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Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen[1]

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[1] The full-length sequence of the Th/04_KhonKaen virus has been deposited in GenBank under accession number FJ040215.

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Abstract

Phylogenetic analysis of recently identified bovine "atypical" pestiviruses, performed based on different gene regions, has revealed unclear relationships with other established species, therefore, their phylogenetic position could not been determined so far. In this study, the atypical pestivirus Th/04_KhonKaen was recovered from serum of a naturally infected calf and the complete genome sequence was determined and analysed, as means to define its position. The viral genome is 12 337 nucleotides (nt) long, and comprises a 5´ UTR of 383 nt, a 3´ UTR of 254 nt and an open reading frame of 11700 nt, without duplication of viral sequences or insertions of cellular sequences. The phylogenetic analyses of the full-length sequence, performed by Neighbor-joining, maximum likelihood, and the Bayesian approach, unanimously placed Th/04_KhonKaen in a single lineage, distinct from the established pestivirus species, and close to bovine viral diarrhea virus types 1 and 2. Furthermore, Th/04_KhonKaen and two previously reported atypical pestiviruses D32/00_'HoBi' and CH-KaHo/cont formed a well-supported monophyletic clade in trees based on the complete N^{pro} and E2 gene regions. The finding provides conclusive classification of the Th/04_KhonKaen virus and confirms the standing of the bovine "atypical" pestiviruses as a novel pestivirus species.

Keywords: BVDV, genotyping, pestivirus, phylogeny, Th/04_KhonKaen

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1. Introduction

The pestivirus genome is a single-stranded, positive-sense RNA molecule, which comprises two untranslated regions (UTRs) at the 5' and 3' ends, and one open reading frame (ORF) encoding a large polyprotein. This polyprotein is co- and post-translationally processed into 11-12 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 [(NS2-3 (or NS2 and NS3), NS4A, NS4B, NS5A, and NS5B)] (reviewed by Rümenapf and Thiel, 2008). Recombination or point mutations in the viral genome have been shown to correlate with induction of cytopatogenic effects (CPE) on cell culture following infection with BVDV (Thiel et al., 2006). Accordingly, two biotypes of BVDV can be distinguished: the non-cytopathogenic (ncp), that replicates without cytopathic effect, and cytopathogenic (cp), that leads to death of infected cells (Thiel et al., 1996).

The genus *Pestivirus* of the family *Flaviviridae* includes four recognized species: *Bovine viral diarrhea virus 1* (BVDV-1), *Bovine viral diarrhea virus 2* (BVDV-2), *Border disease virus* (BDV), *Classical swine fever virus* (CSFV), and a fifth tentative species Pestivirus of giraffe (Seventh Report of the International Committee on Taxonomy of Viruses; http://www.virustaxonomyonline.com). A recently identified new pestivirus, D32/00_'HoBi', was isolated from a batch of fetal calf serum originating from Brazil and was proposed as a sixth pestivirus species (Schirrmeier et al., 2004). Additional atypical pestiviruses of bovine-origin have also been described, including: Brz buf 9, originally isolated from a buffalo in Brazil; CH-KaHo/cont, a cell culture contaminant possibly

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originating from a batch of fetal calf serum produced in South America (Stalder et al., 2005); and Th/04_KhonKaen from a naturally infected calf in Thailand (Ståhl et al., 2007). All these pestiviruses were shown to be closely related to D32/00_'HoBi', based on trees generated from the 5´UTR and N^{pro} regions, however their final phylogenetic classification could not be determined.

The Th/04_KhonKaen virus was first detected by ELISA tests and preliminarily characterized by virus neutralization and partial sequence analysis of the 5' UTR (Ståhl et al., 2007). Further genetic characterization of this pestivirus required a virus isolate, but the fact that the serum had been inactivated hampered virus isolation by conventional methods. In this study, a viable Th/04_KhonKaen virus was recovered by transfection of bovine turbinate cells with RNA extracted from an inactivated serum sample, and the complete sequence was determined. Comparative sequence analyses show that the Th/04_KhonKaen virus, together with atypical pestiviruses D32/00_'HoBi' and CH-KaHo/cont represent a new species within the pestivirus genus.

2. Materials and Methods

2.1. Virus recovery from an inactivated serum sample

The serum sample was obtained from a calf in a herd in Thailand. According to the legislation in Sweden the sample had to be heat-inactivated at 56°C for 90 min, therefore it was no longer suitable for virus isolation by conventional methods. To recover virus, RNA

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was extracted from this sample using TRIzol Reagent (Invitrogen, Carlsbad, USA), and purified with phenol-chloroform. The RNA was electroporated into BVDV-free bovine turbinate (BT) cells in a cell suspension containing 1×10^6 cells, using a Bio-Rad Gene Pulser Xcell electroporator. The cells were grown in Eagle's minimum essential medium (EMEM) containing 10% horse serum, 1% L-glutamine, and 100 U/ml of penicillin and 100 µg/ml of streptomycin, at 37°C in a CO_2 incubator. Confluent cells were split and grown for four days. The cells were then frozen and thawed and the lysates were clarified by centrifugation. The supernatants were used to infect fresh sub-confluent BT cells, as performed routinely for virus propagation. After incubation at 37°C for four days, total RNA extracted from lysates of the cells was used to confirm presence of viral RNA with a pan-pestivirus RT-PCR targeting the 5′ UTR.

2.2. Immunoperoxidase test

The immunoperoxidase (IPX) test was performed to demonstrate presence of BVDV specific antigens in cells infected with the recovered virus. Briefly, serial dilutions of the supernatant from virus-infected BT cells were used to infect BT cells grown in a 96-well plate. Following incubation at 37°C for 4 days, the cells were washed once with 0.01 M PBS, pH 7.6, fixed with fixation fluid and dried. After re-dehydration of the cells, an anti-serum directed against BVDV-1 was added to each well and the plates were incubated at room temperature for 15 min. The cells were washed three times and 50 µl of diluted rabbit anti-bovine horseradish peroxidase conjugate was added, followed by incubation

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and washing as before. For detection, 50 µl of substrate solution (5 ml of 0.05 M sodium acetate, 6 µl of 3% H_2O_2, and 300 µl of chromogen solution) was used.

2.3. Amplification, sequencing and analysis of the full-length genome

2.3.1. RNA extraction

When presence of virus was confirmed by RT-PCR, infected cells were washed twice with cold PBS without Mg^{2+} and Ca^{2+}, and lysed with a lysis buffer containing 100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 5 mM DTT, 10 mM EDTA, pH 8.0 and 5% Lithium Duodecyl Sulphate. Total RNA was purified from the cell lysates by phenol/chloroform and precipitated in an equal volume of isopropanol. After centrifugation, the RNA pellet was washed once with 70% of ice-cold ethanol. The dried pellet was re-suspended in 30 µl RNase-free water.

2.3.2. Synthesis of double-stranded cDNA

SuperScript II (Invitrogen, Carlsbad, USA) was used to synthesize the first strand cDNA using 2 µM of a gene-specific primer (PAS1) in a 20-µl volume, following conditions recommended by the manufacturer. The second strand was prepared in 150-µl reaction volume, containing the 1st strand reaction, *E. coli* DNA ligase (10 U), E. coli DNA polymerase (40 U), *E. coli* RNAse H (2 U) in 1 × second strand buffer. The reaction was performed at 16°C for 2 hours. The cDNA was purified with phenol/chloroform and

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precipitated in 95% ethanol at –20°C overnight. The cDNA pellet was washed with 75% ethanol, and resuspended in 20 µl of distilled water.

2.3.3. PCR amplification

The primers were designed based on conserved sequences of established pestiviruses and the sequence of the D32/00_HoBi virus. The list of primers for PCR amplification and DNA sequencing is presented in Table 1. The viral genome sequence, excluding the 5´ and 3´ ends, was amplified in two overlapping fragments by PCR using primers TF1-TR1, and TF2-TR2. PCR was performed in a 25-µl volume, containing 0.5 µM of forward and reverse primers, 0.5 mM dNTP mix, 1.25 U of *PfuUltra* High-Fidelity or 2.5 U EXL DNA polymerase (Stratagene, La Jolla, CA, WSA), 1 µl of cDNA, and 1 × reaction buffer with $MgCl_2$. After initial denaturation at 92°C for 2 min, a three-step cycling of 30 cycles was performed with denaturation at 92°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 7 min. Following amplification, the PCR products were separated on 0.8% agarose gels, and the designed band was excised from the gel and purified with the Wizard SV Gel and PCR Clean-Up System (Promega Co., Madison, WI, USA).

2.3.4. Determination of the 5´ and 3´ ends by RNA ligase-mediated RACE

To determine the 5´and 3´ends, a GeneRacer Kit (Invitrogen, Carlsbad, USA) was used, following manufacturer's instruction. In brief, the GeneRacer RNA oligo, and RNA oligo

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2 (Table 1) were ligated to the 5´end and 3´end of the viral genome, respectively. Ten units of T4 RNA ligase were used in the reactions and ligation was performed at 37°C for 1 h. The reactions were then treated with proteinase K, followed by phenol/chloroform purification, and precipitation of the RNA in 95% ethanol in the presence of 0.1M sodium acetate. The RNA was pelleted by centrifugation, washed with 70% ethanol and resuspended in 30 µl of RNase-free water. The GeneRacer 5´primer from the kit and gene-specific primer T5U-R1 were used to amplify the target 5´end using a one-step RT-PCR kit (Qiagen, Hilden, Germany), with the following cycling profile: 50°C for 30 min, 95°C for 15 min, 35 cycles of 95°C for 15 sec, 54°C for 20 sec, and 72°C for 1 min, and a final extension at 72°C for 7 min. To amplify the 3´end, the gene-specific primer T3U-F1 and M13 forward primer (complementary to the 5´end of the RNA oligo) were used. The same reaction was performed as for amplification of the 5´end.

The purified PCR products were cloned into pCR4-TOPO (Invitrogen, Carlsbad, USA). Four clones containing inserts of the 5´ and 3´ends of the genome were used for DNA sequencing with M13 forward and reverse primers (Invitrogen, Carlsbad, USA).

2.3.5. DNA sequencing

An ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used for DNA sequencing. Sequencing reactions were performed in a total volume of 10 µl, containing 1.5 µl of ABI 5 × sequencing buffer, 0.5 µM primer, 1.0 µl of BigDye, and 1.0 µl of the purified PCR product. The thermo profile

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was 25 cycles of denaturation at 96°C for 15 sec, annealing at 50°C for 10 sec, and extension at 60°C for 4 min. To remove contaminating salts and unincorporated dye terminators from the sequencing reactions, the Montage SEQ96 sequencing reaction cleanup kit (Millipore Co., Billerica, MA, USA) was used, following the manufacturer's instructions. Capillary electrophoresis was carried out in an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed with multiple programs of the Lasergene package (DNASTAR, Inc., Madison, WI).

2.3.6. Phylogenetic analysis

For phylogenetic analysis, a total of 24 full-length genome sequences of pestiviruses were retrieved from GenBank, including 9 of CSFV, 3 of BDV, 7 of BVDV-1 and 4 of BVDV-2, and Pestivirus of giraffe. The GenBank accession numbers are included in Table 2. Multiple sequence alignment was performed with software BioEdit version 7.0.5.2 (Hall, 1999), using CLUSTAL W (Thompson et al., 1994). To avoid bias due to different methods, phylogenetic analysis of complete genome sequences was performed with three methods: Neighbor-joining, maximum likelihood and the Bayesian approach. All gaps created by insertions were deleted by the software MEGA prior to the analyses. MEGA 4 (Tamura et al., 2007) was used to reconstruct the Neighbor-joining tree under the nucleotide substitution model of Tamura 3-parameter, and tree reliability was evaluated by bootstrapping 1000 replicates. PHYML (v2.4.4) was used for phylogeny inference according to maximum likelihood criterion. The analysis settings were: Base frequency estimates (ML); Proportion of invariable sites (estimated); Nucleotide substitution model

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(GTR); Number of substitution rate categories (4); Gamma distribution parameter (estimated). Following tree reconstruction, 1000 non-parametric bootstrap analyses were performed to test the robustness of the hypothesis. Bayesian inference analysis was performed with 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 modeled using a γ-distribution with a proportion of sites invariable (rates=invgamma). The MCMC search was run with four chains for 2 000 000 generations, sampling the Markov chain every 1000 generations. The first 25% trees were discarded as "burn-in". Each analysis was performed three times and a representative consensus tree is shown in this paper.

In order to demonstrate the relationships among these recently described atypical pestiviruses, sequences of the complete N^{pro} and E2 coding regions from 64 pestiviruses, corresponding to the GenBank accession numbers listed in Table 2, were analysed with the Bayesian approach. The same parameters applied for the full-length phylogeny analysis were used.

3. Results

3.1. Virus recovery

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The RNA extracted from lysates of infected cells yielded a specific PCR product on amplification with a pan-pestivirus RT-PCR targeting 5´ UTR. The recovered virus was confirmed by the IPX test that showed presence of pestivirus-specific proteins upon propagation following cell transfection. The Th/04_KhonKaen virus reacted with the BVDV-1 antiserum, which also recognizes BVDV-2 and D32/00_'HoBi'. Cytopathic effect (CPE) was not observed on isolation and propagation of the virus, supporting the recovery of a non-cytopathogenic pestivirus from the serum sample.

3.2. Sequence analysis of the viral genome

The full-length sequence was assembled from partial sequences obtained from the two RT-PCR fragments and the 5´ and 3´ ends of the genome. The complete genome contains 12 337 nucleotides (nt), comprising 383 nt at the 5´ UTR, 254 nt at the 3´ UTR and an ORF of 11 700 nt. The ORF involves the 11 gene regions that correspond to predicted products of polyprotein processing: the pestivirus-specific N-terminal protease N^{pro} (504 nt), capsid (303 nt), E^{rns} (681 nt), E1 (585 nt), E2 (1119 nt), p7 (210 nt), NS2-3 (3408 nt), NS4A (192 nt), NS4B (1041 nt), NS5A (1491 nt), and NS5B (2166 nt). Neither duplication of viral sequences nor insertions of cellular sequences were found in the genome. Comparative sequence analysis showed that this virus shares 67.3-68.3% identity with other pestivirus species, with the highest identity of 68.3 to BVDV-1 and -67.9% to BVDV-2, respectively (Table 3).

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The polyprotein (3899 aa) was used as a query protein for searching in the Protein Data Bank (PDB). The protein BLAST revealed several conserved domains, including the N^{pro} endopeptidase C53 that is unique to pestiviruses; the RNase T2 in the Erns region; the pestivirus NS3 polyprotein peptidase S31 and DEXH-box helicases located in NS3; and RNA-dependent RNA polymerase within NS5B. Such analysis provided additional support for the classification of Th/04_KhonKaen as a pestivirus.

3.3. Phylogenetic analysis of Th/04_KhonKaen sequence

Three methods, Neighbor-joining, maximum likelihood, and the Bayesian approach were exploited for phylogenetic analysis. By applying the later method, we have previously shown that a total evidence approach could be used for confident classification of BVDV into subgroups (Xia et al., 2007). All three methods produced a single, reliable phylogenetic hypothesis that was supported by significantly high bootstrap values and posterior probability (Fig. 1). The established species BVDV-1, BVDV-2, BDV, CSFV separated into four monophyletic clades, and the tentative pestivirus of Giraffe formed a single branch. Th/04_KhonKaen also formed a single branch positioned distinctly from the four established pestivirus species and from pestivirus of Giraffe, and closely related to BVDV-1 and BVDV-2. The position of Th/04_KhonKaen in the consensus trees was supported by bootstrap values of 99% (Neighbor-joining), 93% (ML), and posterior probability of 1.00 (Bayesian approach).

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To demonstrate the relationship between the Th/04_KhonKaen virus and other atypical pestiviruses D32/00_'HoBi' and CH-KaHo/cont, single N^{pro} and E2 gene analyses were performed. As shown in Fig. 2 (a and b), all three atypical pestiviruses formed a monophyletic clade strongly supported by bootstrap value and posterior probability. The position of this clade in the tree of E2 gene was same as that of Th/04_KhonKaen virus in the tree based on full-length sequence analysis. As in the tree of Figure 1, this clade is genetically distant from the established bovine pestivirus species BVDV-1 and BVDV-2, as well as distinct from BDV, CSFV and pestivirus of Giraffe. The four recognized species BVDV-1, BVDV-2, BDV and CSFV also formed monophyletic clades.

4. Discussion

Phylogenetic analysis is useful to classify novel viruses, and to reveal their evolutionary history. Particularly for pestiviruses, whole-genome phylogeny has been used to classify two novel species, Giraffe-1 and Reindeer-1 (Avalos-Ramirez et al., 2001), although the latter has been re-classified as BDV (Becher et al., 2003).

In this study the complete genome sequence of the recently identified pestivirus Th/04_KhonKaen was determined and analysed, aiming to establish the phylogenetic positioning of this virus and to determine its relationship with known pestivirus species. Following successful recovery of the virus from an inactivated serum sample, the genome organization of Th/04_KhonKaen was shown to be reminiscent of that of an ncp pestivirus, consistent with the recovery of a virus that did not induce CPE. The full-length

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nucleotide identity of the Th/04_KhonKaen with the recognized BVDV species is around 68%, which is close to the range of 69.5% that separates BVDV-2 from BVDV-1 (Table 3), motivating its discrimination as a distinct pestivirus species of bovine origin.

A single phylogenetic tree topology was produced by all three methods, highly supported by strong bootstrap and posterior probability values, on analysis of the full-length sequence. This analysis unambiguously positioned the Th/04_KhonKaen virus as a unique species in the consensus tree, distinct from the four established pestivirus species and pestivirus of Giraffe, and closely related to the bovine species BVDV-1 and BVDV-2. Remarkably, analysis of sequences of the complete N^{pro} and E2 coding region showed that the three atypical pestiviruses formed a well-supported single clade, demonstrating that these atypical bovine pestiviruses are genetically distinct from the current well established pestivirus species. This relationship has been unequivocally confirmed by the analysis of a sequence dataset combining the 5´UTR, and the complete N^{pro} and E2 gene regions of 56 pestiviruses, published in Liu et al. (2009).

Recovery of the Th/04_KhonKaen virus enables further studies currently in progress to define cross-reactivity patterns with polyclonal and monoclonal antibodies, as well as the characterization of in vitro and in vivo biological properties of this group of viruses.

In conclusion, the atypical pestivirus Th/04_KhonKaen was isolated from an inactivated serum sample. Sequence analysis supported positioning of the Th/04_KhonKaen virus as a new species within the pestivirus genus. Comparisons of sequences of the N^{pro} and E2

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gene regions with those of the atypical pestiviruses D32/00_'HoBi', Brz buf 9, and CH-KaHo/cont confirmed a high sequence similarity among these viruses, demonstrating that they belong to a same, new group of pestiviruses of bovine origin.

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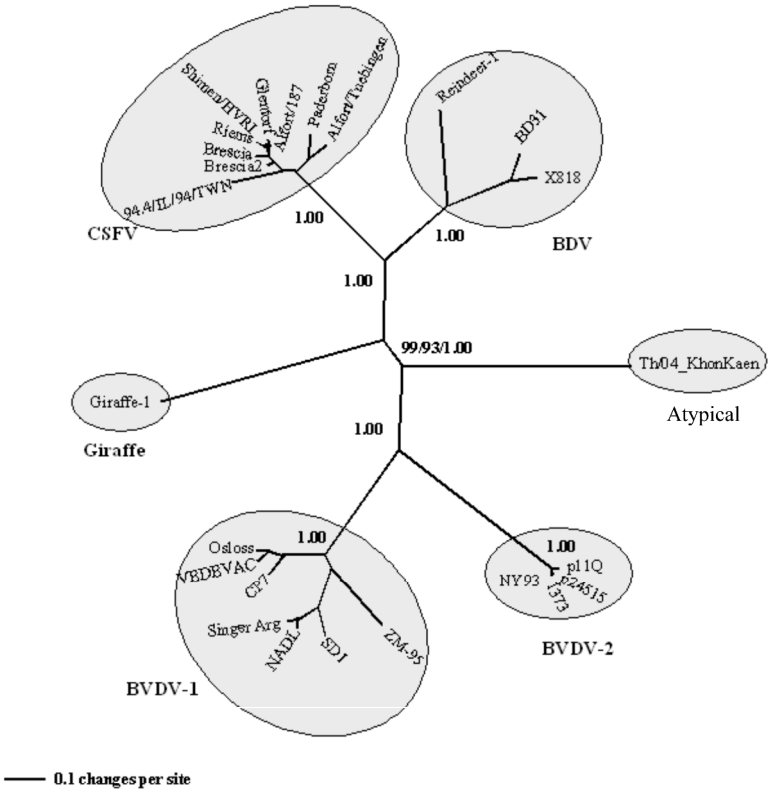

Fig. 1. Consensus phylogenetic tree based on analysis of the full-length sequence of the Th/04_KhonKaen virus and those of pestivirus species available in the GenBank. The Neighbor-joining tree was reconstructed under the nucleotide substitution model of Tamura 3-parameter using MEGA 4. PHYML (v2.4.4) was used for phylogeny inference according to maximum likelihood (ML) criterion, and MrBayes 3.1 for Bayesian inferring.
The robustness of the tree was evaluated by bootstrapping 1000 replicates by Neighbor-joining and maximum likelihood, and by posterior probability from Bayesian analysis. The supporting value for major clades is shown in the order of NJ/ML/PP. ”1.00” indicates 100%/100%/1.00. A representative, un-rooted consensus tree is shown in

the Figure. The bar indicates changes per site.

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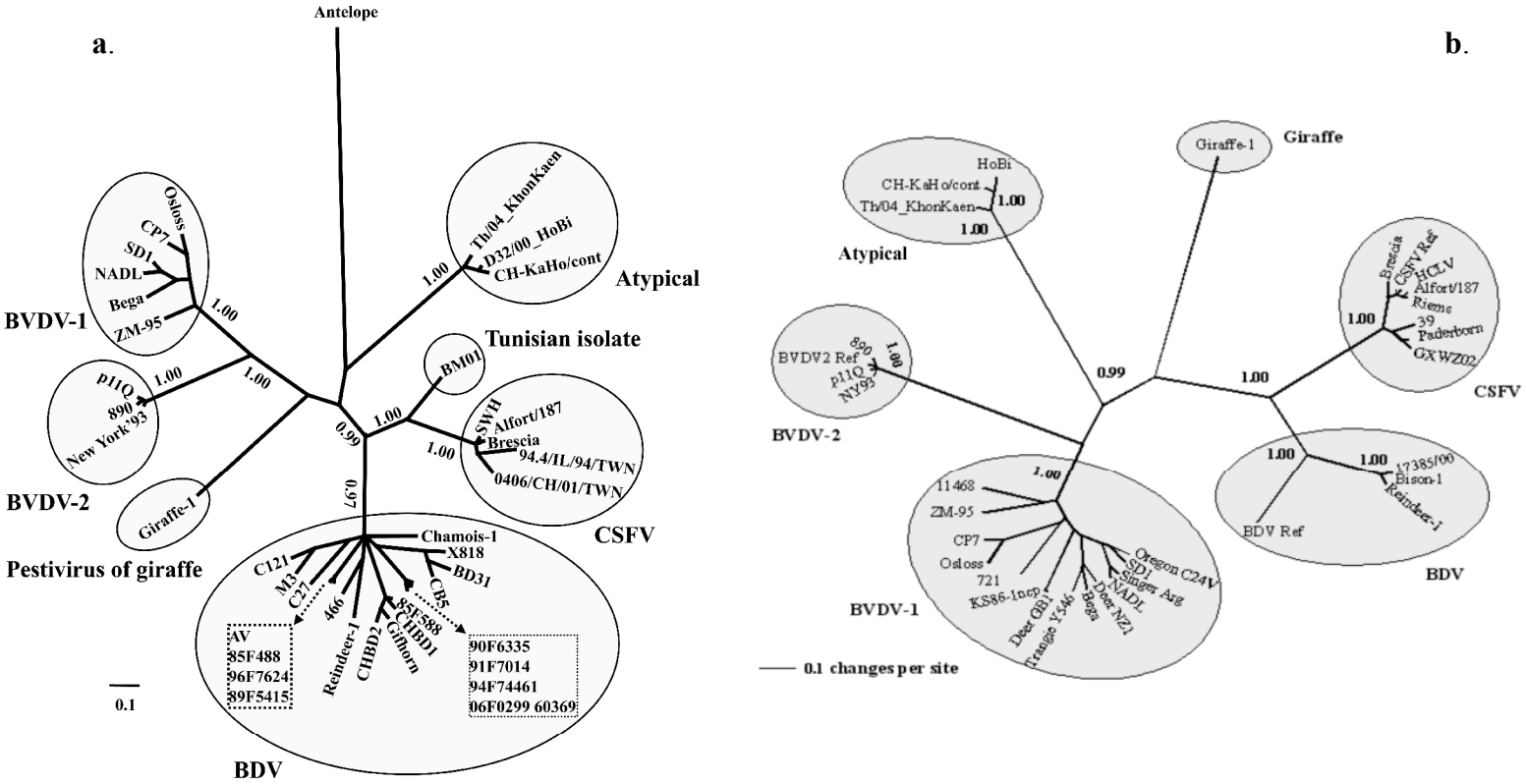

Fig. 2. Phylogenetic trees based on analysis of sequences of the complete N^{pro} (a., left) and E2 (b., right) gene regions. The relationship of atypical pestiviruses Th/04_KhonKaen, D32/00_'HoBi' and CH-KaHo/cont with representatives of established pestivirus species retrieved from GenBank is shown. The un-rooted consensus trees are inferred by Bayesian approach. Numbers indicate posterior probability values for major clades. The bar indicates changes per site.

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Table 1. Nucleotide sequence of primers used for PCR amplification and sequencing of the Th/04_KhonKaen virus.

Primer name	Nucleotide sequence[a]	Position
TF1	G**C**TA**G**CCAT**G**CC**C**TTAGTAGGA	98-119
TR1	CCCTA**A**GTTC**G**GCCCAT**T**GAGTC	5382-5403
TF2	GGGC**C**GAAC**T**AGGGTGGTGT	5390-5409
TR2	**GTGC**G**T**TGAGTGTAGTGTTGTC	12247-12268
TF3	ATGGAGTTGTTAAACTTTGAAC	384-405
TR3	GCAGCTT**C**C**T**ACCCAGATGG	868-878
TF5	GACCT**C**AGTTGTAAGCCTGA**G**	2457-2477
TR5	CCCCCTAGCTCCTTGTTCAGTT	3554-3575
TF4	ACCCCACCAAAAACAAACCAGA	1090-1111
TR4	TGGGCCTATCTCATTGGTCTCA	2495-2516
TF6	TCTTGGGTGGGAGGTATGTGCT	3505-3526
TR6	TTAGGCCCCTTGCTTCTTTCTC	4648-4669
TF7	CAAGGCCCCAGAGCAAAGACG	4431-4451
TF8	CG**A**GC**A**GCGGCAGA**A**TCAGTAT	5913-5934
TR8	GTCCGGCCA**G**TC**C**AACC	7120-7136
TF9	CAAATGCCATAGAATCAGGAGTA	6496-6518
TF10	TGATCACCCGGAACCCATACAG	6923-6944
TF11	AGGCAGGCTGGGGAGTGAAAC	7619-7639
TF12	AGGCAAAAGAATTAGCGGAAAGA	8317-8339
TR9	TGCCCA**G**GCT**G**A**GT**GCYTCYA	9649-9669
TR10	TCCTTCCCGTTCCAGTTGTTCT	10283-10304
TR11	GCGGCTTGTTACCTGAGTGTC	10965-10985
TF13	C**TC**TAAA**A**T**G**AAGAA**A**GGGTGYG	10895-10917
TR12	CTGTGGTTTGCCAGATTCATAC	11327-11348
TR13	GGGGATATACATTTTGCCAGTC	11885-11906
TF14	AGGCACACCAGCAAGAGACTAC	11790-11811
PAS1	CTGTGTGCRTTRARTGTAGTG	12252-12273
T3U-F1	TTATTACTTAGGCTGATGAGAAC	12214-12236
T5U-R1	CAGGGAGCTCACTGCCACCAC	139-159
GeneRacer RNA oligo	CGACUGGAGCACGAGGACACUGACA UGGACUGAAGGAGUAGAAA	
GeneRacer 5´primer RNA oligo 2	CGACTGGAGCACGAGGACACTGA CGCAAAUGGGCGGUAGGCGUGCUGG CCGUCGUUUUAC	

[a] Bold bases indicate mismatches with the sequence of the Th/04_KhonKaen virus.

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Table 2. List of pestiviruses included in the analyses and their GenBank accession numbers

Species[a]	Pestivirus	Genomic sequence	N^{pro}	E2
BDV	X818	NC_003679	g[b]	g
	BD31	U70263	-[c]	-
	Reindeer-1	NC_003677	g	g
	Bison-1	-	-	AF144619
	17385/00	-	-	AY163658
	Chamois_1	-	AY738083	-
	Gifhorn	-	AY163653	-
	CHBD1	-	AY895008	-
	CHBD2	-	AY895009	-
	466	-	AY163650	-
	CB5	-	AF145358	-
	M3	-	DQ273163	-
	C121	-	DQ273159	-
	C27	-	DQ273156	-
	AV	-	EF693962	-
	85F488	-	EF693963	-
	85F588	-	EF693966	-
	89F5415	-	EF693965	-
	90F6335	-	EF693968	-
	91F7014	-	EF693971	-
	94F74461	-	EF693974	-
	96F7624	-	EF693976	-
	06F0299 60369	-	EF696979	-
BVDV-1	ZM-95	AF526381	g	g
	SD1	M96751	g	g
	NADL	AJ133738	g	g
	Singer Arg	DQ088995	-	g
	CP7	U63479	g	g
	VEDEVAC	AJ585412	-	-
	Osloss	M96687	g	g
	Oregon C24V	-	-	AF091605
	Deer NZ1	-	-	AF144614
	Bega	-	AF049221	AF049221
	Trangie Y546	-	-	AF049222
	Deer GB1	-	-	AF144615
	KS86-1ncp	-	-	AB078950
	721	-	-	AF144609
	11468	-	-	AY734488
BVDV-2	p11Q	AY149215	g	g
	p24515	AY149216	-	-
	1373	AF145967	-	-
	NY93	AF502339	g	g
	890	-	g	U18059
	BVDV-2 Ref	-	-	NC_002032
CSFV	94.4/IL/94/TWN	AY646427	g	-
	Brescia	AF091661	g	g
	Brescia-2	M31768	-	-
	Riems	AY259122	-	g
	Shimen/HVRI	AY775178	-	-
	Glentorf	U45478	-	-
	Alfort/178	X87939	g	g
	Paderborn	AY072924	-	g
	Alfort/Tübingen	J04358	-	-
	GXWZ02	-	-	AY367767

[a] Including unclassified pestiviruses
[b] Same as genomic sequence
[c] Sequence is not included in the analysis

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	39	-	-	AF407339
	HCLV	-	-	AF091507
	CSFV Ref	-	-	NC_002657
	SWH	-	DQ127910	-
	0406/CH/01/TWN	-	AY568569	-
Tunisian isolates	BM01	-	AY453629	-
Pestivirus of giraffe	Giraffe-1	NC_003678	g	g
Atypical pestivirus	CH-KaHo/cont	-	AY895011	EU385605
	D32/00_HoBi	-	AY489117	AY604725
	Th/04_KhonKaen	FJ040215	g	g

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Table 3. Identities of Th/04_KhonKaen with full-length sequences of 18 pestiviruses

	BD31	Reindeer-1	BDV/Ref	Oregon C24V	Osloss	SD1	ZM-95	BVDV-1/Ref	1373	890	New York'93	p11Q	BVDV-2/Ref	Alfort/187	Brescia	HCLV	Riems	CSFV/Ref	Giraffe-1
Th_04_KhonKaen	67.4	67.8	67.3	68.3	67.8	68.1	67.6	68.2	67.9	67.8	67.9	67.9	67.7	67.6	67.6	67.4	67.3	67.5	67.8
Giraffe_1	68.3	68.4	68.7	67.8	67.6	67.6	67.5	67.6	67.8	67.6	67.8	67.7	67.6	68	67.9	68	67.9	68.1	
CSFV/Ref	72	71.5	72.1	67.4	66.7	67.4	67.1	67.2	67	66.9	67	67.1	66.8	98	94.8	96.2	96.1		
Riems	71.5	71.1	71.6	67.5	66.7	67.5	66.9	67.3	67	67	67	67.1	66.8	96	93.2	99.2			
HCLV	71.6	71.2	71.6	67.5	66.7	67.5	66.9	67.3	67	66.9	67.1	67.1	66.8	96	93.2				
Brescia	71.7	71.7	72.1	67.5	67.1	67.2	67	67.2	66.7	66.6	66.7	66.7	66.5	94.9					
Alfort/187	71.9	71.5	72.1	67.5	66.8	67.4	67.1	67.4	66.9	66.9	66.9	66.9	66.8						
BVDV-2/Ref	66.9	67.3	66.9	69.4	69.6	69.6	69.6	69.5	96.4	96.3	96.3	96.4							
p11Q	67.1	67.4	67.2	69.6	69.7	69.9	70	69.7	96.8	96.3	96.7								
New York'93	67.2	67.4	67.2	69.6	69.8	69.9	70.1	69.8	99.4	96.1									
890	67.1	67.4	67	69.4	69.7	69.8	69.8	69.7	96.1										
1373	67.1	67.4	67.1	69.6	69.8	69.9	70	69.8											
BVDV-1/Ref	67.4	66.5	67.4	88.3	79.1	88.5	79.5												
ZM-95	67.2	67	67.5	79.9	78.7	79.8													
SD1	67.6	66.9	67.8	90.5	79.7														
Osloss	66.6	66.4	66.6	79.8															
Oregon C24V	67.3	66.8	67.3																
BDV/Ref	89.7	77																	
Reindeer-1	76.9																		

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