COVER SHEET

TITLE: Sequence analysis of Aleutian Mink Disease Virus (AMDV) isolates in captive North American mink

AUTHOR'S NAME: Cristina Vaughan

MAJOR: Biology

DEPARTMENT: Biology

MENTOR: Dr. Kathy Toohey-Kurth

DEPARTMENT: Pathobiological Sciences, School of Veterinary Medicine

MENTOR(2): Jennifer Godhart-Cooper

DEPARTMENT(2): Wisconsin Veterinary Diagnostic Laboratory

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ABSTRACT

Sequence analysis of Aleutian Mink Disease Virus (AMDV) isolates in captive North American mink

Aleutian mink disease virus (AMDV) is a member of the parvoviridae family which primarily affects mink, often resulting in death. Nine viral genomes were amplified in segments using polymerase chain reactions (PCR) and sequenced using Sanger dideoxy method. Sequences were assembled and aligned using CLC Workbench software and compared to known reference strains of AMDV. The deduced amino acid sequences were also analyzed for changes that could affect pathogenicity. Phylogenetic trees were configured for the genomic sequence as well as the all the viral proteins in order to identify strain relatedness. Phylogenetic analysis based on nucleotides revealed clustering based on ranch location, with the strains originating in Idaho and Utah clustered together and the Wisconsin strains forming a second cluster. The identification of similarities and differences between the strains can ultimately be used to help develop diagnostic tools to rapidly classify phylogenetic relationships.

Cristina Vaughan/Biology
Author Name/Major

Dr. Kathy Toohey-Kurth/Pathobiological Sciences, SVM
Mentor Name/Department

Author Signature

Mentor Signature

4/24/14
Date

Sequence analysis of Aleutian Mink Disease Virus (AMDV) isolates in captive North American mink

Senior Honors Thesis for the College of Agriculture and Life Sciences Honors in Research Program, University of Wisconsin-Madison

Cristina Vaughan

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Cristina Vaughan
CALS Honors in Research
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Sequence analysis of Aleutian Mink Disease Virus (AMDV) isolates in captive North American mink

Abstract

Aleutian mink disease virus (AMDV) is a member of the parvoviridae family which primarily affects mink, often resulting in death. A few strains have been identified and genotyped, but full genomic analysis of recent circulating strains has not been published in North America. Nine viral genomes were amplified in segments using polymerase chain reactions (PCR) and sequenced using Sanger dideoxy method. Sequences were assembled and aligned using CLC Workbench software and compared to known reference strains of AMDV. The deduced amino acid sequences were also analyzed for changes that could affect pathogenicity. Phylogenetic trees were conFig.ured for the genomic sequence as well as all the viral proteins in order to identify strain relatedness. Phylogenetic analysis based on nucleotides revealed clustering based on ranch location, with the strains originating in Idaho and Utah clustered together and the Wisconsin strains forming a second cluster. The identification of similarities and differences between the strains can ultimately be used to help develop diagnostic tools to rapidly classify phylogenetic relationships.

Introduction

Aleutian mink disease virus (AMDV) is a parvovirus composed of linear single stranded DNA that is about 5-kb in length that affects both wild and captive mink (Bloom et al, 1980). This

severe progressive disease causes different clinical symptoms in mink, often depending on the host's genotype and age (Hadlow et al, 1983; Alexandersen et al, 1994). This disease has a high rate of mortality in captive mink ranches, consequently causing high economic losses. A factor that makes it difficult to eradicate is its high genetic diversity; a study in Sweden obtained sequences from 35 clinical samples and compared them to five published samples, resulting in the identification of three phylogenetic subgroups while some ranches revealed more than one genotype present (Olofsson et al, 1999). Interestingly, AMDV has been found to rarely infect humans (Jepsen et al, 2009).

There are two structural proteins (VP1 and VP2) and three non-structural proteins (NS1, NS2, and NS3) that the single-stranded AMDV genome codes for (Bloom et al, 1994; Christensen et al, 1995). NS1 is the major nonstructural protein of parvoviruses, and it is responsible for the regulation of transcription, viral DNA replication, and some enzymatic activities (Best et al, 2003; Christensen et al, 1995). The NS2 and NS3 proteins have been found to play critical roles in viral replication during infection (Huang et al, 2014). VP2 capsid proteins perform capsid assembly and DNA packaging roles, and have been suggested to have sequencing differentiation that can indicate the strain's virulence but it is not an obligate determinant of pathogenicity (Oie et al, 1996). Both the NS1 gene and VP2 gene have been found to contain a high amount of variability, but the exact functions of these changes have not been conclusively determined (Gottschalck et al, 1994; Oie et al, 1996; Olofsson et al, 1999). Previous studies based on analysis of the hypervariable region of the VP2 gene at amino acid positions 230-240 performed in Europe indicate that there may be three main types (Gottschalck et al, 1991), but there is no predictable pattern based on virulence, geographic origin, or year of isolation (Olofsson et al, 1999; Schuierer et al, 1997).

Isolates of AMDV have been genetically analyzed, mainly from areas outside of North America (Gottschalck et al, 1994; Knuuttila et al, 2009; Schuierer et al, 1997; Olofsson et al, 1999; Shackelton et al, 2007). These studies have determined that AMDV has a large genetic diversity, but they have been limited to using only a small sequence of the viral genome. Within North America, the only fully sequenced strain is the non-virulent AMDV-G, and the only mostly-complete strain is the virulent AMDV-Utah (Bloom et al, 1988). From this point onward, these strains will be referred to as the reference strains.

To date there has not been a complete genomic sequence analysis of multiple strains of AMDV circulating in North America. By sequencing the majority of the genome from nine isolates of AMDV, we hope to gain a more in-depth analysis as to how the virus changes. Ultimately, we will be able to better understand the biological behavior of AMDV and the relation it has to the natural history of infection in ranch mink, as well as help create more cost-effective diagnostic methods of diagnosing this disease.

Material and Methods

Samples of infected mink tissues were obtained from various mink ranches in Wisconsin, Idaho, and Utah from 2008 to 2013. We extracted DNA from the tissues samples using Qiagen's DNeasy Tissue Extraction Kit. We determined that these samples were positive for AMDV by using a real-time PCR protocol developed by the Wisconsin Veterinary Diagnostic Laboratory (WVDL). To amplify the DNA, we used 15 sets of overlapping primers developed using CLC Main Workbench 7 software (CLC bio, Aarhus, Denmark), based on the AMDV-G strain published by Bloom et al (1980). We then used a modified master mix developed in house to amplify the viral DNA through conventional PCR. The fragments were

sequenced using Sanger sequencing, and then aligned to known AMDV sequences using CLC Main Workbench 7.

Tissue sampling and DNA extraction

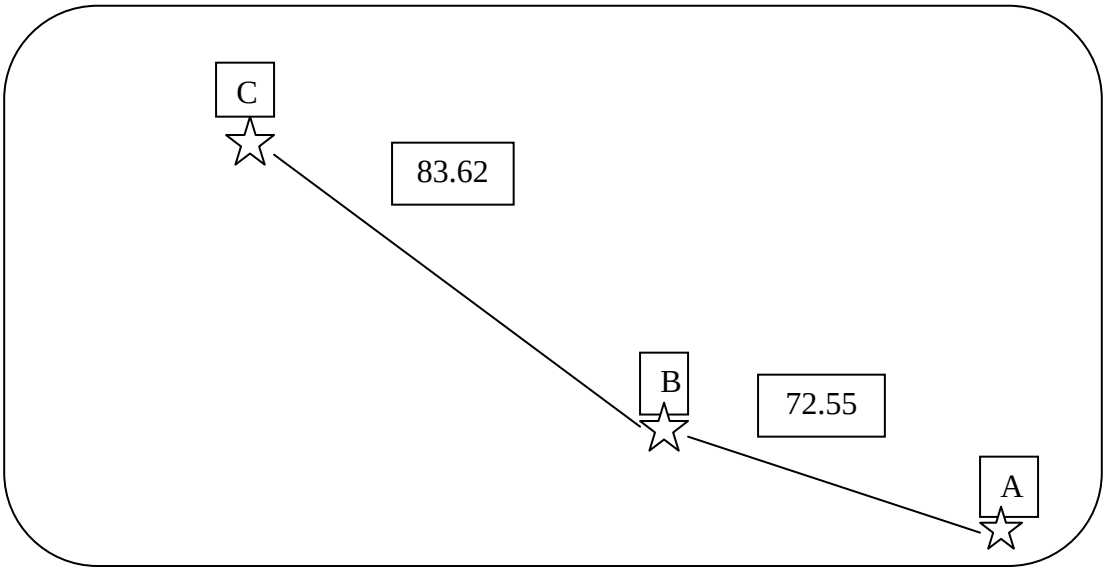
Samples of infected mink tissues were obtained from five mink ranches in Wisconsin (**Fig. 1**), Idaho, and Utah from 2008 to 2013 (**Table 1**). The tissues were submitted to WVDL by veterinarians as suspect AMDV cases based on clinical signs, pathological examination, and past farm history. Samples were taken from various tissues (brain, lung, spleen, kidney, liver, and/or lymph nodes) according to a WVDL protocol. Samples taken from mink from different ranches were assigned letters A-E to maintain ranch privacy. DNA was extracted from the tissues samples using Qiagen's DNeasy Tissue Extraction Kit (Qiagen Inc, Valencia, CA, USA). We determined that these samples were positive for AMDV by using a real-time PCR protocol developed by the WVDL. We chose samples with lower cycling thresholds (CT) because this indicated a higher amount of virus present.

Table 1 Description of ADV isolates

Isolate Number	Isolate ID	Tissue	Clinical Signs	Pathogenic by Clinical Signs
WI1	WI/A*/1/Feb2008	Brain	Off feed, depression, death within 4 days	Yes
WI2	WI/A*/5/Feb2008	Lung/Lymph node		
WI3	WI/A*/6/Feb2008			
WI4	WI/A/12/Apr2008	Lung	Acute death	Yes
WI5	WI/B/14/Oct2008	Spleen/Lymph node/Liver	Slight depression, off feed, death within 24-48 hours	Yes
WI6	WI/B/15/Nov2008	Mix (Not specified)	Testing second part of ranch after first part tested positive for ADV	Yes
WI7	WI/C/18/Aug2010	Spleen	No clinical signs specified	Unknown
UT8	UT/D/10/Jan2013	Kidney/Liver	None, monitoring for ADV	No
ID9	ID/E/22/Jan2011	Lung/Liver		No

Isolate Legend
Location/Ranch/Animal number/MonthYear
* Same accession

Figure 1 Wisconsin ranch locations and approximate distances in miles



PCR

The PCR kit used was developed by ABI Life Sciences and modified by us to achieve optimal results. The PCR primers were designed using CLC Genomics Workbench 7 based on the AMDV genome map (GenBank accession number NC_001662) (**Table 2**).

For primer set P1, each 50 µl reaction contained 30.75 µl of Nuclease free water, 5 µl of 10x PCR Buffer, 4 µl of dNTPs, 2.5 µl of each forward and reverse primers, 0.25 µl of Amplitaq, and 5 µl of template DNA. For primer sets P2, P5, P14, each 50 µl reaction contained 34.75 µl of Nuclease free water, 5 µl of 10x PCR Buffer, 4 µl of 10mM dNTPs, 1.5 µl of each forward and reverse primers, 0.25 µl of Amplitaq and 3 µl of template DNA. For primer sets P1.1, P3, P4, P6, P7, and P11, each 50 µl reaction contained 33.75 µl of Nuclease free water, 5 µl of 10x PCR Buffer, 4 µl of 10mM dNTPs, 1 µl of each forward and reverse primers at a 20mM concentration, 0.25 µl of Amplitaq, and 5 µl of template DNA.

For P1, P1.1, P2, P3, P6, P7, P11, P12, P13, and P14, the thermocycling parameters used were 1 cycle of incubation at 94ºC for 5 minutes, followed by 40 cycles of denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute, and elongation at 72ºC for 1 minute, with

a final 72ºC incubation for 7 minutes. Primer set P4 had identical cycling parameters except for a 45ºC annealing temperature.

An AccuPrime™ GC-Rich DNA Polymerase (Life Technologies, Carlsbad, CA, USA) was used to amplify the G-C rich region (bp 1942-2897). For primer sets P8, P9, and P10, each 25 µl reaction contained 14 µl of Nuclease free water, 5 µl of 5x PCR Buffer A, 0.25 of 20mM of each forward and reverse primer, 0.5 µl of AccuPrime™ GC-Rich DNA Polymerase, and 5 µl of template DNA. The thermocycling parameters for P8 and P9 were 1 cycle of incubation at 95 ºC for three minutes, followed by 40 cycles of denaturation at 95 ºC for 30 seconds, annealing at 50 ºC for 30 seconds, and elongation at 72 ºC for 1 minute, with a final 72 ºC incubation for 10 minutes. Primer set P10 had identical cycling parameters except for a 45ºC annealing temperature.

The final amplified PCR products were then subjected to gel electrophoresis in a 1.2% agarose gel with ethidium bromide.

Sequencing and genomic analysis

The PCR product was submitted to Functional Biosciences (Functional Biosciences, Madison, WI for sequencing using the Sanger sequencing method. The resulting sequences were aligned to each other and to other known AMDV sequences using CLC Genomics Workbench 7. The protein coding genes (NS1, NS2, NS3, VP1, and VP2) were translated using CLC Genomics Workbench 7 and compared to the deduced translations of the two reference strains (Bloom et al, 1988). These isolates were then joined into six possible phylogenetic trees, one for each viral protein and one for the overall nucleotide sequence.

Table 2 Sequences and locations of primers with respect to ADMV-G reference strain used to amplify AMDV isolates

Primer Set	Primer	Primer Position	Primer Sequence (5'-3')	Amplified Fragment Size (bp)
P1	1F	129	TGTGGAATGAGGAAGTAG	330
	1R	459	TCCCCAAGGTTAGTTTAC	
P1.1 (Specific for isolates UT8 & ID9)	1.1F	127	CCTGTGGAATGAGGAAGT	399
	1.1R	526	AGCATCCCATTTGTGACT	
P2	2F	386	TTAGCTTTTGACTCTATTGAAGAGA	701
	2R	1087	ACTAGTTCCTGTTTTTGTGGTTGCT	
P3	3F	997	AATGGATGCTAATATAGACTGGGAAGA	868
	3R	1865	TAGGGTTGATGGTTTTCATGCA	
P4	4F	999	TGGATGCTAATATAGACTGGG	346
	4R	1345	GGCAGTAAGGTTGCATGA	
P5	5F	1208	GGTTGCTTTACTCCAGAAGAT	614
	5R	1822	TTAAGTGGCTCTGCGTG	
P6	6F	1777	AACTGTTGGATGTGTGGAAACAA	516
	6R	2293	CTTTTTCCCAGGATAGTGCT	
P7	7F	2293	AGCACTATCCTGGGAAAAAG	463
	7R	2755	ACCCCAAGCGTTGCTATCTA	
P8	8F	2170	GATGCCTGAACTGTACTT	1109
	8R	3279	CAGGGTTAGTTTGCTTCT	
P9	9F	2293	CAAGAGACCTCGGCATGA	462
	9R	2755	GTTATTAAAGCCGCCAGT	
P10	10F	2424	GAACAAATGGACACTGAG	473
	10R	2897	TACCTTGGTTGGTTTCTG	
P11	11F	2755	TAGATAGCAACGCTTGGGGT	524
	11R	3279	AGCTTAAGGTTAGTTTACATGGTTTACT	
P12	12F	3028	CAACCAAACCAACCCAATATAGG	617
	12R	3645	TGTCTCCACCGTGGTTTCTG	
P13	13F	3433	GTAAGAAAAACGCAGAGAT	630
	13R	4063	GGGTTGTTTTTACATACAAATGG	
P14	14F	3900	ATGCTGCTCAGATGAAAAAGC	573
	14R	4473	TTTATTTTAATCCGCCACT	

Results

We constructed one phylogenetic tree (**Fig. 2**) based on the genomes of our nine isolates, the two reference strains of AMDV, and two other fully sequenced strains from China and Germany. The nucleotides used were from bp 150-4463, based off of the reference AMDV-G genome. The sequences for AMDV-G, AMDV-SL3, AMDV-Utah, and the China isolate were obtained from GenBank. This tree showed that our Wisconsin isolates from ranch A formed one cluster while the other Wisconsin isolates from ranches B and C formed a second cluster. All of the other strains and isolates formed a third cluster. From this phylogenetic tree, it appears that the strains are related based on geographic location. Overall, the only isolates that were 100% identical in all five genes were WI2 and WI3 (**Table 3**). The Wisconsin isolates were more homologous to each other than to any other isolates.

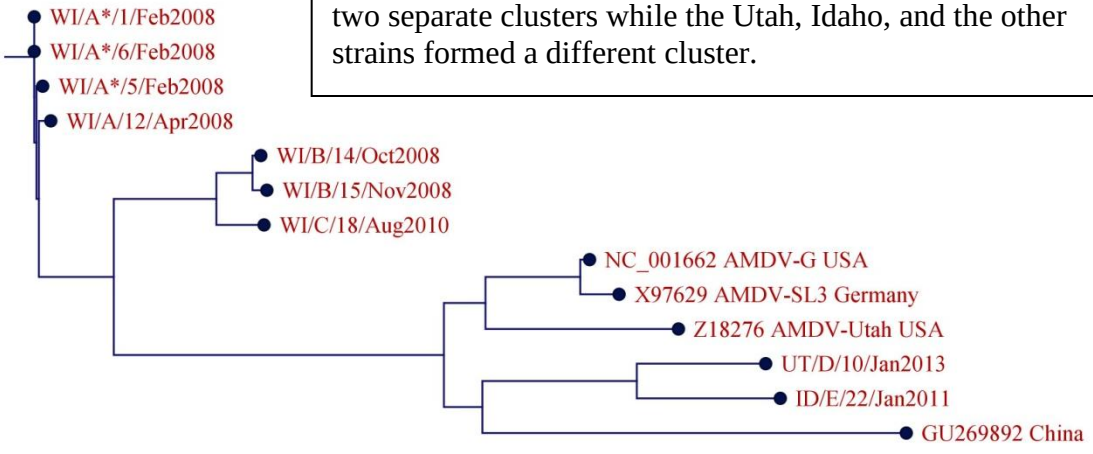


Figure 2 Phylogenetic tree of nucleotides 150-4463 of multiple isolates of AMDV. The Wisconsin isolates formed two separate clusters while the Utah, Idaho, and the other strains formed a different cluster.

Table 3 Total genome homology from nucleotides 150-4463 of the nine isolates (purple cells), as well as the number of differences (green cells) among the isolates.

Total genome (nt 150-4463), Number of differences		Total genome (nt 150-4463), % Homology										
		ADV-G	**ADV-Utah**	**WI/A*/1/Feb2008**	**WI/A*/5/Feb2008**	**WI/A*/6/Feb2008**	**WI/A/12/Apr2008**	**WI/B/14/Oct2008**	**WI/B/15/Nov2008**	**WI/C/18/Aug2010**	**UT/D/10/Jan2013**	**ID/E/22/Jan2011**
	ADV-G		97.59	95.67	95.64	95.64	95.71	94.74	94.69	94.69	96.71	96.62
	ADV-Utah	104		94.6	94.56	94.56	94.56	94.58	94.53	94.62	95.16	95.16
	WI/A*/1/Feb2008	187	233		99.93	99.93	99.86	98.12	98.05	98.08	94.46	94.16
	WI/A*/5/Feb2008	188	235	3		100	99.84	98.08	98.01	98.03	94.44	94.14
	WI/A*/6/Feb2008	188	235	3	0		99.84	98.08	98.01	98.03	94.44	94.14
	WI/A/12/Apr2008	185	235	6	7	7		97.98	97.91	97.98	94.46	94.11
	WI/B/14/Oct2008	227	234	81	83	83	87		99.81	99.26	93.28	93.3
	WI/B/15/Nov2008	229	236	84	86	86	90	8		99.19	93.21	93.26
	WI/C/18/Aug2010	229	232	83	85	85	87	32	35		93.19	93.28
	UT/D/10/Jan2013	142	209	239	240	240	239	290	293	294		97.8
	ID/E/22/Jan2011	146	209	252	253	253	254	289	291	290	95	

NS1

At the nucleotide level, this gene showed the most variation of nucleotides among the nine strains with 248 mutations out of the 1926 base pairs that code for the NS1 gene from the nine isolates. All of the examined isolates showed a higher homology to non-pathogenic AMDV-G than to pathogenic AMDV-Utah (**Table 4**). Ranch A isolates were between 99.7-100% homologous to each other, with the biggest difference of four nucleotides between WI1 and WI4 and the highest homology occurring between WI2 and WI3 where the samples were identical. Isolates from ranch B showed a homology of 99.6% among each other with eight nucleotide differences. They were most homologous to WI7 and least homologous to UT8. The WI7 was most homologous to WI5 and least homologous to UT8. The two western isolates from Utah and Idaho showed the highest homology with each other and were least homologous to isolate WI7.

At the amino acid level, this gene showed the most variation of amino acids with 131 mutations among the 641 amino acid residues of the NS1 protein from the nine isolates (**Fig. 3**). Isolates from ranches A, C, and D had an equal homology to AMDV-G and AMDV-Utah, while

isolates from ranch B had a higher homology to AMDV-G than AMDV-Utah, and the isolate from ranch E had a higher homology to AMDV-Utah than AMDV-G (**Table 4**). Ranch A isolates had homologies between 99.38-100% among each other. They were most homologous to the isolate from ranch C, and least homologous to the western isolate from ranch E. The isolates from ranch B were most homologous to the ranch C isolate and least homologous to the western isolate from ranch D. The western isolates were most homologous to each other and least homologous to the isolates from Wisconsin ranch B.

Table 4 Percent homology of the nucleotides (purple cells) and amino acid (green cells) sequence of the partial NS1 gene of ADV

		NS1 Nucleotides, % Homology										
		AMDV-G	**AMDV-Utah**	**WI/A*/1/Feb2008**	**WI/A*/5/Feb2008**	**WI/A*/6/Feb2008**	**WI/A/12/Apr2008**	**WI/B/14/Oct2008**	**WI/B/15/Nov2008**	**WI/C/18/Aug2010**	**UT/D/10/Jan2013**	**ID/E/22/Jan2011**
NS1 Amino Acids, % Homology	**AMDV-G**		99.17	92.73	92.68	92.68	92.73	92.89	92.78	92.83	94.44	94.18
	AMDV-Utah	98.29		92.58	92.52	92.52	92.47	92.68	92.58	92.68	94.29	94.08
	WI/A*/1/Feb2008	88.79	88.79		99.95	99.95	99.79	98.49	98.34	98.55	90.39	89.88
	WI/A*/5/Feb2008	88.63	88.63	99.84		100	99.74	98.44	98.29	98.49	90.34	89.82
	WI/A*/6/Feb2008	88.63	88.63	99.84	100		99.74	98.44	98.29	98.49	90.34	89.82
	WI/A/12/Apr2008	88.63	88.63	99.53	99.38	99.38		98.29	98.13	98.34	90.39	89.88
	WI/B/14/Oct2008	88.79	88.63	96.73	96.57	96.57	96.26		99.58	98.81	89.98	89.93
	WI/B/15/Nov2008	88.79	88.63	96.42	96.26	96.26	95.95	98.91		98.65	89.82	89.82
	WI/C/18/Aug2010	89.1	89.1	97.2	97.04	97.04	96.73	97.66	97.51		89.93	90.03
	UT/D/10/Jan2013	90.34	90.34	85.67	85.51	85.51	85.83	84.89	84.74	84.89		96.68
	ID/E/22/Jan2011	90.19	90.5	85.36	85.2	85.2	85.51	85.2	85.05	85.2	93.77	

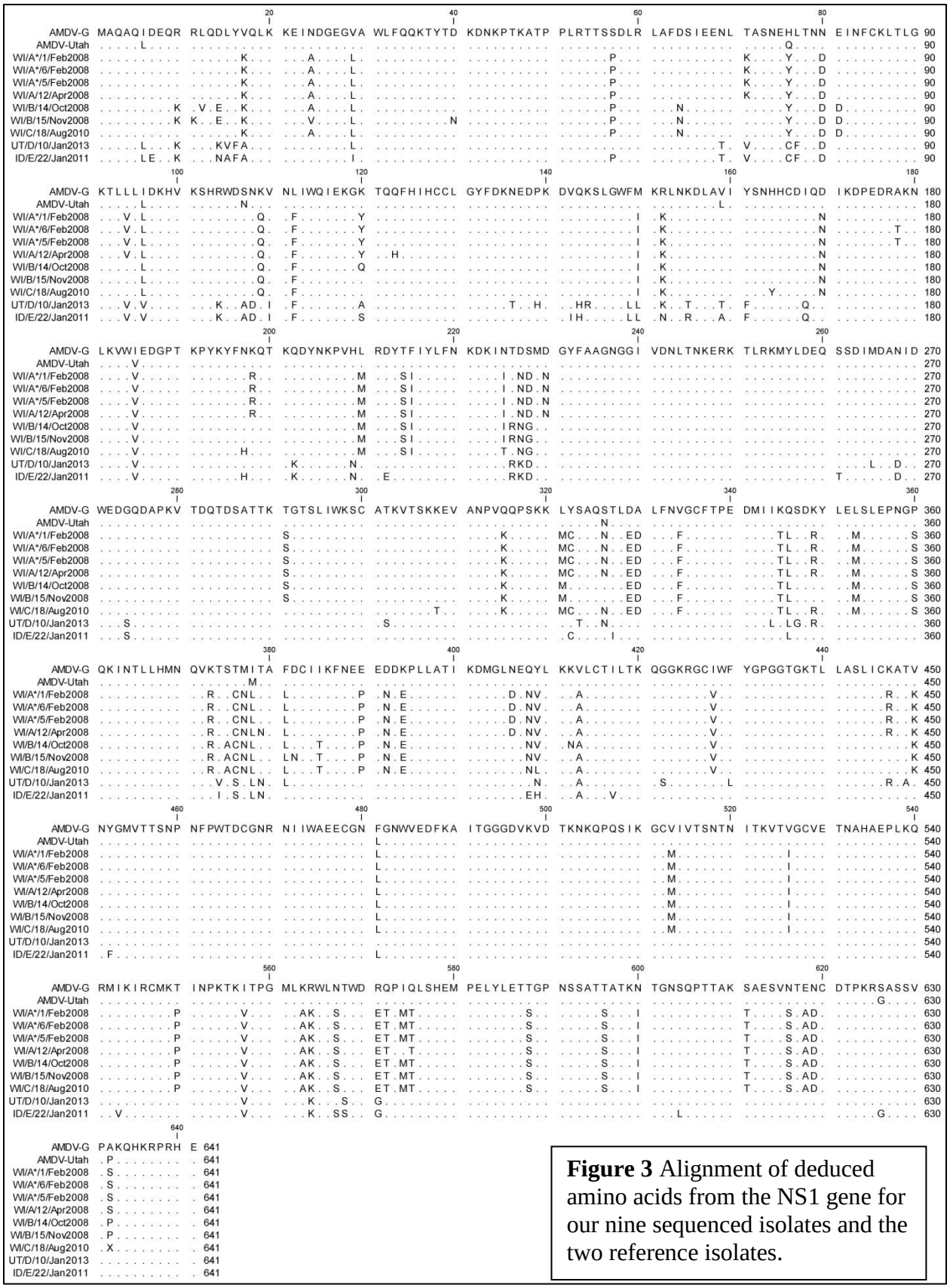


Figure 3 Alignment of deduced amino acids from the NS1 gene for our nine sequenced isolates and the two reference isolates.

We constructed a phylogenetic tree (**Fig. 4**) based on the 11 full-length NS1 gene nucleotide sequences to examine the phylogenetic relationships between our sequenced Wisconsin isolates and two isolates from Utah and Idaho, and the AMDV-G and AMDV-Utah strains. The sequences for AMDV-G and AMDV-Utah were obtained from GenBank. The tree was constructed by the neighbor-joining method using CLC Main Workbench 7 software. The results showed that the Wisconsin isolates from ranch A form their own cluster, the isolates from ranches B and C form a second cluster, the isolates from ranches D and E as well as the two reference strains formed a third more distant cluster.

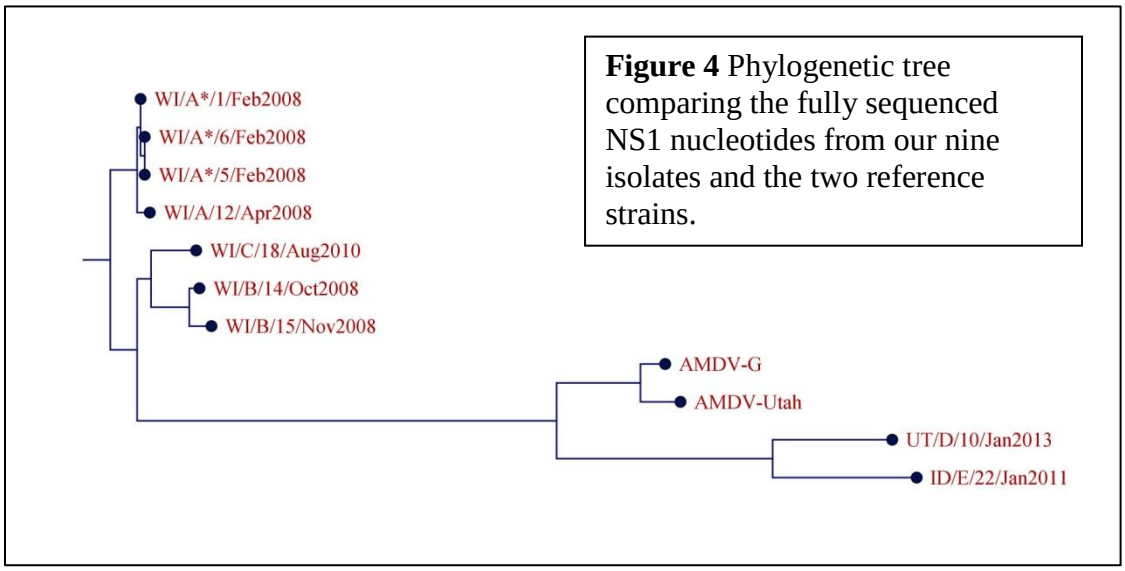


Figure 4 Phylogenetic tree comparing the fully sequenced NS1 nucleotides from our nine isolates and the two reference strains.

A second phylogenetic tree (**Fig. 5**) was constructed from 49 partial sequences of the NS1 gene to examine the relationships between our North American isolates to other AMDV strains around the globe. Like our previous phylogenetic tree, the Wisconsin isolates form their own unique clusters based on ranch locations, with ranch A forming one cluster and ranches B and C forming a second cluster. The closest possibly related isolates were from Denmark. The isolates from Utah and Idaho formed another, more distant cluster that showed more relatedness to AMDV-Utah and AMDV-G than to the Wisconsin isolates.

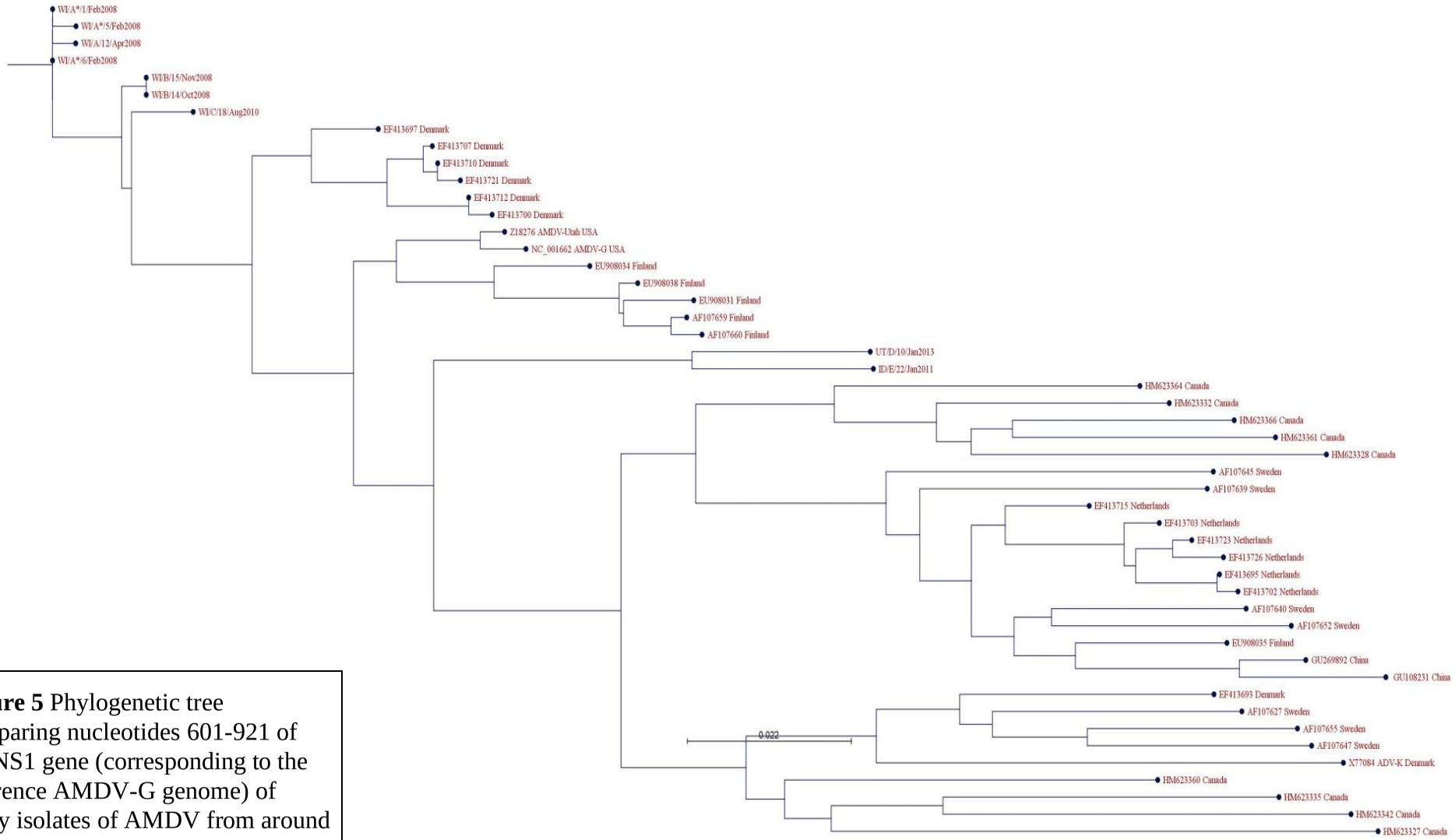


Figure 5 Phylogenetic tree comparing nucleotides 601-921 of the NS1 gene (corresponding to the reference AMDV-G genome) of many isolates of AMDV from around the globe to our isolates. The isolate GenBank number is given, as well as its geographic origin.

NS2

At the nucleotide level, this gene had a total of 47 mutations among the 342 base pairs that code for the NS3 gene from the nine isolates. Isolates from ranches A and C showed equal homologies to AMDV-G and AMDV-Utah, while isolates 5 and 8 were more homologous to AMDV-G than AMDV-Utah, and isolates 6 and 9 were more homologous to AMDV-Utah than AMDV-G (**Table 5**). The isolates from ranch A were all identical to each other, and they were most homologous to the isolate from ranch C while they were least homologous to the western isolate from ranch E. The isolates from ranch B were 97.68% homologous to each other, with both isolates showing a greater homology to the isolate from ranch C than to each other. They were least homologous to the isolate from ranch E. The isolate from ranch C was most homologous to the isolates from ranch A and least homologous to the isolate from ranch E. The isolates from ranches D and E was most homologous to each other and least homologous to the WI6.

At the amino acid level, there were a total of 23 mutations among the 114 amino acid residues of the NS2 protein from the nine isolates (**Fig. 6**). This gene showed the most percent homology differences among the samples. The isolates from ranches A and D showed more homology to AMDV-G than AMDV-Utah (**Table 5**). The isolates from ranch B showed equal homology to AMDV-G and AMDV-Utah. The isolates from ranches C and E showed a greater homology to AMDV-Utah than AMDV-G. All of the isolates from ranch A and the isolate from ranch C had identical homologies, and they were least homologous to the isolate from ranch E. The isolates from ranch B were 96.52% homologous to each other, and they were most homologous to the ranch A isolates and least homologous to the isolates from ranch E. The isolates from ranches D and E were most homologous each other and least homologous to the isolates from ranch A.

Table 5 Percent homology of the nucleotides (purple cells) and amino acid (green cells) sequence of the NS2 gene of ADV.

		NS2 Nucleotides, % Homology										
		AMDV-G	**AMDV-Utah**	**WI/A*/1/Feb2008**	**WI/A*/5/Feb2008**	**WI/A*/6/Feb2008**	**WI/A/12/Apr2008**	**WI/B/14/Oct2008**	**WI/B/15/Nov2008**	**WI/C/18/Aug2010**	**UT/D/10/Jan2013**	**ID/E/22/Jan2011**
NS2 Amino Acids, % Homology	**AMDV-G**		98.55	93.62	93.62	93.62	93.62	92.75	92.46	93.62	94.49	93.62
	AMDV-Utah	96.52		93.62	93.62	93.62	93.62	93.04	92.75	93.62	94.2	93.91
	WI/A*/1/Feb2008	90.43	89.57		100	100	100	98.55	97.97	99.13	89.86	88.99
	WI/A*/5/Feb2008	90.43	89.57	100		100	100	98.55	97.97	99.13	89.86	88.99
	WI/A*/6/Feb2008	90.43	89.57	100	100		100	98.55	97.97	99.13	89.86	88.99
	WI/A/12/Apr2008	90.43	89.57	100	100	100		98.55	97.97	99.13	89.86	88.99
	WI/B/14/Oct2008	87.83	87.83	95.65	95.65	95.65	95.65		97.68	98.26	89.57	88.7
	WI/B/15/Nov2008	86.96	86.96	93.91	93.91	93.91	93.91	96.52		97.68	89.28	88.41
	WI/C/18/Aug2010	91.3	90.43	100	100	100	100	96.52	94.78		89.86	89.57
	UT/D/10/Jan2013	93.04	91.3	86.09	86.09	86.09	86.09	86.09	85.22	86.96		95.94
	ID/E/22/Jan2011	91.3	90.43	85.22	85.22	85.22	85.22	85.22	84.35	86.09	93.91	

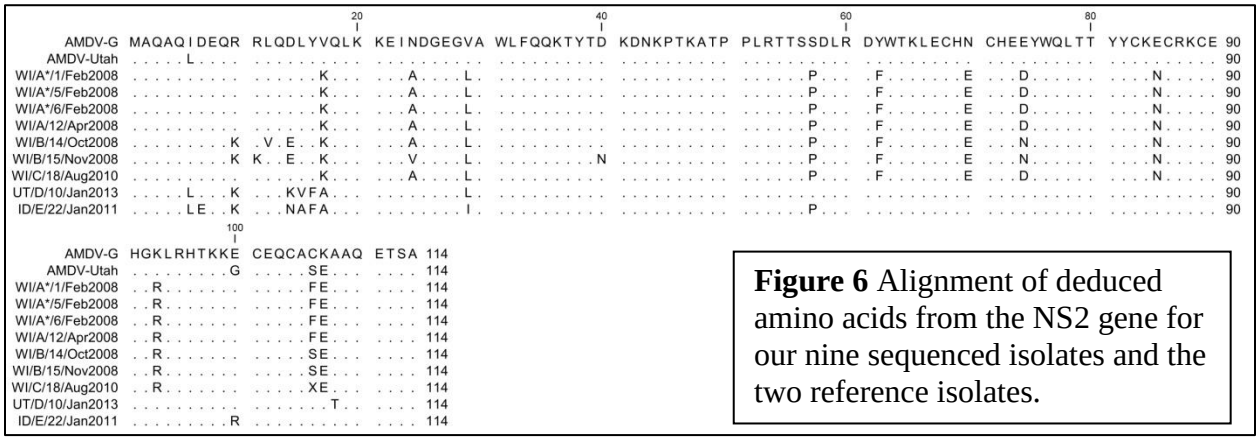


Figure 6 Alignment of deduced amino acids from the NS2 gene for our nine sequenced isolates and the two reference isolates.

We constructed a phylogenetic tree (**Fig. 7**) based on the 11 full-length NS2 gene nucleotide sequences to examine the phylogenetic relationships between our sequenced Wisconsin isolates and two isolates from Utah and Idaho, and the AMDV-G and AMDV-Utah strains. The sequences for AMDV-G and AMDV-Utah were obtained from GenBank. The tree was constructed by the neighbor-joining method using CLC Main Workbench 7 software. The results showed that the Wisconsin isolates from ranch A form their own cluster, the isolates from ranches B and C form a second cluster, the isolates from ranches D and E as well as the two reference strains formed a third more distant cluster.

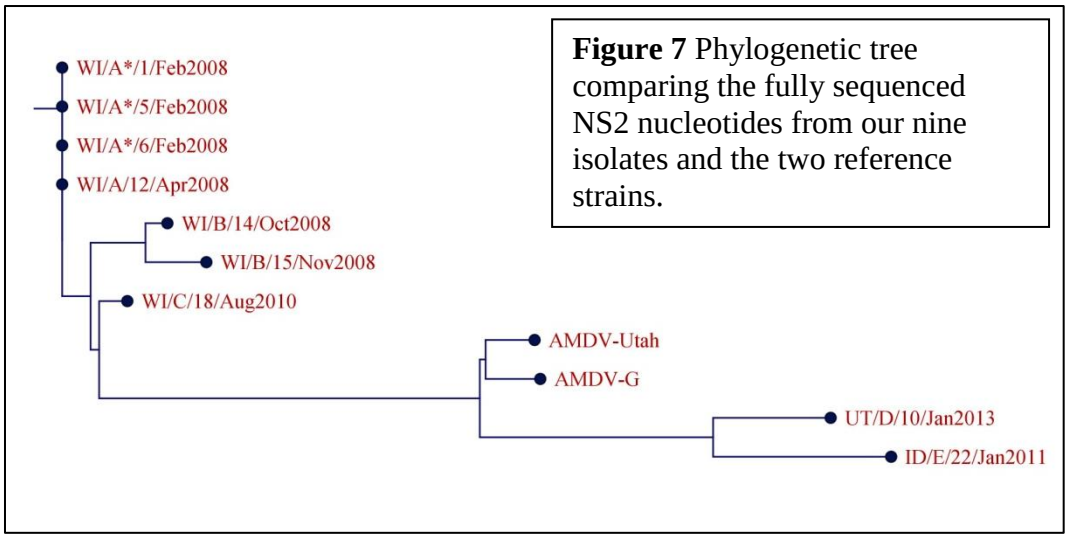


Figure 7 Phylogenetic tree comparing the fully sequenced NS2 nucleotides from our nine isolates and the two reference strains.

NS3

At the nucleotide level, this gene had a total of 33 mutations among the 261 base pairs that code for the NS3 gene from the nine isolates. The isolates from ranch A, B, and C showed equal homology between the AMDV-G and AMDV-Utah strains (**Table 6**). Both isolates from ranches D and E showed a higher homology with AMDV-Utah than AMDV-G. The ranch A isolates all showed identical homologies to each other, while they were most homologous to isolate WI5 and the isolate from ranch C and least homologous to the isolate from ranch E. The isolates from ranch B were 98.48% homologous to each other, they were most homologous to the isolates from ranch A and least homologous to the isolate from ranch E. The isolate from ranch C was most homologous to the isolates from ranch A and least homologous to the isolate from ranch E. The isolates from ranches D and E were most homologous to each other and least homologous to WI6.

At the amino acid level, there were a total of 14 mutations among the 87 amino acid residues of the NS3 protein from the nine isolates (**Fig. 8**). The isolates from ranches A, B, C, and D all showed a greater homology to AMDV-G than AMDV-Utah, while the isolate from ranch E showed a greater homology to AMDV-Utah than AMDV-G (**Table 6**). The isolates from

ranches A and C all showed identical amino acid sequences, and they were most homologous to the isolates from ranch B and least homologous to the isolate from ranch E. The isolates from ranch B had identical amino acid sequences to each other, while they had the most homology to the isolate from ranch C and the least homology to the isolate from ranch E. The isolates from ranches D and E showed the greatest homology to each other and least homology to isolate WI6.

Table 6 Percent homology of the nucleotides (purple cells) and amino acid (green cells) sequence of the NS3 gene of AMDV.

		NS2 Nucleotides, % Homology										
		AMDV-G	**AMDV-Utah**	**WI/A*/1/ Feb2008**	**WI/A*/5/ Feb2008**	**WI/A*/6/ Feb2008**	**WI/A/12/ Apr2008**	**WI/B/14/ Oct2008**	**WI/B/15/ Nov2008**	**WI/C/18/ Aug2010**	**UT/D/10/ Jan2013**	**ID/E/22/ Jan2011**
NS2 Amino Acids, % Homology	**AMDV-G**		98.55	93.62	93.62	93.62	93.62	92.75	92.46	93.62	94.49	93.62
	AMDV-Utah	96.52		93.62	93.62	93.62	93.62	93.04	92.75	93.62	94.2	93.91
	WI/A*/1/Feb2008	90.43	89.57		100	100	100	98.55	97.97	99.13	89.86	88.99
	WI/A*/5/Feb2008	90.43	89.57	100		100	100	98.55	97.97	99.13	89.86	88.99
	WI/A*/6/Feb2008	90.43	89.57	100	100		100	98.55	97.97	99.13	89.86	88.99
	WI/A/12/Apr2008	90.43	89.57	100	100	100		98.55	97.97	99.13	89.86	88.99
	WI/B/14/Oct2008	87.83	87.83	95.65	95.65	95.65	95.65		97.68	98.26	89.57	88.7
	WI/B/15/Nov2008	86.96	86.96	93.91	93.91	93.91	93.91	96.52		97.68	89.28	88.41
	WI/C/18/Aug2010	91.3	90.43	100	100	100	100	96.52	94.78		89.86	89.57
	UT/D/10/Jan2013	93.04	91.3	86.09	86.09	86.09	86.09	86.09	85.22	86.96		95.94
	ID/E/22/Jan2011	91.3	90.43	85.22	85.22	85.22	85.22	85.22	84.35	86.09	93.91	

Figure 8 Alignment of deduced amino acids from the NS3 gene for our nine sequenced isolates and the two reference isolates.

```
                                20                  40                  60                  80
                                I                   I                   I                   I
AMDV-G      MAQAQIDEQR RLQDLYVQLK KEINDGEGVA WLFQQKTYTD KDNKPTKATP PLRTTSSDLR LCDCNKQHQH NQSNCWMCGN KRSRRAT 87
AMDV-Utah   .....L.... .......... .......... .......... .......... .......... .......... .......... ..... 87
WI/A*/1/Feb2008 .......... ......K... ...A....L. .......... .......... .....P.... .Y........ ....H..... ..... 87
WI/A*/5/Feb2008 .......... ......K... ...A....L. .......... .......... .....P.... .Y........ ....H..... ..... 87
WI/A*/6/Feb2008 .......... ......K... ...A....L. .......... .......... .....P.... .Y........ ....H..... ..... 87
WI/A/12/Apr2008 .......... ......K... ...A....L. .......... .......... .....P.... .Y........ ....H..... ..... 87
WI/B/14/Oct2008 .........K .V.E..K... ...A....L. .......... .......... .....P.... .Y........ ....H..... ..... 87
WI/B/15/Nov2008 .........K K..E..K... ...V....L. .........N .......... .....P.... .Y........ ....H..... ..... 87
WI/C/18/Aug2010 .......... ......K... ...A....L. .......... .......... .....P.... .Y........ ....H..... ..... 87
UT/D/10/Jan2013 .....L...K ...KVFA... .........L .......... .......... ..... ..N..... .......... ..... 87
ID/E/22/Jan2011 .....LE..K ...NAFA... .........I .......... .....P.... ..N..... .......... ..... 87
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We constructed a phylogenetic tree (**Fig. 9**) based on the 11 full-length NS3 gene nucleotide sequences to examine the phylogenetic relationships between our sequenced Wisconsin isolates and two isolates from Utah and Idaho, and the AMDV-G and AMDV-Utah

strains. The sequences for AMDV-G and AMDV-Utah were obtained from GenBank. The tree constructed by the neighbor-joining method using CLC Main Workbench 7 software. The results showed that the Wisconsin isolates from ranch A form their own cluster, the isolates from ranches B and C form a second cluster, the isolates from ranches D and E as well as the two reference strains formed a third more distant cluster.

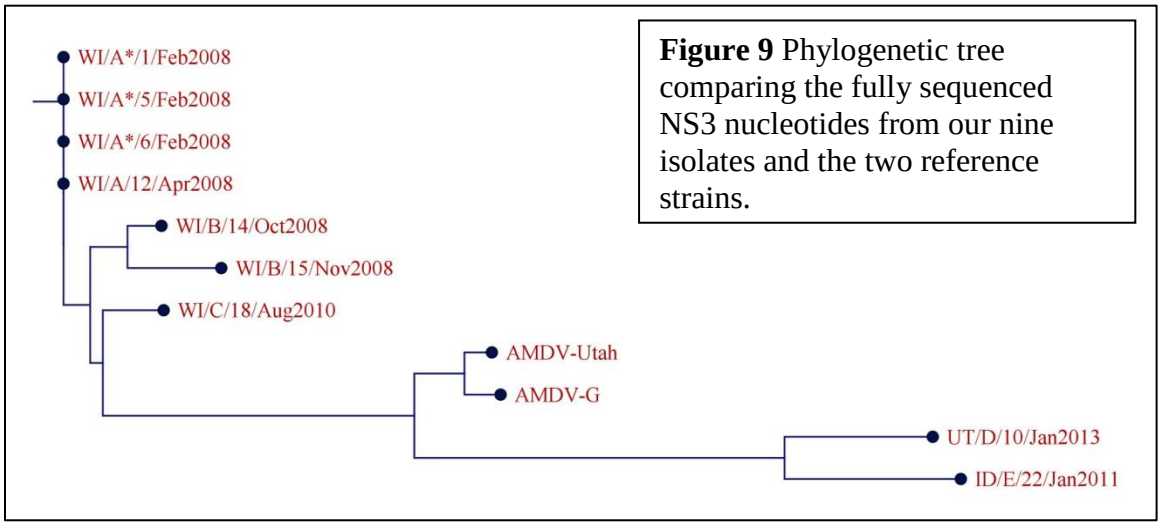

Figure 9 Phylogenetic tree comparing the fully sequenced NS3 nucleotides from our nine isolates and the two reference strains.

VP1

At the nucleotide level, there were a total of 134 mutations among the 2070 base pairs that code for the VP1 gene from the nine isolates. Isolates from ranches A, B, D, and E had a greater homology with AMDV-G than AMDV-Utah, while the isolate from ranch C had a slightly greater homology to AMDV-Utah than AMDV-G (**Table 7**). Isolates from ranch A were only identical in WI2 and WI3, while the other isolates WI1 and WI4 shared an identity of 99.9%. Interestingly, ranch A isolates were the most homologous to isolates from ranches D and E, while they were the least homologous to the isolate from ranch C. Ranch B isolates were identical to each other, and they had the greatest homology to the isolate from ranch C, and the least homology with the isolate from ranch D. The ranch C isolate had the greatest homology

with the isolates from ranch B, while it had the least homology to the isolates from ranch D. The two western isolates from ranches D and E had a homology of 98.65% to each other, while they were least homologous to the isolate from ranch C.

At the amino acid level, there were a total of 37 mutations among the 690 amino acid residues of the VP1 protein from the nine isolates (**Fig. 10**). The isolates from ranches A, D, and E had a higher homology to AMDV-G than AMDV-Utah, while the isolates from ranches B, and C had a higher homology to AMDV-Utah than AMDV-G. The isolates from ranch A had homologies between 99.7-100% to each other (**Table 7**). They were also most homologous to the isolate from ranch D, and least homologous to the isolate from ranch C. The isolates from ranch B were identical to each other. They showed the most homology to the isolate from ranch C and least homologous to the isolates from ranch A. The isolate from ranch C showed the greatest homology to the isolates from ranch B and the least homology the isolate from ranch D. The isolates from ranches D and E showed the greatest homology to each other (98.7%) and the least homology to the isolates from ranch A.

Table 7 Percent homology of the nucleotides (purple cells) and amino acid (green cells) sequence of the VP1 gene of AMDV.

		VP1 Nucleotides, % Homology										
		AMDV-G	**AMDV-Utah**	**WI/A*/1/Feb2008**	**WI/A*/5/Feb2008**	**WI/A*/6/Feb2008**	**WI/A/12/Apr2008**	**WI/B/14/Oct2008**	**WI/B/15/Nov2008**	**WI/C/18/Aug2010**	**UT/D/10/Jan2013**	**ID/E/22/Jan2011**
VP1 Amino Acids, % Homology	**AMDV-G**		96	98.46	98.46	98.46	98.55	96.43	96.43	96.38	98.7	98.79
	AMDV-Utah	96.09		96.48	96.43	96.43	96.48	96.33	96.33	96.43	95.71	96
	WI/A*/1/Feb2008	98.12	96.38		99.9	99.9	99.9	97.64	97.68	97.54	98.22	98.22
	WI/A*/5/Feb2008	98.12	96.38	99.71		100	99.9	97.64	97.64	97.49	98.22	98.22
	WI/A*/6/Feb2008	98.12	96.38	99.71	100		99.9	97.64	97.64	97.49	98.22	98.22
	WI/A/12/Apr2008	98.41	96.53	99.71	99.71	99.71		97.59	97.59	97.54	98.22	98.12
	WI/B/14/Oct2008	96.82	97.68	97.83	97.83	97.83	97.68		100	99.57	96.24	96.43
	WI/B/15/Nov2008	96.82	97.68	97.83	97.83	97.83	97.68	100		99.57	96.24	96.43
	WI/C/18/Aug2010	96.67	97.54	97.68	97.68	97.68	97.83	99.57	99.57		96.09	96.29
	UT/D/10/Jan2013	98.26	96.53	98.84	98.84	98.84	98.99	97.68	97.68	97.4		98.65
	ID/E/22/Jan2011	98.12	96.38	98.55	98.55	98.55	98.26	97.54	97.54	97.11	98.7	

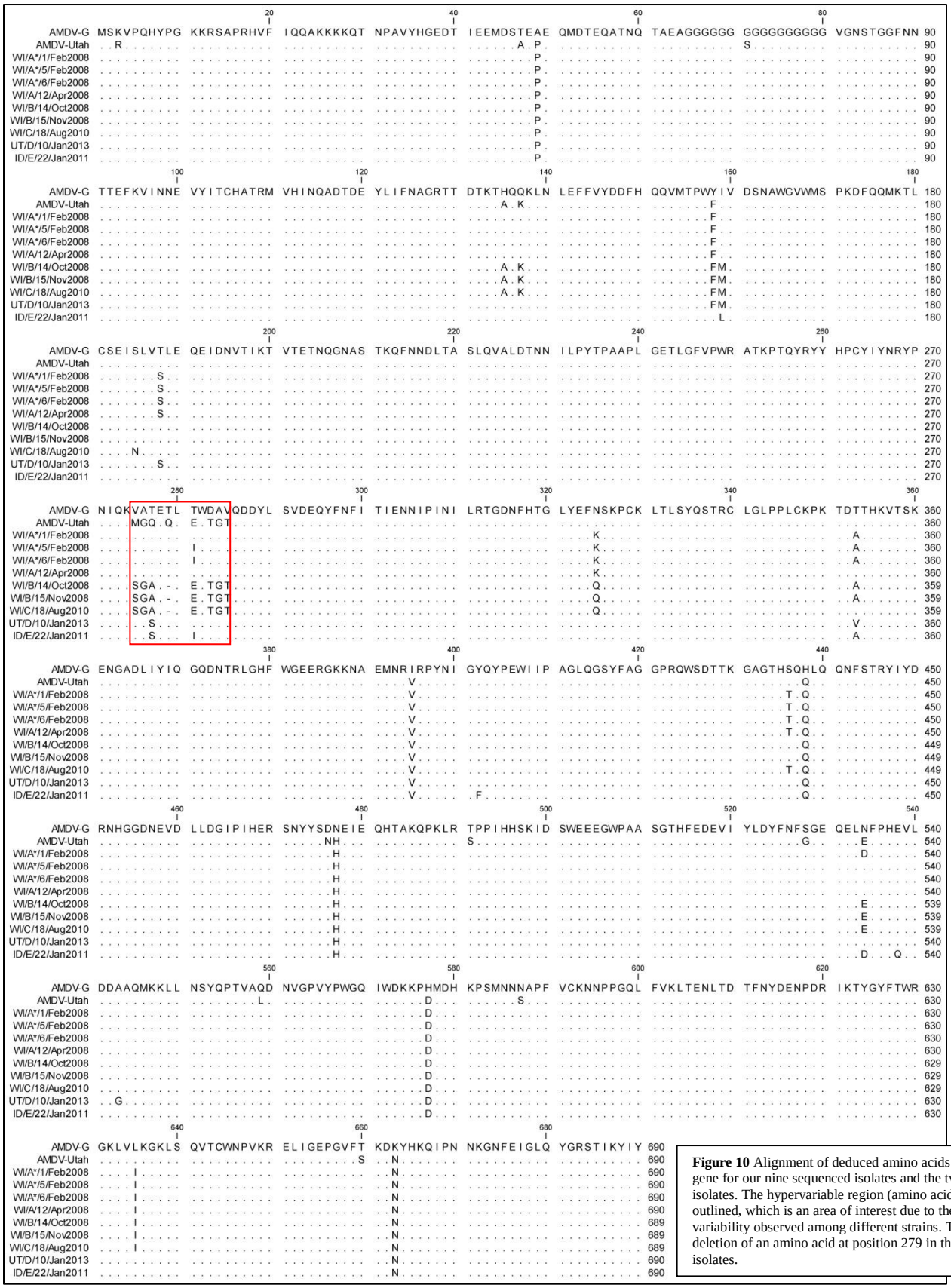

Figure 10 Alignment of deduced amino acids from the VP1 gene for our nine sequenced isolates and the two reference isolates. The hypervariable region (amino acids 275-285) is outlined, which is an area of interest due to the high variability observed among different strains. There is a deletion of an amino acid at position 279 in three of the nine isolates.

We constructed a phylogenetic tree (**Fig. 11**) based on the 11 full-length VP1 gene nucleotide sequences to examine the phylogenetic relationships between our sequenced Wisconsin isolates, the two isolates from Utah and Idaho, and the AMDV-G and AMDV-Utah strains. The sequences for AMDV-G and AMDV-Utah were obtained from GenBank. The tree was constructed by the neighbor-joining method using CLC Main Workbench 7 software. The results showed that the Wisconsin isolates from ranch A form their own cluster, the isolates from Utah and Idaho form another cluster with AMDV-G, and the Wisconsin isolates from ranches B and C form another cluster with AMDV-Utah.

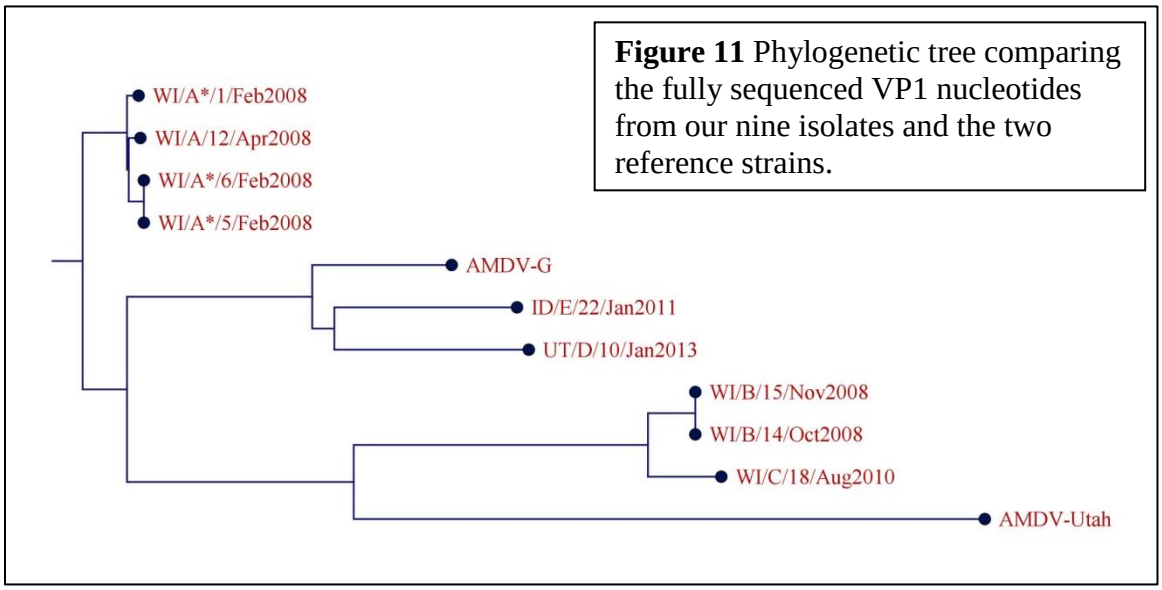

Figure 11 Phylogenetic tree comparing the fully sequenced VP1 nucleotides from our nine isolates and the two reference strains.

VP2

At the nucleotide level, there were a total of 126 mutations among the 1941 base pairs that code for the VP2 gene from the nine isolates. The isolates from ranches A, B, D, and E had a higher homology to AMDV-G than AMDV-Utah, while the isolate from ranch C had a higher homology to AMDV-Utah than AMDV-G (**Table 8**). The isolates from ranch A share a homology of 99.9-100%. They are most homologous to the isolate from ranch D and least homologous to the isolate from ranch C. The isolates from ranch B are identical. They are most

homologous to the isolate from ranch C and least homologous to the isolates from ranch A. The isolate from ranch C is most homologous to the isolates from ranch B and least homologous to the isolate from ranch D. The isolates from ranches D and E are most homologous to each other (98.61%) and least homologous to the isolate from ranch C.

At the amino acid level, there were a total of 36 mutations among the 647 amino acid residues of the VP1 protein from the nine isolates (**Fig. 12**). Isolates from ranches A and E have a higher homology to AMDV-G than AMDV-Utah, while isolates from ranches B, C, and D have a higher homology to AMDV-Utah than AMDV-G (**Table 8**). The isolates from ranch A are between 99.69-100% homologous to each other. They are most homologous the isolate from ranch D and least homologous to the isolate from ranch C. The isolates from ranch B are identical to each other. They are most homologous to the isolate from ranch C and least homologous to the isolate from ranch E. The isolate from ranch C is most homologous to the isolates from ranch B and least homologous to the isolate from ranch E. The isolates from ranches D and E were most homologous to each other and least homologous to the isolate from ranch C.

Table 8 Percent homology of the nucleotides (purple cells) and amino acid (green cells) sequence of the VP2 gene of AMDV.

		VP2 Nucleotides, % Homology										
		AMDV-G	**AMDV-Utah**	**WI/A*/1/Feb2008**	**WI/A*/5/Feb2008**	**WI/A*/6/Feb2008**	**WI/A/12/Apr2008**	**WI/B/14/Oct2008**	**WI/B/15/Nov2008**	**WI/C/18/Aug2010**	**UT/D/10/Jan2013**	**ID/E/22/Jan2011**
VP2 Amino Acids, % Homology	**AMDV-G**		95.99	98.41	98.41	98.41	98.51	98.35	96.35	96.35	98.66	98.71
	AMDV-Utah	95.99		96.45	96.4	96.4	96.45	96.4	96.4	96.4	95.73	95.99
	WI/A*/1/Feb2008	97.99	96.3		99.9	99.9	99.9	97.63	97.63	97.53	98.2	98.15
	WI/A*/5/Feb2008	97.99	96.3	99.69		100	99.9	97.58	97.58	97.48	98.2	98.05
	WI/A*/6/Feb2008	97.99	96.3	99.69	100		99.9	97.58	97.58	97.48	98.2	98.15
	WI/A/12/Apr2008	98.3	96.45	99.69	99.69	99.69		97.53	97.53	97.53	98.2	98.05
	WI/B/14/Oct2008	96.6	97.69	97.69	97.69	97.69	97.53		100	99.69	96.19	96.35
	WI/B/15/Nov2008	96.6	97.69	97.69	97.69	97.69	97.53	100		99.69	96.19	96.35
	WI/C/18/Aug2010	96.45	97.53	97.53	97.53	97.53	97.69	99.54	99.54		96.09	96.24
	UT/D/10/Jan2013	98.15	96.45	98.77	98.77	98.77	98.92	97.53	97.53	97.22		98.61
	ID/E/22/Jan2011	97.99	96.3	98.46	98.46	98.46	98.15	97.38	97.38	96.91	98.61	

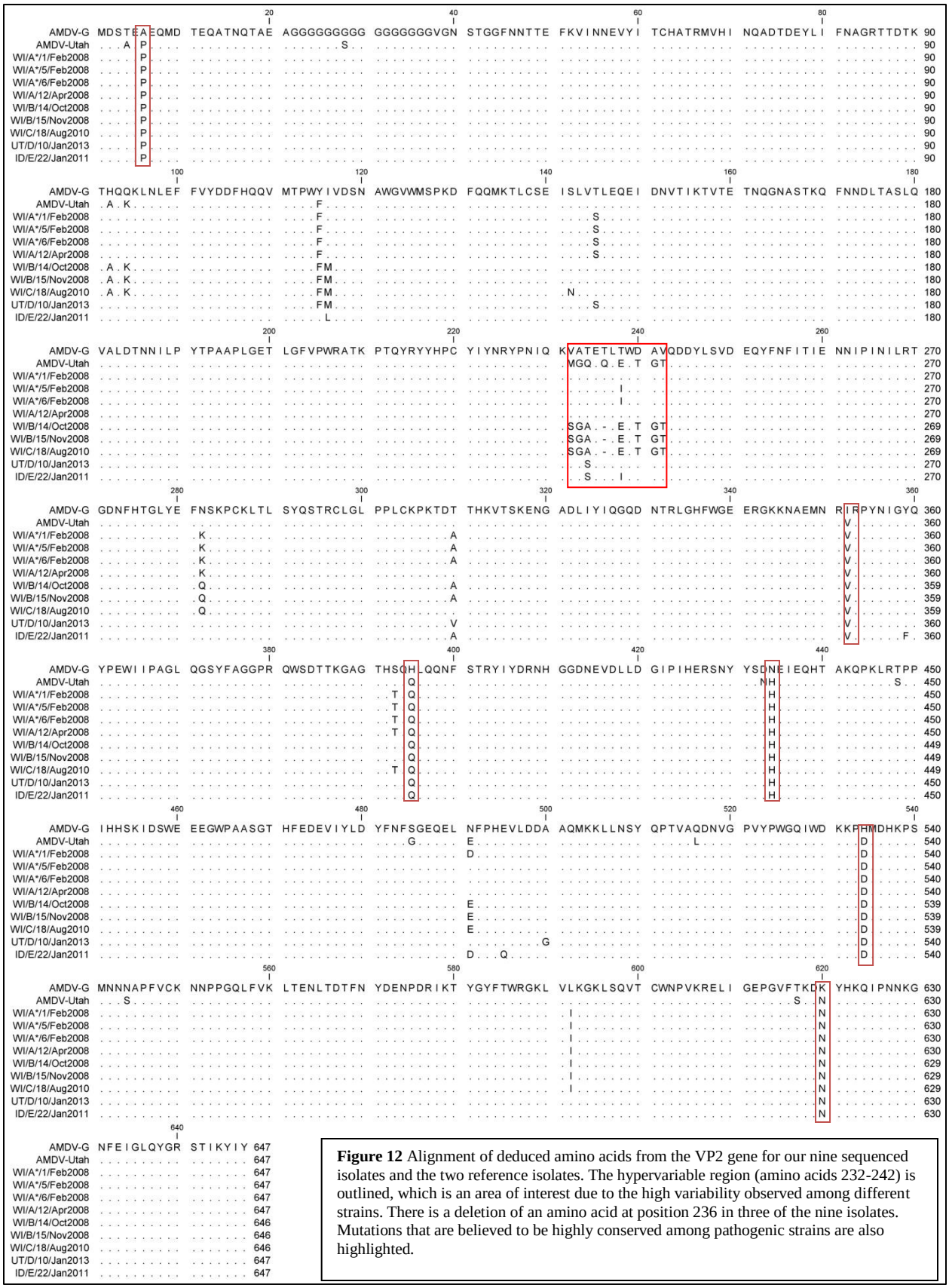

Figure 12 Alignment of deduced amino acids from the VP2 gene for our nine sequenced isolates and the two reference isolates. The hypervariable region (amino acids 232-242) is outlined, which is an area of interest due to the high variability observed among different strains. There is a deletion of an amino acid at position 236 in three of the nine isolates. Mutations that are believed to be highly conserved among pathogenic strains are also highlighted.

We constructed a phylogenetic tree (**Fig. 13**) based on the 11 full-length VP2 gene amino acid sequences to examine the phylogenetic relationships between our sequenced Wisconsin isolates, the two isolates from Utah and Idaho, and the AMDV-G and AMDV-Utah strains. The sequences for AMDV-G and AMDV-Utah were obtained from GenBank. The tree was constructed by the neighbor-joining method using CLC Main Workbench 7 software. The results showed that the Wisconsin isolates from ranch A form their own cluster, the isolates from Utah and Idaho form another cluster with AMDV-G, and the Wisconsin isolates from ranches B and C form another cluster with AMDV-Utah.

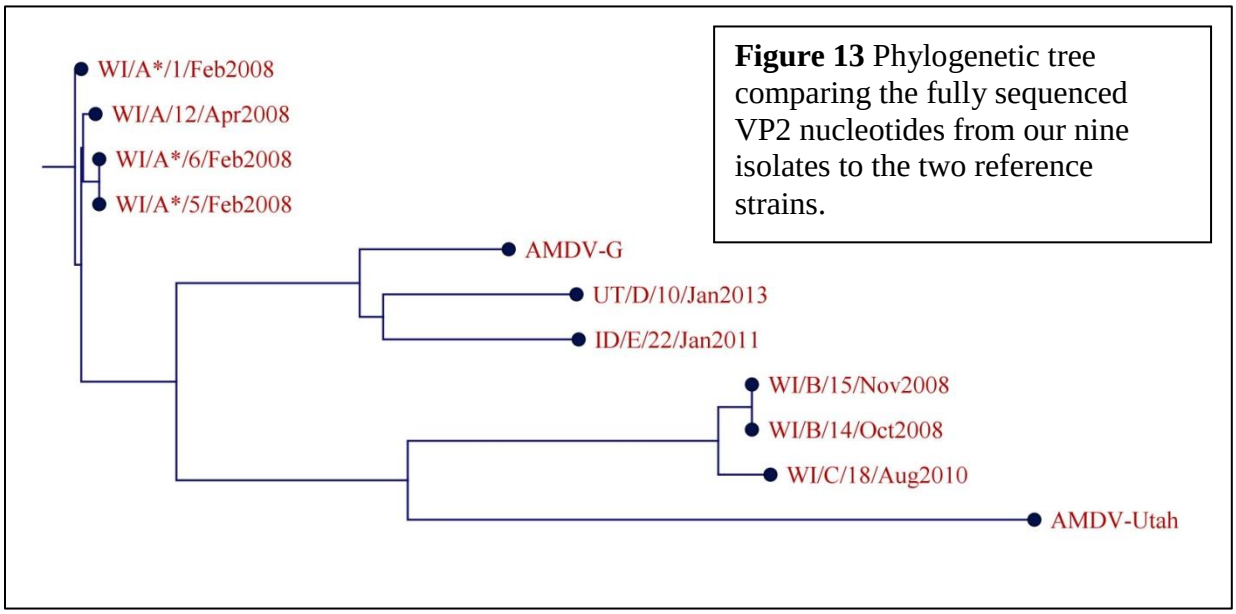

Figure 13 Phylogenetic tree comparing the fully sequenced VP2 nucleotides from our nine isolates to the two reference strains.

A second phylogenetic tree (**Fig. 14**) was constructed from 40 partial sequences of the VP2 gene (bp 2731-3241) to examine the relationships between our North American isolates to other AMDV strains around the globe. Once again we see the Wisconsin isolates from ranch A form their own cluster, with an isolate from Ireland (DQ630718) and another from Wisconsin (AMU39013, AMDV-TR) appearing the be the most closely related. It also appears that the Wisconsin isolates from ranch A share a more common ancestor with the non-pathogenic

AMDV-G than the pathogenic AMDV-Utah. The Wisconsin isolates from ranches B and C appear to form their own cluster that appears to share a more common ancestor with the pathogenic AMDV-Utah than the non-pathogenic AMDV-G. The isolate from ranch D in Utah appears to be more closely related to an isolate from Wisconsin (AMDV-TR) and to AMDV-G than AMDV-Utah or the other Wisconsin isolates. The isolate from ranch E in Idaho seems to be very closely related to another isolate from Wisconsin (AMDV-TH5) and AMDV-G than to any of the isolates from Wisconsin that we sequenced.

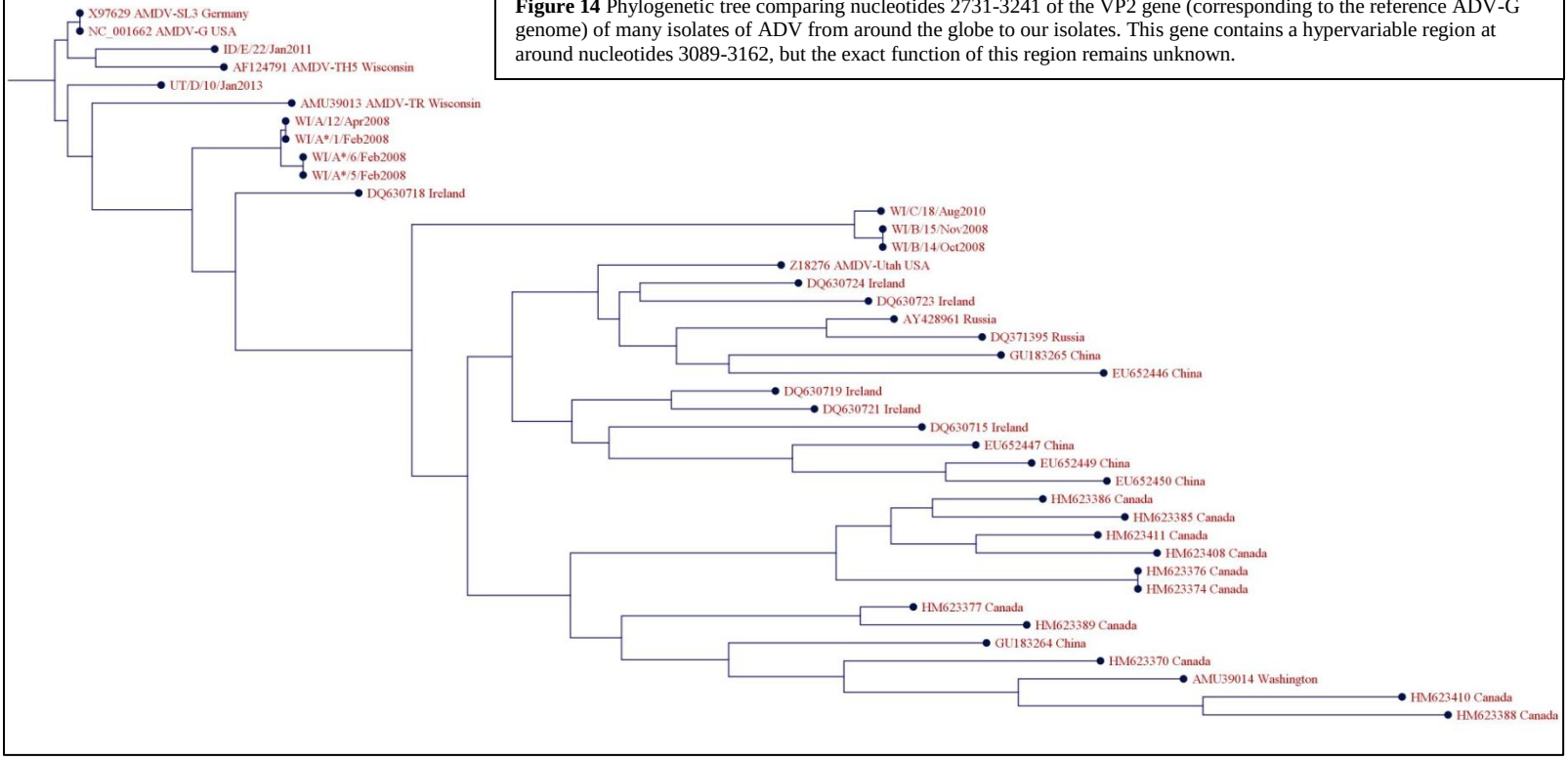

Figure 14 Phylogenetic tree comparing nucleotides 2731-3241 of the VP2 gene (corresponding to the reference ADV-G genome) of many isolates of ADV from around the globe to our isolates. This gene contains a hypervariable region at around nucleotides 3089-3162, but the exact function of this region remains unknown.

Discussion

To date, there have not been many studies that have fully sequenced different isolates of AMDV in North America. AMDV has been found to exhibit unusually high amounts of variability among isolates in Canada, Europe, and China (Nituch et al, 2011; Gottschalck et al, 1991; Gottschalck et al, 1994; Knuuttila et al, 2008; Olofsson et al, 1999; Li et al, 2012).

Molecular epidemiology can provide meaningful information to help control this infectious disease. In this study, we wanted to evaluate whole genomes of multiple isolates to ensure that the best regions are chosen from phylogenic analysis. The genome without the 5' and 3' termini of the nine North American isolates sequenced were 4,313 bp in length. Each full-length sequence of the structural and non structural genes were analyzed and compared to the two reference strains, and our isolates showed 94.5-96.7% nucleotide homology to AMDV-G and AMDV-Utah. A phylogenetic analysis revealed that the Western isolates were more closely related to AMDV-G and AMDV-Utah than the Wisconsin isolates, which formed their own cluster.

Our phylogenetic analyses revealed that clustering based on full-length genomic analysis tends to occur due to geographic location. However, the limited number of full-length sequences available mean that more isolates from around the globe would need to be fully sequenced to support this hypothesis. The Wisconsin isolates consistently clustered in unique clades across all the phylogenetic trees, indicating that this geographic location has unique sequences not identified previously. While temporal clustering was also observed in our isolates, it cannot be conclusively determined because of the short time span and limited number of samples that this study covered. Further studies that sample infected mink from the same ranch over a larger period of time would help identify how the virus changes over time.

Of our nine isolates, six (WI1-WI6) were classified as pathogenic due to the clinical signs exhibited by the mink and the mink mortality, while two (UT8, ID9) were classified as non-pathogenic because the mink did not exhibit any clinical signs and the ranches were only being tested as part of a routine screening procedure. One isolate (WI7) did not have any information about the animal's clinical signs, so the pathogenicity is unknown. AMDV-Utah is a known pathogenic strain, while AMDV-G is a non-pathogenic strain (Porter et al, 1969; Bloom et al,

1980). The major molecular difference between the two strains occurs in the hypervariable region of the VP2 gene; 8 out of 11 amino acid changes occur in this region (Bloom et al, 1988). Since this region occurs in an area that may determine viral host range (McKenna et al, 1999), it is reasonable to hypothesize that this area may be a determinant of pathogenicity and is more thoroughly discussed in the VP2 section.

Previous studies have detected multiple strains of AMDV in infected mink (Gottschalck et al, 1991). Due to financial constraints, we were unable to use next-generation sequencing to validate the presence of only one strain per isolate. Interestingly, when we used Sanger sequencing to sequence the PCR products, some products revealed different nucleotide sequences when compared to other products that sequenced the same fragment. This could indicate that two different strains were sequenced each time we amplified that particular segment, but further work with either cloning or next generation sequencing would need to be done to verify this.

Our analysis of the deduced proteins for the isolates in this study was confounded by earlier transcript analyses, which proved to be misleading in comparison to more recent work (Alexandersen et al, 1988; Qui et al, 2005; Huang et al, 2014). This discussion will presume that the most recent transcript analyses are correct. The older transcript analyses led to nonsensical deduced proteins; for example, using the start codon predicted by the old transcript analyses led to a stop codon early in the protein. Using the updated transcription map by Qui et al (2005) led to non-truncated proteins.

The NS genes have been found to contain the largest amount of variability of all the genes in the AMDV genome (Gottschalck et al, 1991; Gottschalck et al, 1994; Nituch et al, 2011). NS1 is the major non-structural protein in AMDV that along with NS2 and NS3 have been found to play key roles in viral replication and perhaps influence pathogenicity (Huang et

al, 2014; Best et al, 2002). The highest amount of variation among our isolates was found in the NS1 coding region, with the homology among the amino acids ranging between 88.6-100% among the isolates and reference strains. The variability that we observed in our isolates indicates that the NS1 gene should be targeted for sequence analysis when it is necessary to do epidemiological tracing.

The co-localization of the NS2 protein with the NS1 protein and viral DNA has proved to be an important factor in viral replication. The NS3 protein however does not co-localize with the NS1 protein and is not required for viral replication (Huang et al, 2014). The variation among both the NS2 and NS3 has not been explored. With our isolates, the NS2 and NS3 proteins were identical on ranch A; the isolates from ranches B and C were also identical in these proteins. These results indicate that NS2 and NS3 proteins may be highly conserved among geographical locations. It may also indicate an epidemiological link between ranch B and C. We did not observe a pattern between viral pathogenicity and the residues in the NS genes.

VP1 is a structural protein whose transcript analysis and protein sequence has recently been reanalyzed (Qui et al, 2005). Previous studies of what was formally considered to be the VP1 protein may no longer be relevant. Our analysis is based on the more recent transcript data, and with this deduced VP1 protein it is clear that there is little variability in our isolates except for the hypervarible region which is synonymous with the hypervariable region in VP2 (Qui et al, 2005). VP1 shares the same amino acids with VP2 except for the first 43 amino acids at the amino terminus.

An example of how these gene and protein analyses can help with the epidemiological tracing of strain emergence, is the recently published Chinese study in which a fully sequenced VP2 gene showed that the isolates were genetically similar to AMDV strains in other countries (Sang et al, 2012). This likely indicates that the disease was introduced at some point through the

international mink trade. However, the virus showed enough differences that it likely evolved into its own clade over time and tended to differ according to physically separated mink farming areas (Sang et al, 2012). Our results show that the VP2 region of our isolates also tended to form clusters based on geographic ranch locations.

Several amino acid mutations in the VP2 gene have been identified as highly conserved among pathogenic isolates (Oie et al, 1996; Cheng et al, 2010), and of those mutations there were four positions (352, 395, 434, and 534) were conserved across all of our isolates. There was one additional mutation at codon 620 that was highly conserved among all of our isolates and AMDV-Utah that has not been identified as belonging to strains of high pathogenicity. Interestingly, even the isolates that did not present with clinical symptoms (UT8 and ID9) had this mutation, indicating they are not obligate determinants of pathogenicity. One position at 491 has been described to be highly conserved in pathogenic isolates (Oie et al, 1996; Cheng et al, 2010), but our isolates showed three different amino acid residues possible at this location. A study by Stevenson et al (2001) discovered that a valine residue at codon 352 in the VP2 capsid protein affects in vivo viral replication and pathogenicity, and this valine residue was present in all of our nine isolates, even the ones determined to be non-pathogenic.

The hypervariable region in the VP1 and VP2 gene sequences has been described to be located between nucleotides 3100 to 3132 of the AMDV-G genome, which is a region where a high number of mutations are observed among different AMDV strains (Oie et al, 1995; Dyer et al, 2000). Our isolates WI5, WI6, and WI7 all showed a deletion of three base pairs which led to the deletion of one amino acid at codon 235 of the VP2 gene. This same deletion was identified in two Chinese strains (Sang et al, 2012). The function of this deletion is unknown and should be researched to determine if it has an effect on the virus pathogenicity.

Cheng et al (2010) identified a capsid cleavage site at the 420 codon position of the VP2 protein. The capsid protein can be cleaved by caspase at this site, and the mutation of a single amino acid residue at D420 removed the site-specific cleavage. It was predicted that this cleavage of the capsid proteins may have an effect in regulating persistent AMDV infection by reducing progeny virus production (Cheng et al, 2010). However this particular mutation was not observed in any of our isolates and with the absence of the caspase site, it would be predicted that our isolates would be able to replicate freely and thus be pathogenic. With all the observed differences in amino acids observed in the WVDL isolates, ideally future studies using site-directed mutagenesis to make chimera viruses may elucidate the contribution of each mutation to pathogenicity using in vivo models.

In conclusion, AMDV isolates do not appear to be grouped based solely on virulence, geographical origin, or year of isolation when examined from full genomic or individual gene analyses. The full genome analysis of multiple isolates that we undertook has provided valuable information for improving diagnostics and epidemiological tools in this country. This analysis indicated that our Wisconsin isolates showed unique sequences in all of the genes. More studies that involve fully sequencing the entire genome of different AMDV isolates should be performed over larger time spans and greater geographical areas in order to accurately determine phylogenetic relationships. This work has also led to the identification of the ideal site to target for an improved diagnostic assay for phylogenetic analyses. The variability of the NS1 region shows it will be a very good target to identify origin of strains; likewise, the hypervariable region in VP1 and VP2 will be a second region to target to better understand the emergence and transmission of strains.

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