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Genetisk variation i *ASIP* och *MC1R* i nutida och forntida gotländska får

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SUMMARY

Ovine black coat colour is determined by the *MC1R* (Melanocortin 1 receptor) and *ASIP* (Agouti signalling peptide) genes at the Agouti and Extension loci. The black phenotype is caused by production of eumelanin by melanocytes, whereas yellow-tan or light phenotype is the result of phaeomelanin synthesis. The ovine *MC1R* gene has two known alleles: the wildtype (E^+) and the dominant black (E^D) alleles. Two missense mutations (c.218T>A and c.361G>A) constitute E^D. The presence of a third allele, the *e allele*, is proposed and believed to give rise to phaeomelanic phenotype. There are many alleles in the *ASIP* gene, the two most important are the dominant white (A^{wt}) and the most recessive black (A^a). Two mutations are alleged to cause recessive black phenotype: a missense mutation g.5172T>A and a nonsense mutation, a five base pairs (bp) deletion (D_5). However, not all black sheep, lacking the dominant black allele, are homozygous for at least one of the above mentioned *ASIP* mutations. A third mutation, a nine bp deletion (D_9), is identified but its complete phenotypic influence is unknown.

The aim of this study was to compare the presence of E^D and the *ASIP* mutations in past and present sheep native to the Swedish island of Gotland in the Baltic Sea, to gain knowledge regarding the coat colour phenotype in these sheep. Fragments of the genes mentioned above concerning parts of interest were sequenced from present Gute Sheep and Gotland Pelt Sheep (breeds native to Gotland) using Sanger sequencing and from ovine remains contextually dated to the Medieval Times and the Iron Age found on Stora Karlsö, Gotland, (as well as some sheep remains from the Swedish mainland) using pyrosequencing. All present sheep possessed the E^+E^+ genotype. The three black sheep included in this study were homozygous for at least one of g.5172T>A or D_5. Eighty-two grey present sheep possessed several diplotypes, but none was homozygous for g.5172T>A or D_5. The ancient individuals possessed TT of c.218 and GG of c.361G>A. Two ancient individuals possessed the A of c.361G>A of E^D, indicating that this mutation was indeed present in medieval sheep of Gotland and in mainland sheep of the 7th century. While some of the ancient sheep possessed the wildtype genotype of D_5, no genotype could be determined for g.5172T>A. The full genetic background for recessive black coat colour could not be elucidated since some black sheep, that lack E^D, are not always homozygous for D_5 and/or g.5172TT>A.

SAMMANFATTNING

Svart ullfärg hos får bestäms av två gener: *MC1R (*melanocortin 1 receptor) och *ASIP* (Agouti signaling peptide) på Extension och Agouti loci. Den svarta fenotypen orsakas av melaninproduktion från melanocyter, medan ljus fenotyp sker när melanocyterna syntetiserar faeomelanin. Det finns två kända alleler i fårens *MC1R*: vildtypen (E^+) och den dominant svarta (E^D). E^D består av två punktmutationer: c.218T>A och c.361G>A. Det föreslås förekomma ytterligare en allel, *allel e,* som tros ge upphov till faeomelanin-produktion. Det finns flera alleler i *ASIP*. De två viktigaste är den mest dominanta vita (A^{wt}) och den mest recessiva svarta (A^a). Två mutationer i *ASIP* genen orsakar recessiv svart ullfärg: g.5172T>A och en 5 baspars deletion (D_5). Alla svarta får (som saknar E^D) är ändå inte homozygota för en av *ASIP* mutationerna. En tredje mutation, en 9 baspars deletion (D_9) är identifierad, men dess fulla påverkan på nedärvning av svart ullfärg är inte känd.

Målet med denna studie var att jämföra förekomsten av E^D och *ASIP* mutationerna hos nutida och forntida gotländska får för att kunna avgöra ullfärgen hos dessa får. Intressanta delar av DNA hos de ovannämnda generna sekvenserades från blodprover från nutida gutefår och gotlandsfår (gotländska raser) och från kvarlevor från får som kontextuellt hade daterats till tidig medeltid och järnåldern som hittats på Stora Karlsö utanför Gotland. Även några kvarlevor från fastlandet sekvenserades. Sanger sekvensering användes för de nutida fåren och pyrosekvensering för de gamla fåren. Alla nutida får hade genotypen E^+E^+. De tre svarta nutida fåren som ingick i studien var homozygota för en av g.5172T>A och D_5. De grå fåren hade olika diplotyper. Några av de gamla fåren var homozygota för TT (c.218T>A) och GG (c.361G>A) Två individer hade genotypen AG på position c.361. Detta resultat antyder att denna mutation, som utgör en del av E^D, fanns hos svenska får på Gotland under tidig medeltid och på fastlandet redan på 600-talet. Några av de gamla fåren hade den normala vildtypen av D_5 (de saknade deletionen). Inga genotyper för g.5172T>A kunde bestämmas. Den fulla genetiska bakgrunden för recessiv svart ullfärg kunde inte klargöras då svarta får, som saknar den dominanta svarta allelen, inte behöver vara homozygota för någon av ASIP mutationerna.

CONTENTS

INTRODUCTION

Ever since ancient times farmers has bred sheep on the Swedish island of Gotland and its neighboring island Stora Karlsö (Hallander, 2010). Ovine remains have been found in the cave Stora Förvar on Stora Karlsö. The aim of this study is to analyze for the presence of mutations in the Extension (*MC1R*) and Agouti (*ASIP*) loci, that underlies the black coat colour phenotype in sheep and correlate this to the coat colour of ancient sheep of Gotland and present sheep breeds native to Gotland (Gute Sheep and Gotland Pelt Sheep). This study may elucidate how the coat colour of sheep of Gotland has changed over time. The English translations of breed denotations are those suggested by the respective breed associations (Ingvarsson B, Föreningen Gutefåret, personal notification, 141024; Håård M, Gotlandsfårföreningen, personal notification, 141024).

LITERATURE REVIEW

The origin and domestication of sheep

The evolution of the primordial sheep started in Eurasia approximately 2.5 million years ago (Ryder, 1983). The early sheep were the size of oxen, but were replaced by sheep of today's statue at the conclusion of the ice ages. These sheep were native to Europe and Asia, but eventually spread to North America and North Africa.

Domestication is believed to have occurred in the *Fertile Crescent* (Niemi *et al.,* 2013; Chessa *et al.,* 2009; Ryder, 1983) in the Near East approximately 11 000 years ago based on archaeological evidence (Zeder, 2008). An isolated European domestication centre for European breeds has been proposed (Ryder, 1983). The identity of the Near East as the domestication centre is supported by the fact that current wild and domesticated sheep of today are divided into five distinct maternal (mitochondrial) lineages or haplogroups based on their mitochondrial DNA (mtDNA) (Meadows *et al.,* 2007; Demirici *et al.,* 2013). Current sheep native to the Near East carry all haplogroups. Sheep breeds living further away only have a few, because migrated sheep only possessed a part of the genetic diversity of the domestication centre.

Consensus regarding which wild sheep subspecies is the ancestor of the domesticated sheep is yet to be reached. The wild breed native to the Near East, the Urial, historically has been suggested due to its geographical origin (Ryder, 1983). However studies of mtDNA indicate otherwise. The Asiatic Mouflon is proposed since it possesses all five mitochondrial lineages possessed by current domesticated sheep (Demirci *et al.,* 2013). The European Mouflon shares its mitochondrial haplogroup with European domesticated breeds, implying that this wild sheep was a separate progenitor of European breeds (Wood and Phua, 1996 see Meadows *et al.,* 2007; Hiendler *et al.,* 1998). The overall mtDNA of domesticated sheep are more similar to the European Mouflon compared to that of the Urial and the Argali wild sheep, indicating subspecies of the European Mouflon as ancestors of two lines of domesticated sheep (Demirci *et al.,* 2013).

Most commercial domesticated breeds of today are descendants of a second generation of sheep (Chessa *et al.*, 2009). These sheep were bred for the purpose of producing meat and wool and spread from the Near East domestication centre approximately 5 000 years ago. However relatives of the early primordial sheep remain in the Mouflon, the Soay, Okeny and Nordic short-tailed sheep (Chessa *et al.*, 2009), from which the Swedish local breeds derive their origin (Hallander, 2010).

Sheep of Gotland

Sheep have subsisted on Gotland and its neighboring island of Stora Karlsö from time immemorial (Hallander, 2010). Colour, size and if the sheep had horns varied. Until the 19th century the sheep of Gotland were divided into two types. *Bolamb* were small flocks of sheep kept close around the farms and housed indoors during the winter months. *Utegångsfår* were sheep that lived in great numbers at pasture year-round. The sheep of the latter type were mixed with imported breeds (for instance Spanish Merino, Southdown and Cheviot) in the 18th and 19th century. Beyond short peaks of popularity the import and breeding discontinued with the closure of the last purebred Cheviot farm in 1964. The descendants of these native sheep of Gotland (Gotland Pelt Sheep and Gute Sheep) are *local breeds* (a translation from the Swedish word *lantras*), meaning old native breeds that have been found in their habitat a long time, interbreed freely (under human supervision) with each other and are not influenced by the extensive selection of today's commercial breeds.

Gotland Pelt Sheep (*Gotlandsfår*)

The early sheep of Gotland had a heavy build and were bred as a meat producer during the self-sufficiency of World War I (Hallander, 2010). Attempts to improve their wool quality were made via interbreeding with Rya Sheep. Sheep possessing the lock (the curly wool that is the signum of today´s Gotland Pelt Sheep) were at first considered faulty. However since 1935 Gotland Pelt sheep have been bred for this type of wool and for producing a sheepskin of good quality at an age where the sheep also can be slaughtered. In 1971 Gotland Pelt Sheep were pedigreed as a separate breed.

Gotland Pelt Sheep are commonly born black and turn grey in adulthood, while the head and extremities remain black (Gotlandsfårföreningen, 2009). Other phenotypes (such as white or grey-black) are rare, but were possessed by the early sheep of this breed (Hallander, 2010). White sheep remains as an artefact from interbreeding with Rya Sheep in the first half of the 20th century. Both the ewes and the rams are hornless (Gotlandsfårföreningen, 2009). Over 18 000 ewes and 34 000 lambs were reported in 2013 to a Swedish database collecting information about Swedish sheep herds (Elitlamm, 2013).

Gute Sheep (*Gutefår*)

The horned sheep of this breed are the descendants of the fewj remaining horned sheep that could be found on Gotland after World War I. After the war the sheep of Gotland had been bred to be hornless since sheep with horns were considered to have worse wool quality than heir hornless counterparts (Hallander, 2010). The remaining horned sheep constituted the foundation for a breeding program with the aim of preserving horned sheep. In 1974 Gute

Sheep was recognized as a specefic breed. Gute Sheep vary in colour: from black to brown and from grey to white (Föreningen Gutefåret, 2014). Spotting is allowed. 2700 ewes and 4200 lambs were reported in 2013 (Elitlamm, 2013).

Alleles coding for black sheep coat colour

The genes coding for black coat colour

Several genes, at different loci in the ovine genome, code for proteins influencing ovine coat colour phenotype (Sponenberg, 1997; Fontanesi *et al*., 2011). Two genes determine if black coat colour will occur. The Melanocortin 1 receptor (*MC1R*) gene at the Extension locus codes for its namesake, the Melanocortin 1 receptor (MC1R) (Sponenberg, 1997; Royo *et al.*, 2008; Fontanesi *et al*., 2011). This seven transmembrane G-coupled receptor is the receptor for α-melanocyte-stimulating hormone (α-MSH). The Agouti signaling peptide (*ASIP*) gene at the Agouti locus codes for a peptide also designated ASIP. It is a small paracrine molecule that binds to MC1R (Fontanesi *et al*., 2010). The Extension locus shows epistasis over the Agouti locus. Black coat colour is determined by the accumulation of eumelanin in melanocytes, which is produced when MC1R-dependant signaling is active as seen in Figure 1 (Sponenberg, 1997). The other type of melanin, phaeomelanin, causes pale (Fontanesi *et al*., 2011), reddish-brown or yellowish-tan colour and is produced when the MC1R is inactive (Sponenberg, 1997). Some overlap between dark phaeomelanin and light eumelanin phenotype is possible and may be indistinguishable to the naked eye, but only one melanin is produced at a given moment by a certain melanocyte (Sponenberg, 1997). Eumelanin is produced when α- MSH acts on its melanocytic receptors. In absence of binding of α-MSH to its receptors phaeomelanin is produced (Sponenberg, 1997).

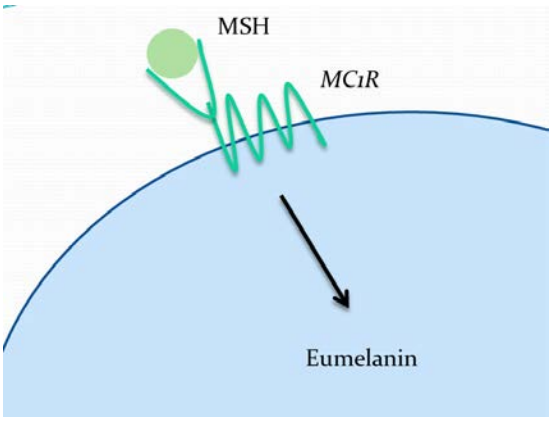


Figure 1. *Binding of α-MSH to MC1R results in an active MC1R, activation of secondary messengers and eumelanin synthesis. After Sponenberg, 1997.*

The Extension locus

The two identified alleles of the ovine Extension locus are the dominant black (E^D) resulting in eumelanin synthesis and black coat colour and the wild type (E^+) (Sponenberg, 1997; Fontanesi *et al*., 2011; Våge *et al*., 2003). According to Sponenberg (1997) and Fontanesi *et al.* (2011) in the presence of E^+ coat colour segregates at the Agouti locus. However in other species such as the horse the wildtype allele (*E*) is the dominant one and code for black coat colour (Marklund *et al.*, 1996). The *e allele* is the recessive allele of the equine *MC1R*. The ovine mutation c.199C>T (p.R67C) is believed to signify the ovine recessive *e allele* and as in the horse codes for the phaeomelanic phenotype (Fontanesi *et al*., 2011). It is only identified in the Valle del Belice breed and is suspected to disrupt the secondary structure of MC1R and influence its translation and RNA stability (Fontanesi *et al*., 2011). Rochus *et al.* (2014) have

not found the *e allele* in Swedish sheep breeds. The Extension locus is mapped to ovine chromosome 14 (Våge *et al.*, 2003).

Two missense mutations of the Extension locus constitute E^D (Våge *et al.*, 2003; Fontanesi *et al.*, 2011) and are present in several sheep breeds (Våge *et al.*, 2003; Våge *et al.*, 1999). They are p.M73K (c.218T>A) and p.D121N (c.361G>A) (Fontanesi *et al.*, 2010). Våge *et al.* (2003) suggest that either one of the two mutations can cause activation of MC1R independently and eumelanin synthesis or that the presence of just one of them induces a weaker stimulation of the receptor. In a study of five Swedish Sheep breeds (Gute Sheep, Swedish Finewool Sheep, Klövsjö Sheep, Roslags Sheep and Värmlands Sheep) only Swedish Finewool Sheep were polymorphic at c.218T>A and c.361G>A (Rochus *et al.*, 2014). These black sheep had the genotype AA-AA or TA-GA. The authors suggested that the Finewool sheep has gained this allele from breeding with foreign breeds and that E^D is not a part of the old Swedish sheep breed gene pool.

Four additional *MC1R* mutations have been identified by Fontanesi *et al.* (2010): c.-31G>A in the 5´-untranslated region (5'-UTR) and three synonymous mutations in the coding region: c.429C>T, c.600T>G and c .735T>C. Of these the last three have been identified in Swedish sheep breeds (Rochus *et al.*, 2014). The position of the known mutations of *MC1R* is given in Figure 2.

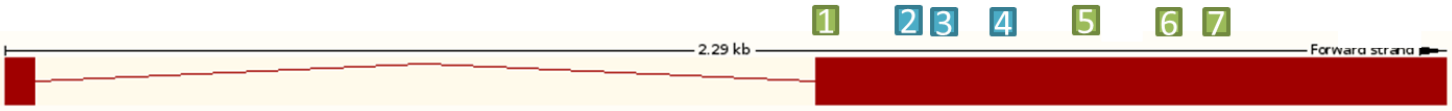


Figure 2. *The MC1R gene and the known mutations are marked. Green are synonymous mutations and blue are coding mutations. Sequence ENSOARG00000002239 modified from www.ensembl.org.* ***1.****c.-36G>A* ***2.*** *c.199C>T* ***3.*** ***c.****218T>A* ***4.*** *c.361G>A* ***5.*** *c.429T>A* ***6.****c.600T>G* ***7.*** *C.735T>C.*

The Agouti locus

ASIP is a paracrine molecule (Fontanesi *et al.*, 2011) that hampers the interaction of α-MSH on its melanocytic receptors as described in Figure 3 (Sponenberg, 1997). As a result the melanocytes cannot synthesize eumelanin and instead phaeomelanin is produced. The *Agouti* locus is mapped to ovine chromosome 13 (Parsons *et al.*, 1999).

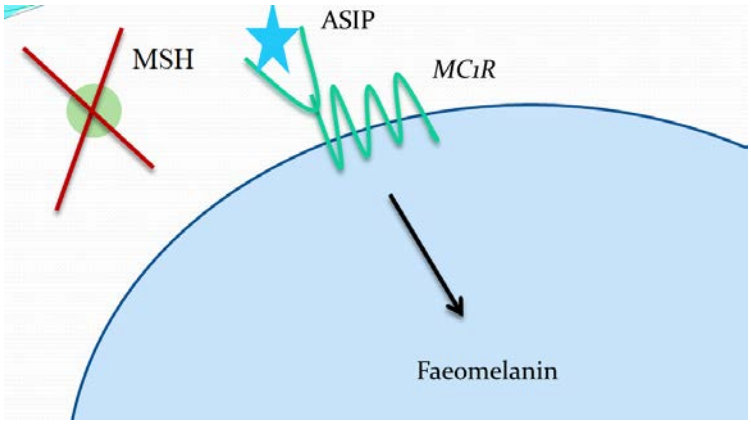


Figure 3. *Faeomelanin synthesis occurs when ASIP blocks the binding of MSH to MC1R. The receptor is then inactive and faeomelanin is produced instead of eumelanin. After Sponenberg, 1997.*

There are several known alleles of the ovine *Agouti* locus and they determine a diversity of coat colours (Sponenberg, 1997). The most dominant is the white or tan (A^{Wt}) allele and the most recessive is the non-agouti (A^a) alleles (Sponenberg, 1997; Royo *et al.*, 2008; Fontanesi *et al.*, 2011). The

A^{Wt} causes phaeomelanic phenotype and is according to Sponenberg (1997) the most common cause for white coat colour of sheep. The non-agouti allele gives rise to eumelanic phenotype (Sponenberg, 1997; Fontanesi *et al.*, 2011) as the inactive ASIP enables MC1R function (Gratten *et al.*, 2009). The intermediate alleles show reciprocal dominance over each other in the mouse, which is the species where the genetics of ASIP is best studied (Sponenberg, 1997).

A five bp deletion in exon 2 (g.100-105delAGGAA or D_5) was the first mutation determined as the cause for recessive black coat colour (Royo *et al.*, 2008). All the black sheep in their study lacked E^D. This study revealed that D_5 was not the only cause for recessive black coat colour, since not all black sheep were homozygous for D_5. Another cause for recessive black coat colour, a single nucleotide polymorphism (SNP) in exon 4 (g.5172T>A), was identified the same year (Norris and Whan, 2008). g.5172T>A causes a change of cysteine to serine at amino acid 123 and thus disrupt the protein structure of ASIP. D_5 results in a frameshift and the formation of a premature stop codon 63 amino acids downwards from the start position. Two additional mutations are recognized: a synonymous mutation (g.5051G>C) and nine bp deletion belonging to exon 2 (named g.10-19delAGCCGCCTC or D_9). The first mutation does not interfere with ASIP function, whereas D_9 causes the removal of a tripeptide that could decrease ASIP function. The positions of the known *ASIP* mutations are pictured in Figure 4.

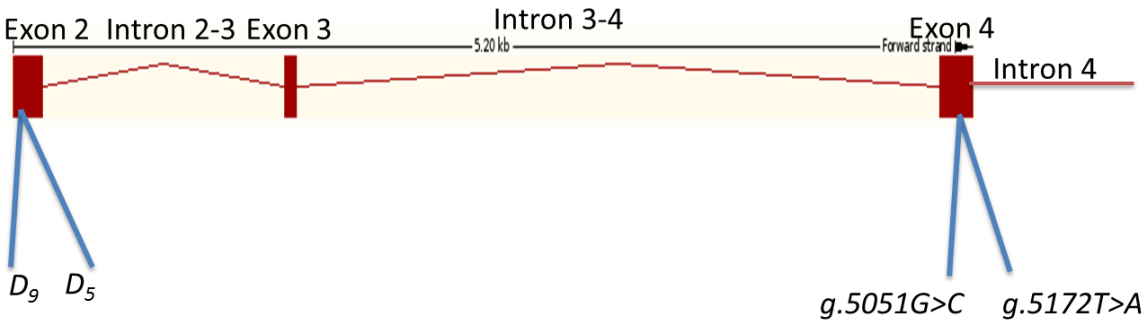


Figure 4. *Positioning of the known mutations of the ASIP gene. Modified* ENSOART00000010326.1 *from* www.ensembl.org. *The names of the exons are according to the nomenclature used in the mouse (Fontanesi* et al., 2011).

The presence of at least one non-mutated variants of g.5172T>A, the D_5 deletion and D_9 deletion (that is T, N_5 and N_9 where N_5 and N_9 is the denotation given to the respective non-mutated alleles of D_5 and D_9) are necessary for the white phenotype (Norris and Whan, 2008). A duplicated *ASIP* gene also causes white phenotype, whereas black sheep only have one copy. The existence of a duplication was confirmed by Fontanesi *et al.* (2010). However of 37 of their 41 Massese sheep with a duplicated genes were grey, implying that duplicated ASIP genes code for grey coat colour in Massese Sheep.

Of the above mentioned mutations D_5 and g.5172T>A have been found in the five Swedish sheep breeds by Rochus *et al.* (2014). Some black sheep included in that study were either homozygous for both these mutations or homozygous for at least one. However some sheep

of this study were either black or white and possessed the following genotypes: N_5D_5-TA, N_5D_5-TT or N_5N_5-TA. Possible haplotypes are N_5-T, D_5-A and N_5-A. One white individual was homozygous for the wildtype alleles (N_5 and T). The authors conclude that the full genetic background for black recessive coat colour of sheep is not elucidated. An unnamed regulatory mutation of ASIP (not yet directly identified) is reported by Norris and Whan (2008). This regulatory mutation is inactive in black sheep and thus inhibits ASIP function and may cause eumelanistic phenotype.

Hidden facts revealed by ancient DNA

Ancient DNA (aDNA) is DNA extracted from ancient remains, such as bone or hair (Paijmans *et al.,* 2013). Analysis of aDNA has been used for phylogenetic studies (Paijmans *et al.,* 2013), examining the relationship between extinct species and their extant living relatives (Paijmans *et al.,* 2013; Hofreiter *et al.,* 2001). Studies of aDNA have clarified several relationships, such as the Neanderthals' relationship with modern humans (combined studies of ancient mitochondrial DNA (mtDNA) and nuclear DNA suggest that some genetic exchange between these species might have transpired) (Paijmans *et al.,* 2013). Studies of aDNA are also used to investigate the diet of our ancestors, the transmission and occurrence of past diseases and to describe early farming (Mitchell *et al.,* 2005).

Despite the advances made within this field the research of aDNA is faced with several challenges (Mitchell *et al.,* 2005; Paimans *et al.,* 2013; Hofreiter *et al.,* 2001). After death the DNA of an individual is degraded by several endogenous processes (for example nucleases) and exogenous ones (for example oxidation and background radiation) (Hofreiter *et al.,* 2001). The deamination of cytosine leads to the insertetion of the wrong base, a T, during PCR. Oxidation of thymines and cytosines result in hindering DNA polymerases during PCR. The consequence is that few and fragmented aDNA molecules remains for extraction (Paimans *et al.,* 2013). Due to degradation, aDNA sequences are usually less than 100 bp long (Shapiro and Hofreiter, 2014). Furthermore, the material used is often treasured, and usually only limited amounts of material can be used for DNA extraction to ensure the intact morphological statue of the sample (Pajmans *et al.,* 2013). Moreover, contamination from environmental DNA (such as earth bacteria and fungi that contaminate the samples when they are in the earth) and from human modern DNA (that is contaminated from humans working with the samples after they are digged up) is a great risk, since this DNA is more abundant than the ancient one (Emma Svensson, personal notification, 20150112). Steps to overcome contamination are the use of protected clothing when working with aDNA and criteria used to validate aDNA sequences (Shapiro and Hofretier, 2014). The criteria are that a result from sequencing of aDNA should be able to be repeated (replicability) and all the sequences obtained from one sample should be the same (reliability). Moreover frequently only few individuals from any temporal zone can be found, giving rise to few samples to examine and draw conclusions from (Paijmans *et al.,* 2013). The environment where the sample has been found impacts the survival of its DNA (Hofreiter *et al.,* 2001; Paijmans *et al.,* 2013): cold environments such as permafrost and high salt concentrations are beneficial (Hofretier *et al.,* 2001). However with time the chance of DNA survival and possible extraction declines

(Hofretier *et al.*, 2001; Paijmans *et al.*, 2013), although the before mentioned factors are more important (Emma Svensson, personal notification, 20150112).

The analysis of aDNA has in the past mainly focused on mtDNA (Paijmans *et al.*, 2013; Hofreiter *et al.*, 2006). Because mtDNA exists in multiple copies in a single cell more of the same DNA sequence survives degradation (Svensson *et al.*, 2007). However in 1999 the first analysis of nuclear DNA was reported (Paijmans *et al.*, 2013) and since then the research has increased and is used frequently today (Svensson *et al.*, 2007; Baker *et al.*, 2012; Svensson *et al.*, 2012).

MATERIALS AND METHODS

Modern sheep

Sampling and DNA extraction

Before the start of this project blood samples had been collected from 144 Gute Sheep and 100 Gotland Pelt Sheep and their coat colour documented. Of these 48 of each breed were randomly selected to be a part of this study. All Gotland Pelt Sheep except three had the typical breed phenotype: grey wool with black head and extremities. The other three were white. The Gute Sheep had a wide variation of colour: 37 grey, two black, one with black wool, two tobiano, one white, two deer coloured and one spotted grey. Because of the low number of only black or white individuals also sheep with other phenotypes were included in the study. The QiaSymphony® robot had previously extracted DNA from 1.7 ml blood using the DNA Midi Kit (Qiagen®, Hilden, Germany). DNA was kept in -20° C until the start of this project. Then aliquots of the DNA samples were diluted to four ng/µL.

DNA sequencing

The BigDye® Direct Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used for sequencing both *MC1R* and *ASIP*. The primers were selected from literature (*MC1R* from Fontanesi *et al.* (2010) and *ASIP* from Gratten *et al.* (2010)), see appendix C, but were modified by adding M13-21 (5'-TGTAAAACGACGGCCAGT-3') and M13-29 (5'-CAGGAAACAGTCTATGACC-3') 5' tails. PCR and cycle sequencing were performed on Applied Biosystems® Gene Amp® PCR System 9700 (Applied Biosystems) or the Applied Biosystems ProFlex PCR systems (Applied Biosystems) using PCR programs optimized for the BigDye® Direct Cycle Sequencing Kit. The BigDye XTerminator® Purification Kit (Applied Biosystems) was used for sequence products purification. The DNA sequences were determined using capillary electrophoresis on the Applied Biosystems® 3500xL Dx Genetic Analyzer (Life Technologies, Foster City, CA, USA).

Analysis

Assembly, alignments, editing and visual inspection of the sequences were performed using the CodonCode Aligner (CondonCode Corporation, Dedham, MA, USA). NCBI reference sequences used were FN600553.1 and EU420022.1 for *MC1R* and *ASIP* respectively (http://www.ncbi.nlm.nih.gov/).

Statistical analysis

Statistical analyses were performed using Fisher's exact test. The computer software used was R http://www.R-project.org/.

Ancient sheep

Sampling

Twenty-six ancient samples were included in this study. Of these twelve were from Gotland, seven from Runsaborg and seven from York, England. Samples (right radius) from Gotland were collected from the cave Stora Förvar on Stora Karlsö and were contextually dated to the Early Middle Ages (five samples) and the Iron Age (eight samples) by osteologist Dr. Jan Storå. The mandibulae of the sheep from Runsaborg, dated to the 4^{th}- 7^{th} century, originated from an excavation of Runsaborg, 10 kilometers northwest of Upplands-Väsby, osteological analysis was performed by Dr. Ylva Telldahl. The mandibualae from York date to the 8^{th}-11^{th} century, osteological analysis was performed by Professor Terry O'Connor.

DNA extraction and amplification

After UV irradiation the DNA from the samples were extracted using a modification of Yang *et al.* (1998) with modifications according to Svensson *et al.*, 2007). For PCR amplification new primers, adapted to the short nature of of aDNA sequences, were used (see Table ii Appendix C). They were optimized (see Appendix D) using a total volume of 25 µl and using the AmpliTaq Gold® DNA polymerase (Life Technologies). The presence of PCR product was visually determined using agarose gel electrophoresis on a 2 % agarose gel. The conventional PCR program for MC1R-snp1 was determined by testing the same annealing temperature (60 °C) that was used on the PyroMark® PCR Kit and protocol (see Appendix E) with the $MgCl_2$ concentration that worked on the touchdown PCR on ancient samples (2.5 mM).

The ancient samples W and Ö were used to determine how well the primers amplified aDNA using MC1R-snp2 and both the PyroMark® PCR Kit and PCR protocol and conventional PCR using the Amplitaq Gold DNA polymerase (see Appendix E and F) on the 2720 PCR thermal cycler (Applied Biosystems). Amplification of all twenty-six samples, from which aDNA had been extracted, were carried out using MC1R-snp2 primers and the PyroMark® PCR Kit (Qiagen) and PCR protocol. For the twelve samples that the presence of a PCR product was confirmed with electrophoresis four duplicates for each sample and primer pair were carried out using the PyroMark® Kit (all primers but MC1R-snp1) or conventional PCR (MC1R-snp1). Negative controls for every fourth sample were included during DNA extraction. Some of those negative controls were amplified in the PCRs. beta, -6 and -5 were included in all four PCRs carried out for MC1R-snp 1 and MC1R-snp2 respectively. Two more (-3 and -15) negative controls were included for ASIP-del and ASIP-snp. In these cases (see Table 3-5) beta, -6 and -5 were included in one PCR out of four for ASIP-snp and -6 in one out of four of ASIP-del respectively. -3 was included in one PCR of ASIP-del and in two of ASIP-snp. -15 was included in three PCRs of ASIP-del and one PCR of ASIP-snp.

However at least one negative control was included in the four PCRs for ASIP-del and ASIP-snp.

DNA genotyping

DNA genotyping was performed using pyrosequencing on the PSQ 96MA System for pyrosequencing (Qiagen) following the Sample Preparation Guidelines for PSQTM96 and PSQ 96MA Systems (Qiagen). Also the negative controls previously used during DNA extraction and PCR amplification were sequenced.

RESULTS

Modern sheep

Ten of 96 sheep analysed for *MC1R* (two Gotland Pelt Sheep and eight Gute Sheep) and nine of the 96 analysed for *ASIP* (three Gotland Pelt Sheep and six Gute Sheep) were determined unanalysable due to the fact that both the reverse and forward strand of one of the primer pairs yielded a nucleotide sequence that was of such low quality that no clear result could be obtained. In those cases (10 % of *ASIP* and 7 % of *MC1R* strands) that just one of the strands of a primer pair (either the reverse or the forward one) were defective a result were obtained from the good one. The primer pair ASIP_4F and ASIP_4R yielded contradictory results regarding g.5172T>A in 23 of 96 sheep (fifteen Gotland Pelt and eight Gute Sheep), where the forward primer gave rise to a TT genotype and the reverse primer to an AT genotype. These sheep have still been included in the genotype frequencies for the other genotypes they possessed, but not for the g.5172T>A genotype frequency nor in the association of coat colour and diplotype.

Neither of the SNPs constituting dominant black coat colour (E^D) nor the *e allele* were possessed by the sheep of this study. All sheep possessed the wildtype (E^+E^+) genotype for *MC1R*. All modern sheep also possessed the A^a allele of *ASIP*. The diplotypes constituting A^a is given in Table 1 in association with the coat colours of the sheep. Fisher's exact test shows a significant association between *ASIP* genotype and coat colour, see Appendix A for calculations.

Table 1. *The numbers of sheep possessing a certain coat colour and ASIP diplotype divided into breeds. GP=Gotland Pelt Sheep, GS= Gute Sheep. Some coat colours are not included since their DNA did not yield analysable results.*

		Breed		
Coat colour	**Diplotype**	**GP**	**GS**	**Total**
Grey	N_9D_9-N_5N_5-AT	27	25	52
	N_9D_9-N_5D_5-AT	6	2	8
Black	N_9N_9-D_5D_5-AT	-	1	1
	N_9N_9-D_5D_5-TT	-	1	1
Black wool	N_9D_9-N_5D_5-AA	-	1	1
Deer	N_9D_9-N_5N_5-AT	-	1	1
Tobiano	N_9D_9-N_5D_5-TT	-	1	1

Spotted grey	N_9D_9-N_5D_5-TT	-	1	1
Brown with black mane	N_9N_9-N_5N_5-AA	-	1	1

Three novel mutations were found in the *MC1R* gene: c.452G>A of the coding sequence and c.970T>C and c.990G>T of the 3'-UTR. Four novel mutations of *ASIP* were identified: g.242G>A) of intron 2-3, g.4982A>C of intron 3-4, g.4960C>G of intron 3-4 and g.5266C>A of intron 4. The number of sheep possessing a certain genotype and their genotype frequency is given in Table 2 and 3.

Table 2. *The number of sheep possessing a certain mutation of MC1R. Genotype frequency is given in brackets. GP=Gotland Pelt Sheep, GS=Gute Sheep.*

Mutation	**Genotype**		
c.-31G>A	*GG*	*AA*	*AG*
GP	0 (0%)	46 (100%)	0 (0%)
GS	24 (60%)	4 (10%)	12 (30%)
c.199C>T	*CC*	*TT*	*CT*
GP	46 (100%)	0 (0%)	0 (0%)
GS	40 (100%)	0 (0%)	0 (0%)
c.218C>T	*CC*	*TT*	*CT*
GP	46 (100%)	0 (0%)	0 (0%)
GS	40 (100%)	0 (0%)	0 (0%)
c.361G>A	*GG*	*AA*	*AG*
GP	46 (100%)	0 (0%)	0 (0%)
GS	40 (100%)	0 (0%)	0 (0%)
c.429C>T	*CC*	*TT*	*CT*
GP	0 (0%)	46 (100%)	0 (0%)
GS	23 (57.5%)	4 (10%)	13 (32.5%)
c.452G>A	*GG*	*AA*	*GA*
GP	2 (4,3%)	0 (0%)	44 (95.6%)
GS	28 (70%)	0 (0%)	12 (30%)

c.600T>G	*TT*	*GG*	*TG*
GP	0 (0%)	46 (100%)	0 (0%)
GS	23 (57.5%)	5 (12,5%)	12 (30%)

c.735T>C	*TT*	*GG*	*TG*
GP	45 (97,8 %)	1 (2.1%)	0 (0%)
GS	9 (22,5%)	23 (57.5 %)	8 (20%)

c.970T>C	*TT*	*CC*	*CT*
GP	0 (0%)	46 (100%)	0 (0%)
GS	0 (0%)	40 (100%)	0 (0%)

c.990G>T)	*GG*	*TT*	*GT*
GP	0 (0%)	46 (100%)	0 (0%)
GS	0 (0%)	40 (100%)	0 (0%)

Table 3. *The number of sheep possessing a certain mutation of ASIP. Genotype frequency is given in brackets. GP=Gotland Pelt Sheep, GS=Gute Sheep.*

D_9	N_9N_9	D_9D_9	N_9D_9
GP	0 (0%)	0 (0%)	45 (100%)
GS	2 (4.8%)	0 (0%)	40 (95.2%)

D_5	N_5N_5	D_5D_5	N_5D_5
GP	34 (75.5 %)	0 (0%)	11 (24.5 %)
GS	30 (71.4%)	2 (4.8%)	10 (23.8 %)

g.242G>A	*GG*	*AA*	*GA*
GP	6 (13.3%)	2 (4.4%)	37 (82.2%)
GS	14 (33.3%)	1 (2.4%)	27 (64.2%)

g.4960G>T	*GG*	*TT*	*GT*
GP	11 (23.4%)	0 (0%)	36 (76.6%)
GS	14 (41.2 %)	0 (0%)	20 (58.8%)

g.4992A>C	*AA*	*CC*	*AC*
GP	15 (33.3 %)	0 (0%)	30 (66.7%)
GS	23 (56.1%)	1 (2.4%)	17 (41.5%)

g.5051G>C	*GG*	*CC*	*GC*

GP	9 (20%)	0 (0%)	36 (80%)
GS	25 (59.5%)	1 (2.4%)	16 (38.1%)

g.5172T>A	*TT*	*AA*	*TA*
GP	0 (0%)	0 (0%)	32 (100%)
GS	28 (84.8%)	2 (6.1%)	3 (9.1%)

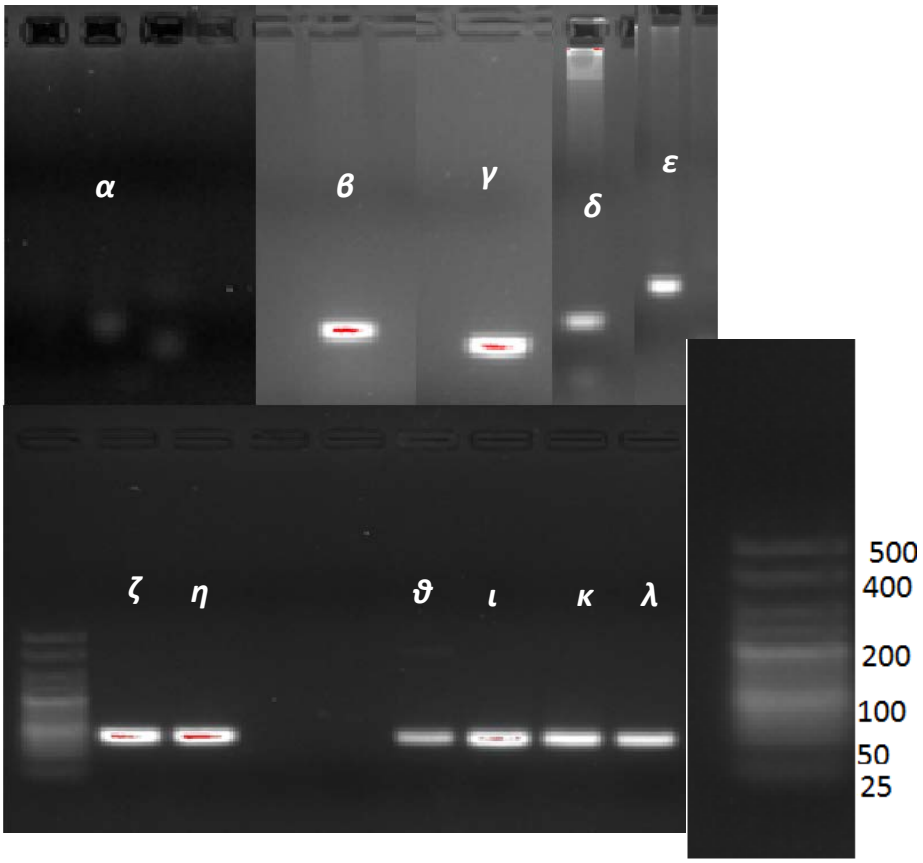
Ancient sheep

Amplification and agarose gel electrophoresis

The optimal annealing temperature and $MgCl_2$ concentration of the primer pairs for conventional PCR using AmpliTaq Gold DNA polymerase were: for MC1R-snp1 60 °C and 2.5 mM (see Gel D wells 13-14), for MC1R-snp2 60 °C and 2 mM (see Gel A well 36), for ASIP-snp and ASIP-del 53°C and 3 mM (see Gel C well 20 and 25 respectively). However when these conditions were tested with aDNA only MC1R-snp1 and MC1R-snp2 worked. However all four primer pairs gave amplifiable products using the PyroMark® PCR Kit.

A summary of the result of the agarose gels are given in Figure 5. For all gels see Appendix G.

Figure 5. *A summary of different agarose gels showing presence of PCR products for some samples.* ***α*** *Modern sample with primer pair MC1R-snp1 at an annealing temperature of 60 °C and $MgCl_2$ of 2 mM from Gel A (for PCR program see table i in Appendix D).* ***β*** *Modern sample using primer pair MC1R-snp1 using touchdown PCR at an $MgCl_2$ of 2.5 mM (see Table ii Appendix D) from Gel B.* ***γ*** *Ancient sample using primer pair MC1R-snp2 and the PyroMark® PCR Kit (see Appendix E) from Gel B.* ***δ*** *Modern sample using ASIP-snp and conventional PCR at an annealing temperature of 53 °C and $MgCl_2$ concentration of 3mM (for PCR program see Appendix D) from Gel C.* ***ε*** *Modern sample using ASIP-del and conventional PCR at an annealing temperature of 53 °C and $MgCl_2$ concentration of 3mM (for PCR program see Appendix D) from Gel C.****ζ-λ*** *Ancient samples using primer pair MC1R-snp2 and the PyroMark® PCR Kit (see Appendix E) form Gel D. The picture to the right is of the ladder (Hyperladder V from Bioline), showing which of its steps corresponds to a number of bp. The picture is not in the same scale as the rest of the gel pictures. All PCR products should be a little less than 100 bp long.*



Of the 26 extracted samples only twelve yielded amplifiable nuclear DNA. Of these eight were from Gotland (five dated to the Iron Age and three to the Early Middle Ages), three from Runsaborg and one from York.

The successrate of all PCR amplifications (that is the number of PCRs in which a product was amplified for aDNA) for all primers (including those that tested if a primer pair could amplify aDNA) was 74 %. The successrate for each primer with ancient samples that could be amplified (not counting test runs with primers) were: for MC1R-snp1 43%, for MC1R-snp2 87.5 %, for ASIP-del 97.6% and for ASIP-snp 90%. Thirty-one percent of the negative controls used during DNA extraction and PCR amplification showed one or more bands on agarose gel electrophoresis. Two ancient samples (S and Y) gave rise to more than one band on electrophoresis in at least one case for primer pairs MC1R-snp2 (one case for Y and two for S out of four), ASIP-del (one case each out of two for S and one for Y) and ASIP-snp (one case each out of one). For both the negative controls and samples S and Y one band was the same size as the amplified ancient DNA PCR products. If other bands were present they were smaller in magnitude.

Genotype

The genotypes obtained for the ancients samples are listed in tables 3-5. No genotypes were obtained for the ASIP mutation g.5172T>A. Examples how the results given by pyrosequencing are given in Figures 6-7. No negative controls (including those which gave rise to a PCR products at the gels) gave rise to any result.

Table 3. *The genotypes at c.218T>A (sequenced with primer pair MC1R-snp1) possessed by the ancient sheep. Samples designations given are their lab denotations used in the lab (Lab id) and their gel denotations (Gel id). The number of PCR run means which one of the four PCRs that was run for every primer and sample (here also called "duplicate") that a result was obtained/ means that the duplicate of the sample never were amplified and thus not pyrosequenced.* ***?*** *Means that the result is*

uncertain. Minus a number means the sample was a negative control. In the last two columns (time and place) the time to which the sample has been dated and the place where it was found are given.

		Number of PCR run					
Lab id	**Gel id**	**1**	**2**	**3**	**4**	**Time**	**Place**
12	*W*	TT	TT	-	TT?	Iron Age	Stora Förvar
23	*Ö*	/	/	/	/	Middle Ages	Stora Förvar
26	*D*	/	-	TT	TT	Middle Ages	Stora Förvar
4	*E*	TT	-	-	TT	Iron Age	Stora Förvar
5	*F*	/	-	-	-	Iron Age	Stora Förvar
1	*G*	TT	TT	TT	-	Iron Age	Stora Förvar
24	*H*	/	/	/	/	Middle Ages	Stora Förvar
7	*J*	/	/	/	/	Iron Age	Stora Förvar
FR7	*O*	/	/	-	/	4th-7th century	Runsaborg
FR8	*P*	/	/	/	/	4th-7th century	Runsaborg
FR6	*S*	/	-	-	-	4th-7th century	Runsaborg
FY6	*Y*	/	/	/	/	8th-11th century	York
-1	*beta*	/	/	/	/	Negative Control	
-6	*-6*	-	-	-	-	Negative Control	
-5	*-5*	-	-	-	-	Negative Control	

Table 4. *The genotypes possessed by the ancient sheep at the c.361G>A (sequenced with primer pair MC1R-snp2). For information about samples designations and abbreviations see table 3.*

		Number of PCR run			
Lab id	**Gel id**	**5**	**6**	**7**	**8**
12	*W*	-	GG	-	-
23	*Ö*	-	-	AG	-
26	*D*	-	-	-	-
4	*E*	GG	-	GG	-
5	*F*	GG	-	-	-
1	*G*	-	-	GG	AG?
24	*H*	/	/	-	/
7	*J*	/	/	-	/
FR7	*O*	AG	-	-	-
FR8	*P*	GG?	-	-	-
FR6	*S*	-	-	-	-
FY6	*Y*	-	-	-	-
-1	*Beta*	/	-	/	-
-6	*-6*	/	-	/	-
-5	*-5*	-	-	-	-

Table 5. *The genotypes of the D_5 deletion (sequenced with primer pair ASIP-del) possessed by the ancient sheep. For information about samples designations and abbreviations see table 3.*

		Number of PCR run			
Lab id	**Gel id**	**9**	**10**	**11**	**12**
12	*W*	N_5D_5?	N_5D_5?	-	-
23	*Ö*	N_5N_5	?	N_5N_5	-
26	*D*	-	N_5D_5?	-	-
4	*E*	N_5D_5?	N_5D_5?	N_5D_5?	N_5D_5?
5	*F*	N_5N_5	N_5N_5	N_5N_5	N_5N_5
1	*G*	N_5D_5?	N_5D_5?	N_5D_5?	N_5D_5?
24	*H*	-	-	-	-
7	*J*	-	-	-	-
FR7	*O*	-	N_5N_5	N_5N_5	N_5N_5
FR8	*P*	N_5N_5	-	/	/
FR6	*S*	-	-	/	/
FY6	*Y*	N_5D_5?	-	/	/
-1	*beta*	-	/	/	/
-6	*-6*	-	/	/	/
-3	*-3*	/	-	/	/
-15	*-15*	/	-	/	-

Figure 6. *Pyrogram from negative control -5,6. This has no peaks, meaning no genotype. Genotyping of c.361G>A.*

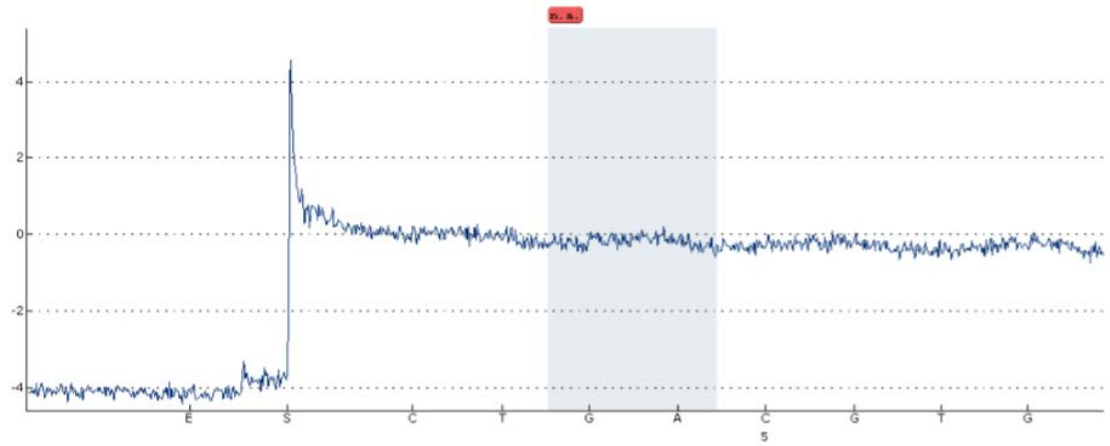
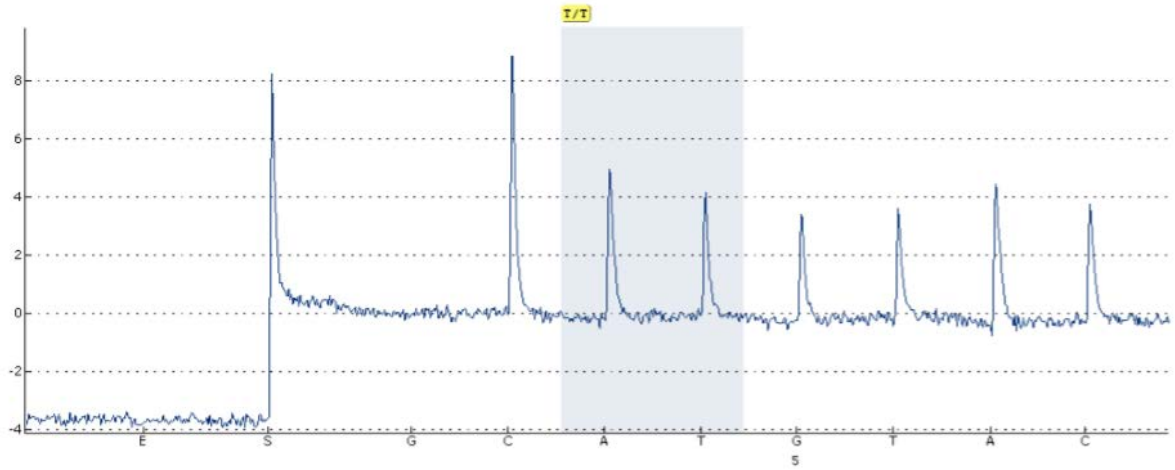


Figure 7. *Pyrogram from W7 at c.218T>A.*



DISCUSSION

All the modern sheep included in this study were homozygous for wildtype allele (E^+E^+) of *MC1R*. Thus the black coat colour of Gute Sheep (no Gotland Pelt Sheep were black) was determined by the Agouti locus. This result corresponds with the results and conclusion of Rochus *et al.* (2014) in which the authors concluded that E^D is not a part of the old Swedish gene pool and only possessed by Swedish Finewool Sheep, which might have inherited this allele from foreign ancestors. However the number of black individuals included in this study and that of Rochus *et al.* (2014) are few and no definite conclusions can be drawn. Also agreeing with the results of Rochus *et al.* (2014) the *e allele* (c.199C>T) was not identified. This is a rare mutation only found in the Valle del Belice breed by Fontanesi *et al.* (2010) and is seemingly not possessed by Swedish sheep breeds.

Three *ASIP* diplotypes gave rise to black coat colour in this study (N_9N_9-D_5D_5-AT and N_9N_9-D_5D_5-TTand N_9D_9-N_5D_5-AA). Black sheep possessed a variety of diplotypes in the study of Rochus *et al.* (2014): N_5D_5-TA, N_5D_5-TT, N_5N_5-TA, D_5D_5-AA, D_5Df_5-TT and N_5D_5-AA. Although sheep homozygous for either D_5 or AA were black not all black sheep were homozygous for at least one of these mutations. Thus I conclude, as did Rochus *et al.* (2014) that the full genetic background of black recessive coat colour of sheep is not elucidated. D_9 was identified in this study in individuals of different coat colours, but none were homozygous. This mutation might disrupt ASIP function by removal of a tripeptide (Norris and Whan, 2008) and could theoretically cause black coat colour. However the effect on phenotype of D_9 needs to be further investigated in other studies, preferably on homozygous individuals. The unnamed regulatory mutation indirectly recognized by Norris and Whan (2008) can be the yet unknown determinant of ovine recessive coat colour. Further studies are needed to directly identify this mutation.

A gene duplication affecting coat colour is described in other species, such as the *KIT* gene at the Dominant white locus of pigs (Möller Johansson *et al.*, 1996; Pielberg *et al.*, 2002). In pigs this phenomenon is well studied. The porcine white coat colour is (like the ovine) associated with a duplicated *KIT* gene as well as a splice mutation of intron 17 (causing exon 17 not to be translated). This splice site mutation only needs to be in one of the gene duplicates to cause white coat colour. A pig possessing only one copy of *KIT* and a splice site mutation does not survive. No such lethal combination has yet been reported in the sheep. Two *KIT* copies without a mutation cause patches. Compared to the well-studied duplications of pigs the *ASIP* tandem duplication of sheep needs to be further elucidated. Fontanesi *et al.* (2010) could confirm the presence of a duplication in grey sheep, but were unable to tell which exact haplotypes a certain allele copy contained. Norris and Whan (2008) too, were unable to identify the exact haplotypes of each duplicated allele. However they have shown that the duplicates of white sheep express genotypes common to white sheep: that is N_9 and N_5. The exact haplotypes of the duplications needs to be elucidated in further studies. A future study should elucidate the reason that Fontanesi *et al.* (2010) associated *ASIP* duplications with grey sheep while Norris and Whan (2008) associated them clearly with white coat colour.

Three novel mutations in the *MC1R* gene and four in the *ASIP* gene were identified in this study. Only one of MC1R mutations results in a missense mutation, c.452G>A, that gives rise to serine instead of glycine. c.970T>C and c.990G>T are located in 3'-UTR of the Extension locus. The novel mutations of *ASIP* are all situated within introns and as such may more accurately be detonated genetic variation then a true mutation. 11 known sequences of the *MC1R* posted on http://www.ncbi.nlm.nih.gov were analysed for the presence of the novel mutations found in Gute Sheep and Gotland Pelt Sheep. None of them has been identified in these sequences. The reference sequence used in this study when analysing *MC1R* (FN600553.1 by Fontanesi *et al.* (2010)) is the only *MC1R* sequence found on http://www.ncbi.nlm.nih.gov that contains the 3'-UTR. The introns of the ASIP gene is only included on the similar sequences EU420022.1 and ENSOARG00000009483 and thus no analyses for the presence the *ASIP* mutations in other sequences were possible.

The mutations that constitute A^a some haplotypes appear more frequently with some coat colours. It can be theorized that the wildtype variants of A^a all belong to the same haplotype (that is one wildtype haplotype is N_9-N_5-T). Using this theory the grey sheep possess the wildtype haplotype and two recombinant ("mutated") haplotypes: D_9-N_5-A and D_9-D_5-A. Grey coat colour thus seems to be associated with one wildtype haplotype and a recombinant haplotype with one mutation. This means that no grey sheep is homozygous for the D_9, D_5 or g.5172T>A, but one of the mentioned mutations might be necessary for grey coat colour. To this discussion is not accounted the influence of gene duplications discussed below. The black individuals possess one recombinant haplotype with one mutation (N_9-D_5-T or N_9-N_5-A) and one recombinant haplotype with two (N_9-D_5-A) or three (N_9-D_5-A) mutations. Only one individual possessing the other coat colours were included in this study and no conclusions regarding haplotypes can be certain. However all of them possess except for the brown sheep with black mane possess the wildtype haplotype.

Four haplotypes encompassing all known mutations of the *MC1R* gene (including those identified in this study) can be deduced: G-C-C-G-C-G-T-G-C-T, A-C-C-G-T-G-G-T-C-T, A-C-C-G-C-G-T-T-C-T and G- C-C-G-T-A-G-T-C-T (written in order of mutations: c.-31G>A, c.199C>T, c.218C>T, c.361G>A, c.429C>T, c.452G>A, c.600T>G, c.735T>C, c.970T>C and c.990G>T). For the mutations coding for a protein (c.199C>T, c.218C>T and c.361G>A) all the modern sheep of this study possessed two copies of the wildtype haplotype (C-C-G). This haplotype is thus associated with non-black sheep. The other mutations (except for c.452G>A) do not code for a protein, have no effect on coat colour and is for that reason unnecessary to associate with coat colour. The effect of c.452G>A remains unkown. Both its assumed wildtype (G) and mutated variant (A) is possessed by grey, black, deercoloured and tobiano sheep.

In this study a mutation was considered present if it appeared on either the forward or reverse strand if the complementary strand failed. In reality a mutation should be identified on both strands. For the failing strands the laboratory work for those strands needs to be redone. However this was not applied due to lack of economy and time. However, the high frequency of some of the mutations (for example c.-31G>A and c.429C>T) on both the forward and

reverse strand indicate that the mutations are often present and for that reason also believable for those that were only identified on one strand.

If both the forward and reverse strand were present but showed different result for a mutation the result was not documented. This was the case for g.5172T>A, where 21% of the samples rendered contradictory results (the forward strand showed TT and the reverse AT). In this case the laboratory work for the contradictory samples should be redone for the primer pair ASIP_4F and ASIP_4R (that yielded the strands on which g.5172T>A was found) to double-check for experimental errors. Due to lack of time and economy this was not applied. If the result is correct the primers should be redesigned, because primers that fail to bind to their primer binding sites of the DNA template may cause the contradictory results. As an example, an individual possesses two chromosomes, one has A and the other T at position g.5172. If the reverse strand primer binding site of the A- chromosome is mutated the primer will not bind to that reverse strand. Without a primer binding to the DNA strand no replication of that strand will be achieved by DNA polymerase. As a result the reverse strand on CodonCode Aligner will only show the result from the other chromosome (T). However on the forward strand both A and T will be replicated and shown on CodonCode Aligner. Another explanation for the contradictory results is the presence of two duplicates of *ASIP*. Duplications of *ASIP* has been reported by Norris and Whan (2008) and Fontanesi *et al.* (2010). The duplicates may possess different genotypes for g.5172T>A. A mutated binding site for one of the primers of one of the duplicates leads to that only one direction of that duplicate is replicated. This leads to different results of the forward and reverse strands since the duplicates are processed in CodonCode Aligner as one gene and not two duplicates due to their similarity. If there had been time the results could have redone using pyrosequencing and the other primers used for pyrosequencing.

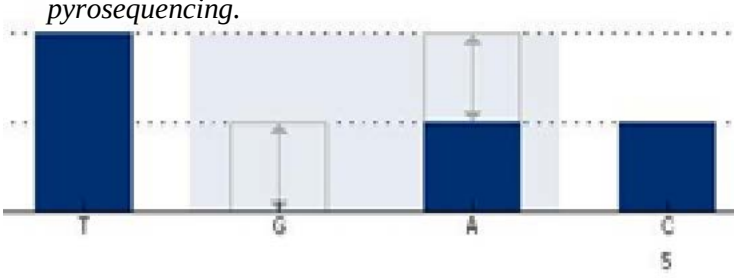
The genotype frequency of GA of c.452G>A and N_9D_9 of D_9 is or close to 100%. This result does not agree with the Mendelian inheritance for heterozygous individuals. At least 50% of the populations should be homozygous for either GG or AA (or N_9N_9 and D_9D_9). Since this was not the case it might be due to a genotyping error.

When *ASIP* was analysed g.5172T>A was found 5170 bp and g.5051G>C 5049 bp from the start codon of exon 2, but should have been found 5172 and 5051 base pair from the start codon according to the nomenclature specified in the supplemental facts to the article by Norris and Whan (2008) (Norris and Whan, 2008b**)**. The SNPs were both identified and named by Norris and Whan (2008). Their sequence (EU420022.1) was used as reference sequence when the *ASIP* gene was analysed in this study. Yet their denotations do not match with the actual number of base pair in their own reference sequence. This sequence aligns with the *ASIP* sequence from www.ensembl.org (ENSOARG00000009483), which has labelled the number base pair of respective exons and introns. The number of base pair in each exon and introns of this sequence (and thereby EU420022.1) differs from those given in Figure 1 in the article by Norris and Whan (2008).

Of the sheep included in this study only three black individuals were encompassed because of

the low availability of black individuals. Yet these breeds were selected, since they are native to Gotland as are most of the ancient samples. The ancient sheep of Gotland possessed a diversity of coat colours (Hallander, 2010) and this is true for the Gute Sheep today. Thus the two breeds are the best alternative to compare with the ancient individuals due to their shared regional ancestry and the diversity of the coat colour phenotypes (meaning that Gute Sheep are likely to carry the same genetic background as their ancestors that also possessed a variety of coat colours).

Figure 8. *The theoretical histograms of how the c.361G>A genotype look when genotyped with pyrosequencing.*



Only few genotypes could be obtained from the ancient samples. However PCR products were amplified for more ancient samples than genotypes were obtained. This could be due several reasons: such as that the amplified products shown on gel were the result of contamination (for example from ancient bacteria or from environment) or failure of the pyro machine to sequence. The machine and the assays used had not previously been tested using modern ovine DNA and thus its settings might not have been optimal. The histogram in Figure 8 shows what the theoretical outcome for the the sequence to be genotyped looks like. The genotyped sequence as the peaks of a graph can be seen in Figure 6-7. The relative height of the peaks of the graph needs to correspond to the height of the theoretical histogram. Some results obtained were questionable (those marked with question marks), since the peaks were lower than required by the software or that the non-polymorphic sites (the sequence around the mutation) did not yield clear peaks.

The reason for the uncertainty about the correct results of D_5 is that it the presence or absence of several nucleotides must be determined by the pyrosequencing reaction. The difference of peak heights between a homozygote and a heterozygote may be small and the risk for misreading by the pyrosequencing reaction high. However the results in which N_5N_5 were determined for all four duplicates the certainty of correct result is higher due to the fact that all duplicates showed the same result.

Due to the incomplete results no firm conclusions about the coat colour of the ancient sheep can be deduced.

The presence of the mutated allele of c.361G>A (one of those that constitute E^D) in two Swedish individuals (one mainland sheep from the 4^{th} – 7^{th} century and one from Gotland from the Early Middle Ages) proves the presence of the mutation in the Swedish ovine gene pool as early as the 7^{th} century. This result opposes the conclusion of Rochus *et al.* (2014) that the mutations of the E^D allele had recently entered the Swedish gene pool (in Swedish Finewool Sheep) due to interbreeding with foreign breeds. The ancient sheep may have gained this allele from interbreeding with ancient foreign breeds during trade or be a natural part of the very ancient Swedish gene pool. The result of this study also indicates that this

allele was present in Gotland during the Early Middle Ages. It is quite probable that this allele remains in the descendants of the ancient sheep of Gotland, but was not detected in this study since only three black individuals were included in this study. However the other mutation of E^D (c.218G>A) was not found in this study, but only few ancient individuals were genotyped. This mutation might also be present, but not detected.

The aDNA of the ancient samples should be amplified separately at least four times, as was done in this study, to ensure replicability and reliability (Shapiro and Hofreiter, 2014). Four duplicates also are usually enough to ensure the detection of allelic drop out (that is that the allele of a heterozygous individual is "lost" during genotyping and the individual appear homozygous for the other allele). However too few genotypes were obtained to calculate allelic drop out in this study.

The common source of error when working with aDNA is contamination from human modern DNA and that the DNA is so degraded that it is not amplifiable (Hofreiter *et al.,* 2001; Paijmans *et al.,* 2013). The risk for contamination was reduced by wearing protected clothing, showering after visiting other labs, strict cleaning of the lab and change of equipment when changing samples. SNPs that are polymorphic between different species can be used to distinguish ovine aDNA from hominid contaminants. Degradation of aDNA is dependent on several factors such as the temperature, salt concentration and pH in which the sample is preserved (Hofreiter *et al.,* 2001). The ancient samples from Gotland must have been better preserved (in a better environment and climate) than the samples of Runsaborg and York, since more samples from Gotland gave rise to an amplifiable product.

When the first PCR optimization was carried out, only MC1R-snp2 gave rise to a PCR product. For that reason another optimization was performed, the touchdown PCR for the remainder of the primers. In this case touchdown PCR was chosen since it gives strong and specific amplification. Since the three primers had not given rise to any PCR product during the first optimization a more specific method was chosen. The touchdown PCR indicated the optimal $MgCl_2$ for MC1R-snp1. The optimal annealing temperature for MC1R-snp1 were achieved by using the same annealing temperature (60 °C) in conventional PCR as when the PyroMark® PCR Kit were used on ancient samples.

The successrate, 74 %, was lower than that obtained by Svensson *et al.* (2007), who also worked with samples dated to the Middle Age and Iron Age. However there were only few samples included in this study due to lack of time. This and the uncertainty regarding the ASIP-snp and ASIP-del PCR products described below the successrate might have been higher or lower in reality. If this is the true successrate it was a bit low. The successrate for the respective primer pairs used on aDNA was better for those used with the PyroMark® PCR Kit than conventional PCR. But by comparing MC1R-snp1 that used conventional PCR and MC1R-snp2 that used PyroMark® PCR Kit (and both these have been tested and been able to amplify ancient samples using both PyroMark® PCR Kit and conventional PCR) the PyroMark® PCR Kit is superior to conventional PCR. This makes it optimal for use of ancient samples that is much harder to amplify (due to degraded DNA) than modern samples.

The presence of band on the gel when the negative controls and presence of more than one band when S and Y were run on agarose gel electrophoresis is not considered contamination since the amplified product could not be pyrosequenced. However some DNA (ancient or modern) must have been present since the primers did bound to “something containing DNA” and for that reason facilitated amplification of the “something”.

Except for the ancient samples’ sensitivity to environmental and bacterial contamination the source of errors are similar when working with ancient and modern DNA. These include erroneous pipetting causing mixture of DNA from two individuals, pipetting of the wrong amount of reagents and using the wrong tip for the wrong pipetting causing pipetting of the wrong volume. All errors mentioned influence the final quantity of reagents or means that the DNA of one individual is contaminated, resulting in that some strands cannot be sequenced. In one instance failure of sequencing could be caused by a malfunctioning PCR thermal cycler that caused the pre-PCR mixture to be set in for 95 degrees for 15 minutes instead of 5 min causing premature denaturation of the DNA and thus denatured templates when the PCR was set on a functioning PCR machine. Lack of sequences might be caused by a too small amount DNA present in the sample. Unreadable chromatograms (that is three or more bases present at a certain position) can be due to failed purification of the DNA with the Big Dye Xterminator® Purification Kit.

CONCLUSION

Although present sheep of Gotland seem to lack the E^D allele, one of the mutations that constitute E^D was present in their ancestors as early as the Early Middle Ages. Since black sheep, lacking the E^D allele, are not homozygous for D_5 and/or g.5172T>A the full genetic background of recessive black coat colour remains to be elucidated. The D_5 deletion might have been present in the ancient sheep of Gotland as early as the Iron Age, but this can at the present time not be determined with certainty. Neither can the presence of g.5172T>A be deducted in this study.

REFERENCES

Baker, D.M., Weigt, L., Fogel, M., Knowlton, N. (2013). Ancient DNA from coral-hosted Symbiodinium reveal a statistic mutualsim over the last 172 years. *Public Library of Science One*, 8 (2): e55057.

Chessa, B., Pereira, F., Arnaud, F., Amorim, A., Goyache, F., Mainland, I., Kao, RR., Pemberton, J.M., Beraldi, D., Stear, M.J, Alberti, A., Pittau, M., Iannuzzi, L., Banabazi, M. H., Kazwala, R. R., Zhang, Y. P., Arranz, J. J., Ali, B. A., Wang, Z., Uzun, M., Dione, M. M., Olsaker, I., Holm, L. E., Saarma, U., Ahmad, S., Marzanov, N., Eythorsdottir, E., Holland, M. J., Ajmone-Marsan, P., Bruford, M. W., Kantanen, J., Spencer, T. E. & Palmarini, M.. (2009) Revealing the history of sheep domestication using retrovirus integrations. *Science*, 324: 532-536.

Demirci, S.,, Koban Baştanlar, E., Dağtaş, N. D., Pişkin, E., Engin, A., Ozer, F., Yüncü, E., Doğan, S. A. & Togan, I. (2013). Mitochondrial DNA diversity of modern, ancient and wild sheep (*Ovis gmelinii anatolica*) from Turkey: new insights on the evolutionary history of sheep. *PLoS One*, doi: 10.1371/journal.pone.0081952. [2014-10-05]

Elitlamm (2013). *Elitlamm Avel 2013*. http://www.elitlamm.com/common/images/ArsstatistikElitlammAvel2013.pdf [2014-11-23]

Fontanesi, L., Beretti, F., Riggio, V., DallÓlio, S., Calascibetta, D., Russo, V. & Portolano, B. (2010). Sequence characterization of the melanocortin 1 receptor (MC1R) gene in sheep with different coat colours and identification of the putative *e* allele at the ovine *Extension* locus. *Small Ruminant Research*, 91: 200-207.

Fontanesi, L., DallÓlio, S., Beretti, F., Portolano, B. & Russo, V. (2011). Coat colours in the Massese sheep breed are associated with mutations in the agouti signalling protein (ASIP) and melanocortin 1 receptor (MC1R) genes. *Animal*, 5:8-17.

Föreningen Gutefåret (2014). *Rasbeskrivning gutefår*. http://test.gutefar.se/MinaFiler/rasbesrkvining%20ig%20ig%20%2027jan%202014.pdf [2014-11-23]

Gotlandsfårföreningen (2009-08-22). *Gotlandsfåret*. http://www.silverlock.se/ras.asp[2014-09-03]

Gratten, J., Pilkington, J. G., Brown, E. A., Beraldi, D., Pemberton, J. M. & Slate, J. (2010). The genetic basis of recessive self-colour pattern in a wild sheep population. *Heredity*, 104: 206-214.

Hallander, H. (2010). *Svenska lantraser*. 2. ed. Dejavu.

Hofreiter, M., Serre, D., Poinar, N. P, Kush, M. & Pääbo, S. (2001). Ancient DNA. *Nature Reviews*, 2: 353-359.

Johansson Möller, M., Chaudhary, R., Hellmén, E., Hoyheim, B., Chowdhary, B., Andersson, L. (1996). Pigs with the dominant white coat colour phenotype carry a duplication of the *KIT* gene encoding the mast/stem cell growth factor receptor. *Mammalian Genome*, 7: 822-830.

Marklund, L., Moller, M.J., Sandberg, K. & Andersson, G.(1996). A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat colour in horses. *Mammalian Genome*, 7 (12): 895-899.

Meadows, J. R. S, Cemal, I., Karaca, O., Gootwine, E. & Kijas, J. W. (2007). Five ovine mitochondrial lineages identified from sheep breeds of the near east. *Genetics Society of America*, doi 10. 1534/genetics. 106.068353. [2014-10-01]

Mitchell, D., Willerslev, E. & Hansen, A. (2005). Damage and repair of ancient DNA. *Mutation Research*, 571 (1-2): 265-276.

Niemi, M., Bläuer, A., Iso-Touru, T., Nyström, V., Harjula., J, Taavitsainen, J.P., Storå, J., Lidén, K. & Kantanen, J. (2013). Mitochondrial DNA and Y-chromosomal diversity in ancient populations of domestic sheep (*Ovis aries*) in Finland: comparison with contemporary sheep breeds. *Genetics Selection Evolution*, 45:2.

Norris B. J. & Whan, V. A. (2008). A gene duplication affecting expression of the ovine *ASIP* gene is responsible for white and black sheep. *Genome Research*, 18: 1282-1293.

Norris B.J. & Whan, V.A. (2008b). *Supplemental Material. A gene duplication affecting expression of the ovine ASIP gene is responsible for white and black sheep.* *http://genome.cshlp.org/content/18/8/1282/suppl/DC1*. [2014-12-06]

Paijmans, J. L. A., Gilbert, M. T. P & Hofreiter, M. (2013). Mitogenomic analyses from ancient DNA. *Molecular Phylogenetics and Evolution*, 69: 404-416.

Parsons, Y. M., Fleet, M. R. & Cooper, D. W. (1999). The *Agouti* gene: a positional candidate for recessive self-colour pigmentation in Australian Merino sheep. *Australian Journal of Agricultural Research*, 50: 1099-1103.

Pielberg, G., Olsson, C., Syvänen, A. C., Andersson., L. (2002). Unexpectadely high diversity at the *KIT* locus causing dominant white colour in the domestic pig. *Genetics*, 106: 305-311.

R Core Team (2014). *R: A language and environment for statistical computing.* http://www.R-project.org/. [2014-11-15]

Rochus, C.M., Mikko, S., Näsholm, A. & Johansson, A. M. (2014). ASIP and MC1R mutations causing black coat colour in five Swedish sheep breeds. *Proceedings, 10th World Congress of Genetics Applied to Livestock Production.*

Royo, L. J., Alvarez, I., Arranz, J., Fernández, I., Rodríguez, A., Pérez-Paradal, L. & Goyache, F. (2008). Differences in the expression of the ASIP gene are involved in the recessive black coat colour pattern in sheep: evidence from the rare Zalda sheep breed. *Animal Genetics*, 39: 290-293.

Ryder, M.L. (1983). *Sheep and man*. London: Gerald Ducksworth & Co. Ltd.

Shaprio, B. & Hofreiter, M. A paleogenomic perspective on evolution and gene function: new insights from ancient DNA. *Science*, doi: 10.1126/science. 1236573. [2014-10-07]

Sponenberg, D.P. (1997). Genetics of colour and hair texture. In: Piper, L & Ruvinsky, A (editor). *The Genetics of Sheep.* Wallingford & New York: CAB International, 51-86.

Svensson, E. M., Anderung, C., Baubliene, J., Persson, P., Malmström, H., Smith, C., Vretemark, M., Daugnora, L. & Götherström, A. (2007). Tracing genetic change over time using nuclear SNPs in ancient and modern cattle. *Animal Genetics*, 38: 378–383.

Svensson, E. M., Telldahl, Y., Sjöling, E., Sundkvist, A., Hulth, H., Sjøvold, T. & Götherström, A. (2012). Coat colour and sex identification in horses from iron age Sweden. *Annals of antatomy*, 194: 82-87.

Våge, D. I., Fleet, M. R., Ponz, R., Olsen, R. T., Monteagudo, L. V., Tejedor, M. T., Arruga, M. V., Gagliardi, R., Postiglioni, A., Nattrass, G. S. & Klungland, H. (2003). Mapping and characterization of the dominant black coat colour locus in sheep. *Pigment Cell Research*, 16 (6): 693-697.

Våge, D. I., Klungland, H., Lu, D. & Cone, R.D. Molecular and pharmacological characterization of dominant black coat color in sheep. *Mammalian Genome*, 10: 39-43.

Wood, N. J., & Phua, S. H. (1996). Variation in the control region sequence of the sheep mitochondrial genome. *Animal Genetics*, 27: 25-33.

Yang D.Y., Eng B., Waye J.S., Dudar J.C. & Saunders S.R. (1998). Technical note: improved DNA extraction from ancient bones using silica-based spin columns. *American Journal of Physical Anthropology*, 105: 539–543.

Zeder, M.A. (2008). Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. *Proceedings of the National Academy of Sciences of the United States*., 105 (33): 11597-11604.

APPENDIX

Appendix A - Statistics

The tables used for statistical calculations using Fishers exact test for r×c contingency table. The nullhypothesis is that there is no association between coat colour and genotype/diplotype. The alternative hypothesis is that there is an association. The significance level is 0.05. In all cases the nullhypothesis can be rejected and there is a statistical significance.

Table i. *The association between coat colour and N_9-D_9 genotype.*

Colour	**N_9N_9**	**D_9D_9**	**N_9D_9**
Black	2	0	1
Grey	0	0	60
Other	1	0	3

p=0.0003549

Table ii. *The association between coat colour and N_5-D_5 genotype.*

Colour	**N_5N_5**	**D_5D_5**	**N_5D_5**
Black	0	2	1
Grey	52	0	8
Other	2	0	2

p=0.0001939

Table iii. *The association between coat colour and A-T genotype.*

Colour	**TT**	**AA**	**AT**
Black	0	1	2
Grey	0	0	60
Other	2	1	1

p=0.00003001

Table iiii. *The association between coat colour and diplotype.*

Diplotype	**Black**	**Grey**	**Other**
D9N9-N5N5-AT	0	52	1
D9N9-N5D5-AT	0	8	0
N9N9-D5D5-AT	1	0	0
N9N9-D5D5-TT	1	0	0
D9N9-N5D5-AA	1	0	0
D9N9-N5D5-TT	0	0	2
N9N9-N5N5-AA	0	0	1

p=0.00000006108

In the below tables the statistical association between a coat colour and a certain ASIP diplotype is given. In some cases a statistical significance could not be determined and the null hypothesis could not be rejected.

Diplotype	**Black**	**Grey**	**Other**
D9N9-N5D5-AT	0	8	0

N9N9-D5D5-AT	1	0	0

p=0.11111

Diplotype	Black	Grey	Other
D9N9-N5D5-AT	0	8	0
N9N9-D5D5-TT	1	0	0

p=0.11111

Diplotype	Black	Grey	Other
D9N9-N5D5-AT	0	8	0
D9N9-N5D5-AA	1	0	0

p=0.11111

Diplotype	Black	Grey	Other
D9N9-N5D5-AT	0	8	0
D9N9-N5D5-TT	0	0	2

p=0.02222

Diplotype	Black	Grey	Other
D9N9-N5D5-AT	0	8	0
N9N9-N5N5-AA	0	0	1

p=0.1111

Diplotype	Black	Grey	Other
N9N9-D5D5-TT	1	0	0
D9N9-N5D5-AA	1	0	0

p=1

Diplotype	Black	Grey	Other
N9N9-D5D5-TT	1	0	0
D9N9-N5D5-TT	0	0	2

p=0.3333

Diplotype	Black	Grey	Other
N9N9-D5D5-TT	1	0	0
N9N9-N5N5-AA	0	0	1

p=1

Diplotype	Black	Grey	Other
D9N9-N5N5-AT	0	52	1
D9N9-N5D5-AT	0	8	0

p=5.766E-11

Diplotype	Black	Grey	Other

D9N9-N5N5-AT	0	52	1
N9N9-D5D5-TT	1	0	0

p=0,03704

Diplotype	Black	Grey	Other
D9N9-N5N5-AT	0	52	1
N9N9-D5D5-TT	1	0	0

p=0,03704

Diplotype	Black	Grey	Other
D9N9-N5N5-AT	0	52	1
D9N9-N5D5-AA	1	0	0

p=0,03704

Diplotype	Black	Grey	Other
D9N9-N5N5-AT	0	52	1
D9N9-N5D5-TT	0	0	2

p=0,00202

Diplotype	Black	Grey	Other
D9N9-N5N5-AT	0	52	1
N9N9-N5N5-AA	0	0	1

p=0,03704

Diplotype	Black	Grey	Other
N9N9-D5D5-AT	1	0	0
N9N9-D5D5-TT	1	0	0

p=1

Diplotype	Black	Grey	Other
N9N9-D5D5-AT	1	0	0
D9N9-N5D5-AA	1	0	0

p=1

Diplotype	Black	Grey	Other
N9N9-D5D5-AT	1	0	0
D9N9-N5D5-TT	0	0	2

p=0,3333

Diplotype	Black	Grey	Other
N9N9-D5D5-AT	1	0	0
N9N9-N5N5-AA	0	0	1

p=1

Appendix B: *MC1R* and *ASIP* sequences

Full cDNA sequence of *MC1R*. Modified from FN600553.1 from http://www.ncbi.nlm.nih.gov/. All mutations are marked. Those in light green and yellow are those reported by Fontanesi *et al.* (2010) (light green are synonymous and yellow are missense), whereas the purple (missense) and dark green (synonymous) are those found in this study.

5′-UTR **c.-31G<A**
CCTGAGAGCAAGCACCCTTTCCTGCTCCCTGCGGGACG

Exon 1
ATGCCTGTGCTCGGCTCCCAGAGGCGGCTGCTGGGTTCCCTTAACTGCACACCCCCAGCCACCCTCCCCCTCACA

CTGGCCCCCAATCGGACAGGGCCCCAGTGCCTGGAGGTGTCCATCCCCGATGGGCTCTTTCTCAGCCTGGGGCTG
c.199C>T **c.218T>A**
GTGAGTCTTGTGGAGAACGTGCTGGTGGTGGCCGCCATCGCCAAGAACCGCAACCTGCACTCCCCCATGTACTAC

TTCATCTGCTGCCTGGCCATGTCCGACCTGCTGGTGAGCGTCAGCAACGTGCTGGAGACGGCAGTCATGCTGCTG
c.361G>A
CTGGAGGCTGGTGTCCTGGCCACCCGGGCGGCCGTGGTACAGCAGCTGGACAATGTCATTGACGTGCTCATCTGC
c.429C>T
AGCTCCATGGTGTCCAGCCTCTGCTTCCTGGGTGCCATCGCTGTGGACCGCTACATCTCCATCTTCTACGCCCTG
c.452G>A
CGGTACCACAGTGTCGTGACACTGCCCCGGGCGTGGAGGATCATTGCAGCCATCTGGGTGGCCAGCATCCTCACC
c.600T>G
AGCGTGCTCTCCATCACCTACTACAACCACACGGTCGTCCTGCTGTGCCTGGTTGGCTTCTTCATAGCCATGCTT

GCCCTGATGGCCGTCCTCTATGTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATCGCCCGGCTCCAG
c.735C>T
AAGAGGCAGCGCCCCATTCATCAGGGCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTC

TTCCTCTGCTGGGGCCCCTTCTTCCTGCACCTCTCGCTCATCGTCCTCTGCCCCCAGCACCCCACCTGTGGCTGC

ATCTTCAAGAACTTCAACCTCTTCCTGGCCCTCATCATTTGCAACGCCATTGTGGACCCCCTCATTTATGCCTTC

CGCAGCCAGGAGCTCCGGAAGACACTCCAAGAGGTGCTGCAGTGCTCCTGGTGA

3′-UTR
c.970T>C **c.990G>T**
GGGTGGCAGTGCCATTGTGTGCCCAGGCCTGGAAGCCTGGGGCAGTCCCTTGA

The full gDNA sequence of *ASIP*. The mutations marked with yellow are the mutations coding for a change of amino acid and light green mark the nonsense mutations previously detected and dark green marks the nonsense mutations identified in this study. Modified variant of ENSOARG00000009483 from www.ensembl.org.

5′-UTR

.....cagaatatagcatatgcaat

gacatacacatttgtccaaaggagccatttatccatgcaccttctcttgagtcttaactc

atgagctttattctcaatactttagtaagacctctgccactgtgaagcattactggggac

ctatcaacaattctgcttaggccttggatctcctggagctaccgacataaaatgtaagaa

aaacagcaccccaccctctggaaggcagagacaattgcaatgcattcttttctctttgc

aatctccctcccttccttgtctttcttcctctcccatctctcccttcctctcttttcct

tcatgagttctccttccatgtcctaagccttgctggtcctccagctccactgggacactg

ggctgtgggatgggggtcagagcaccagcccaaggaaacaaagaaagcaggagggcacat

gcatttgccagacccctggcctacctgactgccttctctgtcgctctcaagcctcctggg

Exon 2

D_9

ATGGATGTCAGCCGCCTCTTCCTGGCTACCTTGCTGGTCTGCCTGTGCTTCCTCAGTGCC

D_5

TACAGCCACCTGGCACCTGAGGAAAAGCCCAGAGATGAAAGGAACCTGAAGAACAATTCT

TCCATGAACCTGTTGGATTTCCCTTCTGTCTCTATCGTGG

Intron 2-3

gtaagtagcctggcctggggcccagcctctgggctctggcccatgagaaggggctgcagg

a

c.242G>A

gggtcaaacactctccaagccgatatcaggatccattgctatcagtcagagctcctttgt

gctcattcctcagaacaattctggaggaatcaagtgtcccttaacttgtttggttaagaa

agctctgcctctcaccaatctagtatgcagcttagcatgatagaggctctgagaaaccat

aaagtcaagacacttggtgaatttggtttaacttcgtgtttctgaaacttactaaatcac

ataactcctatcaatggtaaaatttttagaaaatgctgcagaatataaccaagtctggga

caaactagttctttcatgccagggttgagaattgaaaatgactgctcagaggaaaatatc

aggggtattagagcctagcattggagaatttccggttcaaattcatctgacaataattat

aacatatatgcttattttctgataggtactctgaagtgaatactatttttattcctattt

tatttcacagctaagtaaattaaaagttagaaaagttaagtaactaacatacagtttgtg

attggcagagatggtccatgggtcgcaaagagtaagacatgactgagcgactgaactgaa

ctgactgaactgaatacacacccaggttctgggttaggttctgtttaactccagaatcta

agctcagaactaatattccttactgacttgcctctcacttcaaaaagaggatattctaga

aatatgggaaggcctggagcacattaacattgatctgtgtcagattttccactatataaa

catacctcatttatcctcacaactcttcaaggtaagtataacagtacctacttgcatatg

agaaaactgagtcaaaaggagattaagtaggttgttcaagaccacaaagctgttatgtgg

cagagtcaggattcaaatcaggtctgcctggctccaaagctctgagaaataggaaatagg

aataccggaaacacaagaccatccttgtagagacactgagtccattttccagggccttct

tggattttccctttcactttccttcagaggactctttgctgtcagtctctggctccaggt

ctgcggccaggctgcagaagctgctggcctaagtcccaagatatgatctatccaaccaaa

ccttcatcctctcccccaccctggggcttcctagagcgctctctgctcctccctcttcac

tgcagttaaggaccctcagaaaatggctttgttccttctgtctctctttgaag

Exon 3

CACTGAACAAGAAATCCAAAAAGATCAGCAGAAATGAAGCGGAAAAGAAGAAAAGAGCTT

CCAAG

Intron 3-4

gtaggcctgggagttcacattgtcgggatggggctggacttaaaggggggaggacatccaa

gctctggccagaaactaaatgaaaggttttcagggttgcatgcccagagaaacagaaagc

taccaaaaccttcctgacctcgagccctgaagcagaccgccaagggctgaggtccaggat

gctgagtaaaattgcttacctaaactaaactggagaaggaaatggcaacccactccagtg

ttcttgcctggagaatcccagggacggaggagcctggtgggctaccgtctatggggttgc

acagagtcggacatgactgaagcgtcttagcagcagcagcaaactaaactagcttgaacc

ctgcctaaaataactagctcaaactttctaaactagctcgaacccttcctgaaatttcac

tgtcattcattcttttggatgaacgcgatgtttcccaaacctcagtcatttcagaaccat

ttctacaacttttaccacatctgagttccacttatttaatatttgtttaaaacttaaaaa

caaaaatactatgtaacaggacttccctggtggctgtgtggtaaagaatcctcctgccaa

tgcagaagacatgggttggatccttgggtcggagagaccaccacatgcctcagagcaact

aggcctgtgcgcctcaactattgaacctatgctctagagccggggagctgcaactaacta

ctgaagcctgtgcaccctagagcccatgcaaggcaacaagagaagccactgcagtgagag

gcccaaacactgcgactagagagtagcccccactaaccacaactagagaaaagccctcga

agcaacaagggctctgaacagccaaaaccaaaaataaattaaaaaaaaccaactatataa

tcatgatagaaaaccagtatcacttgacatatataggaagtaaccaagaaaataagtaaa

acgaaaataaaaccttgttagcaaattctagctggttggtgttgcttgccacaagttcta

aatctgagacttgctctctgcattaacaaagaaggattagaaagtgttagggaaacacta

aggatatattgacatcaaactgagcctttccatttgaaagagactgaaaaaagaatcctc

tctcactgtgcgattcagtgtcatttaatattcatgtactgcctaaaattataccaccat

gatacaggttcgagaaatactagattaaggcactgttgaaatgcacagtgttcctatttt

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aggaggcagcacttaaaagactgagaaactctgtaatcttgagccgcagtgttcaatgga

aatgcaacgtgagacacatataaattaaaattccagtagccagattttaagagaaaaaa

acaggtgcaattaatattaatattttatttaacccagtagatcccaaatattatcatttt

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aagtctgacactgtttccccatctatttgccatgaagtgatgggaccagatgccatgatc

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aaggggaaggaggatatgcttagtcccttgtgcgctggagactggacagtaaggggcaa

gggcagcagacagcggccaagcccagggtttcagcggtagtgaagaagtggctagactcc

gaacctaccgtgaaggcagaaccaacagggattgcggatacactggaaggggaagacaac

agagacggaagttaaaaaccgtgcagctgagtgggatcccgtagggagagaagaggccag

gcccgagccagttaagtgttgatggcggagcaggcgaggaccagctagggtgctctgtgg

acatgagtcgagcgggcggtggacagcaggtggggacatctagtccgaggagttcccagg

g.4960G>T **g.4982A>C**

cggggggtgaggacgagtggggcggacgtggatggctcgggcagcctcggcgtttccccac

ag

Exon 4

g.5051G>C

AGAAAGGCTCCGATGAAGAACGTGGCACGGACCCGGCCCCCGCCGCCTACCCCCTGCGTG

GCCACCCGCGACAGCTGCAAGCCACCAGCGCCCGCCTGCTGCGACCCGTGCGCCTTCTGC

g.5172T>A

CAGTGCCGCTTCTTCCGCAGCGCCTGCTCCTGCCGCGTGCTCAACCCCACCTGCTGA

Exon4 and 3′-UTR

gcgcgcctcagggtggcggggcggggctcccgggaccctggggcctttctgccgcgggag

g.5266C>A

agctctggtcggaggggcttccgagaacggagtgggcgcggctattgacgttggggcggg

gcttcaggaagtctcagctagggttgggctaaaatccaaatacatgtagtcttctcgaaa

atgtgtggttgtttctttaagaaacccgaaccattcttttcccttcgcctagtcccctgc

accacacaagttcctcgagctgcgcaactgctgtctcccgggcctggggaaacgcggttg

cttagccctgaggctgcactcctggtaagcgcttttctggatctggccgccagagacagt

gctgccccctacctttaaccctagggacacccgaagtgaagtcgctcagtcgtgtccgac

tctttgtgaccccatggacagtagcctaccaggctccgccatccatgggatttccaggc

aagaatactggagtgggctg.....

Appendix C: Primers

Table i. *The primers for sequencing of MC1R and ASIP of the modern sheep. MC1R after Fontanesi.,* et al. *(2010b) and ASIP after Gratten.* et al. *(2010).*

	Primer name	Foward primer (F) (5´-3´)	Reverse primer (R) (5´-3´)	Use
MC1R	MC1R_F2/R2	agccatgagttgagcaggac	caggacaccagcctccag	Exon 2
	MC1R_F3/R3	gtgagcgtcagcaacgtg	acatagaggacggccatcag	Exon 2
	MC1R_F4/R4	gcctggttggcttcttcata	Tggtctagcgatcctctttg	Exon 2
ASIP	ASIP_F2/R2	acactgggctgtgggatg	agggacacttgattcctcca	Exon 2
	ASIP_F3/R3	ctctttgctgtcagtctctgg	tggtagctttctgtttctctgg	Exon 3
	ASIP_F4/R4	cagctagggtgctctgtgg	agcctcagggctaagcaac	Exon 4

Table ii. *The primers for sequencing of MC1R and ASIP of the ancient samples. Primers designed by Emma Svensson, Uppsala University, using the PyroMark Assay Design Software 2.0.*

	Primer name	Foward primer (F) (5´-3´)	Biotinylated Reverse primer (R) (5´-3´)	Use
MC1R	MC1R_-snp 1 F/R	caagaaccgcaacctgcact	Accagcaggtcggacatg	c.218T>A
	MC1R-snp2 F/R	cgtggtacagcagctggacaat	Agcagaggctggacaccatg	c.361G>A
ASIP	ASIP-snp F/R	gtgcgccttctgccagtg	Caggtggggttgagcacg	g.5172
	ASIP- del F/R	tgaggaaaagcccagagatga	Gagacagaagggaaatccaacag	D_5

Table iii. The primers used for pyrosequencing of MC1R and ASIP of the ancient samples. Primers designed by Emma Svensson, EBC.

Name	5´-3´primer sequence
Snp1 MC1R pyro	gcaacctgcactccc
Snp2 MC1R pyro	cagctggacaatgtca
ASIP-del pyro	aaagcccagagatgaa
ASIP-snp pyro	cgcagcgcctgctcc

Appendix D: PCR-optimization

Table i. *The PCR-program used for optimization of the primers (MC1R-snp1F/R, MC1R-snp2F/R, ASIP-snp F/R, ASIP-del F/R) at $MgCl_2$ 2 mM, 2.5 mM, 3mM and 3.5 mM.*

Stage 1	Stage 2			Stage 3	
95 °C	95 °C	50-55-60 °C	72 °C	72 °C	4 °C
1:00	00:15 min	00:15 min	00:30 min	07:00 min	∞
1 cycle	35 cycles			1 cycle	

Table ii. *Touchdown PCR used for optimization of MC1R-snp1 F/R, ASIP-snp F/R and ASIP-del F/R at $MgCl_2$ 2 mM, 2.5 mM, 3mM and 3.5 mM. *Autodelta, that is a 0.5 °C decrease in temperature for every cycle.*

Stage 1	Stage 2			Stage 2			Stage 3	
95 °C	95 °C	60 °C *	72 °C	95 °C	50 °C	72 °C	72 °C	4 °C
5 min	00:15 min	00:15 min	00:30 min	00:15 min	00:15 min	00:30 min	07:00 min	∞
1 cycle	20 cycles			20 cycles			1 cycle	

Table iii. *The PCR-program used for optimization of the primers for ASIP-snp F/R and ASIP-del F/R at $MgCl_2$ 2 mM, 2.5 mM, 3mM and 3.5 mM and MC1R-snp1 at 2.5 mM.*

Stage 1	**Stage 2**			**Stage 3**	
95 °C	95 °C	47-49-51-53-57-61-65 °C	72 °C	72 °C	4 °C
5:00	00:15 min	00:15 min	00:30 min	07:00 min	∞
1 cycle	35 cycles			1 cycle	

Table iv. *Amounts of reagents used for PCR optimization. All volumes in µl.*

Reagents				
DNA (ca 20-50 ng/ul)	0.5	0.5	0.5	0.5
Foward primer (10uM)	1	1	1	1
Reverse primer (10uM)	1	1	1	1
10Xbuffer	2.5	2.5	2.5	2.5
Mg2+ (25mM)	2	2.5	3	3.5
dNTPs (20mM)	0.25	0.25	0.25	0.25
AT Gold Taq pol	0.125	0.125	0.125	0.125
H_20	19.625	17.125	19.625	19.625

Appendix E: PyroMark® PCR Kit and PCR protocol

Table i. *The protocol used for PCR of aDNA, by PyroMark® PCR kit (Qiagen). All volumes in µl.*

Reagents	**MC1R-snp1**	**MC1R-snp2**	**ASIP-snp and ASIP-del**
Mastermix	12.5	12.5	12.5
CoraLoad Concentrate	2.5	2,5	2.5
$MgCl_2$	1	0.5	1.5
Foward primer	0.5	0,5	0.5
Reverse primer	0.5	0.5	0.5
Rnase free water	3	3,5	0.5
Template aDNA	5	5	5

Table i. *The PCR-program used for PCR of ancient samples with MC1R-snp2 and the PyroMark® PCR kit (Qiagen).*

Stage 1	**Stage 2**			**Stage 3**	
95 °C	94 °C	60 °C	72 °C	72 °C	12 °C
15:00 min	0:30 min	0:30 min	0:30 min	10:00 min	∞
1 cycle	43 cycles			1 cycle	

Appendix F: Conventional PCR programs used for aDNA

Table i. *The PCR-program used for aDNA for MC1R-snp1 and MC1R-snp2 on the PCR thermal cycler. $MgCl_2$ is 2.5 mM and 2 mM respectively.*

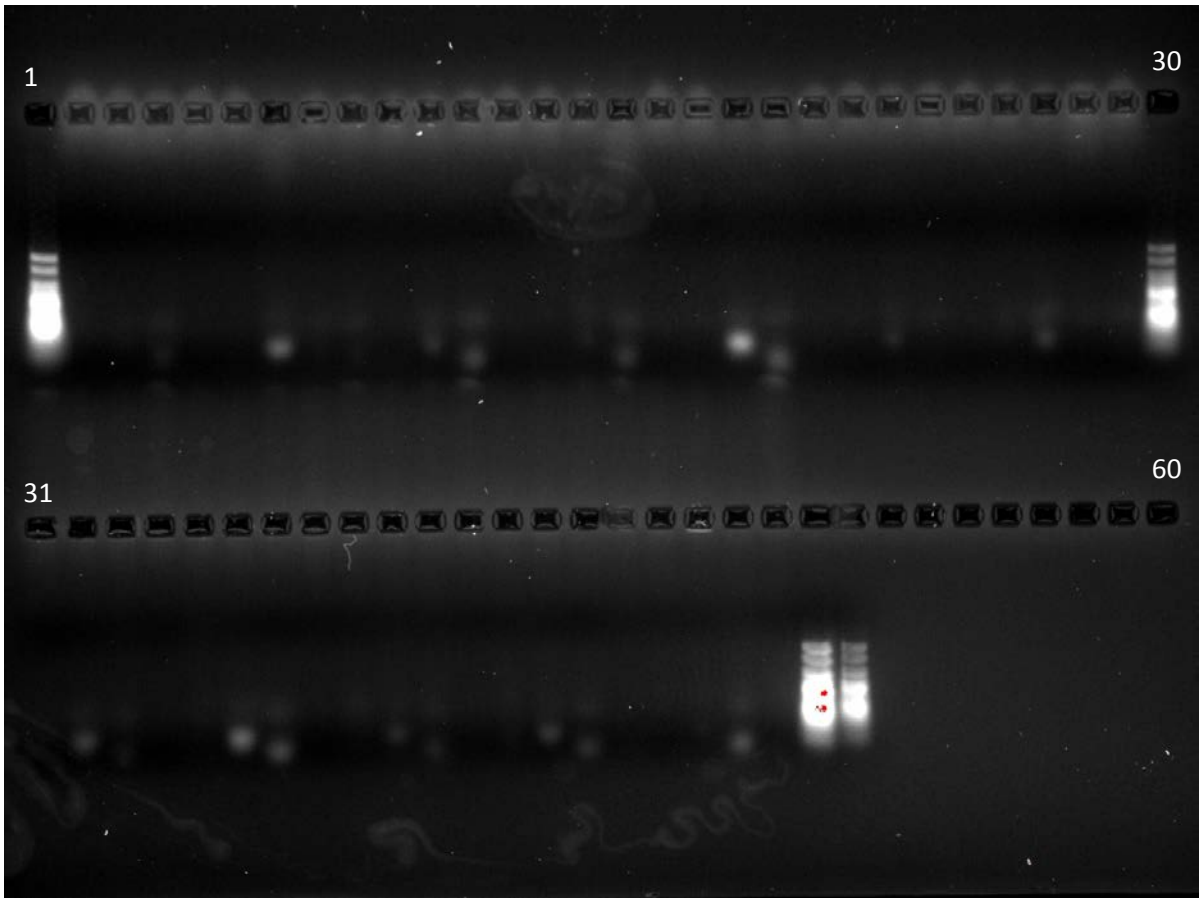
Stage 1	**Stage 2**			**Stage 3**	
95 °C	95 °C	60 °C	72 °C	72 °C	12 °C
15:00 min	0:30 min	0:30 min	0:30 min	10:00 min	∞
1 cycle	43 cycles			1 cycle	

Table i. *The PCR-program used for aDNA for ASIP-snp and ASIP-del on the PCR thermal cycler. $MgCl_2$ is for both 3 mM.*

Stage 1	**Stage 2**			**Stage 3**	
95 °C	95 °C	60 °C	72 °C	72 °C	12 °C
15:00 min	0:30 min	0:30 min	0:30 min	10:00 min	∞
1 cycle	43 cycles			1 cycle	

Appendix G- Agarose gel electrophoresis

Gel A

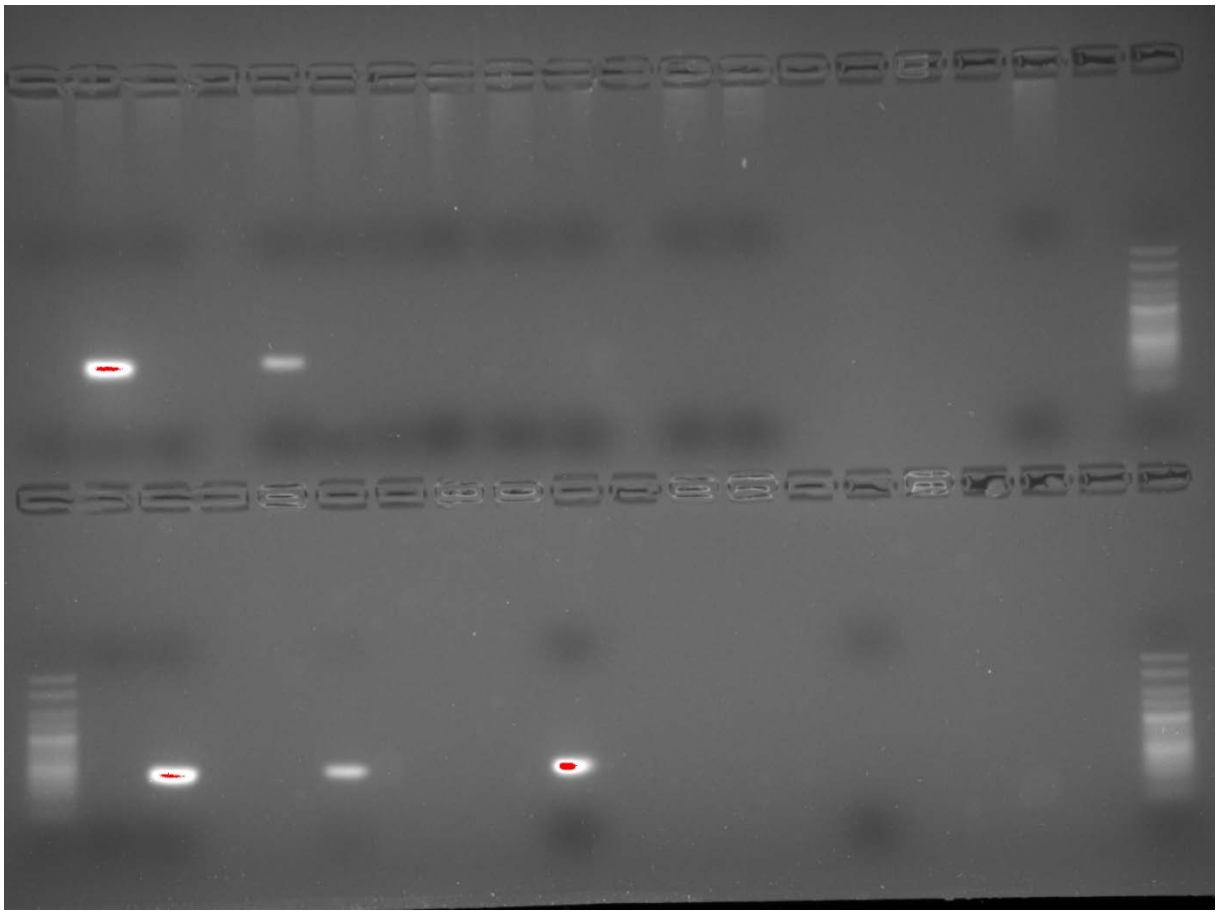


Gel A. Shows the first PCR optimization (table i Appendix D) of primer pairs MC1R-snp1, MC1R-snp2, ASIP-snp and ASIP-del on modern DNA. The name of each sample is written as following: annealing temperature, name of primer pair and $MgCl_2$ concentration.

1: Hyperladder V
2: 50 °C, MC1R-snp1, 2 mM
3: 50 °C, mc1r, snp2, 2 mM
4: 50 °C, asip-snp, 2 mM
5: 50 °C, asip-del,2 mM
6: 50 °C, mc1r-snp1, 2.5 mM
7: 50 °C, mc1r, snp2, 2.5 mM
8: 50 °C, asip-snp, 2.5 mM
9: 50 °C, asip-del,2.5 mM
10: 50 °C, mc1r-snp1, 3 mM
11: 50 °C, mc1r, snp2, 3 mM
12: 50 °C, asip-snp, 3 mM
13: 50 °C, asip-del,3 mM
14: 50 °C, mc1r-snp1, 3,.5 mM
15: 50 °C, mc1r, snp2, 3.5 mM
16: 50 °C, asip-snp, 3.5 mM
17: 50 °C, asip-del,3.5
18: 55 °C, mc1r-snp1, 2 mM
19: 55 °C, mc1r, snp2, 2 mM
20: 55 °C, asip-snp, 2 mM
21: 55 °C, asip-del,2 mM
22: 55 °C, mc1r-snp1, 2.5 mM

23: 55 °C, mc1r, snp2, 2.5 mM
24: 55 °C, asip-snp, 2,.5 mM
25: 55 °C, asip-del,2.5 mM
26: 55 °C, mc1r-snp1, 3 mM
27: 55 °C, mc1r-snp2, 3 mM
28: 55 °C, asip-snp, 3 mM
29: 55 °C, asip-del, 3 mM
30: Hyperladder V
31: 55 °C, mc1r, smp-1, 3.5 mM
32: 55 °C, mc1r, snp-2, 3.5 mM
33: 55 °C, asip-snp, 3,5 mM
34: 55 °C, asip-del, 3,5 mM
35: 60 °C, mc1r-snp1, 2 mM
36: 60 °C, mc1r-snp2, 2 mM
37: 60 °C, asip-snp, 2 mM
38: 60 °C, asip-del,2 mM
39: 60 °C, mc1r-snp1,2.5 mM
40: 60 °C, mc1r-snp2, 2.5 mM
41: 60 °C, asip snp, 2.5 mM
42: 60 °C, asip-del, 2.5 mM
43: 60 °C, mc1r_snp1,3 mM
44: 60 °C, mc1r-snp2, 3 mM
45: 60 °C, asip-snp2, 3 mM
46: 60 °C, asip-del, 3 mM
47: 60 °C, mc1r-snp1, 3,5 mM
48: 60 °C, mc1r-snp2, 3.5 mM
49: 60 °C, asip-snp, 3.5 mM
50: 60 °C, asip-del, 3,5 mM
51: Hyperladder V
52: Hyperladder V

Gel B

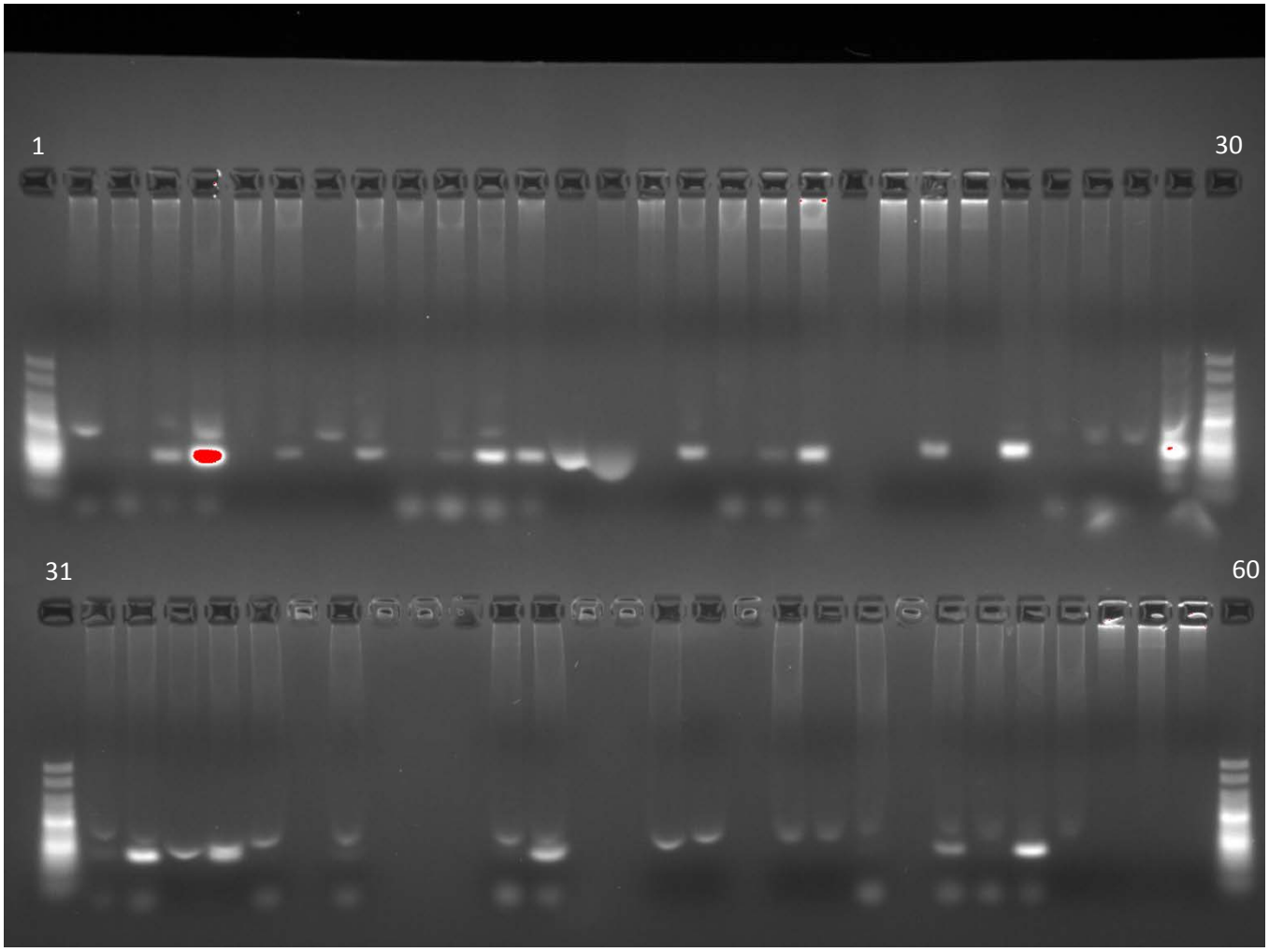


Gel B. Shows the PCR optimization of primers MC1R-snp1, ASIP-snp and ASIP-del using touchdown PCR (table ii Appendix D) on modern DNA and aDNA extracted from sheep of Gotland in wells 22-35 using MC1R-snp2 on the PyroMark® PCR Kit with a $MgCl_2$ concentration of 2mM (Appendix E). The name of each modern sample is written as following: name of primer pair and $MgCl_2$ concentration

1: MC1R-snp1, 2 mM
2: MC1R-snp1, 2.5 mM
3: MC1R-snp1, 3 mM
4:empty
5: MC1R-snp1, 3.5 mM
6:ASIP-snp,2 mM
7: ASIP-snp, 2.5 mM
8: ASIP-snp, 3 mM
9: ASIP-snp, 3.5 mM
10: ASIP-del, 2 mM
11: empty
12: ASIP-del, 2.5 mM
13: ASIP-del, 3 mM
14-17: empty
18: ASIP-del, 3.5 mM
19: empty
20: Hyperladder V
21: Hyperladder V
22: 3
23:23

24-25: empty
26: 12
27-29: empty
30 12
31-34 empty
35: -17
35: empty
40: Hyperladder V

Gel C



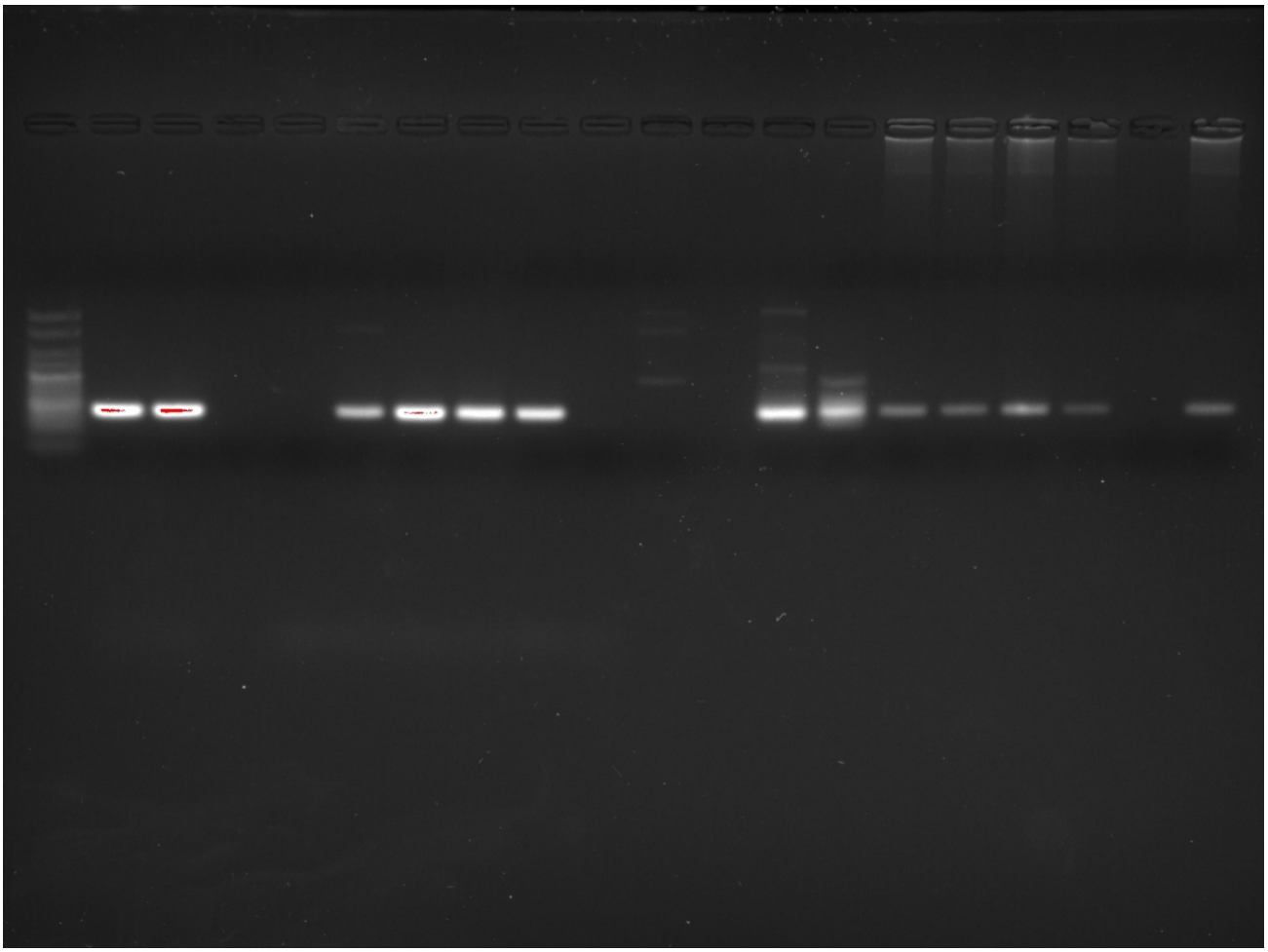
Gel C. Shows the PCR optimization of primers ASIP-snp and ASIP-del (table iii Appendix D). The name of each sample is written as following: name of primer pair, $MgCl_2$ concentration and annealing temperature.

1: Hyperladder V
2: asip-snp, 2 mM, 47 °C
3: asip-snp,2.5 mM, 47 °C
4: asip-snp, 3 mM, 47 °C
5:asip-snp, 3.5 mM, 47 °C
6:asip-del, 2 mM, 47 °C
7:asip-del, 2.5 mM, 47 °C
8: asip-del, 3 mM, 47 °C

9: asip-del, 3.5 mM, 47 °C
10: asip-snp, 2 mM,49 °C
11: asip-snp2, mM,49 °C
12. asip-snp-3 mM,49 °C
13: asip-snp-3.5 mM,49 °C
14: asip-del- 2 mM, 49 °C
15: asip-del-2.5 mM,49 °C
16: asip-del-3 mM,49 °C
17: asip-del, 3.5 mM,49 °C
18: asip-snp. 2 mM, 53 °C
19: asip-snp.2,5 mM,53 °C
20: asip-snp.3 mM, 3 °C
21: empty
22: asip-snp, 3.5 mM, 53 °C
23: asip-del, 2 mM, 53 °C
24: asip-del, 2.5 mM, 53 °C
25: asip-del, 3 mM, 53 °C
26-asip-del, 3.5 mM, 53 °C
27: asip-snp, 2 mM,57 °C
28: asip-snp, 3 mM,57 °C
29: asip-del: 2 mM, 57 °C
30: Hyperladder V
21: Hyperladder V
32: asip-snp, 2.5 mM,57 °C
33: asip-snp, 3.5 mM,57 °C
34: asip-del, 2.5 mM,57 °C
35: asip-del, 3 mM,57 °C
36: asip-del, 3.5 mM, 57 °C
37: empty
38: asip-snp, 2 mM,61 °C
39 - 42: empty
42: asip-snp, 2.5 mM,61 °C
43: asip-snp, 3 mM,61 °C
44-45: empty
46: asip-snp, 3.5 mM,61 °C
47: asip-del, 2 mM, 61 °C
48: empty
49: asip-del, 2.5 mM,61 °C
50: asip-del, 3 mM,61 °C
51: asip-del, 3.5 mM,61 °C
52: empty
53: asip-snp, 2 mM,65 °C
54: asip-snp-2.5 mM,65 °C
55: asip-snp-3 mM,65 °C
56: asip-snp-3.5 mM,65 °C
57: asip-del, 2 mM,65 °C
58: asip-del-2.5- mM,65 °C

59: asip-del, 3.5 mM,65 °C
60: Hyperladder V

Gel D

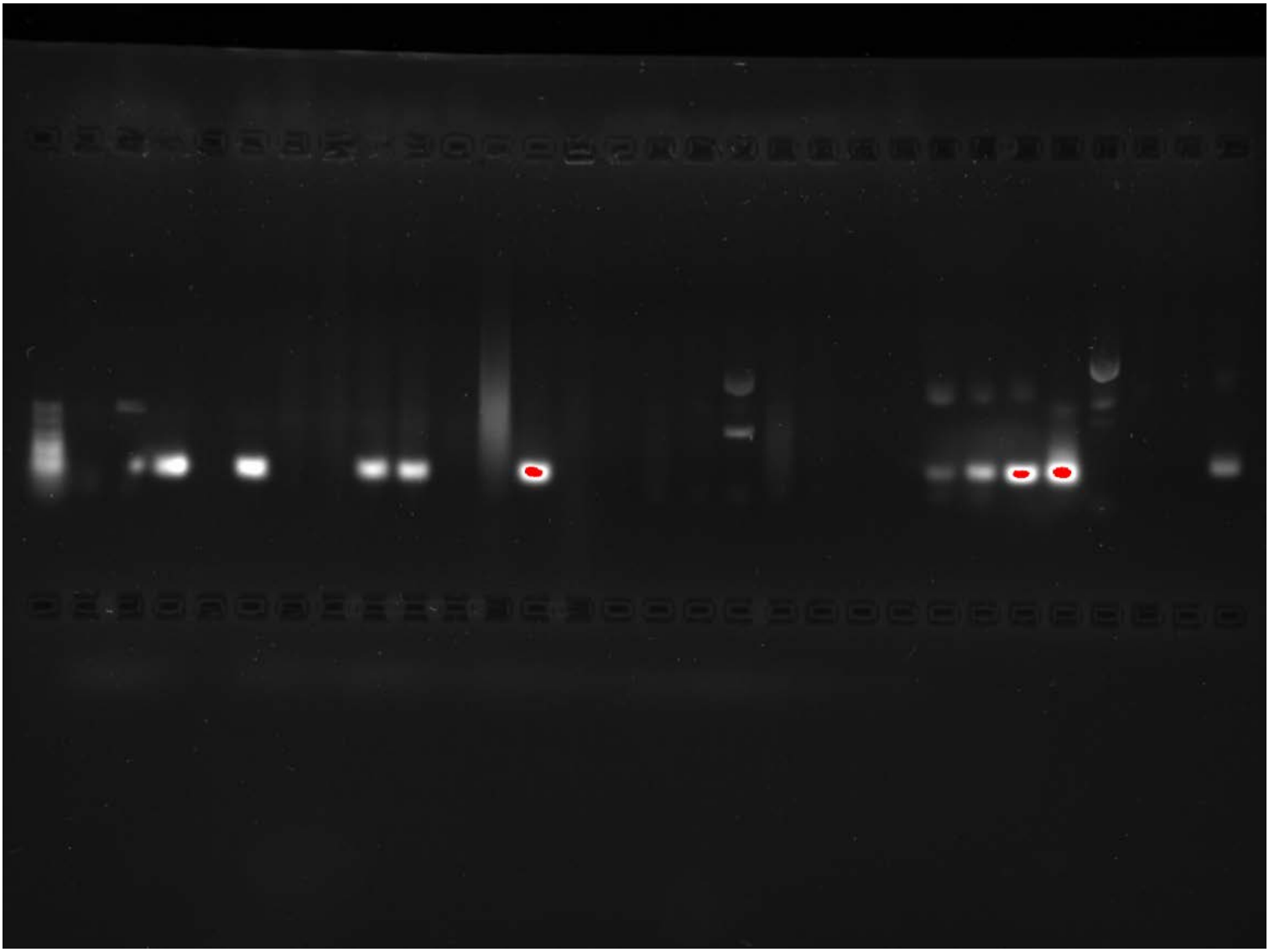


Gel D. Shows the rest of the PCR optimization primer pair MC1R-snp1 at a MgCl2 concentration of 2.5 mM (table iii Appendix D) well 15-20 and ancient samples using primer pair MC1R-snp2 and the PyroMark® PCR Kit (Appendix E) well 5-10.Well 2-3 contain ancient samples using the MC1R-snp1 primer pair and the PyroMark® PCR Kit at a $MgCl_2$ concentration of 2.5 mM. Well 11-12 contain the ancient samples using primer pair MC1R-snp2 with conventional PCR (see table i Appendix F). Well 13-14 ancient samples using primer pair MC1R-snp1 with conventional PCR (see talbe i Appendix F). The name of each modern sample is written as following: name of primer pair, $MgCl_2$ concentration and annealing temperature.

1: Hyperladder V
2: a
3:b
4:empty
5: c
6:d
8:e
9: f
10: g
11: a2
12:b2

13:a3
14:b3
15: mc1r-snp1, 2 mM, 47 **°C**
16: mc1r-snp1, 2 mM, 49 **°C**
17: mc1r-snp1, 2 mM,-53 **°C**
18: mc1r-snp1, 2 mM,57 **°C**
19: mc1r-snp1, 2 mM,61 **°C**
20: mc1r-snp1, 2 mM, 63 **°C**

Gel E

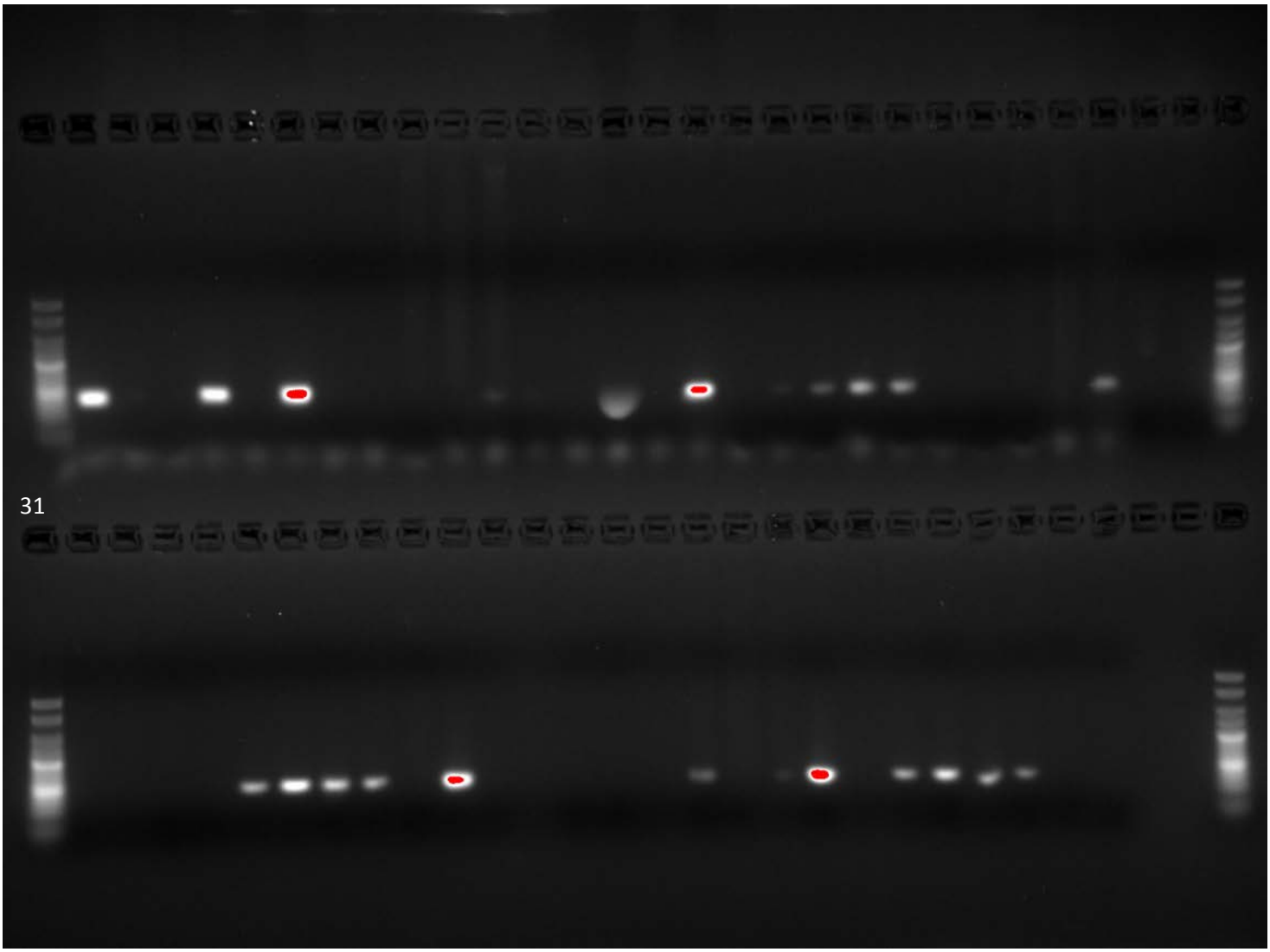


Gel E. Well 2-23 show ancient samples using primer pair MC1R-snp2 and the PyroMark® PCR Kit and PCR program (see Appendix E). Well 24-26 contain ancient samples using MC1R-snp2 and conventional PCR (see Appendix F). Well 27-28 contain ancient samples using ASIP-snp and well 29-30 ancient samples using ASIP-del (see Appendix F).

1:Hyperladder V
2: h
3:i

4:j
5:k
6:l
7:m
8: n
9:o
10:p
11:q
12:r
13:s
14:t
15:u
16:v
17:x
18:y
19:z
20:å
21:beta
22:gamma
23:delta
24:a2:1
25:b2:1
26:g1
27:a4
28:b4
29:a5
30:a5

Gel F

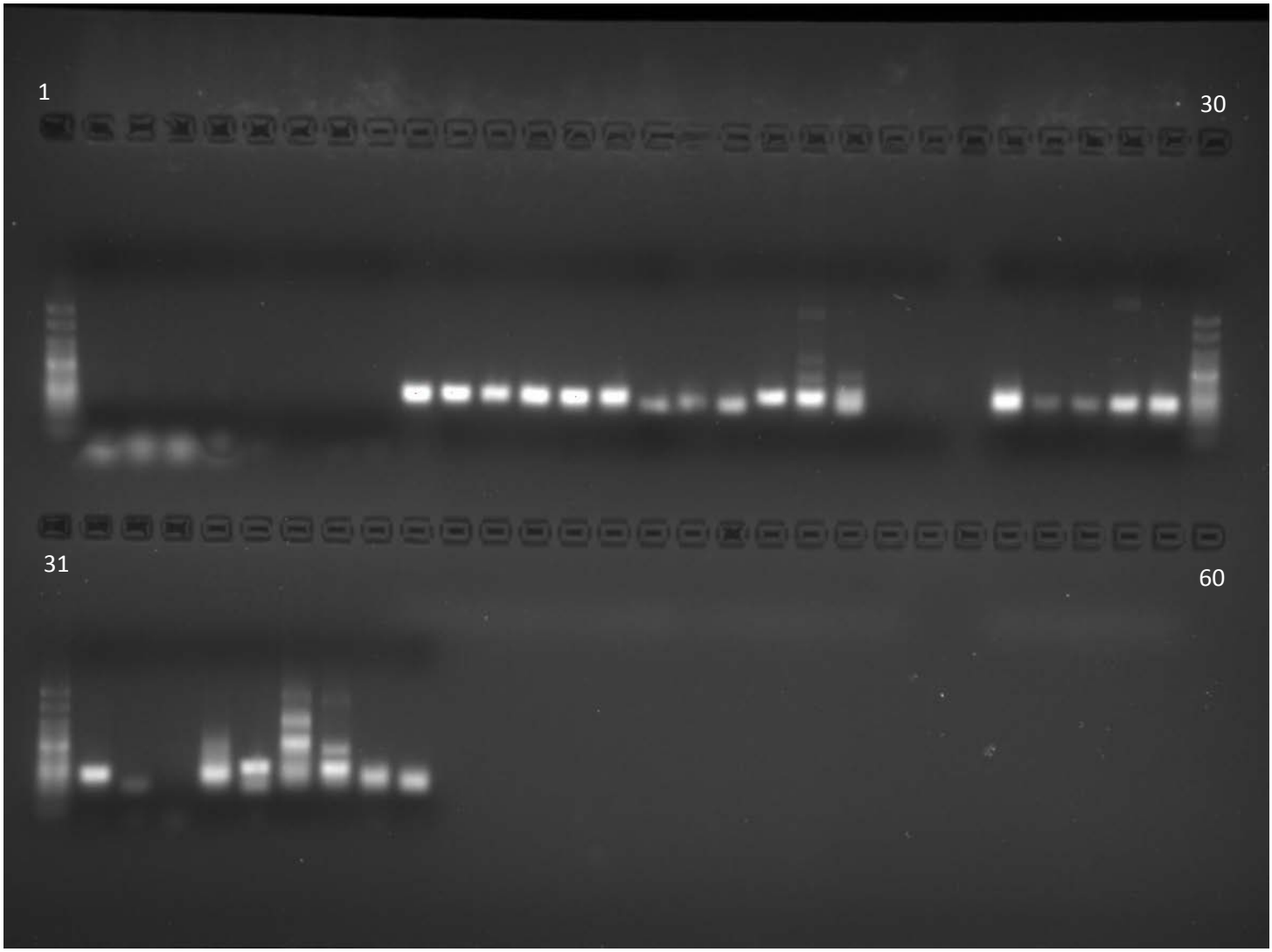


Gel F. Show PCR products of ancient samples using conventional PCR and MC1R-snp1 primer pair (see Appendix F).

1. Hyperladder V
2. w1
3:ö1
4: d1
5:e1
6:f1
7:g1
8:h1
9:j1
10: o1
11:p1
12:s1
13: y1
14: beta1
15:-6,1
16:-5,1
17. w2
18:ö2
19: d2

20:e2
21:f2
22:g2
23:h2
24:j2
25: o2
26:p2
27:s2
28: y2
29: beta2
30: Hyperladder V
31: Hyperladder V
32:-6,2
33:-5,2
34: j3
35:h3
36: g3
37: f3
38:e3
39: d3
40:ö3
41:w3
42:?
43:-5,3
44: -6,3
45:beta3
46:y3
47: s3
48: p3
49: o3
50:w4
51: ö4
52:d4
53: e4
54:f4
55:g4
56:h4
57:j4
58-59: empty
60: Hyperladder V

Gel G



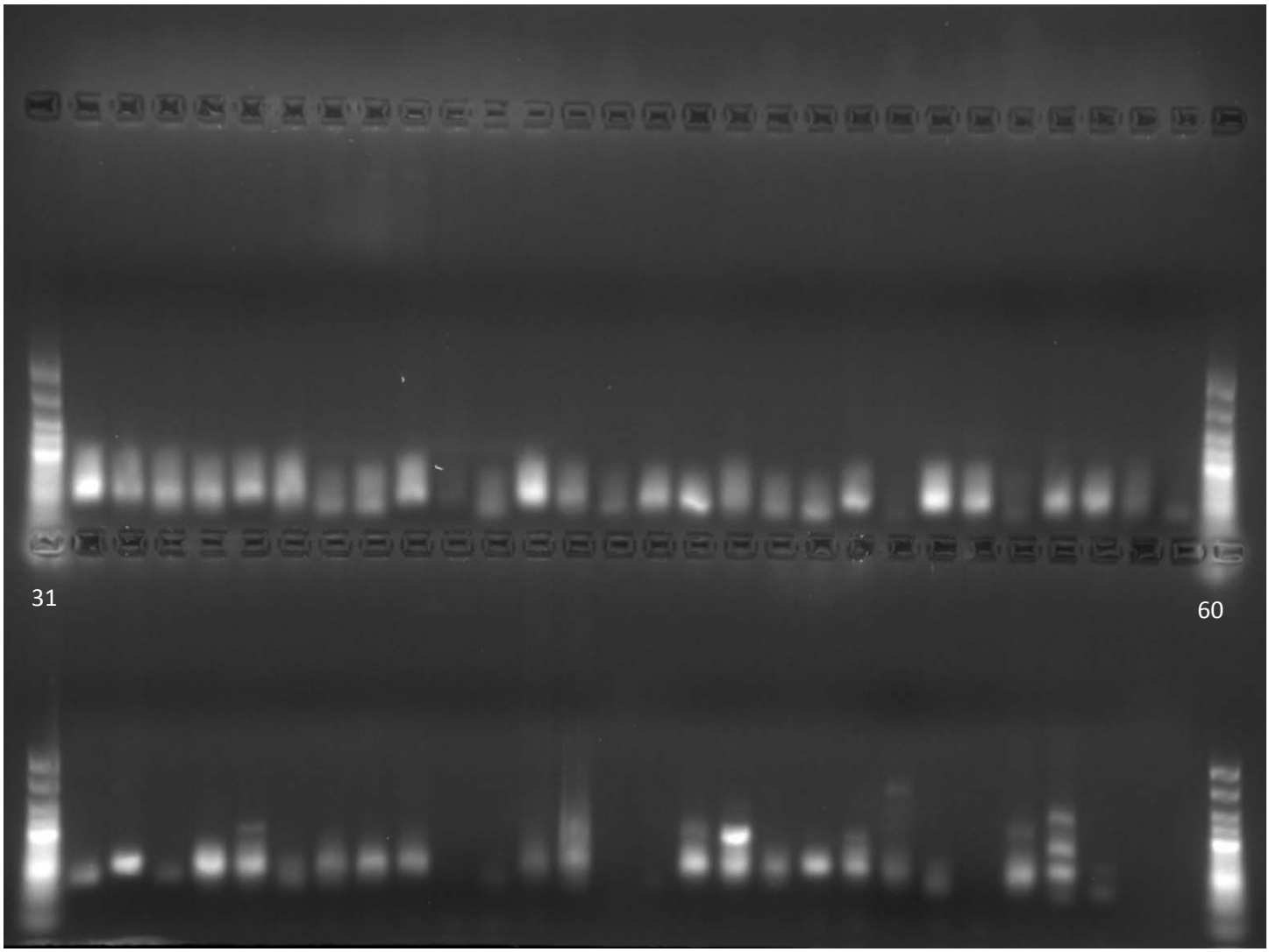
Gel G. Show PCR products of ancient samples using PyroMark® PCR Kit and ASIP-del (well 10-24) and ASIP-snp (well 24- 41) (for PCR program see Appendix F). Well 2-5 contains ancient samples using conventional PCR and ASIP-del, whereas well 6-9 contains ancient samples using conventional PCR and ASIP-snp. For PCR programs see Appendix F.

1:Hyperladder V
2: a6
3:b6
4:d123
5: e123
6:a7
7:b7
8:d143
9:e143
10: w9
11:ö9
12:d9
13:e9
14: f9
15: g9

16: h9

17:j9
18: o9
19: p9
20: s9
21: y9
22: beta9
23: -6,9
24:-5,9 (empty)
25:w13
26: ö13
27: d13
28: e13
29:f13
30:Hyperladder V
31: Hyperladder V
32: g13
33:h13
34:j13
35:o13
36:p13
37: s13
38: y13
39: beta13
40: -6,13
41:-5,13 (empty)

Gel H

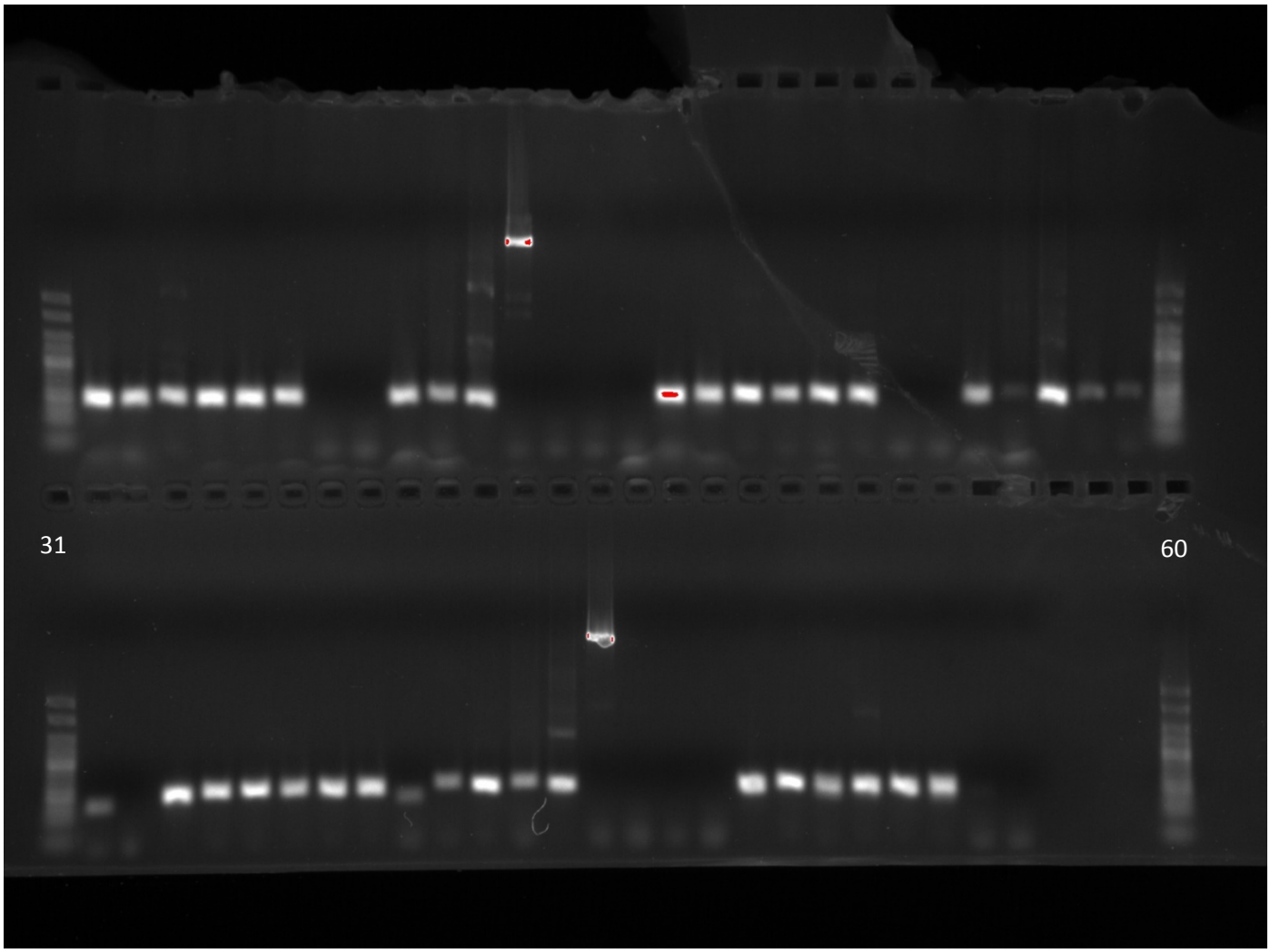


Gel H. Show PCR products of ancient samples using PyroMark® PCR Kit and ASIP-del (well 2-34) and ASIP-snp (well 35- 56) (for PCR program see Appendix E).

1: Hyperladder V
2: w10
3: ö10
4: d10
5:e10
6:f10
7: g10
8: h10:
9: j10
10: o10
11:-3,10
12: -15:10
13: w11
14:ö11
15: d11
16:e11
17: f11
18: g11
19: h11
20: j11

21: o11
22: -15,11
23: w12
24: ö12
25: d12
26: e12
27: f12
28: g12
29: h12
30: Hyperladder V
31: Hyperladder V
32: j12
33: o12
34: -15,12
35: w, 14
36: ö,14
37: d14
38: e14
39: f14
40: g14
41: h14
42: j14
43: o14
44:p14
45: tom
46: -15,14
47: w15
48: ö15
49: e15
50: f15
51: g15
52: h15
53: j15
54: o15
55: p15
56: -15,15
57-59: empty
60: Hyperladder V

Gel I

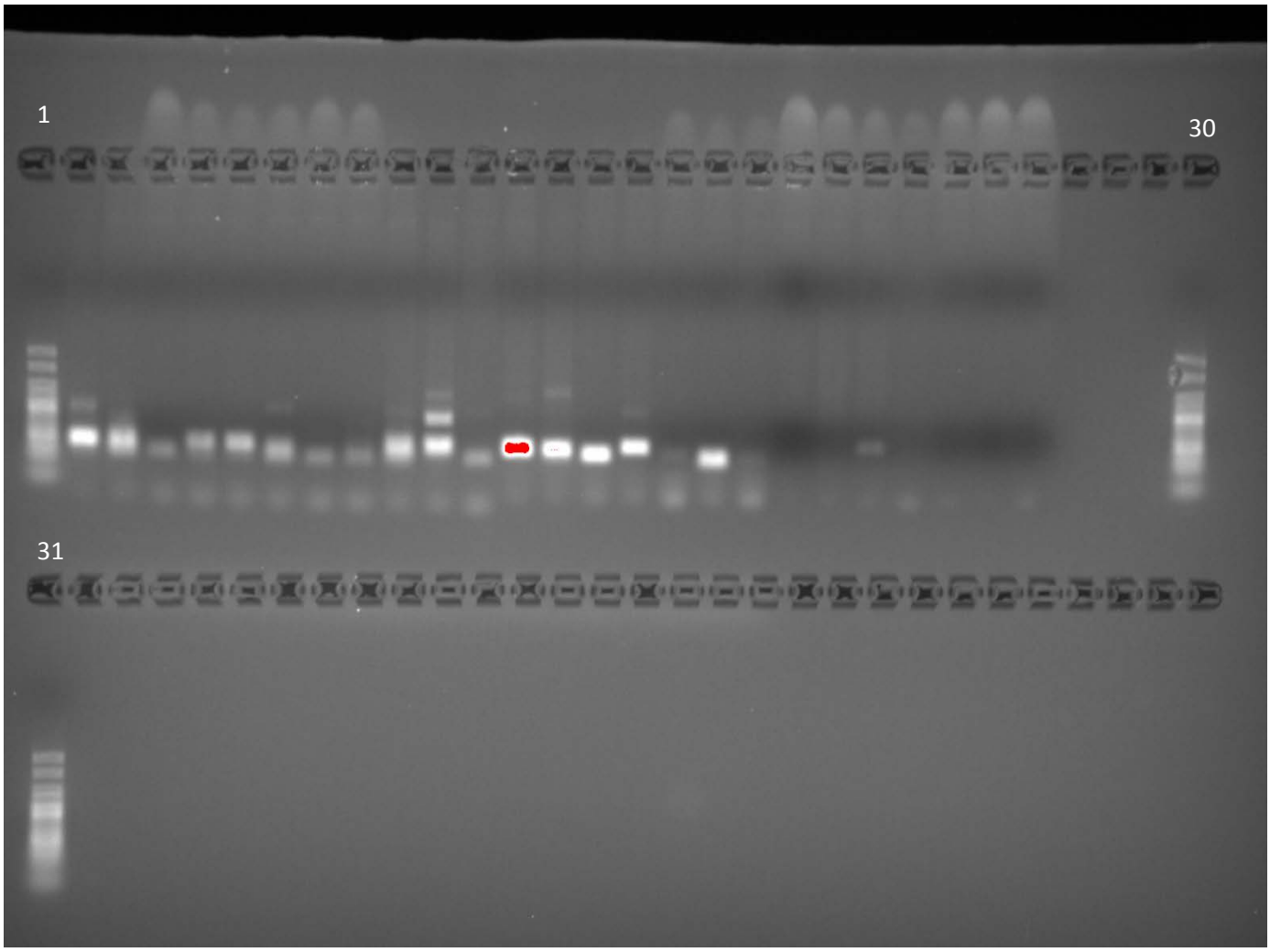


Gel I. Show PCR products of ancient samples using PyroMark® PCR Kit and MC1R-snp2 (see Appendix E).

1: Hyperladder V
2: w5
3:ö5
4: d5
5: e5
6: f5
7: g5
8: h5
9: j5
10: o5
11: p5
12: s5
13: y5
14: beta5
15. -6,5
16. 5,5
17: w6
18:ö6
19: d6
20: e6

21: f6
22: g6
23: h6
24: j6
25: o6
26: p6
27: s6
28: y6
29: beta6
30: Hyperladder V
31: Hyperladder V
32. -6,6
33. 5,6
34: w7
35:ö7
36: d7
37: e7
38: f7
39: g7
40: h7
41: j7
42: o7
43: p7
44: s7
45: y7
46: beta7
47. -6,7
48. 5,7
49: w8
50: ö8
51: d8
52: e8
53: f8
54: g8
55: h8
56: j8
57-59:empty
60: Hyperladder V

Gel J



Gel J. Well 2-12 contains PCR products for ancient samples ASIP-snp and using the PyroMark® PCR Kit and PCR program (see Appendix E). Well12-19 contains ancient samples using MC1R-snp2 and the PyroMark® PCR Kit and PCR program (Appendix E). Well 20-26 contain ancient samples using MC1R-snp1 and conventional PCR (see Appendix F).

1: Hyperladder V
2: w16
3: ö16
4: d16
5: e16
6: f16
7: g16
8: h16
9: j16
10: o16
11: p16
12: -15,16
13: o8
14: p8
15: s8
16: y8
17: beta8
18: -6,8
19: -5,8
20: o4
21: p4
22: s4

23: y4
24: beta4
25: -6,4
26: -5,4
27-29:
30: Hyperladder V
31: Hyperladder V