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PII: S0378-1135(08)00433-1
DOI: doi:10.1016/j.vetmic.2008.09.068
Reference: VETMIC 4199

To appear in: *VETMIC*

Received date: 31-3-2008
Revised date: 11-9-2008
Accepted date: 15-9-2008

Please cite this article as: Hornberg, A., Fernández, S.R., Vogl, C., Vilcek, S., Matt, M., Fink, M., Köfer, J., Schöpf, K., Genetic diversity of pestivirus isolates in cattle from Western Austria, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.068

Genetic diversity of pestivirus isolates in cattle from Western Austria

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Abstract

The genetic diversity of bovine viral diarrhoea virus (BVDV) isolates in infected cattle from Tyrol and Vorarlberg (Austria) was investigated. Blood samples were collected within the compulsory Austrian BVDV control programme during 2005 and 2006. The 5´-untranslated region (5´-UTR) and partially the N-terminal autoprotease (N^{pro}) were amplified by one-step reverse transcriptase-polymerase chain reaction (RT-PCR) and the PCR products were subsequently sequenced. Phylogenetic analysis based on 5´-UTR and N^{pro} sequences demonstrated that almost all isolates (307/310) were of the BVDV-1 genotype. They were clustered into eight different subtypes, here listed by their frequency of occurrence: BVDV-1h (143), BVDV-1f (79), BVDV-1b (41), BVDV-1d (28), BVDV-1e (6), BVDV-1a (4), BVDV-1g (3) and BVDV1-k (3). Two pestivirus isolates were typed as BVDV-2 and one isolate as BDV closely related to Gifhorn strain (BDV-3). Correlation among isolates could only be observed at the farm level, i.e., within a herd. However, no correlation between the genetic and geographical distances could be observed above the farm level. Because of the wide distribution of certain BVDV-1 subtypes and the low prevalence of herd-specific strains, a determination of tracing routes of infection was not possible. Furthermore, recombination events were not detected.

Keywords: pestivirus, BVDV, sequencing, genetic typing

Introduction

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

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

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

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

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

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

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

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

Materials and methods

Serum samples and isolation of RNA

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

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

Real-time RT-PCR

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

RT-PCR

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

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

Nucleotide sequencing

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

Computer-assisted analysis

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

and goats are kept on alps in close proximity with cattle during summer. During this period of grazing contact with wild ungulates is also possible. All these factors might explain the absence of herd-specific strains. Hamers et al. (1998) suggested that a high variability of BVDV sequences may derive from the horizontal transfection from a BVDV-immunocompetent to a susceptible animal, while herd-specific strains may derive from the transmission from PI animals within the farm. In the present work, evidence for geographical clustering of genotypes or subtypes above the farm level was not determined. These findings are in accordance with other previous investigations (Vilcek et al., 1999; Stalder et al., 2005).

In Sweden, where the BVD eradication programme has been running since 1993, the molecular epidemiology is actually used to trace sources and routes of BVDV infections. The comparison of existing BVDV sequences to new cases has been used as an important tool to trace the origin of new outbreaks (Stahl et al., 2005). The samples of this study were collected over two years (2005 and 2006) within the Austrian compulsory BVDV control programme, which was implemented in 2004. So far, it has not been possible to retrace routes of BVDV infections due to the wide geographical distribution of several BVDV subtypes in Tyrol and Vorarlberg and to the low prevalence of herd-specific strains. Tracing the origin of BVDV infections in the future may gain importance during the final phase of the BVD control programme, when the prevalence will be reduced and almost all herds will have a BVDV-free status. The development of a sequence database, as established in Sweden (Stahl et al., 2005), including all characterised isolates from BVDV infected animals from Austria and neighbouring countries, will be a useful tool for eradication and for studying the epidemiology of BVD. Then, genetic typing of BVDV isolates could be used for tracing new infection routes more economically.

Acknowledgements

We thank Wilhelm Berg for the geographical illustrations and Sandra Blome for the gift of the Gifhorn strain. We gratefully acknowledge the laboratory staff from the Department of PCR and Molecular Biology in Moedling for their support and the Department of Serology in Innsbruck for performing the ELISA tests. This work was supported by the Austrian Agency for Health and Food Safety. Stefan Vilcek was supported by the Slovak Research and Development Agency under the contract No. APPV-20-019605.

References

Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea infection. Vet. Clin. North Am. 11 (3), 425–445.

Barros, S.C., Ramos, F., Pauperio, S., Thompson, G., Fevereiro, M., 2006. Phylogenetic analysis of Portuguese bovine viral diarrhoea virus. Virus Res. 118, 192-195.

Becher, P., Orlich, M., Shannon, A.D., Horner, G., König, M., Thiel, H.J., 1997. Phylogenetic analysis of pestiviruses from domestic and wild ruminants. J. Gen. Virol. 78, 1357-1366.

Becher, P., Orlich, M., Kosmidou, A., König, M., Baroth, M., Thiel, H.J., 1999. Genetic diversity of pestiviruses: identification of novel groups and implication for classification.Virology 262, 64–71.

Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., König, M., Schweizer, M., Stalder, H., Schirrmeier, H., Thiel, H.J., 2003. Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. Virology 311, 96-104.

Couvreur, B., Letellier, C., Collard, A., Quenon, P., Dehan, P., Hamers, C., Pastoret, P.P., Kerkhofs, P., 2002. Genetic and antigenic variability in bovine viral diarrhea virus (BVDV) isolates from Belgium. Virus Res. 85, 17-28.

Falcone, E., Cordioli, P., Tarantino, M., Muscillo, M., La Rosa, G., Tollis, M., 2003. Genetic Heterogeneity of Bovine Viral Diarrhoea Virus In Italy. Vet. Res. Commun. 27, 485-494.

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. Distibuted 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., Schirrmeier, 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.

Schöpf, K., Gaede, W., Matt, M., 2005. Erfahrungen zur Bekämpfung der Bovinen Virus Diarrhoe (BVD/MD) mit spezieller Berücksichtigung der Verbreitung von BVDV-Genotypen im Bundesland Tirol. Wien. Tierärztl. Mschr. 92, 254-258.

Stahl, K., Kampa, J., Baule, C., Isaksson, M., Moreno-Lopez, J., Belak, S., Alenius, S., Lindberg, A., 2005. Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. Prev. Vet. Med. 72, 103-108.

Stalder, H.P., Meier, P., Pfaffen, G., Wageck-Canal, C., Rüfenacht, J., Schaller, P., Bachofen, C., Marti, S., Vogt, H.R., Peterhans, E., 2005. Genetic heterogeneity of pestiviruses of ruminants in Switzerland. Prev. Vet. Med. 72, 37-41.

Tajima, M., Frey, H.R., Yamato, O., Maede, Y., Moennig, V., Scholz, H., Greiser-Wilke, I., 2001. Prevalence of genotypes 1 and 2 of bovine viral diarrhea virus in Lower Saxony, Germany. Virus Res. 76, 31–42.

Toplak, I., Sandvik, T., Barlic-Maganja, D., Grom, J., Paton, D.J., 2004. Genetic typing of bovine viral diarrhoea virus: most Slovenian isolates are of genotypes 1d and 1f. Vet. Microbiol. 99, 175–85.

Uttenthal, A., Stedejek, T., Nylin, B., 2005. Genetic diversity of bovine viral diarrhoea viruses (BVDV) in Denmark during a 10-year eradication period. APMIS 113, 536-41.

Vilcek, S., Herring, A.J., Herring, J.A., Nettleton, P.F., Lowings, J.P., Paton, D.J., 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. Virol. 136, 309-323.

Vilcek, S., Drew, T.W., McGoldrick, A., Paton, D.J., 1999. Genetic typing of bovine pestiviruses from England and Wales. Vet. Microbiol. 69, 227–237.

Vilcek, S., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol. 146, 99–115.

Vilcek, S., Durkovic, B., Bobakova, M., Sharp, G., Paton, D.J., 2002. Identification of bovine viral diarrhoea virus 2 in cattle in Slovakia. Vet. Rec. 151, 150-152.

Vilcek, S., Greiser-Wilke, I., Durkovic, B., Obritzhauser, W., Deutz, A., Köfer, J., 2003. Genetic diversity of recent bovine viral diarrhoea viruses from the southeast of Austria (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.
509
510
511

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 bolt 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 bolt. 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).

Fig.3.

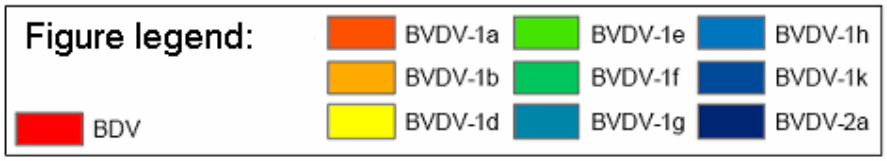
Genetic typing of selected BVDV-1 isolates in the N^{pro} region.

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

Fig.4.

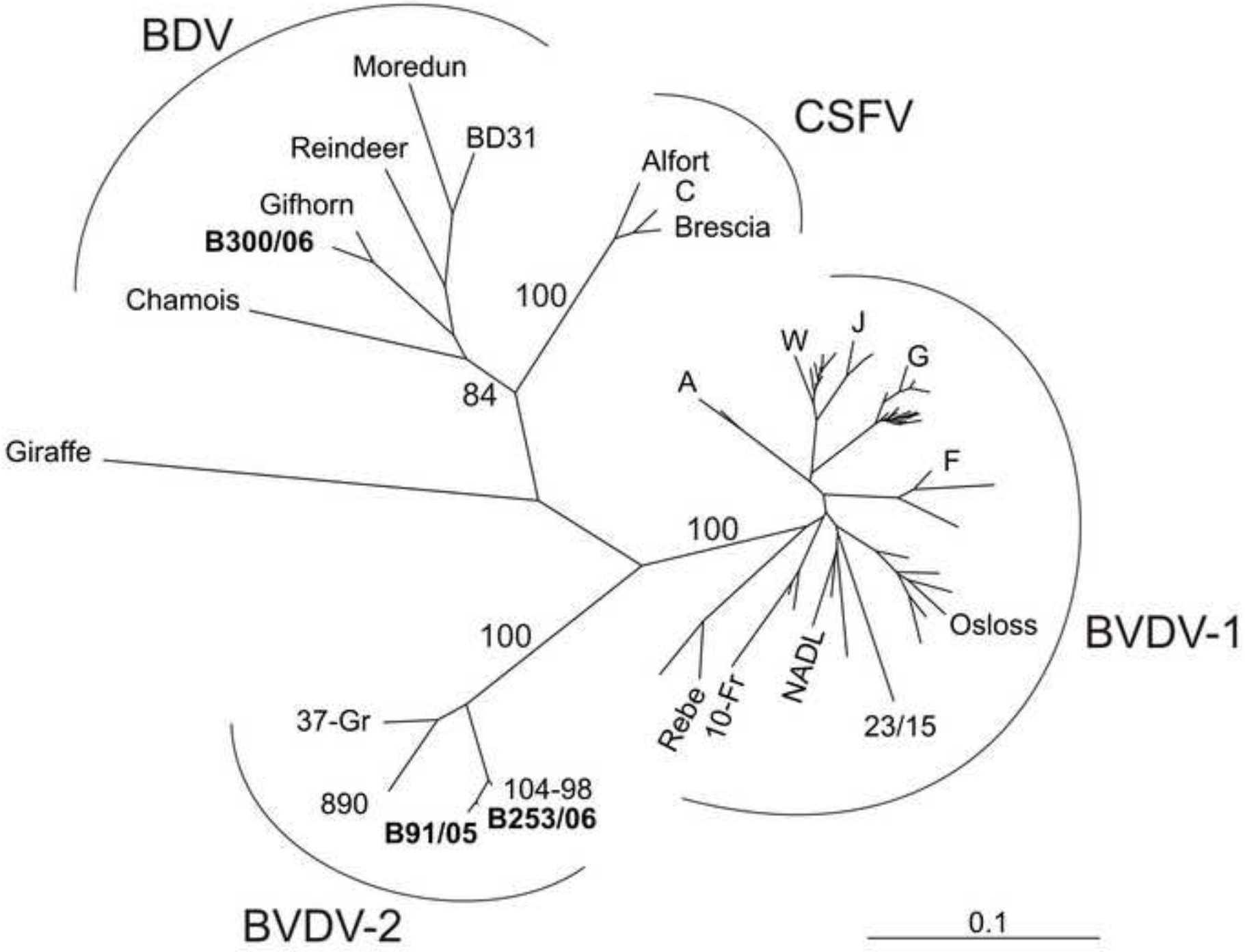
Geographical distribution of all BVDV genotypes and subtypes, respectively, circulating in Tyrol and Vorarlberg.

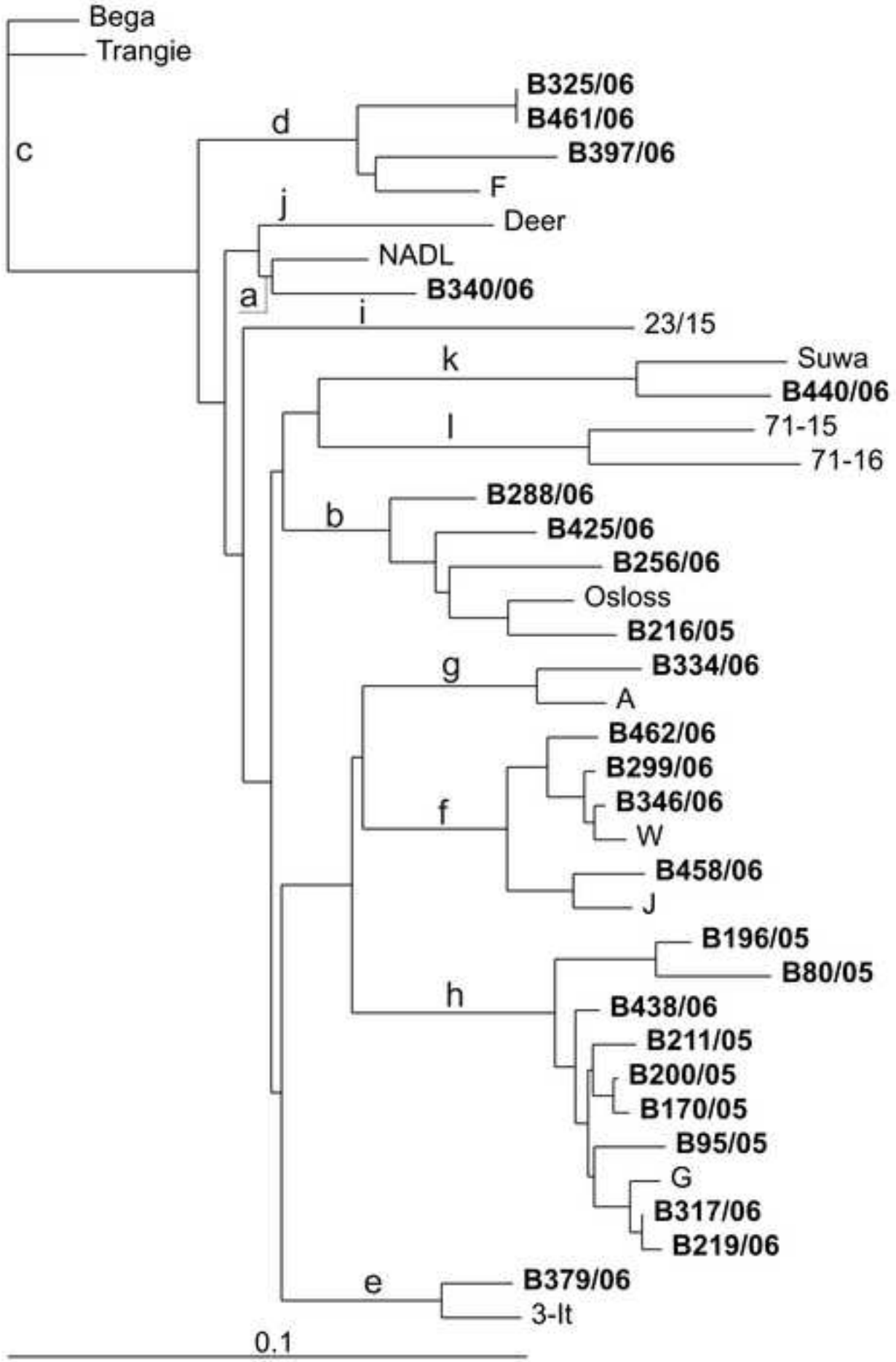
Every spot represents a farm.



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


BDV
Moredun
Reindeer
BD31
Gifhorn
B300/06
Chamois
CSFV
Alfort
C
Brescia
100
84
Giraffe
W
J
G
A
F
100
100
Osloss
BVDV-1
Rebe
10-Fr
NADL
23/15
37-Gr
890
104-98
B91/05
B253/06
BVDV-2
0.1



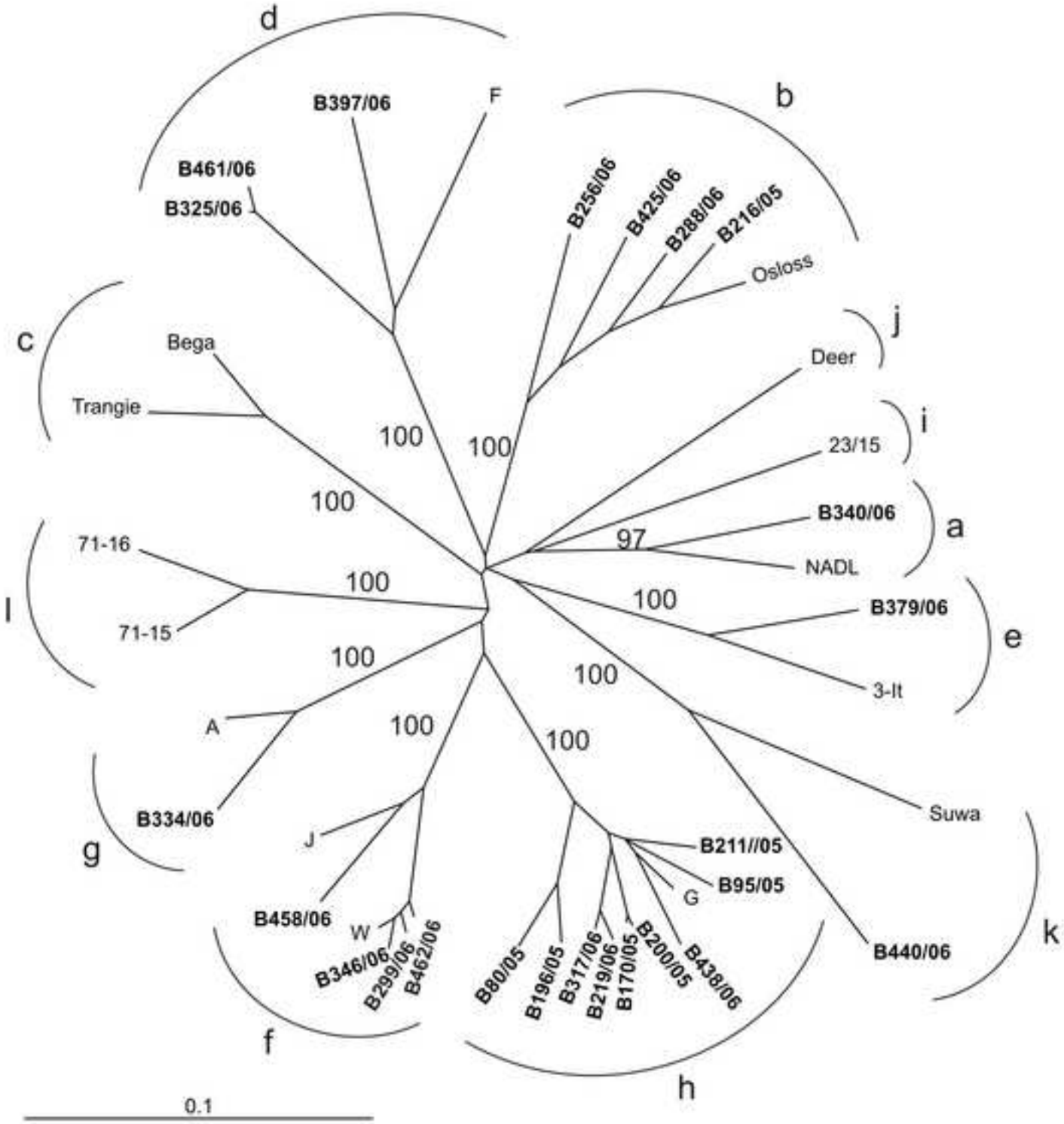


Figure 4