Virology 385 (2009) 351–357

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Phylogeny, classification and evolutionary insights into pestiviruses

Lihong Liu [a,b,1,*], Hongyan Xia [a,b,1], Niklas Wahlberg [c], Sándor Belák [a,b], Claudia Baule [b]

[a] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden
[b] Joint R&D Division in Virology, The National Veterinary Institute & The Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden
[c] Laboratory of Genetics, Department of Biology, University of Turku, 20014 Turku, Finland

ARTICLE INFO

Article history:
Received 16 September 2008
Returned to author for revision
19 October 2008
Accepted 4 December 2008
Available online 24 January 2009

Keywords:
Bayesian approach
BDV
BVDV
Classification
CSFV
Evolution
Maximum likelihood
Pestivirus
Phylogeny

ABSTRACT

The genus *Pestivirus* comprises four established species: *Bovine viral diarrhoea viruses* 1 (BVDV-1) and 2 (BVDV-2), *Border disease virus* (BDV), and *Classical swine fever virus* (CSFV); and a tentative species, Pestivirus of giraffe. Additional pestiviruses have been identified and suggested for recognition as novel subgroups/species. To achieve a reliable phylogeny as the basis for classification of pestiviruses, a molecular dataset of 56 pestiviruses and 2089 characters, comprising the 5′UTR, complete N^{pro} and E2 gene regions was analysed by Maximum likelihood and Bayesian approach. An identical, robust tree topology was inferred, where seven well-supported monophyletic clades and two highly divergent lineages were identified. Dating most recent common ancestor was estimated for major pestivirus lineages and their evolutionary histories were revealed. Accordingly, a new proposal is presented for the classification of pestiviruses into nine species: BVDV-1, BVDV-2, BVDV-3 (atypical bovine pestiviruses), Pestivirus of giraffe, CSFV, BDV, Tunisian sheep virus (TSV; previously termed "Tunisian isolates"), Antelope and Bungowannah.

Introduction

Pestiviruses are single-stranded, positive-sense RNA viruses. The viral genome contains two untranslated regions (UTRs) at the 5′ and 3′ ends, and an open reading frame (ORF) encoding a polyprotein. The polyprotein is co- and post-translationally processed into 12 polypeptides in the following order: N-terminal autoprotease (N^{pro}); capsid protein (C); envelope proteins (E^{rns}, E1, and E2); p7; and, non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (reviewed by Thiel et al., 1996). The genus *Pestivirus* of the family *Flaviviridae* comprises four recognized species: *Bovine viral diarrhoea virus* genotypes 1 (BVDV-1) and 2 (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) (van Regenmortel et al., 2000). A fifth tentative species is represented by the strain H138, isolated from a giraffe in Kenya (Plowright, 1969). Although the natural host of BVDV is bovine, it can infect both domestic and wild animals, including deer (Becher et al., 1999) and pigs (Wang et al., 1996). The natural host of BDV is ovine, however, natural infection of cattle with BDV is reported (Cranwell et al., 2007). The only species that has not been identified outside the natural host is CSFV, which is still restricted to domestic pigs and wild boars. Pestivirus infections can vary from subclinical to manifestation of clinical signs such as: fever, diarrhoea, hemorrhagic syndrome, death, and abortion (reviewed by Thiel et al., 1996).

Besides the established species, there are three groups of recently identified but unclassified pestiviruses. The first group consists of "atypical" pestiviruses of bovine origin detected in contaminated foetal calf serum (FCS) batches, as well as in cattle infected naturally. These include: D32/00_'HoBi', isolated from a batch of FCS originating from Brazil (Schirrmeier et al., 2004); Brz buf 9, originally isolated from a buffalo in Brazil (Stalder et al., 2005); CH-KaHo/cont, a cell culture contaminant possibly originating from a batch of FCS produced in South America (Stalder et al., 2005); and, Th/04_Khon-Kaen, detected from serum of a naturally infected calf in Thailand (Ståhl et al., 2007). All these atypical pestiviruses are closely related to each other. The second group are two divergent, non-bovine origin pestiviruses, including one from a diseased young blind pronghorn antelope in the USA (Vilcek et al., 2005); and Bungowannah virus from pigs, associated with porcine myocarditis syndrome in Australia (Kirkland et al., 2007). These two viruses clustered in a well-supported clade in the phylogenetic trees based on the 5′UTR, and the N^{pro} and E2 protein sequences (Kirkland et al., 2007). The third group is so-called "Tunisian isolates" that have been isolated from both Tunisian sheep and different batches of a contaminated Tunisian sheep pox vaccine (Thabti et al., 2005). The Tunisian

* Corresponding author. Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden. Fax: +46 18 674669.
E-mail address: Lihong.Liu@bvf.slu.se (L. Liu).
[1] These authors have contributed equally to the paper.

0042-6822/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2008.12.004

isolates are phylogenetically closer to CSFV than to BDV: they form a separate branch between BDV and CSFV in the phylogenetic trees of the entire N^{pro}-E2 region and part of E2 gene region (Thabti et al., 2005).

Uncertainty exists in the classification of pestiviruses. According to Schirrmeier et al. (2004), the atypical bovine pestivirus D32/00_'HoBi' is proposed as a "sixth" pestivirus species; Becher et al. (2003) suggest that BVDV-1 and BVDV-2 should be classified as two major genotypes within one species BVDV. The genetic diversity of BDV is greater than other pestivirus species, as up to six major genotypes are reported (Dubois et al., 2008). Furthermore, BDV classification is unclear, for example, genotype-4 has been assigned to two groups of viruses of different host origin: Chamois-1 (Arnal et al., 2004) and Tunisian isolates (Thabti et al., 2005).

The evolutionary relationships between recognised species and unclassified pestiviruses, and among unclassified pestiviruses have not been unambiguously determined. This is particularly pronounced for relationships among atypical bovine pestiviruses, Pestivirus of giraffe and BVDV, as different relationships are inferred from analyses of different gene regions. For example, the atypical bovine pestivirus D32/00_'HoBi' is placed as a sister in three different groups. These include: the group consisting of BDV and CSFV in the tree derived from the N^{pro} region; the group comprising two genotypes of BVDV in the tree constructed from the E2 gene region; and, the giraffe strain with a bootstrap support of 83% in the tree based on the NS3 gene region (Schirrmeier et al., 2004).

In this study, the representative sequences of both recognized species and unclassified pestiviruses were mined, and comprehensive phylogenetic analyses of a combined dataset were performed by Maximum likelihood (ML) and Bayesian approach, with the aim of achieving a reliable phylogeny as the basis for classifying pestiviruses. This strategy resulted in a more stable, well-supported pestivirus phylogeny, where a close relationship between atypical bovine pestiviruses and BVDV-1 and BVDV-2 was, for the first time, unequivocally established, and supported statistically by high posterior probability and bootstrap values. The divergence times of the major pestivirus lineages were also estimated. The evolutionary study established that atypical bovine pestiviruses share the most recent common ancestor with BVDV-1 and BVDV-2, and Tunisian isolates share the most recent common ancestor with CSFV. The analyses provided an additional evolutionary basis for the classification of pestiviruses.

Results

Incongruence–length-difference test and model selection

The incongruence–length-difference (ILD) test revealed that the combined dataset was not significantly incongruent ($p=0.1667$). The GTR+I+G was selected as the best-fit model for the phylogenetic analysis of the combined dataset. At the end of the Bayesian analysis, the standard deviation of split frequencies was less than 0.01 (between 0.006 and 0.007), indicating that four chains had reached convergence.

Phylogeny and classification of pestiviruses

The evolutionary relationships of pestiviruses were reconstructed by Maximum likelihood and Bayesian approach through analysing a combined molecular dataset of 56 pestiviruses and 2089 characters, comprising the 5′UTR, N^{pro} and E2 gene regions. Analysis of this dataset produced an identical, well-supported tree topology, regardless of the methods used (Fig. 1). Both ML and Bayesian methods produce unrooted networks, which in practice are rooted through outgroups. However, as it is uncertain which the closest outgroups of pestiviruses are and that distant outgroups cause biases in phylogenetic analyses (Bergsten, 2005), the exact position of the root for the pestivirus phylogeny is unknown, but is probably located on one of the long branches leading to the two divergent pestiviruses, as indicated in Fig. 1. With this assumption, seven monophyletic clades and two highly divergent lineages, corresponding to both recognised species and unclassified pestiviruses, could be identified. Each clade was strongly supported by the maximum posterior probability value of 1.00 and the highest bootstrap value of 100%. The relationships of the seven major clades were also supported by the maximum posterior probability value of 1.00 and by high bootstrap values of 78–99%. Therefore, this tree topology was regarded as reliable and robust.

The atypical bovine pestiviruses D32/00_'HoBi', CH-KaHo/cont, and Th/04_KhonKaen formed a monophyletic clade, sister to the established species BVDV-1 and BVDV-2 that bifurcate from a common branch. The sister relationship with the established BVDV-1 and BVDV-2 was strongly supported by a posterior probability of 1.00 and a bootstrap value of 78%. Positioning of this clade was identical to the Th/04_KhonKaen lineage in the whole-genome phylogeny (Liu et al., submitted). These results supported the classification of the atypical bovine pestiviruses as a new species, termed genotype-3 BVDV (BVDV-3). The tentative species (Pestivirus of giraffe) had a sister relationship with all three genotypes of BVDV, which was supported by a posterior probability of 1.00 and a bootstrap value of 78%.

In the sister group to the BVDV clade, three monophyletic clades were identified, corresponding to the recognised species CSFV and BDV, and unclassified Tunisian isolates from sheep. In the clade of BDV, BDV-1 formed a well-supported clade. Two single lineages (BDV-3 and BDV-4) and the well-supported BDV-2 clade formed a larger clade that was weakly supported by a posterior probability value of 0.67 and a bootstrap value of 54%. Therefore, the relationship among BDV-2, BDV-3 and BDV-4 could not be resolved. The Tunisian isolates, which have also been termed BDV-4 (Thabti et al., 2005), formed a sister clade to the CSFV lineage and was supported by a bootstrap value of 99% and a posterior probability value of 1.00. This branch was not the sister group of BDV and is termed Tunisian sheep virus (TSV) in this study.

Evolutionary history of major pestivirus lineages

To date the most recent common ancestor (MRCA) for major pestivirus lineages and their evolutionary history, the program BEAST (Drummond and Rambaut, 2007) was used with the relaxed clock model (uncorrelated exponential). The mean MRCA dates and 95% highest posterior density (HPD) were calculated by the software Tracer. As shown in Fig. 2, the pestiviruses, excluding the two highly divergent terminals, began diverging around 1483 (HPDs 600 to 1892). The clade of mainly bovine-origin pestiviruses diverged between 1615 and 1743 to form the four major lineages in the clade, corresponding to the four species: Pestivirus of giraffe, BVDV-3, BVDV-2 and BVDV-1. The clade of mainly ovine- and swine-origin pestiviruses diverged between 1629 and 1736 to form the three species: BDV, TSV and CSFV. In addition, the earliest MRCA date estimates within species were 1802 (HPDs, 1522 to 1939) for BVDV-1; 1890 (HPDs, 1712 to 1988) for BVDV-2; 1880 (HPDs, 1651 to 1993) for BVDV-3; 1861 (HPDs, 1637 to 1961) for Pestivirus of giraffe; 1825 (HPDs, 1564 to 1947) for CSFV; 1748 (HPDs, 1334 to 1952) for BDV; and 1906 (HPDs, 1705 to 1993) for TSV.

Discussion

Phylogenetic analysis of pestiviruses is vital for classifying novel viruses and for revealing their evolutionary history. Although it is generally accepted that the N^{pro} and E2 genes are suitable for

phylogenetic analysis of pestiviruses (Becher et al., 1997, 1999, 2003), we have observed that analysis of the N^{pro} gene produced an unsupported hypothesis where Pestivirus of giraffe, rather than BVDV-3, was the sister group to BVDV-1 and BVDV-2. This phenomenon is reported in other studies on atypical pestiviruses (Schirrmeier et al., 2004; Stalder et al., 2005). Analysis of the single E2 gene revealed a less robust hypothesis where Pestivirus of giraffe and BVDV-3 bifurcated from a common branch without strong support. To overcome the limitations of single gene analyses, a dataset combining 5′UTR, complete N^{pro} and E2 gene regions was analysed by two phylogenetic methods (ML and Bayesian approach) for classifying pestiviruses. The same strategy was used for dating most recent common ancestor.

The three codon positions of the N^{pro} and E2 protein-coding genes may evolve differently. It is possible that substitution saturation in the third positions may occur over time, therefore, the true level of divergence is masked and deeper phylogenetic relationships are obscured to the point of making them unrecoverable (Arbogast et al., 2002; Avise et al., 1987). To investigate the possible effect of this on the reliability of the phylogeny inferred from the combined dataset, the third codon positions were deleted and the first and second codons were subject to the same analyses. The same tree topology was obtained by both ML and Bayesian approach, indicating that analysis of the combined dataset was reliable.

Species demarcation criteria in the genus include nucleotide sequence relatedness, serological relatedness and host of origin (van Regenmortel et al., 2000). By applying these criteria, Schirrmeier et al. (2004) propose that the atypical bovine pestivirus is classified as a new species. The question, however, has been whether to coin a new species name for this taxon or to link it with an established bovine pestivirus species. There are two arguments for suggesting these atypical pestiviruses as a new genotype of BVDV. The first is the close relationship between atypical pestiviruses and BVDV, as revealed in this study through the analysis of the combined dataset. The second is that the Th/04_KhonKaen virus was initially detected by a commercial BVDV Ag-ELISA Kit (Herd Check BVDV Ag/Serum plus, IDEXX Laboratories) (Kampa et al., 2008), indicating a high degree of serological relatedness of the Th/04_KhonKaen virus with BVDV. In a broader context of evolution, this group of bovine pestiviruses appear to have diverged early from the common ancestor of BVDV-1 and BVDV-2, and to have evolved independently in South America and possibly South-East Asia (Thailand). Thus, in this study, the atypical bovine pestiviruses are proposed as BVDV-3.

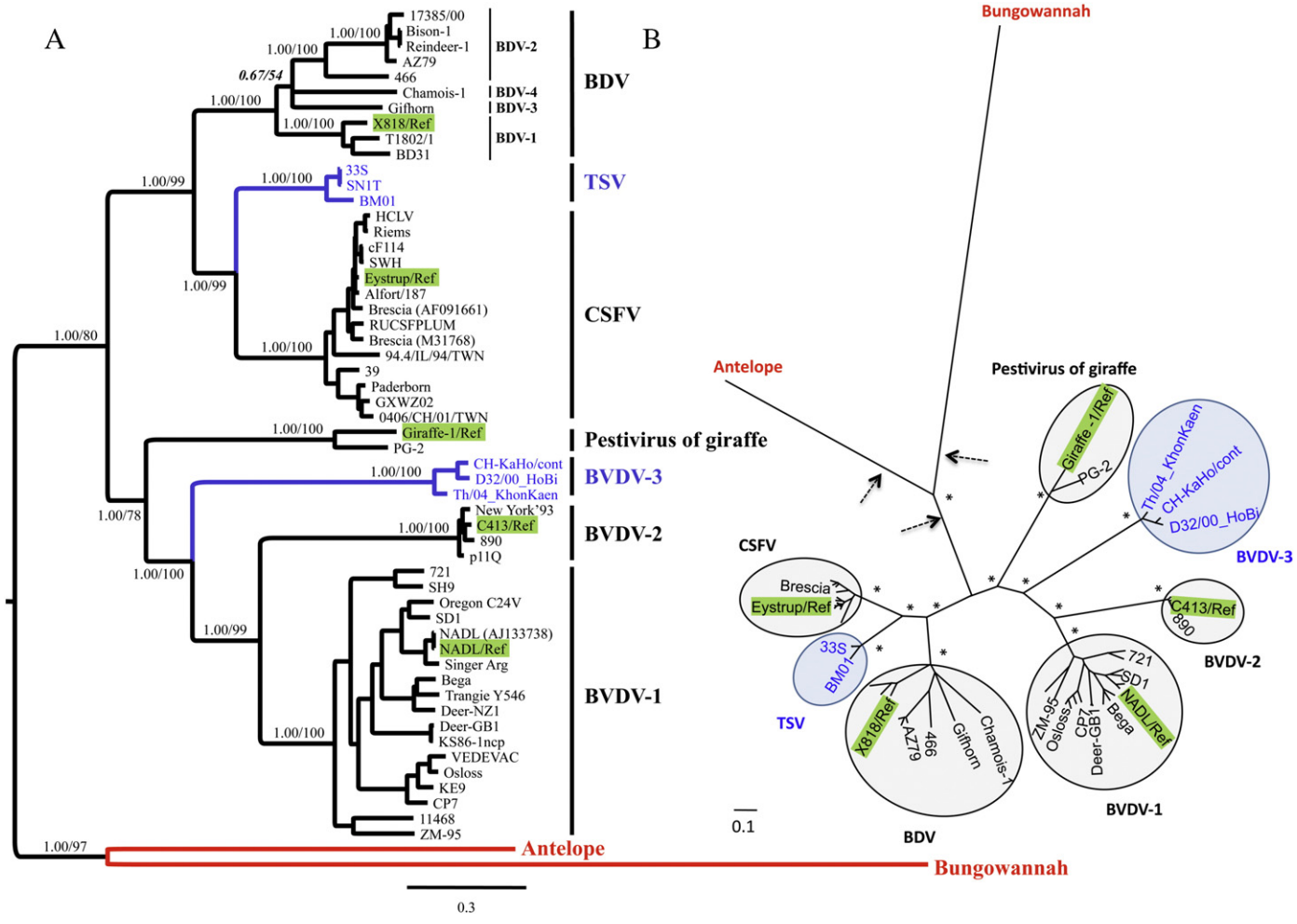

Fig. 1. Phylogeny and classification of pestiviruses by Maximum likelihood and Bayesian approach. The molecular dataset contains 56 sampled pestiviruses and 2089 characters, comprising the 5′UTR, N^{pro} and E2 gene regions. PHYML (v2.4.4) was used for phylogeny inference according to maximum likelihood criterion. MrBayes 3.1 was used for Bayesian analysis. This is a representative consensus tree: mid-point rooted (1a, left) and unrooted (1b, right). The reference sequences are highlighted in green. The new species proposed in this study are in blue (BVDV-3 and TSV) and in red (Antelope and Bungowannah). Fig. 1a presents all sampled pestiviruses and their relationships. The numbers at a node are posterior probability (left) and percentage of 1000 bootstrapping replicates (right). For a clear demonstration, some terminals are not displayed in Fig. 1b. A "*" indicates strong statistical support for a node by a posterior probability value of 0.99–1.00 and by a bootstrap value of 78–100%. The scale bar represents changes per site. The arrows show the probable placements of the root for the given unrooted network.

The evolutionary relationship should also be considered as an additional criterion for species demarcation. The Tunisian isolates, which have been typed as a subgroup of BDV according to antigenic relatedness and host of origin criteria, are proposed in this study as representing another new species, TSV. The closer phylogenetic relationship of TSV with CSFV rather than with BDV indicated an evolutionary history of these isolates independent of BDV. Indeed, TSV shared the most recent common ancestor with CSFV rather than with BDV. Therefore, based on the evolutionary relationship of TSV with CSFV, naming these isolates either as a new genotype of BDV or as a subgroup of BDV appears inappropriate.

Becher et al. (2003) suggest BVDV-1 and BVDV-2 as one species. In the new classification scheme proposed here, BVDV-1 and BVDV-2 were not considered as one species. Firstly, each of the species BVDV-1, BVDV-2, and the proposed BVDV-3 formed a well-supported monophyletic clade in the phylogenetic tree, and the separation between species was statistically supported. This was in sharp contrast to only weakly supported separation between the considered major genotypes of BDV. Secondly, the evolutionary distance calculated by Neighbour-joining method may not reflect the true distance. For example, the ratio of evolutionary distances between CP7 (BVDV-1) and 890 (BVDV-2) to that between BDV strains Gifhorn and T1802 (Fig. 1) was approximately 2.17; whereas, when calculated with the Neighbour-Joining method, it was approximately 1.44 (Fig. 2 in Becher et al., 2003). This would suggest either a smaller genetic distance between two species BVDV-1 and BVDV-2, or a larger distance between two BDV strains within one species based on a Neighbour-joining tree, therefore, masking the real evolutionary distance. It is unclear if this is an isolated observation or a difference between two methods in general.

Based on the estimates of the dates of divergence (Fig. 2), the diversification of major pestiviruses started around 1483 (HPDs, 600 to 1892), approximately 520 years before present. Pestiviruses of two origins may have been present: one of mainly bovine-origin, comprising BVDV-1, BVDV-2, BVDV-3 and Pestivirus of giraffe; and the other of mainly swine- and ovine- origins, consisting of CSFV, BDV and TSV. At this time, pestiviruses of bovine-origin separated from those of mainly swine- and ovine-origins. The first evolutionary event for pestiviruses of mainly bovine-origin was when Pestivirus of giraffe diverged and evolved independently, probably in Africa, from the common ancestor around 1615 (HPDs, 1017 to 1904). The second event happened to the BVDV-3 lineage that diverged in South America or Eastern Asia around 1681 (HPDs, 1210 to 1912), approximately 330 years before present. As this group of pestiviruses was detected in either contaminated FCS batches (D32/00_'HoBi', CH-KaHo/cont) or persistently infected calf (Th/04_KhonKaen), they are of non-cytopathogenic biotype, which has enabled them to establish persistent infection in cattle. The third event was the separation of BVDV-2 and BVDV-1 around 1743 (HPDs, 1373 to 1926), when BVDV-2 evolved independently in North America. In general, this group of viruses are highly virulent (Rümenapf and Thiel, 2008), for example, a bovine viral diarrhoea epidemic in the province of Quebec, Canada, resulted in the death of 32 000 out of 143 000 (22.4%) animals in the 1993 veal calf crop (Pellerin et al., 1994). Finally, the type species of the *Pestivirus*

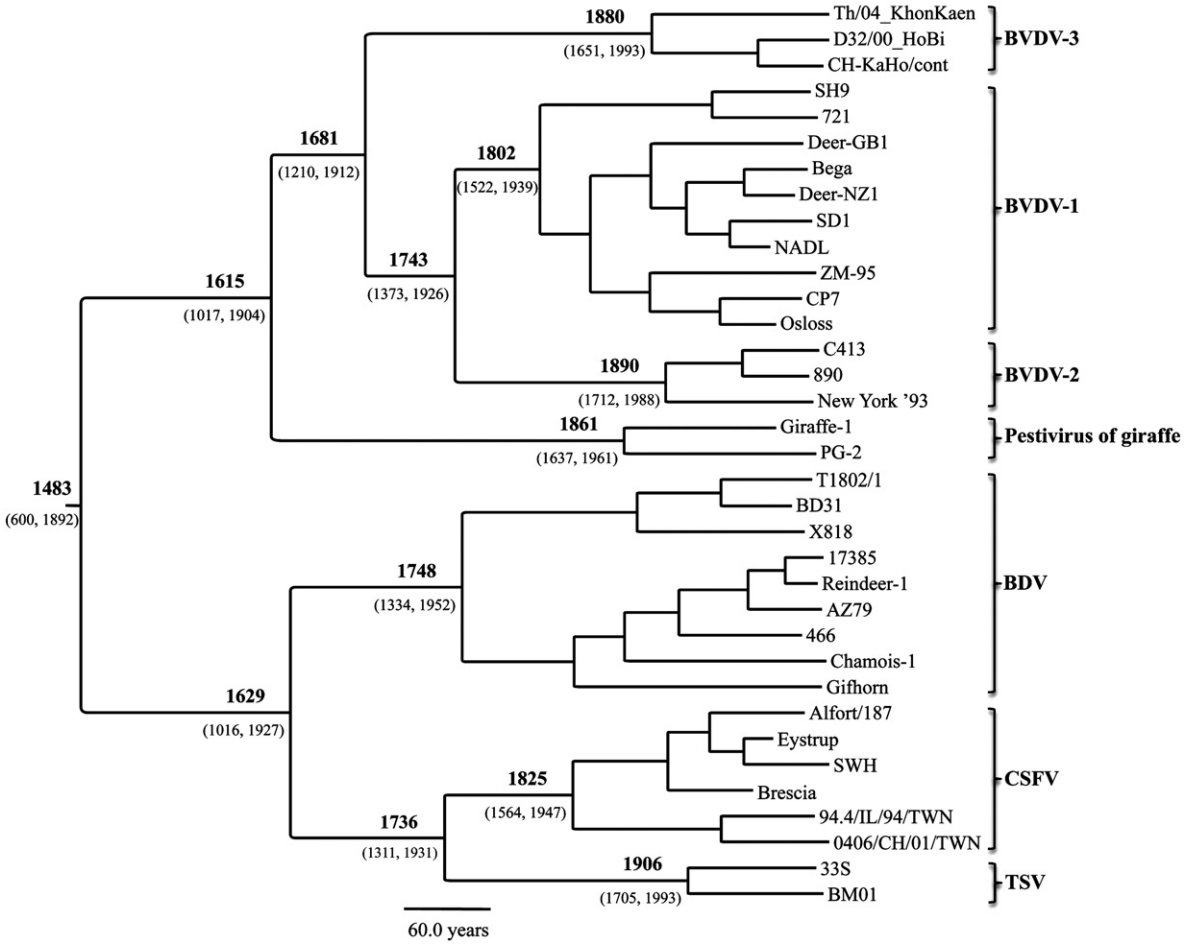

Fig. 2. Divergence dates of major pestivirus lineages in a phylogenetic tree based on analysis of 2037 characters (35 terminals) comprising the 5′UTR, N^{pro} and E2 gene regions. Numbers at branch nodes indicate the divergence dates, with the 95% HPDs in brackets.

genus, BVDV-1, first diverged from BVDV-2 around 1743 and then diversified around 1802, approximately 150 years before the first description of the disease in 1946 (Olafson et al., 1946). Since then, BVDV-1 has spread and been detected worldwide.

The separation of swine and ovine pestiviruses occurred at 1629 (HPDs, 1016 to 1927), when BDV speciated in ovine hosts and CSFV speciated in swine hosts. The BDV clade began diversifying around 1748 (HPDs, 1334 to 1952), approximately 210 years before the first report of the disease from the border region of England and Wales in 1959 (Hughes et al., 1959). The disease has been reported in most sheep-rearing countries, such as UK, Australia, New Zealand, Spain, USA. Although most of the isolates are non-cytopathogenic, a highly virulent strain X818 is reported (Becher et al., 1994). The latest event of pestivirus speciation was the separation of TSV and CSFV occurring around 1736 (HPDs, 1311 to 1931).

As large HPDs were associated with most of the dates, the mean MRCA date probably does not reflect the exact real date; thus, caution should be exercised when interpreting the results. However, the estimates coincided with historical observations. One particular case is the date for CSFV, which is estimated to have diversified around 1825. No exact data of the first outbreak of the disease (CSF) exists, but a report of the USDA Bureau of Animal Industry from 1887–1888 indicates that the disease (then named hog cholera) was first noted in Ohio, USA, in 1833 (Liess, 1981). Other reports suggest that the disease was already present in Europe in the first part of the 19th century (Beynon, 1962). These records agreed with the estimated age of the virus.

The evolutionary history of the major pestivirus lineages was inferred from analysis of the combined dataset. It was determined that BVDV-1, BVDV-2, atypical bovine pestivirus (BVDV-3), and Pestivirus of giraffe shared a common ancestor. These pestiviruses have evolved separately and formed independent lineages after possibly being moved to specific regions of the world. They are mainly of bovine origin, with the exception of the giraffe strain that was isolated from a giraffe in Africa. However, the isolate PG-2 that clustered with giraffe in the phylogenetic tree was isolated from a bovine cell culture in Africa (Becher et al., 2003). Therefore, it is reasonable that all the pestiviruses of bovine origin are found in the same larger clade (Fig. 1). In the sister clade, TSV separated from CSFV branch. As this relationship is independent of the gene regions analysed (Thabti et al., 2005) and the methods used, it is unlikely that recombination events have created this species. The relationship of the two most divergent pestivirus species to the remaining species is still unclear, as the position of the root of the pestivirus phylogeny is not yet known, although it is possible that the root of the phylogeny lies on the branch of one of the divergent species (Bungowannah or Antelope). This can only be ascertained when the sister group to pestiviruses is identified. The E2 gene sequences of Hepatitis C virus are the closest to pestivirus E2 gene, but these sequences were not alignable to pestivirus sequences in any meaningful way; thus, rendering them unsuitable as outgroups.

In conclusion, a reliable pestivirus phylogeny is inferred from a molecular dataset combining the 5′UTR, N^{pro} and E2 gene regions by Maximum likelihood and Bayesian approach. The phylogenetic relationships among atypical bovine pestiviruses, Pestivirus of giraffe and two recognised species BVDV-1 and BVDV-2 are established for the first time, and statistically supported by high posterior probability and bootstrap values. The dates for most recent common ancestor of major pestivirus lineages have been estimated and their evolutionary histories have been revealed. Accordingly, a proposal is presented for the genetic classification of pestiviruses into nine species: BVDV-1, BVDV-2, BVDV-3, Pestivirus of giraffe, CSFV, BDV and TSV, each corresponding to a well-supported monophyletic clade in the stable, robust phylogenetic tree; and two most divergent pestiviruses: Antelope and Bungowannah. This study provides a guideline for classification of newly detected pestiviruses, and has a potential application in phylogenetic analysis of other viruses.

Materials and methods

PCR amplification and sequencing of the E2 gene

The complete sequence of the E2 gene of the pestivirus CH-KaHo/cont (kindly provided by Dr. H.P. Stalder, Institute of Veterinary Virology, University of Bern, Switzerland) was determined in this study. Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, USA), and cDNA was synthesised with random priming by SuperScript II (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. The E2 gene region was amplified with *PfuUltra* High-Fidelity DNA polymerase (Stratagene) by primers F1 (1-21): 5′-GACCTCAGTTGTAAGCCTGAG-3′, and R1 (1098-1119): 5′-CCCCCTAGCTCCTTGTTCAGTT-3′. The amplification reaction and sequencing of the cloned PCR product in the vector pCRII-TOPO (Invitrogen) are described previously (Xia et al., 2008). The nucleotide sequence of the E2 gene was deposited in GenBank under the accession number EU385605.

Incongruence–length-difference (ILD) test and model selection

The 5′UTR and the complete N^{pro} and E2 gene sequences from 56 pestiviruses were retrieved from GenBank. The virus name, year of isolation, country and accession numbers are presented in Table 1. Multiple sequence alignment of each genetic region was done with CLUSTAL W (Thompson et al., 1994). The ILD test was performed using software WinClada ver 1.00.08. The 5′UTR, and the complete N^{pro} and E2 gene sequences were then combined. As some sequences of the 5′ UTR had not been deposited in the GenBank, they were treated as missing data. The final dataset of 56 pestiviruses and 2089 characters was used to select the best-fit model of evolution with software MrModelTest V.2.2 (Nylander, 2004), as previously described (Xia et al., 2007).

Phylogenetic analysis of a molecular dataset

PHYML v2.4.4 (Guindon and Gascuel, 2003) was used for phylogeny inference according to Maximum likelihood criterion. Analysis settings were: Base frequency estimates (ML); Proportion of invariable sites (estimated); Nucleotide substitution model (GTR); Number of substitution rate categories (4); Gamma distribution parameter (estimated). After tree reconstruction, the robustness of the hypothesis was tested through 1000 non-parametric bootstrap analyses. Bayesian inference analysis used the software MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), as previously described (Xia et al., 2007). In brief, the model settings were a Dirichlet prior for both substitution rates (Nst=6) and state frequencies (# states=4). Rate variation across sites was modelled with a γ-distribution (rates=invgamma). The Markov chain Monte Carlo (MCMC) search was run with four chains for 2 million generations, sampling the Markov chain every 1000 generations. At the end of run, the convergence of the chains was inspected through the average standard deviation of split frequencies. The first 500 (25%) trees were discarded as "burn-in". Each analysis was performed at least three times and a representative consensus tree is presented in this paper.

Estimation of divergence dates

A sub-dataset (Table 1, not in boldface) of 35 pestiviruses representing all the major lineages determined in phylogenetic analyses and 2037 characters was used for generating the BEAST input file by BEAUti within the BEAST package (Drummond and

Table 1
List of pestiviruses

Species[a]	Virus name	Accession numbers[b]			Virus isolation[c]		Reference
		5′ UTR	N^{pro}	E2	Year	Country/region	
BVDV-1	NADL/Ref	NC_001461	–	–	1963	USA	Gutekunst and Malmquist, 1963
	SD1	M96751	–	–	1992	USA	Deng and Brock, 1992
	Deer-NZ1	m	U80903	AF144614	1980	New Zealand	Becher et al., 1997
	Bega	AF049221	–	–	1989	Australia	Mahony et al., 2005
	Deer-GB1	m	U80902	AF144615	1986	UK	Becher et al., 1997
	CP7	U63479	–	–	1985	USA	Corapi et al., 1988
	Osloss	M96687	–	–	1967	Germany	Liess, 1967
	ZM-95	AF526381	–	–	1995	China	Wang et al., 1996
	721	m	AF144463	AF144609	1996	Germany	Becher et al., 1999
	SH9	m	AF144473	AF144616	1991	Germany	Becher et al., 1999
	NADL	**AJ133738**	**–**	**–**	**1963**	**USA**	**Gutekunst and Malmquist, 1963**
	KS86-1ncp	**AB078950**	**–**	**–**	**1986**	**Japan**	**Nagai et al., 2001**
	Singer Arg	**DQ088995**	**–**	**–**	**2006**	**Argentina**	**Jones et al., 2006**
	Trangie Y546	**AF049222**	**–**	**–**	**na**	**Australia**	**Mahony et al., 2005**
	KE9	**EF101530**	**–**	**–**	**2007**	**Germany**	**Meyers et al., 2007**
	VEDEVAC	**AJ585412**	**–**	**–**	**na**	**Hungary**	**Vaccine strain**
	Oregon C24V	**AF041040**	**–**	**–**	**1960**	**USA**	**Gillespie et al., 1960**
	11468	**m**	**AY735458**	**AY734488**	**na**	**na**	**Cedillo Rosales and Koenig (unpublished)**
BVDV-2	C413/Ref	NC_002032	–	–	1997	USA	Chen and Berry (unpublished)
	New York'93	AF502399	–	–	1993	USA	Ridpath et al., 2006
	890	U18059	–	–	1990	USA	Bolin and Ridpath, 1992
	P11Q	**AY149215**	**–**	**–**	**na**	**na**	**Goens et al. (unpublished)**
BVDV-3	Th_04/ThonKaen	FJ040215	–	–	2004	Thailand	Ståhl et al., 2007
	CH-KaHo/cont	m	AY895011	EU385605	2000	South America	Stalder et al., 2005
	D32/00_HoBi	AY489116	AY735486	AY604725	2004	Brazil	Schirrmeier et al., 2004
Pestivirusof giraffe	Giraffe-1/Ref	NC_003678	–	–	1967	Kenya	Plowright, 1969
	PG-2	M	AY163647	AY163654	1995	Africa	Becher et al., 2003
	Antelope	**AY781152**	**–**	**–**	**2000**	**USA**	**Vilcek et al., 2005**
	Bungwannah	**DQ901402**	**DQ901403**	**DQ901404**	**2003**	**Australia**	**Kirkland et al., 2007**
BDV	X818/Ref	NC_003679	–	–	1987	Australia	Becher et al., 1994
	BD31	U70263	–	–	1978	USA	Clarke and Osburn, 1978
	T1802/1	U65046	AY163649	AY163656	1992	UK	Becher et al., 2003
	466	m	AY163650	AY163657	1985	Germany	Becher et al., 2003
	AZ79	m	AY163652	AY163659	1999	Germany	2003
	17385/00	m	AY163651	AY163658	2000	Germany	Becher et al., 2003
	Reindeer-1	NC_003677	–	–	1996	Germany	Becher et al., 1999
	Gifhorn	m	AY163653	AY163660	1999	Germany	Becher et al., 2003
	Chamois-1	AY738080	AY738083	AY738082	2001	Andorra	Arnal et al., 2004
	Bison-1	**m**	**AF144476**	**AF144619**	**1996**	**Germany**	**Becher et al., 1999**
TSV	33S	AF462002	AY452485	AY452485	1995	Tunisia	Thabti et al., 2005
	BM01	AF462006	AY452482	AY452482	2000	Tunisia	Thabti et al., 2005
	SN1T	**AF461997**	**AY452484**	**AY452484**	**1995**	**Tunisia**	**Thabti et al., 2005**
CSFV	94.4/IL/94/TWN	AY646427	–	–	1994	Taiwan	Lin et al., 2007
	Brescia	M31768	–	–	1945	Italy	Greiser–Wilke et al., 1998
	Eystrup/Ref	NC_002657	–	–	1964	Germany	Greiser-Wilke et al., 1998
	Alfort/187	X87939	–	–	1987	France	Greiser-Wilke et al., 1990
	SWH	DQ127910	–	–	2004	China	Li et al., 2006
	0406/CH/01/TWN	AY568569	–	–	2001	Taiwan	Deng et al., 2005
	GXWZ02	**AY367767**	**–**	**–**	**2002**	**China**	**Li et al., 2006**
	cF114	**AF333000**	**–**	**–**	**2001**	**China**	**Li et al., 2006**
	Riems	**AY259122**	**–**	**–**	**na**	**na**	**Vaccine strain**
	RUCSFPLUM	**AY578688**	**–**	**–**	**2001**	**USA**	**Risatti et al., 2005**
	HCLV	**AF091507**	**–**	**–**	**1999**	**China**	**Vaccine strain**
	Brescia	**AF091661**	**–**	**–**	**na**	**na**	**Kyle et al. (unpublished)**
	39	**AF407339**	**–**	**–**	**2001**	**China**	**Li et al., 2006**
	Paderborn	**AY072924**	**–**	**–**	**1997**	**Germany**	**Greiser-Wilke et al., 1998**

[a] Species are named according to this study.
[b] "–" stands for the same accession number as for the 5′UTR; "m" stands for sequence is not available and treated as missing data.
[c] "na" stands for not available; the year of isolation, when uncertain, is taken as the year of publishing the sequence or paper.

Rambaut, 2007). Based on previous phylogenetic analysis, the taxon sets were selected to include all major clades and major lineages within clades. The two most divergent terminals (Bungowannah and Antelope) were not included in the final analysis as isolated long branches confound phylogenetic analyses in general (Bergsten, 2005), and their effect on times of divergence analyses is unknown. The dates for all sampled pestiviruses were specified as years from some time in the past. The best-fit models "General Time Reversible (GTR)" for nucleotide substitution and "Gamma+Invariant sites" for site heterogeneity were used. The selection of the relaxed uncorrelated exponential distribution molecular clock model (Drummond et al., 2006) and tree prior coalescent (exponential growth) was based on several initial tests, in which the MCMC chain was run for 50 to 150 million generations. "Auto optimise" was used in an attempt to tune the operators to maximum efficiency. In the final four independent BEAST analyses, the MCMC chains were run for 150 to 200 million generations and the ESS values (except "coefficient of variation) were greater than 100, as analysed by software Tracer v1.4 (Rambaut and Drummond, 2007). The analysis with maximum log tree likelihood value of -3.107×10^4 was selected and presented in this

paper. The tree samples were analysed with the program TreeAnnotator v1.4.8 (Drummond and Rambaut, 2007), with the first 10% (18555) trees discarded as "burn-in". The tree with maximum log clade credibility value of −3.0275 was selected and visualized by FigTree v1.1.2 (Rambaut, 2008).

References

Arbogast, B.S., Edwards, S.V., Wakeley, J., Beerli, P., Slowinski, J.B., 2002. Estimating divergence times from molecular data on phylogenetic and population genetic timescales. Annu. Rev. Ecol. Syst. 33, 707–740.

Arnal, M., Fernandez-de-Luco, D., Riba, L., Maley, M., Gilray, J., Willoughby, K., Vilcek, S., Nettleton, P.F., 2004. A novel pestivirus associated with deaths in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). J. Gen. Virol. 85, 3653–3657.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst. 18, 489–522.

Becher, P., Shannon, A.D., Tautz, N., Thiel, H.-J., 1994. Molecular characterization of border disease virus, a pestivirus from sheep. Virology 198, 542–551.

Becher, P., Orlich, M., Shannon, A.D., Horner, G., Konig, 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., Konig, M., Baroth, M., Thiel, H.-J., 1999. Genetic diversity of pestiviruses: identification of novel groups and implications for classification. Virology 262, 64–71.

Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., Konig, 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.

Bergsten, J., 2005. A review of long-branch attraction. Cladistics 21, 163–193.

Beynon, A.G., 1962. Swine fever in Great Britain. Bull. Off. Int. Epiz. 57, 1461–1487.

Bolin, S.R., Ridpath, J.F., 1992. Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am. J. Vet. Res. 53, 2157–2163.

Clarke, G.L., Osburn, B.I., 1978. Transmissible congenital demyelinating encephalopathy of lambs. Vet. Pathol. 15, 68–82.

Corapi, W.V., Donis, R.O., Dubovi, E.J., 1988. Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. J. Virol. 62, 2823–2827.

Cranwell, M.P., Otter, A., Errington, J., Hogg, R.A., Wakeley, P., Sandvik, T., 2007. Detection of Border disease virus in cattle. Vet. Rec. 161, 211–212.

Deng, R., Brock, K.V., 1992. Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. Virology 191, 867–869.

Deng, M.C., Huang, C.C., Huang, T.S., Chang, C.Y., Lin, Y.J., Chien, M.S., Jong, M.H., 2005. Phylogenetic analysis of classical swine fever virus isolated from Taiwan. Vet. Microbiol. 106, 187–193.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214–221.

Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, 699–710.

Dubois, E., Russo, P., Prigent, M., Thiéry, R., 2008. Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006. Vet. Microbiol. 130, 69–79.

Gillespie, L.H., Baker, J.A., McEntee, K., 1960. A cytopathogenic strain of virus diarrhea virus. Cornell Vet. 50, 73–79.

Greiser-Wilke, I., Moennig, V., Coulibaly, C.O., Dahle, J., Leder, L., Liess, B., 1990. Identification of conserved epitopes on a hog cholera virus protein. Arch. Virol. 111, 213–225.

Greiser-Wilke, I., Depner, K., Fritzemeier, J., Haas, L., Moennig, V., 1998. Application of a computer program for genetic typing of classical swine fever virus isolates from Germany. J. Virol. Methods 75, 141–150.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Gutekunst, D.E., Malmquist, W.A., 1963. Separation of a soluble antigen and infectious particles of Bovine viral diarrhea viruses and their relationship to Hog Cholera. Can. J. Comp. Med. Vet. Sci. 27, 121–123.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Hughes, L.E., Kershaw, G.F., Shaw, I.G., 1959. 'B' or Border disease, an undescribed disease of sheep. Vet. Rec. 71, 313–316.

Jones, L.R., Zandomeni, R.O., Weber, E.L., 2006. A long distance RT-PCR able to amplify the Pestivirus genome. J. Virol. Methods 134, 197–204.

Kampa, J., Alenius, S., Emanuelson, U., Chanlun, A., Aiumlamai, S., 2008. Bovine herpesvirus type 1 (BHV-1) and bovine viral diarrhoea virus (BVDV) infections in dairy herds: Self clearance and the detection of seroconversions against a new atypical pestivirus (doi:10.1016/j.tvjl.2008.07.006).

Kirkland, P.D., Frost, M.J., Finlaison, D.S., King, K.R., Ridpath, J.F., Gu, X., 2007. Identification of a novel virus in pigs—Bungowannah virus: a possible new species of pestivirus. Virus Res. 129, 26–34.

Liess, B., 1967. Die ätiologische Abgrenzung selbständiger Virusinfektionen, insbesondere der Virusdiarrhoe-Muscosal-Disease im sogenannten "Mucosal-Disease-Komplex" bei Rindern. Dtsch. Tieräztl. Wschr. 74, 46–49.

Liess, B., 1981. Hog cholera. In: Gibbs, E.P.J. (Ed.), Virus Diseases of Food Animals: A World Geography of Epidemiology and Control, vol. II. Academic Press, London, pp. 627–650.

Lin, Y.J., Chien, M.S., Deng, M.C., Huang, C.C., 2007. Complete sequence of a subgroup 3.4 strain of classical swine fever virus from Taiwan. Virus Genes 35, 737–744.

Li, X., Xu, Z., He, Y., Yao, Q., Zhang, K., Jin, M., Chen, H., Qian, P., 2006. Genome comparison of a novel classical swine fever virus isolated in China in 2004 with other CSFV strains. Virus Genes 33, 133–142.

Mahony, T.J., McCarthy, F.M., Gravel, J.L., Corney, B., Young, P.L., Vilcek, S., 2005. Genetic analysis of bovine viral diarrhoea viruses from Australia. Vet. Microbiol. 106, 1–6.

Meyers, G., Ege, A., Fetzer, C., von Freyburg, M., Elbers, K., Carr, V., Prentice, H., Charleston, B., Schürmann, E.M., 2007. Bovine viral diarrhea virus: prevention of persistent fetal infection by a combination of two mutations affecting E^{rns} RNase and N^{pro} protease. J. Virol. 81, 3327–3338.

Nagai, M., Ito, T., Sugita, S., Genno, A., Takeuchi, K., Ozawa, T., Sakoda, Y., Nishimori, T., Takamura, K., Akashi, H., 2001. Genomic and serological diversity of bovine viral diarrhea virus in Japan. Arch. Virol. 146, 685–696.

Nylander, J.A.A., 2004. MrModeltest, v.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Olafson, P., McCallum, A., Fox, F., 1946. An apparently new transmissible disease of cattle. Cornell Vet. 36, 205–213.

Pellerin, C., van den Hurk, J., Lecomte, J., Tussen, P., 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260–268.

Plowright, W., 1969. Joint Campaign Against Rinderpest. First Technical Review Meeting, Phase IV, Mogadiscio, Kenya.

Rambaut, A., 2008. FigTree v1.1.1: Tree figure drawing tool. Available: http://tree.bio.ed.ac.uk/software/figtree. Accessed 29 November 2008.

Rambaut, A., Drummond, A.J., 2007. Tracer v1.4: MCMC trace analyses tool. Available: http://beast.bio.ed.ac.uk/Tracer. Accessed 29 November 2008.

Ridpath, J.F., Neill, J.D., Vilcek, S., Dubovi, E.J., Carman, S., 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Vet. Microbiol. 114, 196–204.

Risatti, G.R., Borca, M.V., Kutish, G.F., Lu, Z., Holinka, L.G., French, R.A., Tulman, E.R., Rock, D.L., 2005. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J. Virol. 79, 3787–3796.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rümenapf, T., Thiel, H.-J., 2008. Molecular biology of pestiviruses. In: Mettenleiter, T.C., Sobrino, F. (Eds.), Animal Viruses: Molecular Biology. Caister Academic Press, Norfolk, UK, pp. 39–96.

Schirrmeier, H., Strebelow, G., Depner, K., Hoffmann, B., Beer, M., 2004. Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. J. Gen. Virol. 85, 3647–3652.

Ståhl, K., Kampa, J., Alenius, S., Persson Wadman, A., Baule, C., Aiumlamai, S., Belák, S., 2007. Natural infection of cattle with an atypical 'HoBi'-like pestivirus-implications for BVD control and for the safety of biological products. Vet. Res. 38, 517–523.

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.

Thabti, F., Letellier, C., Hammami, S., Pépin, M., Ribière, M., Mesplède, A., Kerkhofs, P., Russo, P., 2005. Detection of a novel border disease virus subgroup in Tunisian sheep. Arch. Virol. 150, 215–229.

Thiel, H.-J., Plagemann, P.G.W., Moenning, V., 1996. Pestiviruses, In: Fields, B.N., Knipe, D. M., Howley, P.M. (Eds.), 3rd ed. Fields Virology, Vol. 1. Lippincott–Raven, Philadelphia, PA, pp. 1059–1073.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B., 2000. Seventh Report of the International Committee on Taxonomy of Viruses. http://www.virustaxonomyonline.com.

Vilcek, S., Ridpath, J.F., Van Campen, H., Cavender, J.L., Warg, J., 2005. Characterization of a novel pestivirus originating from a pronghorn antelope. Virus Res. 108, 187–193.

Wang, X., Tu, C., Li, H., Jin, K., Xuan, H., Chang, G., Sun, H., Zhu, W., Fei, E., Yin, Z., 1996. Detection and isolation of bovine viral diarrhea virus from classical swine fever suspected pigs. Chin. J. Vet. Sci. 16, 341–345.

Xia, H., Liu, L., Wahlberg, N., Baule, C., Belák, S., 2007. Molecular phylogenetic analysis of bovine viral diarrhoea virus: a Bayesian approach. Virus Res. 130, 53–62.

Xia, H., Liu, L., Linde, A.M., Belák, S., Norder, H., Widén, F., 2008. Molecular characterization and phylogenetic analysis of the complete genome of a hepatitis E virus from European swine. Virus Genes 37, 39–48.