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A Comparison of the AMELY Gene Sequence in Argali (*Ovis ammon*) and Domestic (*Ovis aries*) Sheep

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A COMPARISON OF THE AMELY GENE SEQUENCE IN ARGALI (OVIS AMMON) AND DOMESTIC (OVIS ARIES) SHEEP

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Abstract

Amelogenin (AMEL) is a gene responsible for tooth bud development. It is located on the X-chromosome, thus called AMELX, in mammals. AMEL has been shown to be present in fish, amphibians, and reptiles, though the exact location on the chromosome has not been determined. Amelogenin-like gene (AMELY), an AMEL homolog encoded on the Y-chromosome in some mammals, including sheep, cattle, deer, bears, humans, and some primates, is shorter than the sequence on the X-chromosome. It is unknown whether AMELY is transcriptionally active, but it has been found to be useful for human sexing purposes in forensics, archaeology and prenatal diagnosis, as well as in animal and beef sexing by the use of a simple polymerase chain reaction (PCR) with primers designed to flank the deletion region of AMELY.

This project involved isolating and sequencing the deletion region of AMELY from male Argali sheep (*Ovis ammon*) DNA. Research methods included culture of the Argali cells, extraction and purification of genomic DNA from the cells, PCR of a portion of the AMEL sequence, gel electrophoresis and purification of the PCR product bands, and preparation and analysis of the sample for sequencing. It has been hypothesized that there is a DNA sequence polymorphism between the deletion region of the wild Argali AMELY sequence compared to the previously published AMELY sequence of the domestic sheep (*Ovis aries*).

Results discussed in this paper show that no evidence of a DNA polymorphism between the wild and domestic sheep species is present. The Argali AMELY sequence appears to be identical to that of the domestic sheep.

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Introduction and Review of Literature

Amelogenin proteins play a critical role in the developing tooth bud in mammals. Amelogenins form the extracellular matrix of the fetal enamel tissue (Termine *et al.* 1980). Since the amelogenin protein is only expressed in the developing tooth, cDNA samples of the gene were hard to come by in humans, therefore much of the research of AMEL was limited to cattle and mice (Nakahori *et al.* 1990). In 1989, Lau *et al.* reported finding the chromosomal location of AMEL in the human and mouse genomes by Southern blot analysis. They determined that in humans, AMEL is located on the short arm of the X-chromosome (thus called AMELX) in the p22.1-p22.3 region, and on the Y-chromosome (called AMELY) in the proximal long arm (Yq11) region near the centromere. In the mouse, AMEL is located solely on the X-chromosome.

Sasaki and Shimokawa (1995) summarized two reports about a variety of other mammalian species that have the AMEL gene and its homolog located on both the X- and Y-chromosomes like in humans, or, as found in the mouse, only localized on the X-chromosome. Old world monkeys, such as the Japanese monkey, rhesus monkey, crab-eating macaque and talapoin, and a new world monkey, the tufted capuchin monkey, have AMEL on both sex chromosomes. Also in this group are the gorilla, chimpanzee, and orangutan. Animals with AMEL solely on the X-chromosome include old world monkeys: hamadryas baboon, patas monkey, and green monkey, and a new world monkey, the cotton-headed tamarin. Rats, like mice, have AMEL on the X-chromosome only.

Several species of wild and domestic cattle, including European domestic cattle (Gibson *et al.* 1991), banteng, gaur, European bison, water buffalo, African buffalo and

anoa (Weikard *et al.* 2006) have been shown to possess both AMELX and AMELY genes. Sheep (Pfeiffer and Brenig 2005, Weikard *et al.* 2006), European red deer (Pfeiffer and Brenig 2005), Japanese black bear (Yamamoto 2002), goat (Weikard *et al.* 2006), pig (Ikawa *et al.* 2005) and horse (Hasegaw *et al.* 2000) are other mammalian species that have been reported to have both AMEL genes on the X- and Y-chromosomes.

In marsupial (wallaby) and monotreme (platypus and echidna) studies of AMEL, it was found that the gene is located on autosomal chromosomes rather than on one or both sex chromosomes as in the examples described previously (Sasaki and Shimokawa 1995, Toyosawa *et al.* 1998). Toyosawa *et al.* (1998) also studied reptile (caiman) and amphibian (African clawed frog) species. They found that AMEL is present in these species, and in the case of the African clawed frog, AMEL is transcribed from two distinct loci. However, the precise chromosomal location of AMEL in reptiles and amphibians was not discussed.

It has been suggested that AMEL is even present in the bony fish species *Anarrhichas lupus* or wolf fish. An experiment using wolf fish DNA hybridized to a mouse AMEL probe under stringent conditions exhibited one band at 18 kb. It is not known, however, whether the AMEL gene in this fish is expressed or not (Sasaki and Shimokawa 1995).

Sex Determination Using the Amelogenin Locus

The fact that many species have both X- and Y-specific homologs of AMEL has become

useful to scientists for determining the sex of an unknown DNA sample. Because the deletion region of the sequence on the Y-chromosome makes AMELY a shorter sequence than AMELX, a simple polymerase chain reaction (PCR) involving a single primer pair spanning the deletion region can easily and quickly identify the sex of a sample. If gel electrophoresis of the amplified PCR product shows two bands, the sample is said to be genetically male since the primers have amplified two different lengths of AMEL from both the X- and Y-chromosomes. A female sample shows only one band of the larger size (the X-specific product) because only X-chromosomes are present to act as template for the reaction (Ennis and Gallagher 1994, Weikard *et al.* 2006). Such PCR experiments have been carried out successfully using tiny amounts of genomic DNA from meat, hair, bone, embryo biopsies (Weikard *et al.* 2006), blood, and skin samples.

The ease, accuracy and robustness of the AMEL PCR method of sex determination have been cited numerous times by scientists. Previous sexing methods used similar PCR techniques to amplify Y-specific gene sequences. In this case, if the sample was male, a product would be amplified and seen as a band on an agarose gel. Female samples would have no amplified product. Problems occurred when PCRs failed, thus showing no product band, and the samples were incorrectly identified as female. A separate set of primers, homologous to internal sequences present on both the X- and Y-chromosomes, were needed to control for PCR failure. Multiplex PCRs come with their own set of problems when oftentimes one or more of the primer sets fails to amplify, thus leading to false results. The AMEL PCR method uses only one set of primers, common to both male and female samples, and thus eliminates the

problems associated with multiple primer reactions and the false negatives that come with them (Ennis and Gallagher 1994).

Applications for the use of AMELX and AMELY to determine both the species and sex of an individual are many. Animal production, forensics, population research, and monitoring within the family *Bovidae* were reasons cited by Weikard *et al.* (2006), and similar applications can be applied to any of the species mentioned above.

In Europe, male beef meat is considered to be a higher quality than female beef and thus costs more. By using the AMEL PCR method, Zeleny *et al.* (2002) discovered that they could accurately, reliably, and quickly determine the sex of their bovine meat samples. Quality control measures like beef sexing help to prevent deception in the European beef market and to ensure that export refunds are properly allocated.

Prenatal diagnosis via PCR testing on the AMEL locus is also possible. Screening for X-linked genetic disorders is a commonly cited reason. For example, the amplification of AMEL via PCR can be used to characterize sex chromosome abnormalities in the case of Klinefelter's syndrome (XXY) (Sasaki and Shimokawa1995). Other uses for the AMEL PCR technique include determining the sex of a fetus in early pregnancy by detection of AMEL in maternal plasma (Zhu *et al* 2005) and identifying the sex of a fetus or infant with ambiguous genitalia (Caenazzo *et al.* 1997-1998).

Amelogenesis Imperfecta

Amelogenesis imperfecta is a human genetic disorder that impacts 1 in 14,000 individuals (Lau *et al*. 1989). This inherited disease affects the formation of tooth enamel in primary and permanent dentition. Clinical manifestations show two

phenotypes in general. The first type of amelogenesis imperfecta described is hypoplastic. This phenotype is characterized by abnormal thickness of enamel during development. The second phenotype, hypomineralization, is distinguished by enamel that is softer than normal (Sasaki and Shimokawa 1995).

Amelogenesis imperfecta appears to be a genetically heterogeneous disorder. Autosomal dominant, autosomal recessive, X-linked dominant and X-linked recessive Mendelian inheritance patterns have been described (Lau *et al.* 1989, Nakahori *et al.* 1991, Sasaki and Shimokawa 1995). It has been suggested that an X-linked type of amelogenesis imperfecta, manifested in heterozygous females by vertical markings of the enamel, is caused by a structural mutation in the AMEL gene (Lau *et al.* 1989, Sasaki and Shimokawa 1995). Affected (hemizygous) males and (heterozygous) females in the same family showed different clinical manifestations. Various cases of families affected by amelogenesis imperfecta demonstrated the genetically heterogeneous nature of the disorder, in which several different kinds of mutations of AMEL on the X-chromosome were implicated (Sasaki and Shimokawa 1995). Autosomal forms of the disorder are probably consequences of structural defects or changed regulation of genes that create other enamel matrix products such as enamelin (Lau *et al.* 1989).

Argali Sheep

The Argali sheep (*Ovis ammon*) is an endangered wild sheep species native to high mountainous regions of central Asia. Argali are the largest wild sheep species in the world, growing between 120-200 cm/ 4-6.6 ft long and 90-120 cm/ 3-4 ft tall (Huffman 2004) and weighing 60-185 kg/ 132-407 lb (Tonda 2002). Males are highly sought after

for their massive cork-screw horns which can reach up to 190 cm/ 6.3 ft when measured on the spiral (Huffman 2004).

Research Objectives

The purposes of this project were (1) to isolate and sequence a portion of the Argali genomic DNA and (2) to align the sequence obtained with other DNA sequences of domestic sheep to find possible polymorphisms between the two species. It was hypothesized that since the two sheep species are different, one being wild and the other being domestic, that the DNA sequences of the AMELY deletion regions would be different for each species.

Materials and Methods

Argali cell culture

An Argali cell line, established from skin biopsies of an adult Argali ram from the People's Republic of China (White *et al.* 1999), and cryopreserved in liquid nitrogen at subculture number three by White's lab, were thawed, and a new culture was started. The cells were cultured in a T-75 flask in Dulbecco's modified Eagle's medium (DMEM) +10% fetal bovine serum (HyClone, Logan, UT) in a humidified 37°C incubator with 5% CO_2 and air. They grew until confluent and ready for harvesting for DNA extraction.

DNA extraction

DNA from the Argali cells was extracted using the Puregene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). Cultured Argali cells were collected in suspension and stored on ice. Cell number was determined by counting with a hemocytometer and Trypan Blue staining. Cells in culture media were added to a 1.5 ml microfuge tube and were centrifuged for 5 sec at 13,000-16,000 x g (all centrifugation steps were performed at this setting) to pellet cells. Supernatant was removed by aspiration, leaving behind 10-20 µl residual liquid. The tube was vortexed vigorously to resuspend the cells in the residual supernatant. 300 µl Cell Lysis Solution was added to the cells, followed by vortexing on high speed for 10 sec to lyse the cells.

RNase treatment of the cell lysate was carried out by addition of 1.5 µl RNase A Solution to the cell lysate. The sample was mixed by inverting the tube 25 times and incubating it at 37°C for 5 min. The sample was cooled to room temperature by placing on ice for 1 min, and then 100 µl Protein Precipitation Solution was added to the RNase

A-treated lysate. The sample was mixed by vortexing at high speed for 20 sec. The tube was then centrifuged for 1 min to pellet precipitated proteins.

The supernatant containing the DNA was poured into a clean 1.5 ml microfuge tube containing 300 µl 100% isopropanol. The sample was mixed by inverting 50 times, followed by a 1 min spin. The supernatant was poured off and the tube was drained on a paper towel. 300 µl 70% Ethanol was added to the sample and the tube was inverted several times to wash the DNA pellet. After a 1 min centrifugation, the ethanol was poured off and the tube was allowed to drain on a paper towel for 15 min.

Following the standard DNA hydration protocol, 50 µl DNA Hydration Solution was added to the dry DNA pellet. DNA was rehydrated by incubating at 65°C for 1 hour and at room temperature overnight. Hydrated DNA was stored at 4°C.

Polymerase Chain Reaction

The genomic DNA sample extracted and purified above was then used as the template in a PCR reaction to amplify the amelogenin-like gene sequence on the Y chromosome. PCR was performed using Epicentre's FailSafe PCR reagents (Madison, WI, USA). A 25 µl reaction containing 0.625 U Enzyme Mix, 0.5 µM each SE 47 (5'-CAGCCAAACCTCCCTCTGC-3') and SE 48 (5'-CCCGCTTGGTCTTG-TCTGTTGC-3') primers (Ennis and Gallagher 1994), 1X PreMix D (100 mM Tris-HCl (pH 8.3, 22°C), 100 mM KCl, 400 µM each dNTP, MgCl2, PCR Enhancer with Betaine), and 380 ng genomic DNA was set up and run in a BioRad Icycler Thermal Cycler (Hercules, CA, USA). An initial denaturation step at 97°C for 3 min was followed by 32 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 59°C, and 1 min extension at 72°C. A final

extension at 72°C for 10 min followed.

Gel Electrophoresis

Separation of PCR products was carried out on a 2% agarose gel stained with 5 mg/ml ethidium bromide. The samples were run next to Fisher Scientific's (Hampton, NH, USA) exACTGene 100 bp ladder in TAE buffer at 100V for 45 min to 1 hour. Bands were visualized using a UV light box, and gels were photographed using an Alpha Innotech MultiImage Light Cabinet (San Leandro, CA, USA). Y-specific bands, 217 bp in length, were excised using a sharp scalpel.

Purification of PCR products

Gel purification of PCR products was carried out using Qiagen's QIAquick Gel Extraction Kit (Hilden, Germany). Excised bands were weighed and placed in a 1.5 ml clear microfuge tube. Three gel volumes of Buffer QG were added to the gel slice and the tube was incubated for 10 min at 50°C. When the gel slice was completely dissolved, one gel volume of 100% isopropanol was added to the sample. The tube was mixed well, and the contents were added to the QIAquick spin column assembly. The sample was centrifuged at ~10,000 x g (all centrifugation steps were carried out at this setting) for 1 min. Flow-through waste was discarded, and the spin column was replaced in the collection tube. Once the entire volume of sample had been applied to the spin column, 500 µl Buffer QG was added to remove all traces of agarose, and the column was centrifuged for 1 min. After discarding the flow-through, 750 µl Buffer PE (wash buffer) was added, and the column was spun for 1 min. Flow-through was

discarded and the column was returned to the centrifuge for an additional 1 min spin. 30 µl Buffer EB (10mM Tris-Cl, pH 8.5) was added directly to the filter in the spin column and was allowed to stand for 1 min before a final 1 min spin to elute the purified DNA into a clean 1.5 ml tube. Sample concentrations were determined using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

DNA sequencing

The Argali DNA samples were delivered to the USU Sequencing Lab and were sequenced using the same primers as were used for PCR.

Sequence Analysis

Using Vector NTI (Invitrogen, Carlsbad, CA, USA) and BioEdit (Ibis Therapeutics, Carlsbad, CA, USA) software, raw DNA sequence obtained from the Argali Y-specific PCR amplicons were aligned to the Ovine AMELX and Ovine AMELY sequences.

Results

Using two different sets of primers (SE 47/48 and SGAM) to amplify the Y-specific deletion region of the AMEL gene proved successful with Argali genomic DNA as shown by clear and distinct bands on the agarose gel (see **Figure 1**, below). Y-specific amplicons from both primer pairs were used for direct sequencing, but the best sequence results were obtained by using the SE 47 and SE 48 primers for amplification and subsequent sequencing, as described in Materials and Methods.

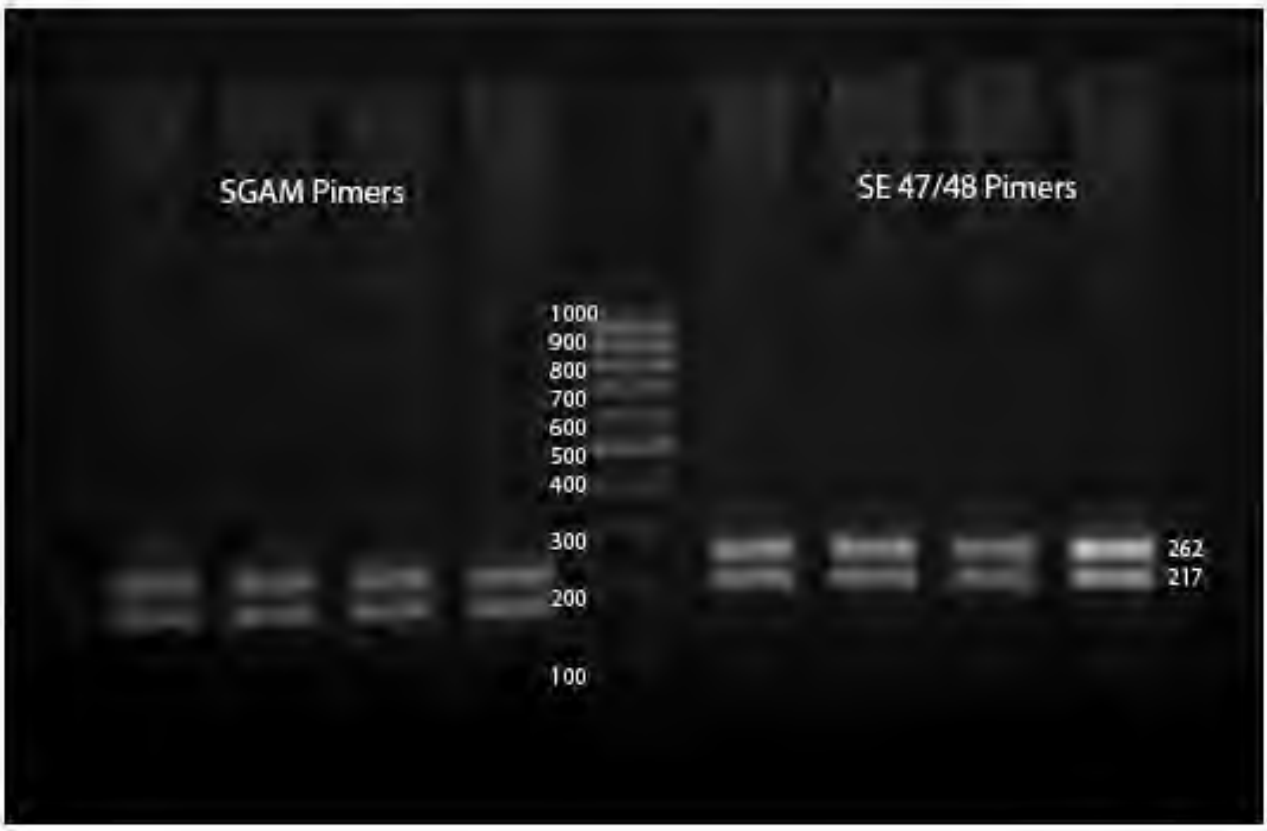

Figure 1. Argali SGAM and SE 47/48 gel.

Results from several attempts at sequencing the deletion region of the Argali Y-specific portion of AMEL did not show evidence of any DNA sequence polymorphisms as compared to the same region of the AMELY sequence of the domestic sheep. As

shown in **Figure 2**, below, there is 100% homology between the wild and domestic sheep sequences in the deletion region amplified by SE 47 and SE 48 primers.

```
                          10         20         30         40
Ovine AMELX:  CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC
Ovine AMELY:  CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC
Argali AMELY:  CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC

                   50         60         70         80
90
OX: CCCAGTCCAT CCAGCCGCAG CCTCACCAGC CCCTGCAGCC CCTGCAGCCC
OY: CCCAGCCCAT CCAGCCACAG CCTCACCAAC CCCTGCAGCC CC * * * * * * * * *
AY: CCCAGCCCAT CCAGCCACAG CCTCACCAAC CCCTGCAGCC CC * * * * * * * * *

                  100        110        120        130
140
OX: ATGCAGCCCT TCGCGCCCTT GCAGCCCCTG CAGCCCCAGC CACCCGTGCA
OY: * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *AGC CACCCGTGCA
AY: * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *AGC CACCCGTGCA

                  150        160        170        180        190
OX: CCCCATCCAG CCCTTGCCGC CACAGCCACC TCTGCCTCCG ATATTCCCCA
OY: CCCCATCCAG CGCTTGCCAC CACAGCCACC TCTGCCTTCA ATATTCCCCA
AY: CCCCATCCAG CGCTTGCCAC CACAGCCACC TCTGCCTTCA ATATTCCCCA

                  200        210        220        230        240
OX: TGCAGCCTCT GCCCCCCATG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA
OY: TGCAACCTCT GCCCCCTGTG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA
AY: TGCAACCTCT GCCCCCTGTG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA

                  250        260
OX: GCAACAGACA AGACCAAGCG GG
OY: GCAACAGACA AGACCAAGCG GG
AY: GCAACAGACA AGACCAAGCG GG
```

Figure 2. Alignment of Argali and domestic sheep AMEL sequences. Asterisks indicate deletions.

Discussion

In previous experiments that compared domestic sheep and goat AMEL sequences, it was found that the 45 bp deletion region of the male sheep was also present in the same position and nearly the same size (44 bp) in the male goat, but the goat had an additional, smaller (15 bp) deletion 38 bp upstream of the common deletion region (see **Figure 3**, below).

```
                                10        20        30        40
Sheep AMELX:  CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC
Sheep AMELY:  CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC
Goat AMELX:    CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC
Goat AMELY:    CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC

                50        60        70        80        90
SX: CCCAGTCCAT CCAGCCGCAG CCTCACCAGC CCCTGCAGCC CCTGCAGCCC
SY: CCCAGCCCAT CCAGCCACAG CCTCACCAAC CCCTGCAGCC CC * * * * * * * * *
GX: CCCAGTCCAT CCAGCCGCAG CCTCACCAGC CCCTGCAGCC CCTGCAGCCA
GY: CACAG * * * * * * * * * * * * * CCTCACCAAC CCCTACAGCC CC * * * * * * * * *

               100       110       120       130       140
SX: ATGCAGCCCT TCGCGCCCTT GCAGCCCCTG CAGCCCCAGC CACCCGTGCA
SY: * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * AGC CACCCGTGCA
GX: TGCAGCCCT TCGCGCCCTT GCAGCCCCTG CAGCCCCAGC CACCCGTGCAC
GY: * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * AGC CACCTGTGCAC

               150       160       170       180       190
SX: CCCCATCCAG CCCTTGCCGC CACAGCCACC TCTGCCTCCG ATATTCCCCA
SY: CCCCATCCAG CGCTTGCCAC CACAGCCACC TCTGCCT TCA ATATTCCCCA
GX: CCCATCCAGC CCTTGCCGCC ACAGCCACCT CTGCCTCCGA TATTCCCCAT
GY: CCCATCCAGC GCTTGCCACC ACAGCCACCT CTGCCT TCAA TATTCCCCAT

               200       210       220       230       240
SX: TGCAGCCTCT GCCCCCCATG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA
SY: TGCAACCTCT GCCCCCT GTG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA
GX: GCAGCCTCTG CCCCCCATGC TTCCTGACCT GCCTCTGGAA GCTTGGCCAG
GY: GCAACCGCTG CCCCCTGTGC TTCCTGACCT GCCTCTGGAA GCTTGGCCAG

               250       260
SX: GCAACAGACA AGACCAAGCG GG
SY: GCAACAGACA AGACCAAGCG GG
GX: CAACAGACAA GACCAAGCGG G
GY: CAACAGACAA GACCAAGCGG G
```

Figure 3. Sheep-goat AMEL deletion region sequence alignment. Nucleotide differences between species are indicated by bold letters. Asterisks specify deletions.

Another observation in these previous experiments was that numerous

nucleotide differences existed between the sequences of the sheep and goat. It was hypothesized that similar DNA polymorphism phenomena, either of multiple deletion regions or nucleotide differences, would be present in the wild Argali sequence when compared with the domestic ovine sequence.

Perhaps with further experimentation, a DNA polymorphism can be found elsewhere in the AMEL gene of the Argali. It is also possible that a significant difference between the two genomes will not be found. Further research with the AMEL gene will include sequencing and comparing the deletion region of various other species, using methods similar to those described above. Species of interest include caribou, elk, yak, bison, musk ox, Dulong cattle, and various other wild sheep species such as Dall's, Mouflon, Bighorn, and Stone's. Comparison of domestic and wild goat species, including American Mountain Goat, Chamois, Tahr and Ibex, would be interesting to study, as well.

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Author's Biography

Kimberly Elwood was born in Logan, Utah and graduated in June 2003 from Logan High School. She is a member of the National Society of Collegiate Scholars and Phi Kappa Phi Honor Society. Kim plans to graduate from Utah State University in December 2006 with a B.S. degree in Animal Science with an emphasis in Biotechnology and minors in Chemistry and Biology. After graduation she plans to start a graduate program in Animal Science with a Molecular Biology emphasis at Utah State.