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21 **Abstract**

22

23 The genetic diversity of bovine viral diarrhoea virus (BVDV) isolates in infected cattle from
24 Tyrol and Vorarlberg (Austria) was investigated. Blood samples were collected within the
25 compulsory Austrian BVDV control programme during 2005 and 2006. The 5'-untranslated
26 region (5'-UTR) and partially the N-terminal autoprotease (N^{pro}) were amplified by one-step
27 reverse transcriptase-polymerase chain reaction (RT-PCR) and the PCR products were
28 subsequently sequenced. Phylogenetic analysis based on 5'-UTR and N^{pro} sequences
29 demonstrated that almost all isolates (307/310) were of the BVDV-1 genotype. They were
30 clustered into eight different subtypes, here listed by their frequency of occurrence: BVDV-1h
31 (143), BVDV-1f (79), BVDV-1b (41), BVDV-1d (28), BVDV-1e (6), BVDV-1a (4), BVDV-
32 1g (3) and BVDV-1k (3). Two pestivirus isolates were typed as BVDV-2 and one isolate as
33 BDV closely related to Gifhorn strain (BDV-3). Correlation among isolates could only be
34 observed at the farm level, i.e., within a herd. However, no correlation between the genetic
35 and geographical distances could be observed above the farm level. Because of the wide
36 distribution of certain BVDV-1 subtypes and the low prevalence of herd-specific strains, a
37 determination of tracing routes of infection was not possible. Furthermore, recombination
38 events were not detected.

39

40 **Keywords:** pestivirus, BVDV, sequencing, genetic typing

41

42 **Introduction**

43

44 The bovine viral diarrhoea virus (BVDV) is an important cattle pathogen and generates
45 significant economic losses (Houe, 1999). Although BVDV infections usually cause no or
46 only mild clinical symptoms such as inappetence, mild diarrhoea, oculo-nasal discharge, oral
47 lesions, transient fever or reduced milk yield, severe forms elicit diarrhoea, pyrexia and
48 decreased milk production (Baker, 1995). Several outbreaks of hemorrhagic syndrome were
49 also described (Baker, 1995). The highest financial damage is caused by transplacental
50 infections (Houe, 1999). Clinical signs are abortion, mummification, congenital defects,
51 stillbirth and birth of weak or persistently infected (PI) calves. The extent depends on the
52 timing of the infection relative to the period of gestation. PI cattle are immunotolerant to
53 BVDV and shed the virus throughout their lifetime. Therefore, they are the main viral
54 reservoir and source for viral transmission. Superinfections of PI animals induce the fatal
55 mucosal disease, which is characterised by profuse and watery diarrhoea, anorexia, pyrexia,
56 weakness and oral lesions (Baker, 1995).

57 The genus Pestivirus belongs to the family *Flaviviridae* and includes the following species:
58 bovine viral diarrhoea virus 1 (BVDV-1), bovine viral diarrhoea virus 2 (BVDV-2), border
59 disease virus (BDV) and classical swine fever virus (CSFV). As a tentative species in the
60 genus, a pestivirus isolated from a giraffe was also added (Fauquet et al., 2005). Pestiviruses
61 were primarily classified according to their host of origin, but many investigations
62 demonstrated interspecies transmission (Paton, 1995). Therefore, the classification of
63 pestiviruses based on genetic and antigenic characteristics is rather appropriate (Becher et al.,
64 1999).

65 Pestiviruses are enveloped viruses of spherical shape. Their genome is a single-stranded,
66 positive-sense RNA of a size of approximately 12.3 kb. The large open reading frame (ORF)

67 encodes a polyprotein of about 3900 amino acids and is flanked by the 5'- and the 3'-
68 untranslated region (UTR) (Fauquet et al., 2005).

69 Most of the phylogenetic analyses of the bovine viral diarrhoea virus have been based on the
70 5'-UTR of the viral genome (Vilcek et al., 1999; Falcone et al., 2003; Hurtado et al., 2003;
71 Vilcek et al., 2003; Kolesarova et al., 2004; Stahl et al., 2005; Uttenthal et al., 2005; Barros et
72 al., 2006; Pizarro-Lucero et al., 2006), but also on the N-terminal autoprotease (N^{pro}) (Becher
73 et al., 1997; Vilcek et al., 2001; Toplak et al., 2004) and on the structural envelope protein E2
74 (Hamers et al., 1998; Tajima et al., 2001). BVDV can be subdivided into two species, the
75 BVDV-1 and the BVDV-2, based on the sequence variations within the highly conserved 5'-
76 UTR (Ridpath et al., 1994; Wolfmeyer et al., 1997). Genetic typing of BVDV-1 isolates from
77 different countries revealed at least eleven subtypes (1a-1k) (Vilcek et al., 2001; Vilcek et al.,
78 2004), whereas recently a new subtype (subtype 1l) was found (Jackova et al., 2008). BVDV-
79 2 is divided into two to four different subtypes (2a-2d) (Giangaspero et al., 2001; Tajima et
80 al., 2001; Flores et al., 2002).

81 BVDV-1 was first isolated in the 1940s (Olafson and Rickard, 1947) and it occurs worldwide.
82 In contrast, BVDV-2 was discovered in the 1990s and was initially identified only in North
83 America (Ridpath et al., 1994). In recent years, it has been also found in Europe (Tajima et
84 al., 2001; Vilcek et al., 2001; Couvreur et al., 2002; Vilcek et al., 2003), Japan (Nagai et al.,
85 2004), Korea (Park et al., 2004) and South America (Jones et al., 2001; Flores et al., 2002;
86 Pizarro-Lucero et al., 2006).

87 In the 1990s, non-obligatory BVDV control schemes were adopted in several provinces of
88 Austria, e.g., Lower Austria, Styria, Tyrol and Vorarlberg. In 2004, a compulsory national
89 BVDV control programme was implemented. The aim of this programme was the annual herd
90 screening to identify PI animals and their elimination without using vaccination (Lindberg
91 and Alenius, 1999; Schöpf et al., 2005).

92 The aim of this study was to type BVDV isolates from infected cattle in Tyrol and
93 Vorarlberg, two provinces of Western Austria. All viruses were typed in 5'-UTR, selected
94 isolates were also analysed in the N^{pro} region. Within the ongoing national BVDV control
95 programme we collected blood samples from the years 2005 and 2006. The highly conserved
96 5'-UTR sequences were tested for recombination using the likelihood permutation test
97 (McVean et al., 2002), since recombination may cause changes in the genetic composition,
98 such that phylogenetic distances become irrelevant. Furthermore, we analysed the correlation
99 between genetic and geographical distances. Therefore, we performed a test based on
100 permutation, comparing the phylogenetic to the geographical distance (Mantel, 1967).

101

102 **Materials and methods**

103 *Serum samples and isolation of RNA*

104

105 Blood samples of 353 cows from 163 different farms in the western Austrian provinces, Tyrol
106 and Vorarlberg, were used for this study. The samples were collected from January 2005 to
107 December 2006 within the national BVDV control programme. The geographical origin and
108 herd information of all specimens were recorded and all samples were tested ELISA positive.
109 The ELISA test was performed at the Austrian Agency for Health and Food Safety (AGES) in
110 Innsbruck by the commercially available ELISA Kit HerdChek BVDV Ag/Serum Plus
111 (IDEXX Scandinavia, Oesterbybruk, Sweden). After serological testing, the samples were
112 sent to the Austrian Agency for Health and Food Safety (AGES) in Moedling for further
113 molecular analysis.

114 Viral RNA was isolated from the 353 sera by using the QIAamp[®] Viral RNA Mini Kit
115 (QIAGEN, Vienna, Austria) following the manufacturer's instructions.

116

117 *Real-time RT-PCR*

118

119 For initial pestivirus RNA detection and in order to estimate the amount of RNA, a real-time
120 reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the 7500
121 Fast Real-Time PCR System (Applied Biosystems, Vienna, Austria) and the QuantiTect
122 Probe RT-PCR Kit (QIAGEN) following the protocol described by Gaede et al. (2005) with
123 the modifications that 0.2 μM TQ-Pesti Probe and 0.5 μM of each primer (Pesti 3 and Pesti 4)
124 (Hyndman et al., 1998) were used. Three μl total RNA was added to the reaction mix.
125 Reverse transcription was performed at 50° C for 30 min followed by 45 cycles of PCR
126 amplification persisting of denaturation at 95° C for 30 s, primer annealing at 60° C for 30 s
127 and elongation at 72° C for 30 s.

128

129 *RT-PCR*

130

131 Both reverse transcription and PCR amplification of the viral RNA prior to sequencing were
132 done in one-step using the One-step[®] RT-PCR Kit (QIAGEN). Template RNA (2.5 μl) was
133 added to the reaction mix (22.5 μl) containing RNase Inhibitor (Invitrogen, Lofer, Austria),
134 1x one-step Buffer, 1 μl RT-PCR Enzym Mix, dNTP Mix (0.4 mM each) and 0.5 μM of each
135 primer. For the 5'-UTR amplification the primer pair 324 and 326 (Vilcek et al., 1994) was
136 used whereas amplification of the N-terminal autoprotease (N^{pro}) was done with the primer
137 pair BD1 and BD3 (Vilcek et al., 2001). Reverse transcription was performed at 50° C for 30
138 min. PCR conditions were as follows: initial denaturation at 95° C for 15 min followed by 40
139 PCR cycles of denaturation at 94° C for 30 s, primer annealing at 50° C for 30 s (primers
140 324/326) or 62° C for 50 s (primers BD1/BD3) and elongation at 72° C for 1 min. The final
141 elongation was extended to 5 min at 72° C. The PCR products of 288 bp (5'-UTR) and 428 bp
142 (N^{pro}) were separated by gel electrophoresis in 1.5 % agarose gel stained with ethidium

143 bromide. The fragments were visualized using a UV transilluminator. DNA bands of the
144 expected sizes were excised from the agarose gel and recovered using the QIAquick® Gel
145 Extraction Kit (QIAGEN) according to the manufacturer's protocol and finally the purified
146 DNA was stored at -20° C.

147

148 *Nucleotide sequencing*

149

150 Sequencing reactions were performed using the BigDye® Terminator v1.1 Cycle Sequencing
151 Kit (Applied Biosystems). Five µl of purified DNA was added to five µl of the sequencing
152 reaction. Standard sequencing reaction was carried out. The RT-PCR primers were also used
153 for sequencing of the 5'-UTR and N^{pro} products. Afterwards, unincorporated dye terminators
154 were removed using the DyeEx™ 2.0 Spin Kit (QIAGEN) following the protocol of the
155 manufacturers. Each PCR products was sequenced from the 5' and from the 3' direction
156 utilizing capillar electrophoresis on the 3130xl Genetic Analyzer (Applied Biosystems).

157

158 *Computer-assisted analysis*

159

160 In our analysis, additional sequences of representative strains of BVDV-1, BVDV-2, border
161 disease virus (BDV) and classical swine fever virus (CSFV) from the NCBI (National Center
162 for Biotechnology Information) GenBank were included. The nucleotide sequences were
163 aligned using the SeqScape® Software Version 2.5 (Applied Biosystems). Phylogenetic trees
164 were constructed using the Dnadist, Neighbor, and Consense programmes of the Phylogeny
165 Inference Package (PHYLIP, Seattle, USA) Version 3.6 (Felsenstein, 2005). For BVDV-1,
166 the strain Suwa (BVDV-1k) served as an outgroup sequence for the 5'-UTR and N^{pro}
167 phylogenetic trees. The 5'-UTR and N^{pro} phylogenetic trees were constructed using 245
168 nucleotides long sequences flanked by 324/326 primers and 348 nucleotides flanked by

169 BD1/BD3 primers, respectively. Bootstrap analysis was performed on 1000 replicates with
170 the Seqboot programme. Representative sequences of the 5'-UTR (n = 26), including two
171 BVDV-2 and one BDV isolate, and of the N^{pro} (n = 14) obtained in this work were deposited
172 in the NCBI GenBank with the following accession numbers: EU224221-EU224260
173 (Table1).

174 To test for the presence of recombination, a composite likelihood permutation test was carried
175 out (McVean et al., 2002). Briefly, first the population recombination rate per site using the
176 composite-likelihood estimation of $4N_e r$ (N_e = effective population size, r = genetic map
177 distance across the region analysed) was calculated, using an extended version of Hudson's
178 composite likelihood estimator and a finite-sites model. Then, the recombination rate of the
179 unpermuted data was compared with a data set where the genomic location of segregating
180 sites was permuted. If the observed recombination rate had a higher value than the permuted
181 data set, recombination would be estimated. This test can also be applied to RNA viruses with
182 high mutation rates.

183 Furthermore, the geographical distance was compared with the genetic distance among
184 several BVDV-1 subtypes using the permutation test (Mantel, 1967). For this purpose, either
185 the samples from the same herd and samples from different herds were differentiated (the
186 compared farm was encoded with 0, all other farms with 1) or the geographical distances
187 between the farms were considered.

188

189 **Results**

190

191 For the identification of pestivirus RNA, we performed both, real-time and conventional RT-
192 PCR. Real-time RT-PCR amplification with the pestivirus specific TaqMan[®] probe and the
193 primer-pair Pesti 3 and Pesti 4 (Hyndman et al., 1998) detected 301 of the 353 ELISA-
194 positive samples. However, by the conventional one-step RT-PCR targeting the 5'-

195 untranslated region (5'-UTR) with the pestivirus specific primers 324 and 326 (Vilcek et al.,
196 1994) we amplified these 301 and nine additional samples. These amplification primers were
197 used for direct DNA sequencing of the PCR products in both directions. For phylogenetic
198 analysis, a 5'-UTR sequence of about 245 bp was used. Sample B293/06, B384/06 and
199 B385/06 could not be sequenced in this region and were therefore excluded from the 5'-UTR
200 comparison.

201 To verify the BVDV-1 classification obtained from the 5'-UTR sequences, 33 samples
202 representing all BVDV-1 subtypes and the three sera B293/06, B384/06 and B385/06 were
203 examined in the N-terminal autoprotease (N^{pro}) with another conventional one-step RT-PCR
204 using the primer pair BD1 and BD3 (Vilcek et al., 2001). All samples were successfully
205 analysed and a phylogenetic tree could be constructed with 348 bp long sequences.

206 The phylogenetic analysis based on the 5'-UTR sequences revealed 304 isolates belonging to
207 eight different BVDV-1 subtypes (1a, 1b, 1d, 1e, 1f, 1g, 1h and 1k). Genetic typing was
208 performed following the nomenclature by Vilcek (2001; 2004). Clustering of the BVDV-1
209 isolates showed a high diversity of the virus. Most isolates belonged to the subtypes BVDV-
210 1h (141) and BVDV-1f (78). Forty-one viruses were classified as BVDV-1b, 28 clustered
211 within the BVDV-1d subtype and three within the subtype BVDV-1g. We identified three
212 BVDV-1 subtypes, never before described in Austria: these are four isolates of the subtype
213 1a, six of the subtype 1e and three of the subtype 1k.

214 Only two isolates (B91/05 and B253/06) were classified as BVDV-2, both showing a 99 %
215 nucleotide similarity to the 5'-UTR region of the German BVDV-2 strain 104-98, found in
216 Lower Saxony (Acc.No. AJ304381, Tajima et al., 2001).

217 One cattle (B300/06) from Innsbruck-Land was infected with a border disease virus (BDV).
218 This isolate showed a 96.7 % similarity at the nucleotide level with the BDV strain Gifhorn
219 and therefore phylogenetically clustered as a BDV-3. This was the first time that a BDV was
220 shown in Austrian cattle.

221 The three samples (B293/06, B384/06 and B385/06), that could not be sequenced at the 5'-
222 UTR, were successfully classified as BVDV-1f (one sample) and BVDV-1h (two samples) by
223 analysing the N^{pro} region. In addition, the BVDV-1 grouping of the other 33 samples could be
224 confirmed.

225 Due to the large number of samples, a graph of a complete phylogenetic tree based on all 5'-
226 UTR isolates would have been too big. Therefore, a tree was built with only selected isolates
227 (Fig. 1). Reference strains were obtained from the NCBI GenBank and were included in the
228 alignment. These strains comprised different BVDV-1 subtypes, representative BVDV-2,
229 BDV, classical swine fever virus (CSFV) and the Giraffe isolates. Bootstrap analysis was
230 performed on 1000 replicates with bootstrap values 100 %, except for the the BDV genotypes
231 (84 %). This phylogenetic tree clearly indicated the identification of two BVDV-2 isolates
232 (B91/05, B253/06) and one BDV isolate (B300/06) in our collection of pestivirus isolates.
233 Furthermore, two other trees were constructed, showing the distinct subtypes of BVDV-1, one
234 based on the 5'-UTR nucleotide sequences (Fig. 2) and the other one on the N^{pro} region
235 sequences (Fig. 3).

236 Following the method of McVean et al. (2002), the recombination rate (ρ) of the 5'-UTR
237 sequences showed a value of $\rho = 14.00$. The recombination rate of the permuted samples was
238 inferred to be higher ($\rho = 16.23$). Hence, we could not find evidence for recombination.
239 Originally, our 310 successfully analysed samples derived from 163 different farms. In 66
240 farms, more than one animal was infected with a pestivirus. In 40 of these cases (60.6 %) the
241 animals were infected with the same BVDV subtype. In 37.9 % of the cases, which represent
242 25 farms, animals from the same herd were infected with two different genotypes or subtypes.
243 In one farm (1.5 %), even three different BVDV-1 subtypes occurred. Fig. 4 shows the
244 distribution of the distinct subtypes in Tyrol and Vorarlberg. To quantitatively investigate a
245 possible geographical correlation of the variation of BVDV, a permutation test was performed
246 (Mantel, 1967). Using this test, a correlation of 0.088 between the geographical and the

247 genetic distance matrices was inferred. When, instead of geographical distances, only
248 individuals from the same or different herds were differentiated, the correlation was 0.109. As
249 the correlation at the farm level was higher than the geographical correlation, we concluded
250 that correlation above farm level was absent.

251

252 **Discussion**

253

254 In this study, the genetic diversity of bovine viral diarrhoea virus (BVDV) isolates from two
255 western provinces of Austria was analysed. By molecular analysis, 353 antigen-capture
256 ELISA positive samples were examined. A total of 301 isolates were determined positive
257 (85.3 %) by real-time RT-PCR. With the conventional one-step RT-PCR nine additional
258 BVDV samples could be detected. The sequences of the nine real-time RT-PCR negative
259 samples were examined, but no relevant mismatches in the primer and probe binding regions
260 were observed (data not shown) (Klein et al., 1999). Therefore, we have no explanation for
261 these real-time RT-PCR negatives. One reason for detecting only 310 out of 353 ELISA
262 positives could be due to a poor RNA quality, but in addition, also ELISA false positives were
263 observed in similarly designed investigations (Schöpf et al., 2005).

264 Initially, we could successfully sequence the 5'-untranslated region (5'-UTR) of 307
265 pestivirus isolates using the primers 324 and 326 (Vilcek et al., 1994). However, we could not
266 sequence the 5'-UTR of three isolates (B293/06, B384/06 and B385/06). In order to exclude
267 mispriming (Klein et al., 1999), another RT-PCR based on the 5'-UTR was repeated with the
268 nested primers A11 and A14 (McGoldrick et al., 1998), but results remained unchanged (data
269 not shown).

270 The most conserved region of the pestivirus genome is the 5'-UTR and therefore Becher et al.
271 (1997) recommended its use for the classification of pestivirus isolates of several genotypes.
272 However, for more detailed subdivision less conserved regions, e.g., the coding N-terminal

273 autoprotease (N^{pro}) region, are better suited. Therefore, for verification of the genetic
274 variability of BVDV-1 observed in the 5'-UTR, a phylogenetic analysis based on the N^{pro}
275 region was carried out with 33 samples, representing different BVDV-1 subtypes. The results
276 of the 5'-UTR typing were confirmed in all samples and additionally, the three samples that
277 could not be sequenced at the 5'-UTR, could be successfully typed as BVDV-1f (B293/06)
278 and BVDV-1h (B384/06 and B385/06).

279 Most pestivirus isolates (307/310) from Tyrol and Vorarlberg were classified as BVDV-1.
280 They represented eight distinct subtypes within this genotype. This high BVDV-1 genetic
281 diversity has never been described before in any country, although former publications
282 confirmed a wide genetic range of BVDV-1 in Austria. In Styria five different subtypes (1b,
283 1d, 1f, 1g and 1h) were identified (Vilcek et al., 2003). In another study, BVDV field samples
284 collected from certain parts of Austria revealed only four distinct subtypes, 1b, 1f, 1g and 1h
285 (Kolesarova et al., 2004). In the present study, the subtype 1h accounted for the largest
286 number of sequenced BVDV isolates (143), but also the subtype 1f (79 isolates) was
287 widespread in Tyrol and Vorarlberg. These results are in accordance with Kolesarova et al.
288 (2004), where three of five samples collected in Tyrol were also typed as BVDV-1h and two
289 as BVDV-1f. However, the subtype 1f appeared to be the most abundant when samples from
290 five different Austrian provinces were considered (Vilcek et al., 2003; Kolesarova et al.,
291 2004). Only three of 310 isolates analysed in this study belong to subtype BVDV-1g. This
292 low prevalence is in agreement with other Austrian investigations (Vilcek et al., 2003;
293 Kolesarova et al., 2004), where only one BVDV-1g isolate was found. Interestingly, all
294 BVDV-1g isolates of this study originated from Tyrol, but from three different districts
295 (Innsbruck Land, Kitzbuehel and Kufstein). The three BVDV-1 subtypes 1a, 1e and 1k
296 represented with four, six and three isolates, respectively, were isolated for the first time in
297 Austria, both in Tyrol and in Vorarlberg. Three isolates of the subtype 1a were found in Tyrol
298 (Schwaz) and one in Vorarlberg (Dornbirn) and four isolates of the subtype 1e were found in

299 Tyrol (Innsbruck Land, Reutte and Schwaz) and two in Vorarlberg (Bregenz). One cow
300 infected with the subtype 1k was born in Tyrol and shared alpine communal pasturing during
301 at least two summers in this province, so transmission of this subtype on the alp was possible.
302 The other two animals, which were persistently infected, were born in the late summer on a
303 farm in Vorarlberg and died with the age of two and three months. Both mother cows were
304 infected in winter and therefore BVDV transmission occurred within animals in this farm. All
305 1k subtypes showed a similarity of 100 % at the nucleotide level.

306 The BVDV-2 genotype was detected in samples B91/05 and B253/06 (Acc.No. EU224242
307 and EU224225). Both isolates showed a nucleotide similarity of 99 % with the German virus
308 strain 104-98 from Lower Saxony (Tajima et al., 2001). This was the first time that BVDV-2
309 was detected in Western Austria. Sample B91/05 was collected in the district Kitzbuehel,
310 whereas sample B253/06 originated from a farm in the district Lienz, where cattle are
311 imported from Bavaria. In 2003, one BVDV-2 isolate was described in Styria, Austria (Vilcek
312 et al., 2003) for the first time (see Fig. 1, isolate 37-Gr), showing a nucleotide similarity of
313 about 93 % with the two BVDV-2 isolates of this study. In Europe, the prevalence of BVDV-
314 2 compared to BVDV-1 was always considered to be very low: in Southern Germany two of
315 61 analysed BVDV isolates were BVDV-2 (Tajima et al., 2001) and in Portugal three of 34
316 typed viruses were BVDV-2 (Barros et al., 2006). In Slovakia, two BVDV-2 positive samples
317 were detected (Vilcek et al., 2002). BVDV-2 was not present in field studies from Spain
318 (Hurtado et al., 2003), Italy (Falcone et al., 2003), Switzerland (Stalder et al., 2005), Slovenia
319 (Toplak et al., 2004), Denmark (Uttenthal et al., 2005) or England and Wales (Vilcek et al.,
320 1999). A higher prevalence of this genotype has been described in Germany and Belgium,
321 where 15.9 % and 24.1 % of the isolates, respectively, were classified as BVDV-2
322 (Wolfmeyer et al., 1997; Couvreur et al., 2002).

323 Surprisingly, one isolate in this study (B300/06, Acc.No. EU224227) was identified as a
324 border disease virus (BDV), genotype BDV-3. So far, four different genotypes (BDV 1-4)

325 have been identified and until now, the genotype BDV-3 was only found in Germany (Becher
326 et al., 2003), Switzerland (Stalder et al., 2005) and Austria (Krametter-Froetscher et al.,
327 2007). The BDV isolate B300/06 showed a 96.7 % similarity at the nucleotide level in the 5'-
328 UTR to the BDV strain Gifhorn. The infected cow derived from a farm in the district
329 Innsbruck Land (Tyrol), where cattle were kept in the neighbourhood of sheep. This was the
330 first time that BDV was found in Austrian cattle, since BDV was only recently characterised
331 in Austrian sheep (Krametter-Froetscher et al., 2007). We suggest that the transmission from
332 sheep to cattle appears to be the most likely route of infection, as an interspecies transmission
333 of ovine pestiviruses has also been described before by Paton (1995). In contrast, in a British
334 study the transmission of BDV from sheep to cattle could not be proved, whereas BVDV
335 transmission from cattle to sheep was not uncommon (Vilcek et al., 1999).

336 Because mutation and recombination have been observed in pestiviruses, this work also
337 aimed to test for RNA recombination. Therefore, we analysed the 5'-UTR sequences using
338 the composite likelihood permutation test (McVean et al., 2002). However, no evidence for
339 recombination could be detected. The high diversity of BVDV found in Tyrol and Vorarlberg
340 might evolve from the intense trade practices in this region.

341 In terms of epidemiological features, the possible geographical correlation to the prevalence
342 of certain BVDV-1 and BVDV-2 subtypes occurring in the Western part of Austria was
343 investigated. Only a low correlation between the genetic and geographical distances at the
344 farm level was found. This is in contrast to other works, where viruses of the same farm were
345 closely related (Hamers et al., 1998; Paton et al., 1995). Principally, this could be due to the
346 communal pasturing and frequent trading with young animals, which are common practices in
347 Western Austria. In contrast to other Austrian regions the average size of 17 animals per herd
348 is relatively low. During the summer period, which lasts from May until September, about
349 60% and of the Tyrolean cattle population is kept on approximately 2,600 alps. In Vorarlberg
350 about 80 % of the cattle population pastures on approximately 500 alps. Additionally, sheep

351 and goats are kept on alps in close proximity with cattle during summer. During this period of
352 grazing contact with wild ungulates is also possible. All these factors might explain the
353 absence of herd-specific strains. Hamers et al. (1998) suggested that a high variability of
354 BVDV sequences may derive from the horizontal transfection from a BVDV-
355 immunocompetent to a susceptible animal, while herd-specific strains may derive from the
356 transmission from PI animals within the farm. In the present work, evidence for geographical
357 clustering of genotypes or subtypes above the farm level was not determined. These findings
358 are in accordance with other previous investigations (Vilcek et al., 1999; Stalder et al., 2005).
359 In Sweden, where the BVD eradication programme has been running since 1993, the
360 molecular epidemiology is actually used to trace sources and routes of BVDV infections. The
361 comparison of existing BVDV sequences to new cases has been used as an important tool to
362 trace the origin of new outbreaks (Stahl et al., 2005). The samples of this study were collected
363 over two years (2005 and 2006) within the Austrian compulsory BVDV control programme,
364 which was implemented in 2004. So far, it has not been possible to retrace routes of BVDV
365 infections due to the wide geographical distribution of several BVDV subtypes in Tyrol and
366 Vorarlberg and to the low prevalence of herd-specific strains. Tracing the origin of BVDV
367 infections in the future may gain importance during the final phase of the BVD control
368 programme, when the prevalence will be reduced and almost all herds will have a BVDV-free
369 status. The development of a sequence database, as established in Sweden (Stahl et al., 2005),
370 including all characterised isolates from BVDV infected animals from Austria and
371 neighbouring countries, will be a useful tool for eradication and for studying the
372 epidemiology of BVD. Then, genetic typing of BVDV isolates could be used for tracing new
373 infection routes more economically.

374

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376

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383

384 **References**

385

- 386 Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhoea infection. *Vet. Clin.*
387 *North Am.* 11 (3), 425–445.
- 388 Barros, S.C., Ramos, F., Pauperio, S., Thompson, G., Fevereiro, M., 2006. Phylogenetic
389 analysis of Portuguese bovine viral diarrhoea virus. *Virus Res.* 118, 192-195.
- 390 Becher, P., Orlich, M., Shannon, A.D., Horner, G., König, M., Thiel, H.J., 1997. Phylogenetic
391 analysis of pestiviruses from domestic and wild ruminants. *J. Gen. Virol.* 78, 1357-
392 1366.
- 393 Becher, P., Orlich, M., Kosmidou, A., König, M., Baroth, M., Thiel, H.J., 1999. Genetic
394 diversity of pestiviruses: identification of novel groups and implication for
395 classification. *Virology* 262, 64–71.
- 396 Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., König, M., Schweizer, M.,
397 Stalder, H., Schirmer, H., Thiel, H.J., 2003. Genetic and antigenic characterization
398 of novel pestivirus genotypes: implications for classification. *Virology* 311, 96-104.
- 399 Couvreur, B., Letellier, C., Collard, A., Quenon, P., Dehan, P., Hamers, C., Pastoret, P.P.,
400 Kerkhofs, P., 2002. Genetic and antigenic variability in bovine viral diarrhoea virus
401 (BVDV) isolates from Belgium. *Virus Res.* 85, 17-28.
- 402 Falcone, E., Cordioli, P., Tarantino, M., Muscillo, M., La Rosa, G., Tollis, M., 2003. Genetic
403 Heterogeneity of Bovine Viral Diarrhoea Virus In Italy. *Vet. Res. Commun.* 27, 485-
404 494.

- 405 Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. Virus
406 Taxonomy. Elsevier Academic Press, San Diego, pp. 988-992.
- 407 Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the
408 author. Department of Genome Sciences, University of Washington, Seattle.
409 <http://evolution.genetics.washington.edu/phylip.html>
- 410 Flores, E.F., Ridpath, J.F., Weiblen, R., Vogel, F.S.F., Gil, L.H.V.G., 2002. Phylogenetic
411 analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: evidence
412 for a subgenotype within BVDV-2. *Virus Res.* 87, 51-60.
- 413 Gaede, W., Reiting, R., Schirrmeyer, H., Depner, K.R., Beer, M., 2005. Nachweis und
414 Spezies-spezifische Differenzierung von Pestiviren mit der real-time RT-PCR. *Berl.*
415 *Münch. Tierärztl. Wschr.* 118 (3/4), 113-120.
- 416 Giangaspero, M., Harasawa, R., Zecconi, A., Luzzago, C., 2001. Genotypic Characteristics of
417 Bovine Viral Diarrhea Virus 2 Strains Isolated in Northern Italy. *J. Vet. Med. Sci.* 63
418 (9), 1045-1049.
- 419 Hamers, C., Lecomte, C., Kulcsar, G., Lambot, M., Pastoret, P.P., 1998. Persistently infected
420 cattle stabilise bovine viral diarrhea virus leading to herd specific strains. *Vet.*
421 *Microbiol.* 61, 177-182.
- 422 Houe, H., 1999. Epidemiological features and economical importance of bovine virus
423 diarrhoea virus (BVDV) infections. *Vet. Microbiol.* 64, 89-107.
- 424 Hurtado, A., Garcia-Perez, A.L., Aduriz, G., Juste, R.A., 2003. Genetic diversity of ruminant
425 pestiviruses from Spain. *Virus Res.* 92, 67-73.
- 426 Hyndman, L., Vilcek, S., Conner, J., Nettleton, P.F., 1998. A novel nested reverse
427 transcription PCR detects bovine viral diarrhoea virus in fluids from aborted bovine
428 fetuses. *J. Virol. Methods* 71, 69-76.
- 429 Jackova, A., Novackova, M., Pelletier, C., Audeval, C., Gueneau, E., Haffar, A., Petit, E.,
430 Rehby, L., Vilcek, S., 2008. The extended genetic diversity of BVDV-1 : typing of
431 BVDV isolates from France. *Vet. Res. Commun.* 32, 7-11.
- 432 Jones, L.R., Zandomeni, R., Weber, E.L., 2001. Genetic typing of bovine viral diarrhea virus
433 isolates from Argentina. *Vet. Microbiol.* 81, 367-375.
- 434 Klein, D., Janda, P., Steinborn, R., Müller, M., Salmons, B., Günzburg, W.H., 1999. Proviral
435 load determination of different feline immunodeficiency virus isolates using real-time
436 polymerase chain reaction: influence of mismatches on quantification. *Electrophoresis*
437 20, 291-299.

- 438 Kolesarova, M., Franz, S., Jackova, A., Vilcek, S., Möstl, K., Benetka, V., Schöpf, K.,
439 Schoder, G., Hofer, J., Baumgartner, W., 2004. Genetic typing of Bovine Viral
440 Diarrhoea Virus from Austrian field samples. *Wien. Tierärztl. Mschr.* 91, 265-268.
- 441 Krametter-Froetscher, R., Kohler, H., Benetka, V., Moestl, K., Golja, F., Vilcek, S.,
442 Baumgartner, W., 2007. Influence of Communal Alpine Pasturing on the Spread of
443 Pestiviruses among Sheep and Goats in Austria: First Identification of Border Disease
444 Virus in Austria. *Zoonoses Public Health* 54, 209-213.
- 445 Lindberg, A.L.E. and Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea
446 virus (BVDV) infections in cattle populations. *Vet. Microbiol.* 64, 197-222.
- 447 Mantel, N., 1967. The Detection of Disease Clustering and a Generalized Regression
448 Approach. *Cancer Res.* 27, 209-220.
- 449 McGoldrick, A., Lowings, J.P., Ibata, G., Sands, J.J., Belak, S., Paton, D.J., 1998. A novel
450 approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic
451 probe (TaqMan). *J. Virol. Methods* 72, 125-135.
- 452 McVean, G., Awadalla, P., Fearnhead, P., 2002. A Coalescent-Based Method for Detecting
453 and Estimating Recombination From Gene Sequences. *Genetics* 160, 1231-1241.
- 454 Nagai, M., Hayashi, M., Sugita, S., Sakoda, Y., Mori, M., Murakami, T., Ozawa, T., Yamada,
455 N., Akashi, H., 2004. Phylogenetic analysis of bovine viral diarrhea viruses using five
456 different genetic regions. *Virus Res.* 99, 103-113.
- 457 Olafson, P. and Rickard, C.G., 1947. Further Observations on the Virus Diarrhea (New
458 Transmissible Disease) of Cattle. *Cornell Vet.*, 37, 104-106.
- 459 Park, J.S., Moon, H.J., Lee, B.C., Hwang, W.S., Yoo, H.S., Kim, D.Y., Park, B.K., 2004.
460 Comparative analysis on the 5'-untranslated region of bovine viral diarrhea virus
461 isolated in Korea. *Res. Vet. Sci.* 76, 157-163.
- 462 Paton, D.J., 1995. Pestivirus diversity. *J. Comp. Path.* 112, 215-236.
- 463 Paton, D.J., Carlsson, U., Lowings, J.P., Sands, J.J., Vilcek, S., Alenius, S., 1995.
464 Identification of herd-specific bovine viral diarrhoea virus isolates from infected cattle
465 and sheep. *Vet. Microbiol.* 43, 283-294.
- 466 Pizarro-Lucero, J., Celedon, M.O., Aguilera, M., de Calisto, A., 2006. Molecular
467 characterization of pestiviruses isolated from bovines in Chile. *Vet. Microbiol.* 115,
468 208-217.
- 469 Ridpath, J.F., Bolin, S.R., Dubovi, E.J., 1994. Segregation of bovine viral diarrhoea virus into
470 genotypes. *Virology* 205, 66-74.

- 471 Schöpf, K., Gaede, W., Matt, M., 2005. Erfahrungen zur Bekämpfung der Bovinen Virus
472 Diarrhoe (BVD/MD) mit spezieller Berücksichtigung der Verbreitung von BVDV-
473 Genotypen im Bundesland Tirol. Wien. Tierärztl. Mschr. 92, 254-258.
- 474 Stahl, K., Kampa, J., Baule, C., Isaksson, M., Moreno-Lopez, J., Belak, S., Alenius, S.,
475 Lindberg, A., 2005. Molecular epidemiology of bovine viral diarrhoea during the final
476 phase of the Swedish BVD-eradication programme. *Prev. Vet. Med.* 72, 103-108.
- 477 Stalder, H.P., Meier, P., Pfaffen, G., Wageck-Canal, C., Rüfenacht, J., Schaller, P., Bachofen,
478 C., Marti, S., Vogt, H.R., Peterhans, E., 2005. Genetic heterogeneity of pestiviruses of
479 ruminants in Switzerland. *Prev. Vet. Med.* 72, 37-41.
- 480 Tajima, M., Frey, H.R., Yamato, O., Maede, Y., Moennig, V., Scholz, H., Greiser-Wilke, I.,
481 2001. Prevalence of genotypes 1 and 2 of bovine viral diarrhea virus in Lower Saxony,
482 Germany. *Virus Res.* 76, 31–42.
- 483 Toplak, I., Sandvik, T., Barlic-Maganja, D., Grom, J., Paton, D.J., 2004. Genetic typing of
484 bovine viral diarrhoea virus: most Slovenian isolates are of genotypes 1d and 1f. *Vet.*
485 *Microbiol.* 99, 175–85.
- 486 Uttenthal, A., Stedejek, T., Nylin, B., 2005. Genetic diversity of bovine viral diarrhoea
487 viruses (BVDV) in Denmark during a 10-year eradication period. *APMIS* 113, 536-41.
- 488 Vilcek, S., Herring, A.J., Herring, J.A., Nettleton, P.F., Lowings, J.P., Paton, D.J., 1994.
489 Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three
490 genogroups using polymerase chain reaction and restriction endonuclease analysis.
491 *Arch. Virol.* 136, 309-323.
- 492 Vilcek, S., Drew, T.W., McGoldrick, A., Paton, D.J., 1999. Genetic typing of bovine
493 pestiviruses from England and Wales. *Vet. Microbiol.* 69, 227–237.
- 494 Vilcek, S., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A.,
495 Rossmanith, W., Vega, S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea
496 virus genotype 1 can be separated into at least eleven genetic groups. *Arch. Virol.* 146,
497 99–115.
- 498 Vilcek, S., Durkovic, B., Bobakova, M., Sharp, G., Paton, D.J., 2002. Identification of bovine
499 viral diarrhoea virus 2 in cattle in Slovakia. *Vet. Rec.* 151, 150-152.
- 500 Vilcek, S., Greiser-Wilke, I., Durkovic, B., Obritzhauser, W., Deutz, A., Köfer, J., 2003.
501 Genetic diversity of recent bovine viral diarrhoea viruses from the southeast of Austria
502 (Styria). *Vet. Microbiol.* 91, 285–91.

503 Vilcek, S., Durkovic, B., Kolesarova, M., Greiser-Wilke, I., Paton, D., 2004. Genetic diversity
504 of bovine viral diarrhoea virus (BVDV) isolates: identification of a new BVDV-1
505 genetic group. *Vet. Res.* 35, 609–615.

506 Wolfmeyer, A., Wolf, G., Beer, M., Strube, W., Hehnen, H.R., Schmeer, N., Kaaden, O.R.,
507 1997. Genomic (5'-UTR) and serological differences among German BVDV field
508 isolates. *Arch. Virol.* 142, 2049-2057.

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512 **TEXT TO TABLE AND FIGURES**

513

514 **Table 1.**

515 Sequences representing Austrian pestiviruses from Tyrol and Vorarlberg and their
516 phylogenetic classification.

517

518 **Fig.1.**

519 Typing of selected Austrian pestivirus isolates in the 5'-UTR.

520 The Austrian isolates B91/05 and B253/06 in the BVDV-2 branch and B300/06 in the BDV
521 branch are written in bold letters. The selected BVDV-1 Austrian isolates are presented as
522 nonlabelled branches only. They are more precisely analysed on Fig. 2. The origin of the 245
523 bp nucleotide sequences taken from the NCBI GenBank: Giraffe: H138 (AB040131); BDV:
524 Moredun (U65022); Reindeer (AF144618), Chamois (AY38080), CSFV: Alfort (J04358),
525 Brescia (M31768), C (Z46258); BVDV-2: 890 (U18059), 37-Gr (EU327594), 104-98
526 (AJ304381); BVDV-1: BVDV-1a – NADL (M31182), BVDV-1b – Osloss (M96687),
527 BVDV-1d – F (AF298065); BVDV-1e – 3-It (AF298062), BVDV-1f – J (AF298067), W
528 (AF298073), BVDV-1g – A (AF298064), BVDV-1h – G (AF298066), BVDV-1i – 23-15
529 (AF298059), BVDV-1k – Rebe (AF299317). The nucleotide sequence for Gifhorn strain was
530 obtained in this work. The tree was computed by the neighbor-joining method (Kimura 2-
531 parameter; transition/transversion ratio: 2.0) using the PHYLIP Dnadist and Neighbor
532 programmes (Felsenstein, 2005). Bootstrap values are given in percentage for 1000 replicate
533 data sets, performed with the PHYLIP Seqboot programme (Felsenstein, 2005).

534

535 **Fig.2.**

536 Genetic typing of selected BVDV-1 isolates in the 5'-UTR.

537 The phylogenetic tree was constructed from 245 bp 5'-UTR sequences of selected BVDV-1
538 isolates. The Austrian isolates analysed in this work are labelled in bold. The tree was
539 generated with the PHYLIP Dnadist, Neighbor and Consense programmes (Felsenstein,
540 2005). Bootstrap values for 1000 replicate data sets were calculated for all BVDV-1
541 subgroups. All branches for the BVDV-1 subtypes were supported with 100 percentage
542 support, except BVDV-1b branch supporting with 84.5 %. The nucleotide sequences were
543 taken from the NCBI GenBank as it is shown in the legend of Fig.1, except Bega
544 (AF049221), Trangie (AF049222), Deer (AB040132), Suwa (AF117699), isolates 71-15 and
545 71-16 were taken from Jackova et al. (2008).

546 **Fig.3.**547 Genetic typing of selected BVDV-1 isolates in the N^{pro} region.

548 The phylogenetic tree was constructed from 392 bp nucleotide sequences from the N-terminal
 549 part of N^{pro}. The Austrian isolates analysed in this work are labelled in bold. Other sequences
 550 were taken from the NCBI GenBank with the following accession numbers: BVDV-1a –
 551 NADL (M31182); BVDV-1b – Osloss (M96687); BVDV-1c – Bega (AF049221), Trangie
 552 (AF049222); BVDV-1d – F (AF287284); BVDV-1e – 3-It (AF287282); BVDV-1f – J
 553 (AF287286), W (AF287290); BVDV-1g – A (AF287283); BVDV-1h – G (AF287285);
 554 BVDV-1i – 23-15 (AF287279); BVDV-1j – Deer (U80902); BVDV-1k – CH-Suwa
 555 (AY894998). The tree was constructed using PHYLIP Dnadist, Neighbor and Consense
 556 programmes (Felsenstein, 2005). The bootstrap values presented in percentage supporting
 557 particular branch were computed with the Seqboot programme for 1000 replicates.

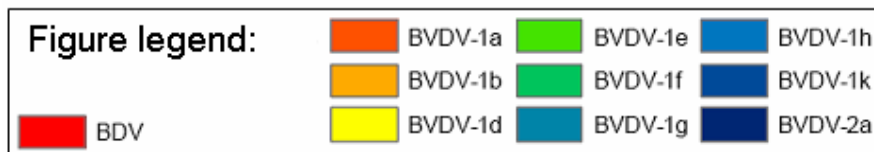
558

559 **Fig.4.**

560 Geographical distribution of all BVDV genotypes and subtypes, respectively, circulating in
 561 Tyrol and Vorarlberg.

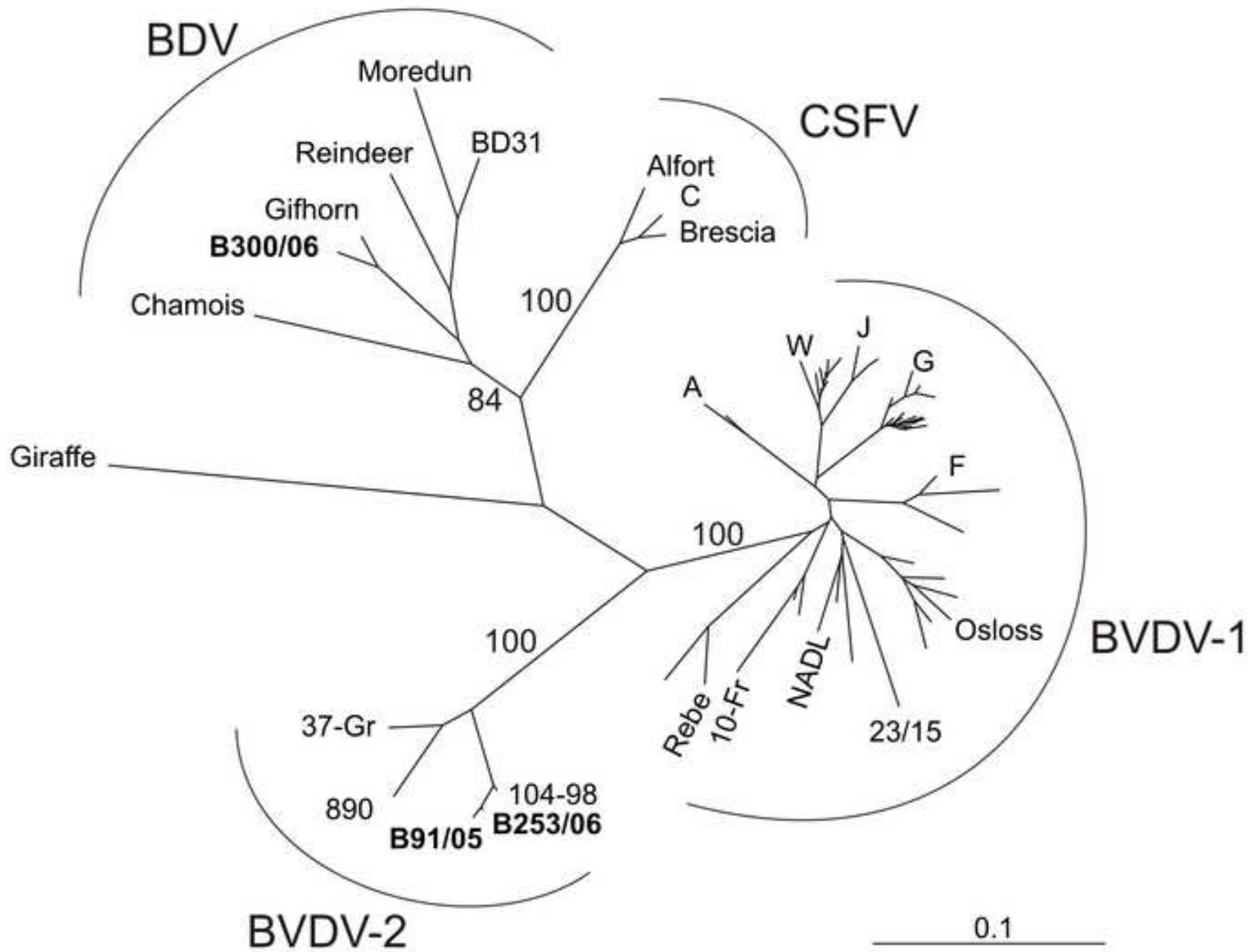
562 Every spot represents a farm.

563



Virus Isolate	Collection Date	Genotype	5'-UTR	Npro	Accession number	Herd Origin	Province
B50/05	2005	BVDV-1	e		EU224244	Kaltenbach im Zillertal	Tyrol
B68/05	2005		b		EU224228	Höchst	Vorarlberg
B75/05	2005			b	EU224254	Kramsach	Tyrol
B80/05	2005		h		EU224239	Weerberg	Tyrol
				h	EU224256	Weerberg	Tyrol
B99/05	2005			f	EU224259	Mieming	Tyrol
B116/05	2005		f		EU224243	St. Johann	Tyrol
B145/05	2005		b		EU224245	Sulz-Röthis	Vorarlberg
B149/05	2005		f		EU224246	Schlitters	Tyrol
B170/05	2005			h	EU224260	Satteins	Vorarlberg
B183/05	2005		f		EU224221	Mieming	Tyrol
B206/05	2005		h		EU224222	Breitenbach am Inn	Tyrol
B211/05	2005			h	EU224247	Volders	Tyrol
B216/05	2005		b		EU224223	Zell am Ziller	Tyrol
				b	EU224248	Zell am Ziller	Tyrol
B248/06	2006		e		EU224224	Reutte	Tyrol
B256/06	2006			b	EU224249	Sulz-Röthis	Vorarlberg
B288/06	2006		b		EU224226	Hohenweiler	Vorarlberg
B306/06	2006		h		EU224229	Lustenau	Vorarlberg
B325/06	2006		d		EU224230	Thüringen	Vorarlberg
				d	EU224250	Thüringen	Vorarlberg
B334/06	2006			g	EU224251	Hochfilzen	Tyrol
B335/06	2006		h		EU224231	Obsteig	Tyrol
B340/06	2006			a	EU224252	Fügen	Tyrol
B341/06	2006		a		EU224232	Fügen	Tyrol
B379/06	2006		e		EU224233	Riezlern im Kleinwalsertal	Vorarlberg
				e	EU224253	Riezlern im Kleinwalsertal	Vorarlberg
B397/06	2006		d		EU224234	Götzis	Vorarlberg
				d	EU224255	Götzis	Vorarlberg
B425/06	2006		b		EU224235	Hopfgarten im Brixental	Tyrol
B434/06	2006		g		EU224236	Reith im Alpbachtal	Tyrol
B440/06	2006		k		EU224237	Rankweil	Vorarlberg
				k	EU224257	Rankweil	Vorarlberg
B444/06	2006		h		EU224238	Anras	Tyrol
B458/06	2006		f		EU224240	Scheffau am Wilden Kaiser	Tyrol
				f	EU224258	Scheffau am Wilden Kaiser	Tyrol
B463/06	2006		h		EU224241	Innsbruck	Tyrol
B91/05	2005	BVDV-2			EU224242	Kirchdorf	Tyrol
B253/06	2006				EU224225	Obertilliach	Tyrol
B300/06	2006	BDV-3			EU224227	Mutters	Tyrol

Figure 1



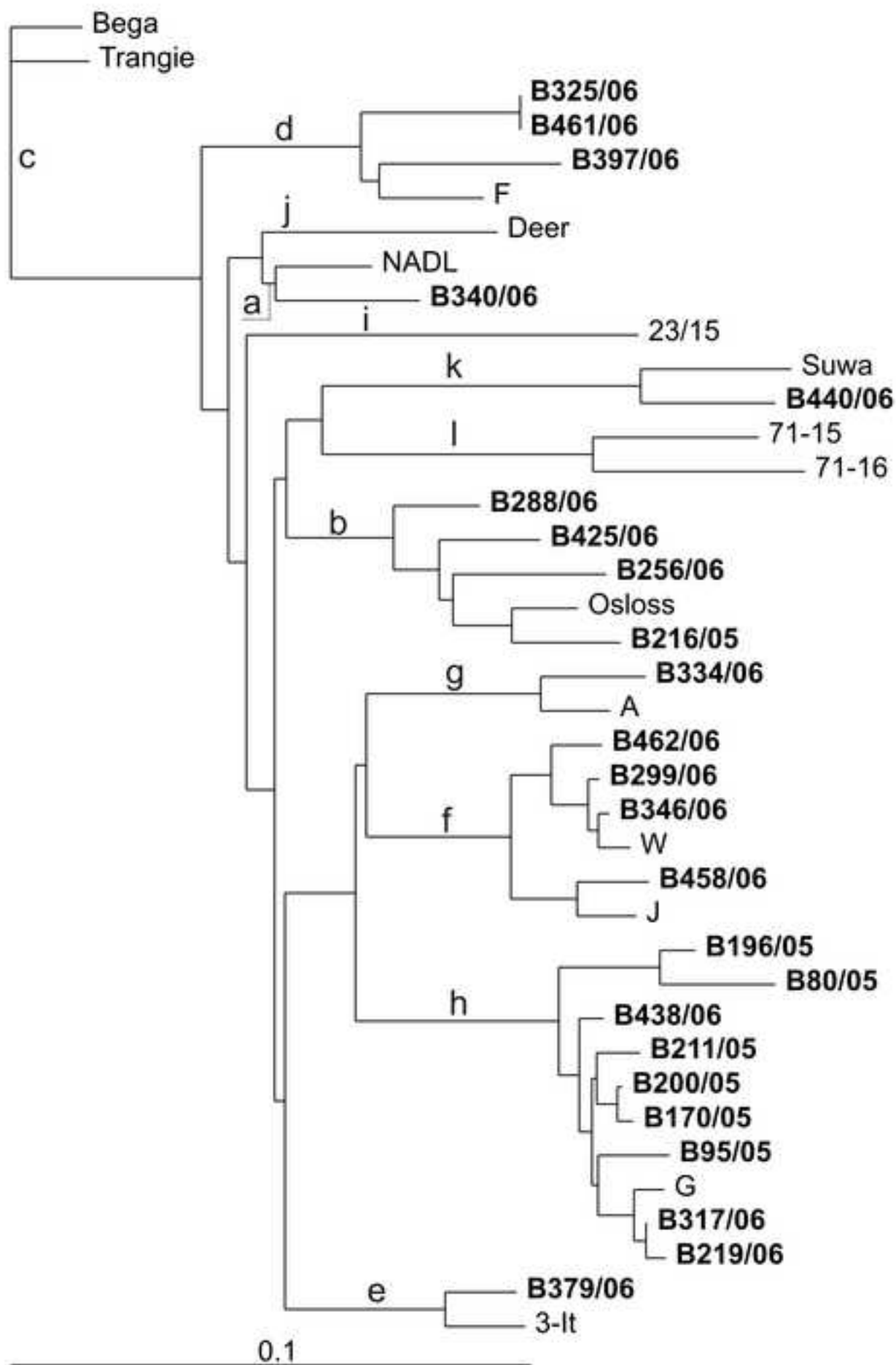


Figure 4

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