoon, SK S7N 5A8, Canada
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     1981 ctctgttctg
```

```
LOCUS       EU715239                280 bp    mRNA    linear   MAM 03-SEP-2008
DEFINITION  Bos taurus beta-defensin 300 (DEFB300) mRNA, complete cds.
ACCESSION   EU715239
VERSION     EU715239.1  GI:197359121
KEYWORDS    .
SOURCE      Bos taurus (cattle)
  ORGANISM  Bos taurus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;
            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 280)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Characterization of K in Cattle
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 280)
  AUTHORS   Dreger,D.L. and Schmutz,S.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-MAY-2008) Animal and Poultry Science, University of
            Saskatchewan, 51 Campus Dr., Saskatoon, SK S7N 5A8, Canada
FEATURES             Location/Qualifiers
     source          1..280
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                     /mol_type="mRNA"
                     /db_xref="taxon:9913"
                     /chromosome="27"
                     /sex="female"
                     /tissue_type="skin"
                     /note="breed: Holstein"
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                     /gene="DEFB300"
     CDS             77..277
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                     /codon_start=1
                     /product="beta-defensin 300"
                     /protein_id="ACH69777.1"
                     /db_xref="GI:197359122"
                     /translation="MRLYYLLFALFLFLLPVPGNGGIISGLQRYYCKIRSGRCALIGC
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```

ORIGIN

```
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       61 tgggaagcca gcagccatga ggctctacta ccttctcttt gcgttgttct tgttcttgct
      121 gcctgttcca ggcaacggcg gcatcataag cgggttacaa aggtattatt gcaaaataag
      181 aagcggccgg tgtgctctga ttggctgcct tccaaaggag gaacagatag gccgctgttc
      241 actgagtggc cgaaaatgct gccggaagaa gaaatgaaaa
```

Appendix E Linkage of microsatellite *BMS995* to *ASIP*

Linkage of *ASIP* to *BMS995* in our lab was accomplished with *ASIP* SNP 2684C>T (Berryere unpublished data) in the Canadian Beef Reference Herd (Schmutz et al. 2001).

cM	0.01	0.02	0.03	0.04	0.05	0.06
LOD	3.551	3.490	3.427	3.364	3.300	3.236

Appendix F Nucleotide alignment of five *β-Defensins* located on BTA27.

```
DEFB300     ATGAGGCTCTACTACCTTCTCTTTGCGTTGCTCTTCTTGTTCTTGCTGC
DEFB7       ATGAGGCTCCATCACCTGCTCCTCGCGCTCCTCTTCCTGGTCCTGTCTG
DEFB8       ATGAGGCTCCATCACCTGCTCCTCGCGCTCCTCTTCCTGGTCCTGTCTG
DEFB1       ATGCAGAGCGCCCCGAGACCCTCTCCGTGTGGAGTGCGCTGGAACCCTC
DEFB        ATGTGCCAGGAAAGGGGGGACCTGCAACCTATCGCCCTGCCCCCTCTAC

DEFB300     CTGTTCCAGGCAACGGCGGCATCATAAGCGGGTTACAAAGGTATTATTG
DEFB7       CTGGGTCAGGATTTACTCAAGGAGTAAGAAATTTTGTAACCTGCCGTAT
DEFB8       CTGGGTCAGGATTTACTCAAGGAGTAAGAAATTTTGTAACCTGCCGTAT
DEFB1       TGTGCGGCCTCCGGGGGACAACCTCGAGCAGCCGTTCCCTTCGTGGGGA
DEFB        AACAGGATCGAAGGCACCTGCTACAGGGGCAAAGCCAAGTGCTGCATCC

DEFB300     CAAAATAAGAAGCGGCCGGTGTGCTCTGATTGGCTGCCTTCCAAAGGAG
DEFB7       AAATAGAGGCTTCTGTGTGCCGATCAGGTGCCCTGGACACAGGAGACAG
DEFB8       AAATAG.....................................
DEFB1       GCAGGACCACACCGAGGTCAGGAGGACTGAGCCCCGGGTGGACGCAGAG
DEFB        GTTGACTCTGAGCTGGGAGCGATGGCAGAGGGCACTCAGGAGCCACGTG

DEFB300     GAACAGATAGGCCGCTGTTCACTGAGTGGCCGAAAATGCTGCCGGAAGA
DEFB7       ATTGGCACCTGTTTAGGGCCCCGAATAAAATGCTGCAGGTAG.......
DEFB8       ..............................................
DEFB1       CCAGGCCCCGCCACTGGGCATGTCCTGTCCCACAGCCCCCTCCCTCCCT
DEFB        GAGTATTTGTAA..................................

DEFB300     AGAAATGA.......................................
DEFB7       ........................................
DEFB8       ........................................
DEFB1       CAGAAGCAGCCTCAGACACTTCAGGGATGTCGGGTGGAAAGATGACGCT
DEFB        ........................................


DEFB300     ..............................................
DEFB7       ..............................................
DEFB8       ..............................................
DEFB1       GGACCAAGTCTTCGGACTCCGGACTTCGGTCCCGGCTCCACCACCGACT
DEFB        ..............................................
```

```
DEFB300     ..............................................
DEFB7       ..............................................
DEFB8       ..............................................
DEFB1       ACAGGTCACGTCCCTTCATGGCGCCTCCGGGCCCTCGTCAGTGAGATGC
DEFB        ..............................................

DEFB300     ..............................................
DEFB7       ..............................................
DEFB8       ..............................................
DEFB1       CAGAGCTGGCCATCGTGGAATTCCTCCCAGAACCTGGGACCTTTATAAA
DEFB        ..............................................

DEFB300     ..............................................
DEFB7       ..............................................
DEFB8       ..............................................
DEFB1       GCGGCAAGAGCAGCCTCTTCTCCAGCATCAGCCGAAGAGCTCGGGACGC
DEFB        ..............................................

DEFB300     ..............................................
DEFB7       ..............................................
DEFB8       ..............................................
DEFB1       CAGCATGAGGCTCCATCACCTGCTCCTCACACTTCTCTTCCTGGTACTG
DEFB        ..............................................



DEFB300     ..............................................
DEFB7       ..............................................
DEFB8       ..............................................
DEFB1       TCTGCTGGGTCAGGATTTACTCAAGGAATAAGTAATCCTCTAAGCTGCC
DEFB        ..............................................

DEFB300     ..........
DEFB7       ..........
DEFB8       ..........
DEFB1       GTCTGAATAG
DEFB        ..........
```