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12 **Abstract**

13 Aleutian mink disease virus (AMDV) is a parvovirus that causes an immune complex-
14 mediated disease in minks. To gain a more detailed view of the molecular
15 epidemiology of mink AMDV in Finland, we phylogenetically analysed 14 new
16 Finnish strains from 5 farms and all 40 strains with corresponding sequences available
17 in GenBank. A part of the major non-structural protein (NS1) gene was amplified and
18 analysed phylogenetically. A rooted nucleotide tree was constructed using the
19 maximum parsimony method. The strains described in this study showed 86%–100%
20 nucleotide identity and were nearly identical on each farm. The ratio of synonymous
21 to non-synonymous substitutions was approximately 2.7, indicating a mild purifying
22 selection. Phylogenetic analysis confirmed that AMDV strains form three groups (I-
23 III), all of which contained Finnish strains. The tree inferred that the three lineages of
24 AMDV have been introduced to Finland independently. The analysis suggested that
25 AMDV strains do not cluster into genotypes based on geographical origin, year of
26 isolation or pathogenicity. Based on these data, the molecular clock is not applicable
27 to AMDV, and within this gene area no recombination was detected.

28

29 *Keywords:* Aleutian mink disease virus; Finland; Molecular epidemiology; NS1;

30 Parvovirus

31

32 1. Introduction

33 Aleutian disease (AD), a potentially fatal immune complex-mediated disease of the
34 mink (*Mustela vison*), is caused by Aleutian mink disease virus (AMDV). It is
35 common in all mink breeding countries and causes significant economic losses to
36 mink farmers. In addition to farmed and feral American minks, AMDV can also infect
37 and cause disease in other mustelids, raccoons and skunks (Kenyon et al., 1978;
38 Porter et al., 1982; Oie et al., 1996; Mañas et al., 2001; Fournier-Chambrillon et al.,
39 2004).

40

41 AMDV is the only member of the genus *Amdovirus*, subfamily *Parvovirinae*, family
42 *Parvoviridae*. It has an approximately 5-kb single-stranded DNA genome (Bloom et
43 al., 1990). The icosahedral non-enveloped virion contains three non-structural (NS1,
44 NS2, putative NS3) and two structural (VP1, VP2) proteins (Bloom et al., 1994; Qiu
45 et al., 2006). NS1 is encoded by ORF1 (Qiu et al., 2006). The NS1 protein of
46 parvoviruses is required for virus replication, regulation of DNA replication and
47 transcription, and it also has several enzymatic activities (reviewed by Christensen et
48 al., 1995). It has been found to be cytotoxic and can induce apoptosis (Vanacker and
49 Rommelaere, 1995; Moffatt et al., 1998). In AMDV, NS1 may also have a role in the
50 restriction of virus replication and pathogenicity (Best et al., 2002). Different strains
51 of AMDV exhibit a high degree of variability in the NS genes (Gottschalck et al.,
52 1994; Olofsson et al., 1999).

53

54 AD has different manifestations – from mild non-progressive to fatal progressive
55 disease in adult minks and acute pneumonia in mink kits – depending on the virus
56 strain and host factors (Hadlow et al., 1983; Bloom et al., 1994). The strains vary

57 from non-pathogenic (AMDV-G) to highly pathogenic (e.g. AMDV-Utah 1, -United,
58 and -K) (Bloom et al., 1980; Hadlow et al., 1983; Alexandersen 1990; Gottschalck et
59 al., 1994). Aleutian genotype minks with blue-grey coat colour are more susceptible
60 and severely affected than other genotypes (Hadlow et al., 1983; Bloom et al., 1994).
61 The most serious form of AD, known as classical AD, is characterised by viraemia,
62 plasmacytosis, hypergammaglobulinaemia, high anti-AMDV-antibody levels,
63 formation of infectious immune complexes and glomerulonephritis (Hadlow et al.,
64 1983; Bloom et al., 1994).

65
66 In Finland, mink farming began in the 1930s. The exact origin of Finnish minks is
67 unclear, but most of the breeding animals were probably initially imported from USA
68 (E. Smeds, personal communication). During the 1940s, Finnish breeders also
69 imported minks from Denmark and Sweden (E. Smeds, personal communication). In
70 general, the main import countries have been Denmark, USA and Canada. From
71 Finland, minks have been exported primarily to Russia, Poland and China (L. Finne,
72 personal communication). Most of the mink farms are situated in Ostrobothnia in
73 Western Finland. In 2006, the seroprevalence of AMDV in Finland was 3%
74 [measured with counter immunoelectrophoresis (CIE) from 384 600 minks].

75
76 Published phylogenetic analyses on AMDV are scarce (Gottschalck et al., 1994;
77 Schuierer et al., 1997; Olofsson et al., 1999; Murakami et al., 2001; Shackelton et al.,
78 2007), and most of them include only a few strains (Gottschalck et al., 1994;
79 Schuierer et al., 1997; Murakami et al., 2001). Furthermore, previous data on Finnish
80 AMDV strains are limited (Olofsson et al., 1999).

81

82 The findings of earlier studies on AMDV evolution suggest that mink AMDV strains
83 can be divided into three major groups (Olofsson et al., 1999), and that ferret strains
84 form a group of their own, separated from the mink strains (Murakami et al., 2001).
85 AMDV strains do not seem to group based on virulence, geographical origin or year
86 of isolation (Schuierer et al., 1997; Olofsson et al., 1999). Results also indicate that
87 the tree topology is very similar in phylogenetic trees constructed from different
88 AMDV proteins (Schuierer et al., 1997). A recent study by Shackelton et al. (2007)
89 shows that trees constructed from separate VP2 regions cut at recombination sites
90 have different topologies.

91

92 To perform a more extensive analysis on the molecular epidemiology and genetic
93 relationships of AMDV, we studied sequences of new field strains from Finland, and
94 included also previously reported strains from several geographic areas and time
95 periods in the analysis. Our aim was to determine possible correlations with temporal
96 and geographical distribution, transmission routes, pathogenicity, and variance. Since
97 there are a limited number of sequence data available of AMDV field strains from
98 minks, we wanted to include previous Swedish and Finnish reference sequences
99 (Olofsson et al., 1999) in our comparison, therefore using a part of the NS1 gene in
100 the analysis.

101

102 **2. Materials and methods**

103 **2.1. Animals and samples**

104 Minks were obtained from six commercial mink farms (A-F) located in the provinces
105 of Ostrobothnia and Northern Ostrobothnia (Finland) in spring 2005. Seventeen
106 minks were included: three from farms A-E and two from farm F. The farms had AD-

107 related clinical problems and AMDV antibody-positive minks. Due to the breeding
108 season, it was not possible to test the minks beforehand for antibodies. Hence, animals
109 showing clinical AD symptoms were chosen. Severe AD outbreaks occurred at three
110 farms: D, E and F. Soon after regaining an AMDV-free status, these farms had
111 encountered problems with rapidly spreading virus. Farm F also reported more severe
112 symptoms than previously.

113

114 Spleen, liver, mesenteric lymph node and blood samples were collected aseptically.

115 Some typical AD gross lesions could be detected, such as splenomegaly, mesenteric
116 lymphadenopathy and coloration and enlargement of kidneys and liver.

117

118 2.2. Detection of antibodies

119 The antibody response to AMDV infection was measured from serum samples by
120 counter immunoelectrophoresis (CIE), which was performed with a commercial
121 antigen following the manufacturer's instructions (Antigen Laboratory of the
122 Research Foundation of the Danish Fur Breeders' Association, Glostrup, Denmark).

123

124 2.3. PCR and sequencing

125 From each animal, 100 mg of spleen, liver and mesenteric lymph node (only spleen
126 and liver from minks FIN05/F16 and FIN05/F17) were homogenised and DNA was
127 extracted with a commercial kit, TriPure (Roche, Indianapolis, USA), following the
128 manufacturer's instructions.

129

130 A 390-nucleotide fragment of the AMDV NS1 gene was amplified from the isolated
131 DNA by semi-nested PCR using previously described primers (Olofsson et al., 1999).

132 The PCRs were performed in 100- μ l volumes containing 5 μ l template DNA for the
133 first and 2 μ l for the second round, 1x PCR buffer with $(\text{NH}_4)_2\text{SO}_4$, 0.2 mM dNTP
134 mix, 1.5 mM MgCl_2 , 0.5 μ M of each primer and 2.5 u Taq polymerase (Fermentas,
135 Burlington, Canada). The first-round amplification mixture was initially incubated at
136 95°C for 10 min, then cycled 35 times through denaturation at 95°C for 30 sec,
137 annealing at 56°C for 30 sec, and elongation at 72°C for 1 min, with a final 10-min
138 elongation step at 72°C. The second-round amplification was identical to the first
139 round, except that the number of cycles was 30 and the annealing temperature 54°C.
140 The PCR products were visualised by agarose gel electrophoresis. Each mink's most
141 prominent PCR product from one organ, usually the spleen, was purified with
142 QIAquick PCR Purification kit (Qiagen, Hilden, Germany) according to the
143 manufacturer's instructions. The purified PCR products were sequenced in both
144 directions.

145

146 2.4. Sequence analysis

147 The acquired nucleotide sequences were assembled and translated with BioEdit 7.0.5.
148 (www.mbio.ncsu.edu/BioEdit/bioedit.html) and GeneDoc 2.6.0.2 (Nicholas and
149 Nicholas, 1997). All 40 available AMDV NS1 sequences were collected from the
150 GenBank database (www.ncbi.nlm.nih.gov/entrez) (sequences available in the
151 GenBank at the time of submission). AMDV strains included are presented in Table 1.
152 Identity matrices of both nucleotide and amino acid sequences were constructed by
153 BioEdit 7.0.5. The analysis of synonymous and non-synonymous nucleotide
154 substitutions was calculated by SNAP (Korber, 2000; <http://www.hiv.lanl.gov>). In this
155 program, the rates of synonymous (ds) vs. non-synonymous (dn) substitutions are

156 calculated based on the method of Nei and Gojobori (1986) for all pairwise
157 comparisons of sequences in an alignment.
158
159 To infer ancestral relationships, the tree was rooted, and human parvovirus B19
160 (accession no. AY903437) and porcine parvovirus (accession no. DQ499631) were
161 used as outgroups. Multiple sequence alignment (Fig. 1) was performed with
162 ClustalW 1.83 (Thompson et al., 1994) by using default values. The final
163 phylogenetic analysis was carried out by both MEGA 3.1 (Kumar et al., 2004) using
164 the maximum parsimony (MP) method with heuristic close-neighbor interchange
165 search and PAUP* 4.0 (Swofford, 2003) with the maximum likelihood (ML) method.
166 Modeltest 3.7 (Posada and Crandall, 1998) run in PAUP* 4.0 (Swofford, 2003) was
167 used to test for the best nucleotide substitution model for ML. The most appropriate
168 model for AMDV was general time reversible + invariant sites + gamma (GTR+I+G).
169 Bootstrap resampling was performed for each analysis.

170
171 Split decomposition analysis was performed with SplitsTree4 (Huson and Bryant,
172 2006) using the HKY85 method (Hasegawa et al., 1985) for computing distances.
173 Existence of molecular clock was tested with TreePuzzle (Schmidt et al., 2002) using
174 ML with GTR and 25 000 puzzling steps. Rate heterogeneity was applied using a
175 uniform rate of nucleotide substitutions, gamma distribution with eight rate categories
176 and mixed model (1 invariable + 8 gamma rates). Outgroups and identical sequences
177 were excluded from the analysis.

178

179 **3. Results**

180 3.1. Serology and PCR

181 Altogether 13 of 17 minks were AMDV antibody-positive by CIE, and 14 of 17
182 minks were AMDV DNA-positive by PCR (Table 2). The acquired consensus
183 sequences varied from 343 to 377 bp in length. The sequences were edited to a length
184 of 336 bp corresponding to nucleotide positions 587–922 of the complete sequence of
185 the AMDV-G. GenBank accession numbers are given in Table 1.

186

187 3.2. Sequence analysis

188 The NS1 gene nucleotide sequences of the newly sequenced strains had 86%–100%
189 identity to each other and 87%–96% to AMDV-G (Table 3). At the amino acid level,
190 the strains were 78%–100% identical to each other and 79%–93% to AMDV-G. The
191 most diverged of the newly sequenced strains were FIN05/D11 and /D12 vs.
192 FIN05/B4-6; where the nucleotide identity was 86% and amino acid identity 78%.
193 Strains FIN05/B5 and /B6, and FIN05/E14, /E15 and /F17 were identical. For all
194 sequences, the mean ratio of synonymous to non-synonymous (ds/dn) substitutions
195 was 2.71 (1.01-5.02). Identities and synonymous and non-synonymous substitutions
196 of all AMDV strains and genogroups are depicted in Table 3.

197

198 3.3. Phylogenetic analysis

199 A rooted nucleotide tree, constructed by MEGA using the MP method, was found to
200 best explain the evolutionary history of AMDV (Fig. 2). The topology was essentially
201 the same with ML in PAUP*. However, the MP tree was more fully resolved in the
202 internal nodes near the taxa. Based on the tree topology, the AMDV strains fell into
203 three groups. Because no official genotyping exists for AMDV at the moment
204 (Fauquet et al., 2005), the genogroups were designated as I, II and III.

205

206 Finnish AMDV strains were distributed in all groups. However, none of the newly
207 sequenced strains were in group II, which consists completely of Nordic strains.
208 Strains from farm D were placed in group I, thus differing from the other Finnish
209 strains described in this study. Group I strains shared a common ancestor with some
210 of the Swedish strains and with the highly pathogenic United strain. All the other
211 strains described in this study – from farms B, C, E and F – shared a common ancestor
212 and belonged to group III. Also G, SL3, highly pathogenic Utah 1 and two previous
213 Finnish strains were placed in this group. None of the Swedish strains were in this
214 group.

215

216 The tree constructed with SplitsTree4 did not show any recombination between the
217 groups of strains within the studied region (data not shown), thus suggesting a tree-
218 like evolution. The conflicting evolutionary signals in the data set would have been
219 presented as a network. Furthermore, a clock-like tree was rejected for all of the tested
220 rate heterogeneity models.

221

222 **4. Discussion**

223 We phylogenetically analysed 54 mink AMDV strains originating from several
224 European countries and USA. The phylogenetic analysis indicated that AMDV strains
225 form three clearly distinct genetic groups. Grouping of the reference strains was
226 consistent with previous analyses (Gottschalck et al., 1994; Schuierer et al., 1997;
227 Olofsson et al., 1999; Murakami et al., 2001).

228

229 The geographical origin of the common ancestor of all of the strains and the possible
230 ancestor of the groups I and II warrant further studies. The common ancestor could

231 originate from USA, Denmark, or even Finland, but due to the North American origin
232 of the species of mink being bred, AMDV has likely been introduced from North
233 America to the rest of the breeding countries and all three genotypes probably already
234 exist there. However, feral origin of AMDV in another mustelid species cannot be
235 excluded.

236

237 The tree inferred that AMDV has been introduced to Finland on several different
238 occasions, at least three times, as Finnish strains could be found in all three groups.
239 Regrettably, exact information on Finnish mink imports and exports decades ago is
240 unavailable; thus, the origin of the ancestors of Finnish strains also remains unclear.
241 Hypothetically, the virus was introduced with the imported breeding minks from
242 North America and Denmark or Sweden at the beginning of mink farming during the
243 1930s and 1940s. More sequence data, especially from North American strains, are
244 needed to compile a clearer picture of the molecular epidemiology of AMDV.

245

246 Although group II comprises only Nordic strains, with more sequence data, AMDV
247 strains from other geographical areas would likely also fall into this group.

248

249 All of the strains appeared to be viable, as several lineages seemed to evolve
250 simultaneously. No correlation according to the year of isolation or geographical
251 origin was evident. Known pathogenic strains existed in all of the groups, i.e., United
252 in group I, K in group II and Utah 1 in group III. Thus, no clustering according to
253 pathogenicity was detected. Highly pathogenic strains seem to appear spontaneously
254 from different genetic backgrounds; from strains of low pathogenicity and vice versa.
255 AMDV-United seems to be ancestral to farm D strains, which could indicate high

256 pathogenicity and explain the rapid spread of the virus at the farm. On the other hand,
257 only a few nucleotide changes in a parvovirus may greatly alter its biological
258 characteristics (Parrish, 1999).

259

260 The strains described here were nearly identical on one farm; however, strains from
261 one farm can fall into different groups (Olofsson et al., 1999). Gottschalck et al.
262 (1991) reported also that several types of AMDV sequence can be found in one
263 animal. They studied strains that had been passaged in mink several times; thus, cross-
264 contamination with other strains is possible. In our study, there was no indication of
265 infection of one animal with multiple strains, as no minor or alternative variants could
266 be seen in the sequence chromatogram data. However, this was not verified by
267 cloning.

268

269 Earlier studies (Gottschalck et al., 1994; Schuierer et al., 1997; Olofsson et al., 1999)
270 have implied that AMDV has a replication bias towards amino acid changes.
271 Gottschalck et al. (1994) reported that the ds/dn ratio for AMDV NS1 is around one,
272 suggesting that there might be positive selection for variation. However, they analysed
273 only four strains. Here we found the ratio to be slightly higher, approximately 2.7,
274 indicating mild purifying selection. Lukashov and Goudsmit (2001) also suggested a
275 ds/dn ratio of over one for AMDV. Ds/dn ratios of ORF1 reported for other
276 parvoviruses are higher, from four to eight (Lukashov and Goudsmit, 2001).

277

278 Another question is whether the virus is evolving slowly or rapidly. Gottschalck et al.
279 (1994) calculated, based on the estimated rate of retained nucleotide sequence
280 substitution per year, that AMDV-G and -K have separated 700, and -G and -Utah 1

281 50 evolutionary years ago, concluding that the observed heterogeneity is due to the
282 long evolutionary history of the virus rather than the result of a high mutation rate.

283 Our data indicated that a molecular clock is not applicable for AMDV, thus a constant
284 rate of evolution cannot be applied and such calculations are unreliable.

285

286 No recombination of AMDV strains was found, possibly because of the short
287 sequence analysed. Shackelton et al. (2007) recently reported putative recombination
288 regions for AMDV at two nucleotide sites of the VP2 gene. Moreover, recombination
289 has been noted to occur in other parvoviruses (Johansen et al., 1998; Lukashov and
290 Goudsmit, 2001; Shackelton et al., 2007).

291

292 Note added in revision: During the submission/revision period, new AMDV NS1
293 sequences derived from strains from Denmark and the Netherlands have become
294 accessible in GenBank. The Dutch strains form a distal lineage within group I, and the
295 Danish strains cluster within either group II or group III. The latter strains are
296 separate from and form a sister taxon with the other group III strains presented here.
297 Trees added with this data do not alter the clustering of the Finnish strains to the three
298 groups. Overall, a wider genetic variability exists within the Finnish strains than in the
299 Danish and Dutch strains.

300

301 **5. Conclusions**

302 In this study, phylogenetic analysis was performed on 54 AMDV strains. According
303 to the analysis, the strains formed three groups (I-III), and Finnish strains could be
304 found in all groups, indicating that AMDV has been introduced at least three times to
305 Finland. The strains described in our study were nearly identical on each farm. No

306 correlation with the year or place of isolation or pathogenicity was detected. Contrary
307 to previous studies, AMDV seemed to be under mild purifying selection. No
308 recombination or molecular clock was verified here.

309

310 We analysed partial NS1 sequences, and more sequence data, possibly from several
311 genes, are needed to construct a more fully resolved AMDV tree. More importantly,
312 to gain more data on AMDV evolution, obtaining strains from mink farms from
313 different countries and strains from ferrets and feral mustelids is necessary.

314

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323

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412 **Tables**

413 Table 1.
414 AMDV strains included in the phylogenetic analysis (n=54).

Strain	Isolation year	Country and region	Reference	GenBank accession no.
FIN05 ^a /B4 ^b	2005	Finland, Nykarleby	This publication	Will be added upon acceptance
FIN05/B5	2005	Finland, Nykarleby	This publication	-
FIN05/B6	2005	Finland, Nykarleby	This publication	-
FIN05/C7	2005	Finland, Sundby	This publication	-
FIN05/C8	2005	Finland, Sundby	This publication	-
FIN05/C9	2005	Finland, Sundby	This publication	-
FIN05/D10	2005	Finland, Yppäri	This publication	-
FIN05/D11	2005	Finland, Yppäri	This publication	-
FIN05/D12	2005	Finland, Yppäri	This publication	-
FIN05/E13	2005	Finland, Hirvlax	This publication	-
FIN05/E14	2005	Finland, Hirvlax	This publication	-
FIN05/E15	2005	Finland, Hirvlax	This publication	-
FIN05/F16	2005	Finland, Hirvlax	This publication	-
FIN05/F17	2005	Finland, Hirvlax	This publication	-
A1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107626
A2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107627
B1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107628
B2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107629
B3	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107630
C1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107631
D1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107632
D2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107633
E1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107634
E2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107635
F1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107636
F2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107637
F3	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107638

G1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107639
H1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107640
H2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107641
H3	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107642
I1	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107643
I2	1995	Sweden, Blekinge	Olofsson et al. (1999)	AF107644
J1	1996	Sweden, Halland	Olofsson et al. (1999)	AF107645
K1	1995	Sweden, Skaraborg	Olofsson et al. (1999)	AF107646
K2	1995	Sweden, Skaraborg	Olofsson et al. (1999)	AF107647
L1	1995	Sweden, Skaraborg	Olofsson et al. (1999)	AF107648
L2	1995	Sweden, Skaraborg	Olofsson et al. (1999)	AF107649
M1	1995	Sweden, Skaraborg	Olofsson et al. (1999)	AF107650
M2	1995	Sweden, Skaraborg	Olofsson et al. (1999)	AF107651
N1	1997	Sweden, Skåne	Olofsson et al. (1999)	AF107652
N2	1997	Sweden, Skåne	Olofsson et al. (1999)	AF107653
N3	1997	Sweden, Skåne	Olofsson et al. (1999)	AF107654
O1	1997	Sweden, Älvsborg	Olofsson et al. (1999)	AF107655
O2	1997	Sweden, Älvsborg	Olofsson et al. (1999)	AF107656
P1	1997	Finland, Vaasa	Olofsson et al. (1999)	AF107657
Q1	1996	Finland, Vaasa	Olofsson et al. (1999)	AF107658
R1	1997	Finland, Vaasa	Olofsson et al. (1999)	AF107659
R2	1997	Finland, Vaasa	Olofsson et al. (1999)	AF107660
AMVD-G ^c	Late 1970s	USA	Bloom et al. (1980, 1990)	M20036
AMDV-Utah 1	1963	USA, Utah	Gottschalck et al. (1994)	X77083
AMDV-United	n/a ^d	USA, n/a	Gottschalck et al. (1994)	X77085
AMDV-K	1982	Denmark, n/a	Gottschalck et al. (1994)	X77084
AMDV-SL3	Early 1980s	Germany, n/a	Schuijer et al. (1997)	X97629

415 ^a Sequences described in this study are indicated with FIN05

416 ^b Farm and number of the animal

417 ^c Cell culture adapted clone of AMDV-Utah 1

418 ^d Data not available

419

420

421 Table 2.
 422 AMDV-antibody and AMDV-DNA findings of the studied minks^a (n=17).

Mink	CIE	PCR		
		Spleen	Mesenteric lymph node	Liver
FIN05/A1 ^b	-	-	-	-
FIN05/A2	-	-	-	-
FIN05/A3	-	-	-	-
FIN05/B4	+	+	+	+
FIN05/B5	+	+	+	+
FIN05/B6	+	+	+	+
FIN05/C7	+	+	-	+
FIN05/C8	+	-	+	-
FIN05/C9	-	+	-	-
FIN05/D10	+	+	+	+
FIN05/D11	+	-	+	+
FIN05/D12	+	+	+	-
FIN05/E13	+	+	+	+
FIN05/E14	+	+	+	+
FIN05/E15	+	+	+	+
FIN05/F16	+	+	n/a ^c	+
FIN05/F17	+	+	n/a	+

423 ^a Antibodies were tested by counter immunoelectrophoresis and DNA by semi-nested NS1 PCR

424 ^b Farm and number of the animal

425 ^c Data not available

426

427

428

429 Table 3.
 430 AMDV NS1 (336-bp) mean sequence identities and substitutions. Range is indicated in parentheses.

	Nucleotide identities	Amino acid identities	Synonymous substitutions (ds)	Non-synonymous substitutions (dn)	Synonymous to non-synonymous substitutions (ds/dn)
All strains	89% (81-100%)	83% (70-100%)	0.17 (0.07-0.37)	0.06 (0.03-0.09)	2.71 (1.01-5.02)
Group I strains	95% (90-100%)	91% (80-100%)	0.05 (0.00-0.14)	0.02 (0.00-0.06)	3.08 (0.82-13.95)
Group II strains	93% (87-100%)	89% (79-100%)	0.11 (0.06-0.19)	0.04 (0.02-0.06)	3.02 (2.39-3.84)
Group III strains	97% (94-100%)	94% (88-100%)	0.03 (0.00-0.10)	0.01 (0.00-0.04)	2.86 (2.14-3.61)

431 When calculating ds, dn and ds/dn, all strains were compared with the consensus of all sequences, and strains within one group
 432 were compared with the consensus sequence of each group. Identical sequences were excluded from the analysis.
 433 Identities were calculated by pairwise comparisons.
 434
 435

436 **Figure captions**

437

438 Fig. 1. Alignment of nucleotide sequences of a 336-bp fragment of the NS1 gene of
439 54 AMDV strains. Only the nucleotides different from the consensus sequence are
440 shown.

441

442 Fig. 2. Phylogenetic tree of 54 AMDV strains based on alignment of the 336-
443 nucleotide fragment of an AMDV NS1 gene. The tree was constructed with MEGA
444 using maximum parsimony. Country of origin is stated in parentheses. The strains fell
445 into three groups, indicated with Roman numerals I–III. Finnish strains are circled.
446 Numbers at nodes indicate bootstrap values. Only values greater than 50% are shown
447 (1000 replications). The scale for genetic distance is provided below. See Table 1 for
448 strain designations.

449

A2 :AG.....A.....A.....C.G.....T... : 90
 B2 :AG.....A.....G.....A.....A.....A... : 90
 TGTTCCTAGGTTACTTTGATAAGAATGAAGATCCTAAGGATGTTCAAAAATCCTTAGGTTGGTTTCTAAAAAATTAATAAAGACCTA

100 * 120 * 140 * 160 * 180
 F1 :T.....A.....C.....C.A...G..... : 180
 F3 :G.T.....A.....C.....A...G..... : 180
 C1 :C.....T.....A.....C.....A...G..... : 180
 E1 :C.....T.....A.....C.....A...G..... : 180
 H1 :C.....T.....A.....C.....C.A...G..... : 180
 H3 :C.....T.....A.....C.....C.A...G..... : 180
 F2 :C.....T.....A.....C.....C.A...G.....A..... : 180
 B1 :C.....T.....A.....C.....C.....G..... : 180
 B3 :C.....T.....A.....C.....C.A...G..... : 180
 N2 :G.....G..T.....A...A.....C.....C.A...G..... : 180
 N3 :G.....G..T.....A...A.....C.....C.A...G..... : 180
 N1 :G.....G..T.....A...A.....C.....C.A...G..... : 180
 E2 :C.....T.....A.....C.....C.A...G..... : 180
 D2 :C.....T.....A...A.C.....A...G..... : 180
 H2 :C.....T.....A...A.C.....A...G..... : 180
 I1 :C.....T.....A...A.C.....A...G..... : 180
 L1 :C.....T.....A...A.C.....A...G..... : 180
 L2 :C.....T.....A...A.C.....A...G..... : 180
 FIN05/D10 :T.....A...A.....C.....A.C.A..... : 180
 FIN05/D11 :T.....A...A.....C.....A.C.A..... : 180
 FIN05/D12 :T.....A...A.....C.....A.C.A..... : 180
 I2 :T.....A...A.....C.....C.A..... : 180
 United :C.....T.....A...A.....C.....C.A..... : 180
 A1 :C.....T...G.....T.A...A.C.....AA...A...A...A..... : 180
 G1 :C.....T.....A...A.C.....A.....A...G..... : 180
 J1 :T.....C.....C.....C.....C.....G..... : 180
 SL3 :C.....A.....A.....A.A.....G...C.A..... : 180
 G :C.....T.....A...A.....A.A.....G...C.A..... : 180
 Utah 1 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 R2 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/B4 :A.....A.....A.....A.A.....G...C.A.....G..... : 180
 R1 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/E14 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/E15 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/F17 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/B5 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/B6 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180
 FIN05/E13 :C.....T.....A...A.....A.A.....G...C.A.....G..... : 180

```

FIN05/F16 : .....A.....A.A.....G..C.A.....G..... : 180
FIN05/C8 : .....A.....A.A.....G..C.A.....G..... : 180
FIN05/C9 : .....A.....A.A.....G..C.A.....G..... : 180
FIN05/C7 : .....A.....A.A.....G..C.A.....G..... : 180
K : .....GG.....A.T...G..AC.A..T...T.G.....G.....A.A..... : 180
Q1 : .....GG.....A.T...G..AC.A..T...T.G.....G.....A.A..... : 180
P1 : .....T.GG.....A.....G..AC.A..T...T.A.....GC.....A..... : 180
K1 : .....G.T.....G..G..AC.A..T...T.G.....G.....A..... : 180
K2 : .....G.T.....G..G..AC.A..T...T.G.....G.....A..... : 180
D1 : .....G.T.....A.G...G..AC.A..T...T.G.....G.....A..... : 180
M1 : .....G.T.....A.G...G..AC.A..T...T.G.....G.....A..... : 180
M2 : .....G.T.....A.G...G..AC.A..T...T.G.....G.....A..... : 180
O1 : .....G.T.....A.G...G..AC.A.....T.G.....A..... : 180
O2 : .....G.T.....A.G...G..AC.A.....T.G.....A..... : 180
A2 : .....G.T.....A.G...G..AC.A.....AAG.....A..... : 180
B2 : ...TG.G.T.....A.G.....A.A.....A.G.....A..... : 180
GCAGTTATCTATAGTAACCATCATTGTGACCTACAAGATATTAAGGATCCTGAGGCTAGAGCTAATAACTTGAAAGTGTGGATTGAAGAT

```

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*      200      *      220      *      240      *      260      *
F1 : .....G..... : 270
F3 : .....C.....G..... : 270
C1 : .....C.C.....C.....T..... : 270
E1 : .....C.C..G.....CC.....G.....T..... : 270
H1 : .....TC.....G.....T.C..... : 270
H3 : .....A.....TC.....G.....T.C..... : 270
F2 : .....G.....A.....C.....G.....T...GC..... : 270
B1 : .....C..G.....T.....C.....G.....T...G..... : 270
B3 : .....C.....C.....C.....G.....T..... : 270
N2 : .....C.C.....C.....C.....T.G.G..... : 270
N3 : .....C.C.....C.....C.....T...G..... : 270
N1 : .....C.C.....C.....C.....T..... : 270
E2 : .....G.....C.....G.....T.C..... : 270
D2 : .....C.....C.....C.....G.....T..... : 270
H2 : .....C.....C.....C.....G.....T..... : 270
I1 : .....C.....C.A...G.....T...G..... : 270
L1 : .....C.....C.....C.....G.....T...G..... : 270
L2 : .....C.C.....C.....C.....G.....T...G..... : 270
FIN05/D10 : .....C.....T.....T.....C...CT..C...T..... : 270
FIN05/D11 : .....C.....T.....G.....C...CT..C...T..... : 270
FIN05/D12 : .....C.....T.....C...CT..C...T..... : 270
I2 : .....C..G.....C...CT..C...ATC..... : 270
United : .....T.....C...T..C...T..... : 270

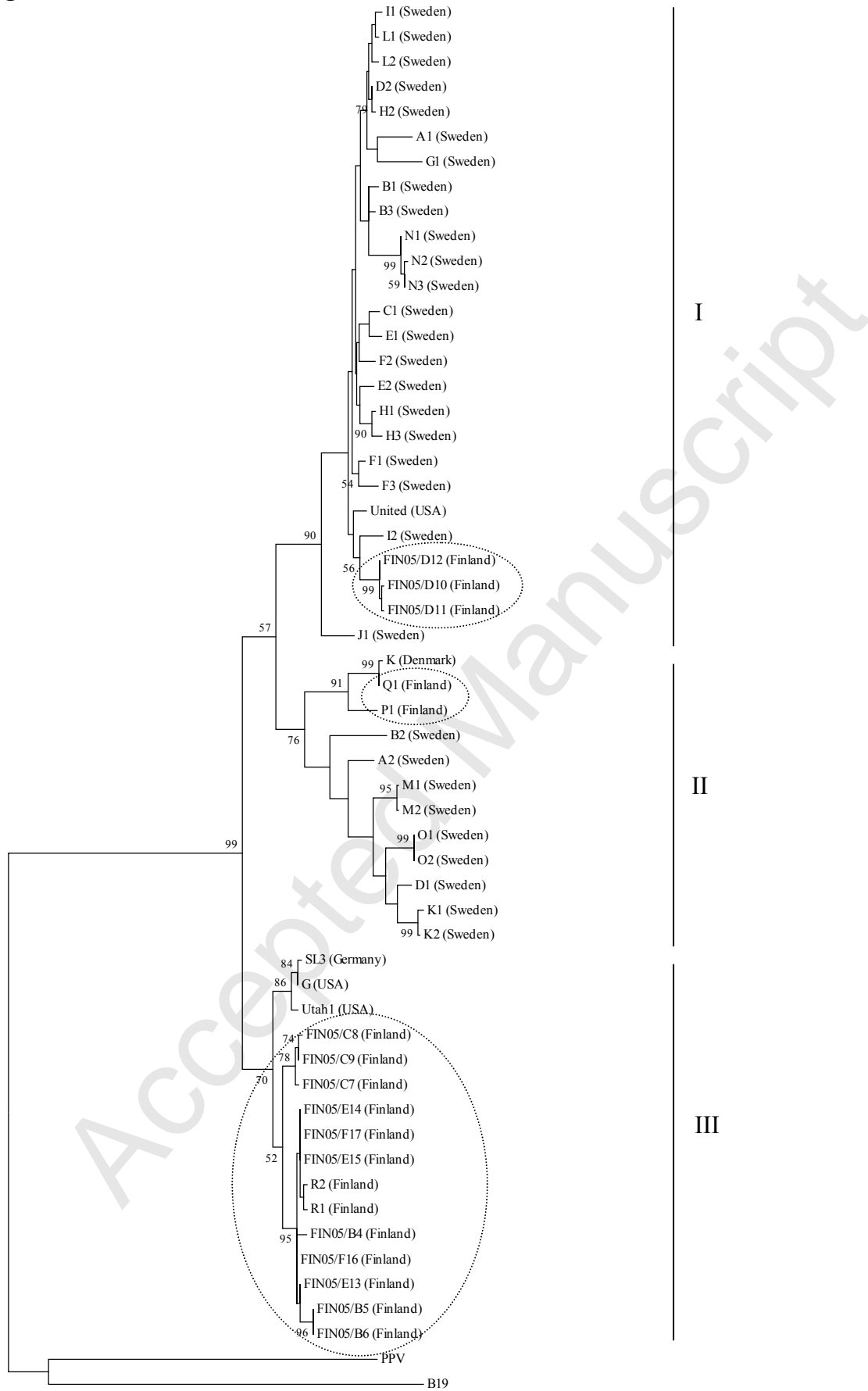
```

A1 :C.A...T.....T..... : 270
 G1 :G...A.AC.T..C...T..C.....T : 270
 J1 :C..C.....A.....A.....T.T.....T..G..... : 270
 SL3 : ..A.....G.....T.T.....T.....C.....C..A..T.C..... : 270
 G : ..A.....G.....T.T.....T.....C.....C..A..T.C..... : 270
 Utah 1 : ..A.....G.....T.T.....C.....C..A..T.C..... : 270
 R2 : ..A.....G.....T.T...G.....T.....G.A.A.....A.GT.G..... : 270
 FIN05/B4 : ..A.....G.....T.T.....G.....T.....A.A.....A.GT.G..... : 270
 R1 : ..A.....G.....T.T.....T.....G.A.A.....A.GT.G..... : 270
 FIN05/E14 : ..A.....G.....T.T.....G.A.A.....A.GT.G..... : 270
 FIN05/E15 : ..A.....G.....T.T.....G.A.A.....A.GT.G..... : 270
 FIN05/F17 : ..A.....G.....T.T.....G.A.A.....A.GT.G..... : 270
 FIN05/B5 : ..A.....G.....T.T.....T.....A.A.A..G..A.GT.G..... : 270
 FIN05/B6 : ..A.....G.....T.T.....T.....A.A.A..G..A.GT.G..... : 270
 FIN05/E13 : ..A.....G.....T.T.....A.A.....A.GT.G..... : 270
 FIN05/F16 : ..A.....G.....T.T.....A.A.....A.GT.G..... : 270
 FIN05/C8 : ..A.....G.....T..C.....C.....G..G..... : 270
 FIN05/C9 : ..A.....G.....T..C.....C.....G..G..... : 270
 FIN05/C7 : ..A.....G.....T.....C.....G..G..... : 270
 K :C.....G..T.....T.....AC...C..C.G..C..A..T.G..... : 270
 Q1 :C.....G..T.....T.....AC...C..C.G..C..A..T.G..... : 270
 P1 :C.....G..T.....T.....TC..GC.....A..T.T..... : 270
 K1 : ..A.....T.....G.....A.....A.....C.T..C..A..C.....T : 270
 K2 : ..A.....T.....G.....A.....A.....G..C.T..C..A..C.....T : 270
 D1 : ..A.A.C.....T.....A.....G.....A..C.T..C..A..C.C.....T : 270
 M1 :C.....T..C.....A.....G.....A..A..C.T..C..A..C.....T : 270
 M2 :C.....T..C.....A.....G.....A..A..C.T..C..A..C.....T : 270
 O1 :C.....T..C.....G.....C.AC.T..C..A..TC..C..T : 270
 O2 :C.....T..C.....G.....C.AC.T..C..A..TC..C..T : 270
 A2 :T..C.....A.....G.....A..C.T.....A..GC.....T : 270
 B2 : ..A..A.....T.....A.....A.....A..C.T..G..CA.....T : 270
 GGGCCTACTAAACCTTACAATACTATAACAACAACCAACCAACCAAGACTACAACAACCAGTTAACTTGAGAGATTATGCAATAATATAC

280 * 300 * 320 *
 F1 :C...G...A.....A.....T : 336
 F3 : ..C.....C...G.....A.....T : 336
 C1 :C...G.....T : 336
 E1 : ..A.....C...G.....T : 336
 H1 : ..A.....C...G.....T : 336
 H3 : ..A.....C...G.....A.....T : 336
 F2 : ..A.....C...G.....T : 336
 B1 :C...G...AA.....T : 336

B3 :C...G...AA.....T.....T : 336
 N2 :G.....C...G...AA.....T : 336
 N3 :G.....C...G...AA.....T : 336
 N1 :G.....C...G...AA.....T : 336
 E2 :C...G...AA.....A.T : 336
 D2 : ..A.....C...G...A.....T : 336
 H2 : ..A.....C...G...A.....T.....T : 336
 I1 :C...G...AA.....T : 336
 L1 :C...G...A.....T : 336
 L2 :C...G...A.....T : 336
 FIN05/D10 :C.....C.....T : 336
 FIN05/D11 :C.....C.....T : 336
 FIN05/D12 :C.....C.....T : 336
 I2 :G...G.....T : 336
 United :C...G.....T : 336
 A1 :G...A.....C...T...A.....T : 336
 G1 :CA...A.....T : 336
 J1 :A...C.GG.....T : 336
 SL3 :C...TA.....T.....T : 336
 G :C...TA.....T.....T : 336
 Utah 1 :C...TA.....T.....T : 336
 R2 :C...T.....C...A.....T : 336
 FIN05/B4 :C...T.....C...A.....T : 336
 R1 :C...T.....C...A.....T : 336
 FIN05/E14 :C...T.....C...A.....T : 336
 FIN05/E15 :C...T.....C...A.....T : 336
 FIN05/F17 :C...T.....C...A.....T : 336
 FIN05/B5 :G...T.A.....C...A.....T : 336
 FIN05/B6 :G...T.A.....C...A.....T : 336
 FIN05/E13 :C...T.A.....C...A.....T : 336
 FIN05/F16 :C...T.....C...A.....T : 336
 FIN05/C8 :C...T.....A.....T : 336
 FIN05/C9 :C...T.....A.....T : 336
 FIN05/C7 :C...T.....A.....T : 336
 K :C.C.G.....T.....G... : 336
 Q1 :C.C.G.....T.....C... : 336
 P1 :C.C.G.....T.....C... : 336
 K1 :CA.G.....A.....AG..... : 336
 K2 :CA.G.....A.....AG..... : 336
 D1 :A...CA.G.....A.....AG..... : 336
 M1 :A...CA.....A.....AG..... : 336
 M2 :A...CA.....A.....A..... : 336
 O1 :CA.G.....A.....AG.....C... : 336
 O2 :CA.G.....A.....AG.....C... : 336
 A2 :C...G...A.....T : 336
 B2 : ..A...A...A...A...G...A.....A... : 336
 CTGTTTAAACAAAGATAAGATAAATAAAGAAGGTATGGATGGTTACTATGCTGCTGGTAACGGTGGC

Figure 2

1
2

20